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Nabel et al.

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(54) **VIRUS-LIKE PARTICLES AND METHODS OF USE**

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(2), (4) Date: **Dec. 27, 2013**

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PCT Pub. Date: **Aug. 9, 2012**

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Related U.S. Application Data

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(51) **Int. Cl.**
C07K 14/005 (2006.01)
A61K 39/12 (2006.01)
C12N 7/00 (2006.01)
A61K 39/00 (2006.01)

(52) **U.S. Cl.**
CPC ***C07K 14/005*** (2013.01); ***A61K 39/12*** (2013.01); ***C12N 7/00*** (2013.01); *A61K 2039/5258* (2013.01); *A61K 2039/53* (2013.01); *A61K 2039/55566* (2013.01); *C12N 2770/36123* (2013.01); *C12N 2770/36134* (2013.01); *C12N 2770/36151* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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Sequence alignment of Seq ID No: 65 with Geneseq database access No. AYC84492 of Akahata et al. In WO 2010/062396 on Jun. 3, 2010.*
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Primary Examiner — Shanon A Foley
(74) *Attorney, Agent, or Firm* — Sheridan Ross P.C.

(57) **ABSTRACT**

The invention features modified alphavirus or flavivirus virus-like particles (VLPs). The invention provides methods, compositions, and kits featuring the modified VLPs. The invention also features methods for enhancing production of modified VLPs for use in the prevention or treatment of alphavirus and flavivirus-mediated diseases. The invention also provides methods for delivering agents to a cell using the modified VLPs.

19 Claims, 177 Drawing Sheets

Figure 1

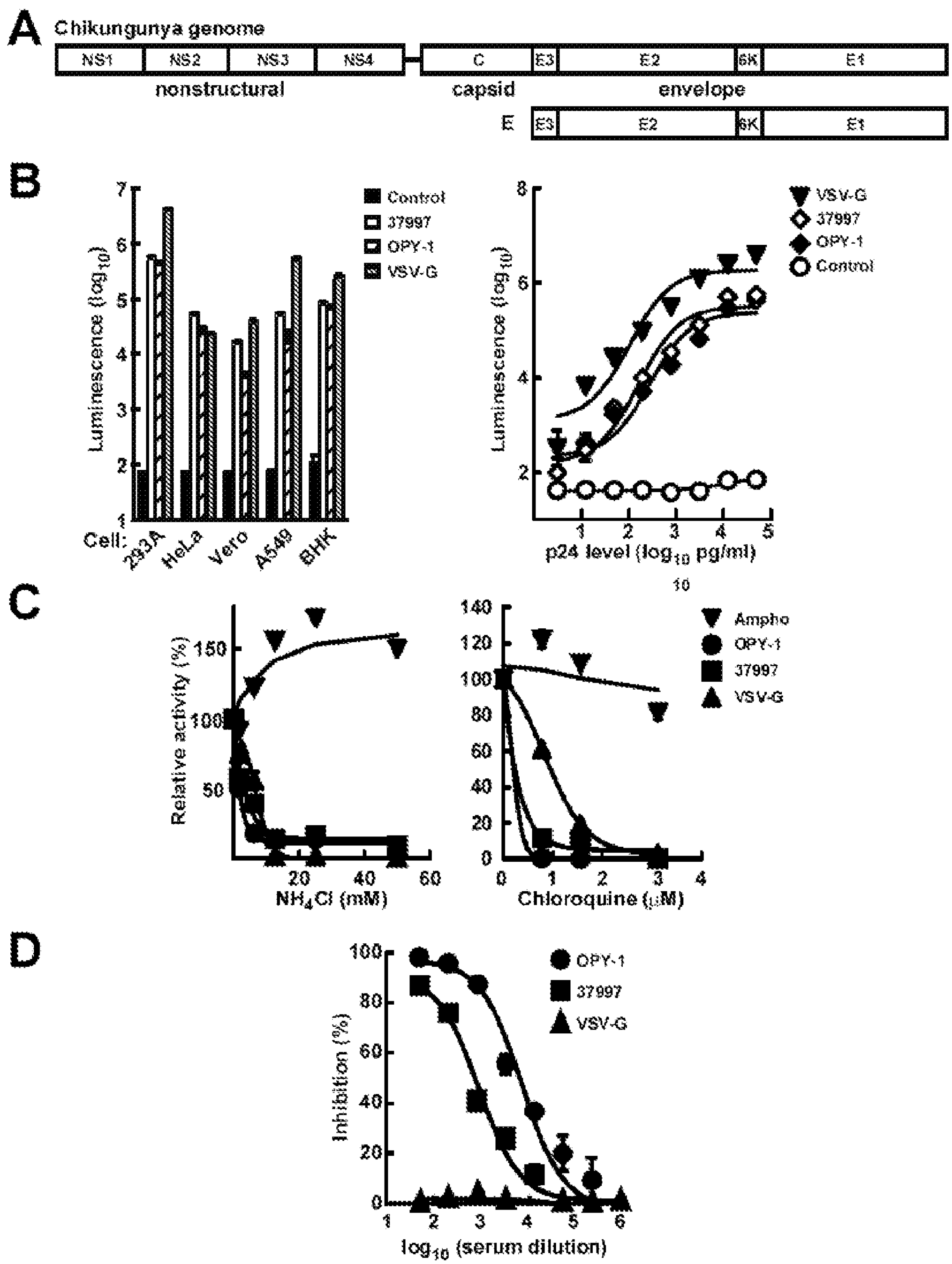
A
Chikungunya genome
NS1
NS2
NS3
NS4
C
E3
E2
6K
E1
nonstructural
capsid
envelope
E
B
Luminescence (log10)
Control
37997
OPY-1
VSV-G
Cell:
293A
HeLa
Vero
A549
BHK
p24 level (log10 pg/ml)
C
Relative activity (%)
NH4Cl (mM)
Ampho
Chloroquine (μM)
D
Inhibition (%)
log10 (serum dilution)

Figure 2

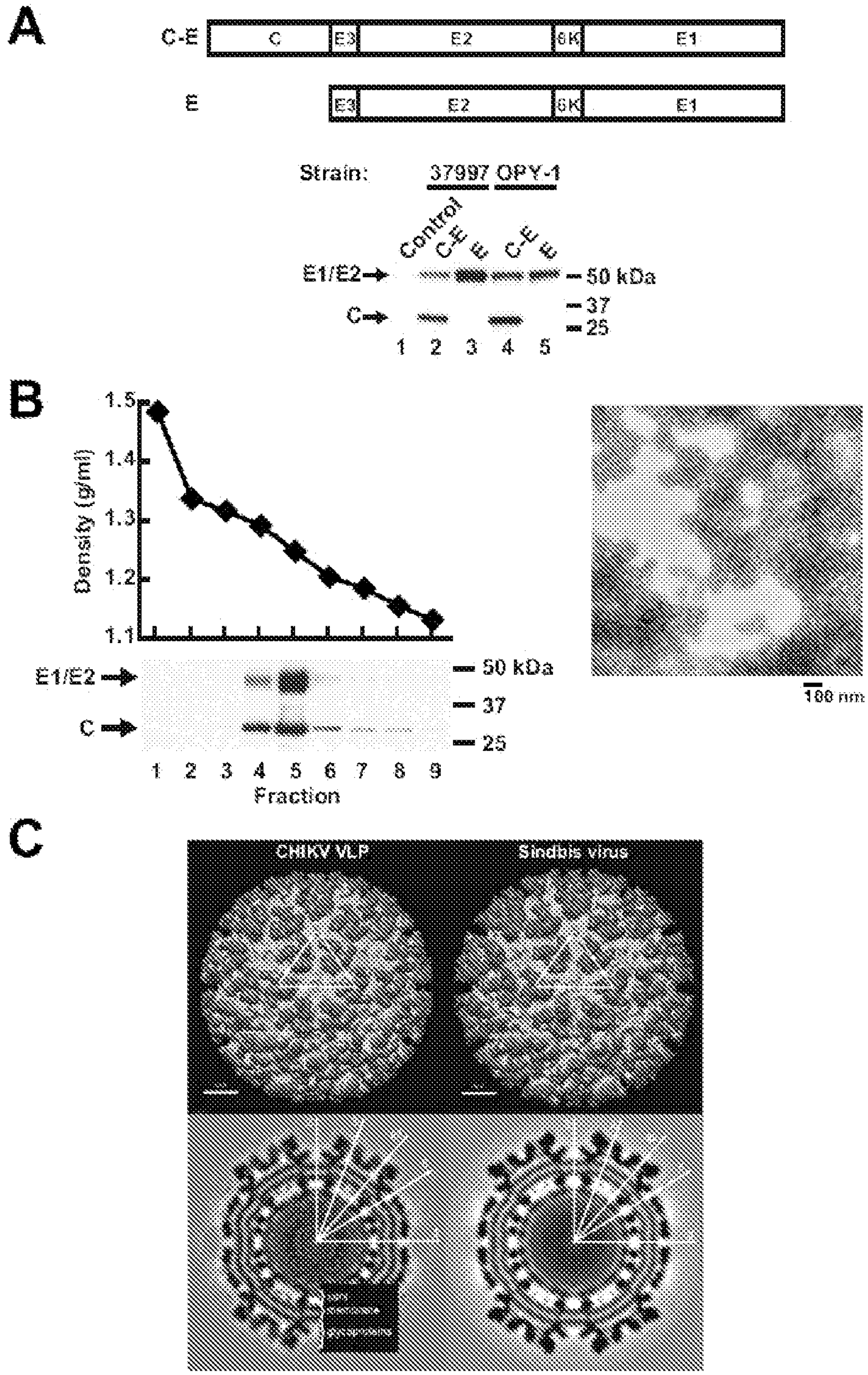
A
C-E
C
E3
E2
6K
E1
E
Strain:
37997
OPY-1
Control
C-E
E
C-E
E
E1/E2
50 kDa
C
37
25
1 2 3 4 5
B
1.5
1.4
1.3
1.2
1.1
Density (g/ml)
E1/E2
50 kDa
37
C
25
1 2 3 4 5 6 7 8 9
Fraction
100 nm
C
CHIKV VLP
Sindbis virus

Figure 3

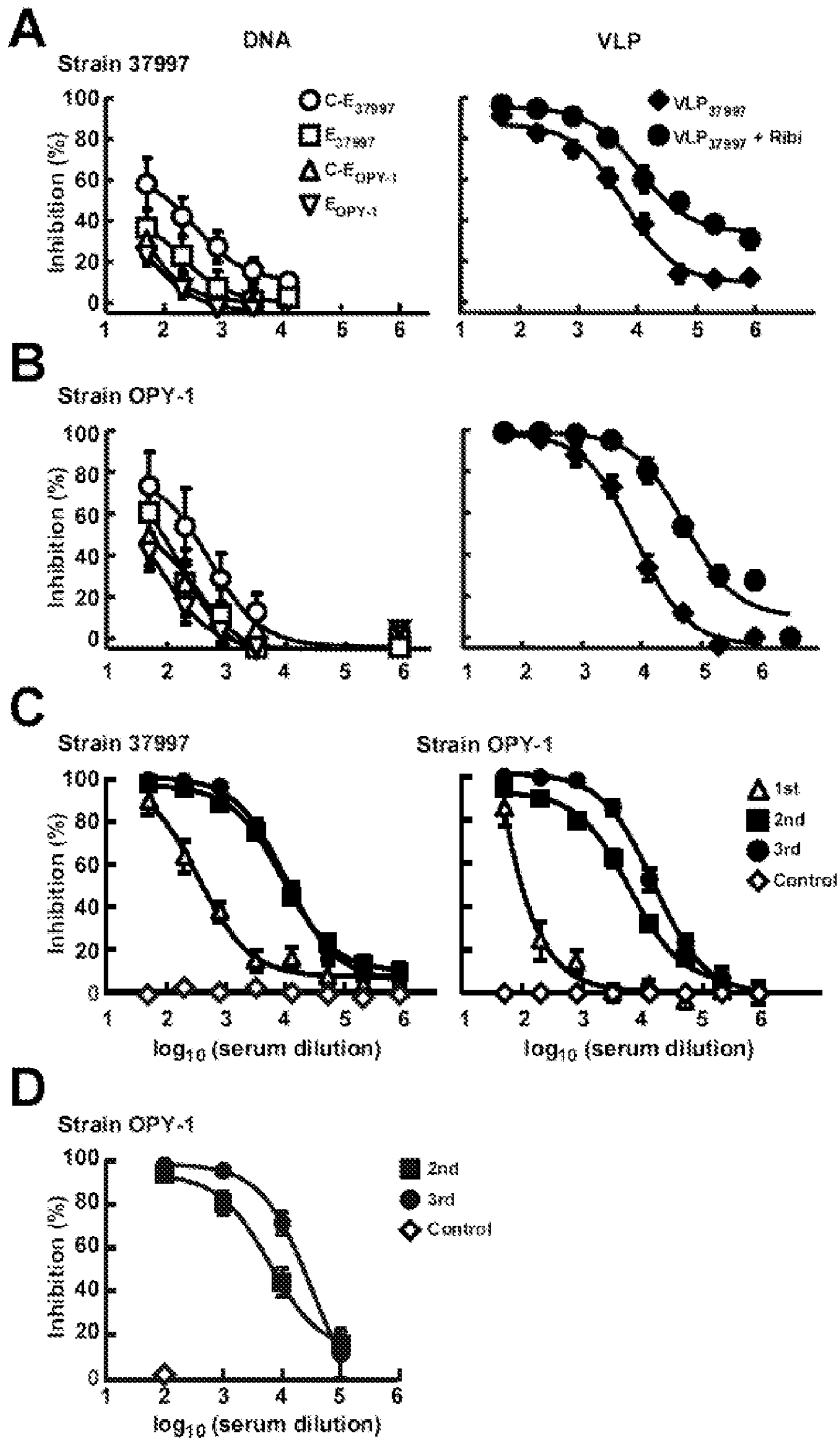
A
DNA
VLP
Strain 37997
C-E37997
E37997
C-EOPY-1
EOPY-1
VLP37997
VLP37997 + Ribi
Inhibition (%)
B
Strain OPY-1
C
Strain 37997
Strain OPY-1
1st
2nd
3rd
Control
log10 (serum dilution)
D
Strain OPY-1
2nd
3rd
Control
log10 (serum dilution)

Figure 4

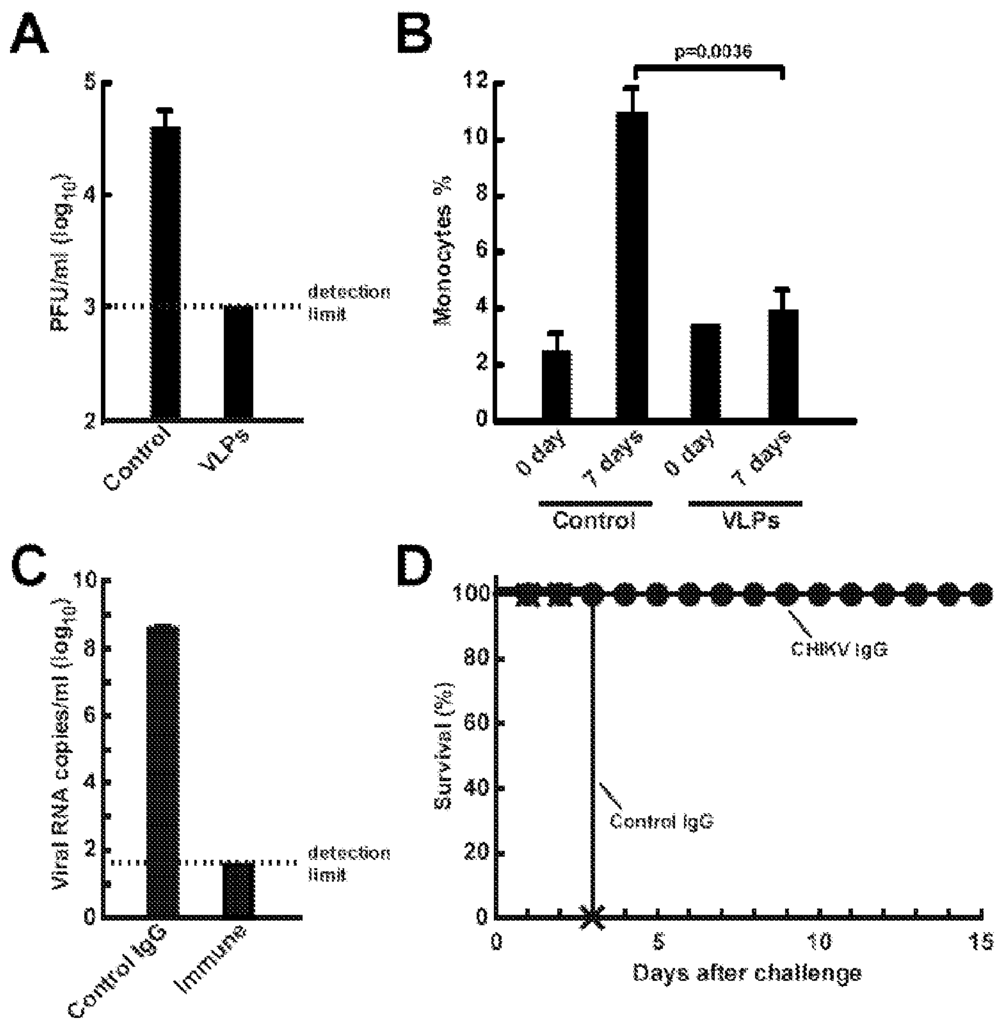
A
PFU/ml (log_{10})
5
4
3
2
detection limit
Control
VLPs
B
p=0.0036
Monocytes %
12
10
8
6
4
2
0
0 day
7 days
0 day
7 days
Control
VLPs
C
Viral RNA copies/ml (log_{10})
10
8
6
4
2
0
detection limit
Control IgG
Immune
D
Survival (%)
100
80
60
40
20
0
CHIKV IgG
Control IgG
0
5
10
15
Days after challenge

Figure 5

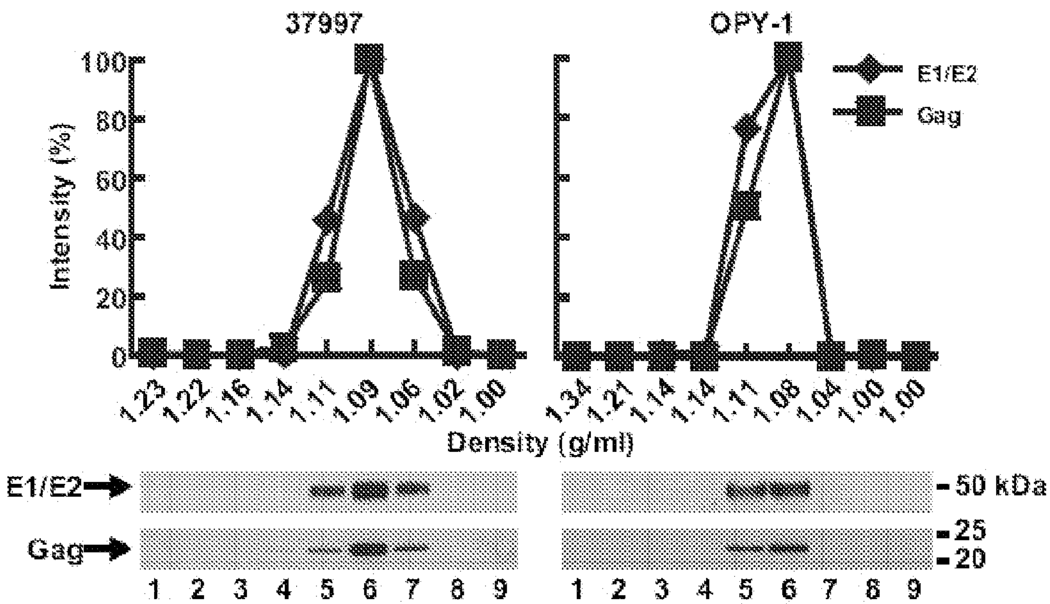
37997
OPY-1
E1/E2
Gag
Intensity (%)
100
80
60
40
20
0
1.23
1.22
1.16
1.14
1.11
1.09
1.06
1.02
1.00
1.34
1.21
1.14
1.14
1.11
1.08
1.04
1.00
1.00
Density (g/ml)
E1/E2
Gag
50 kDa
25
20
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9

Figure 6

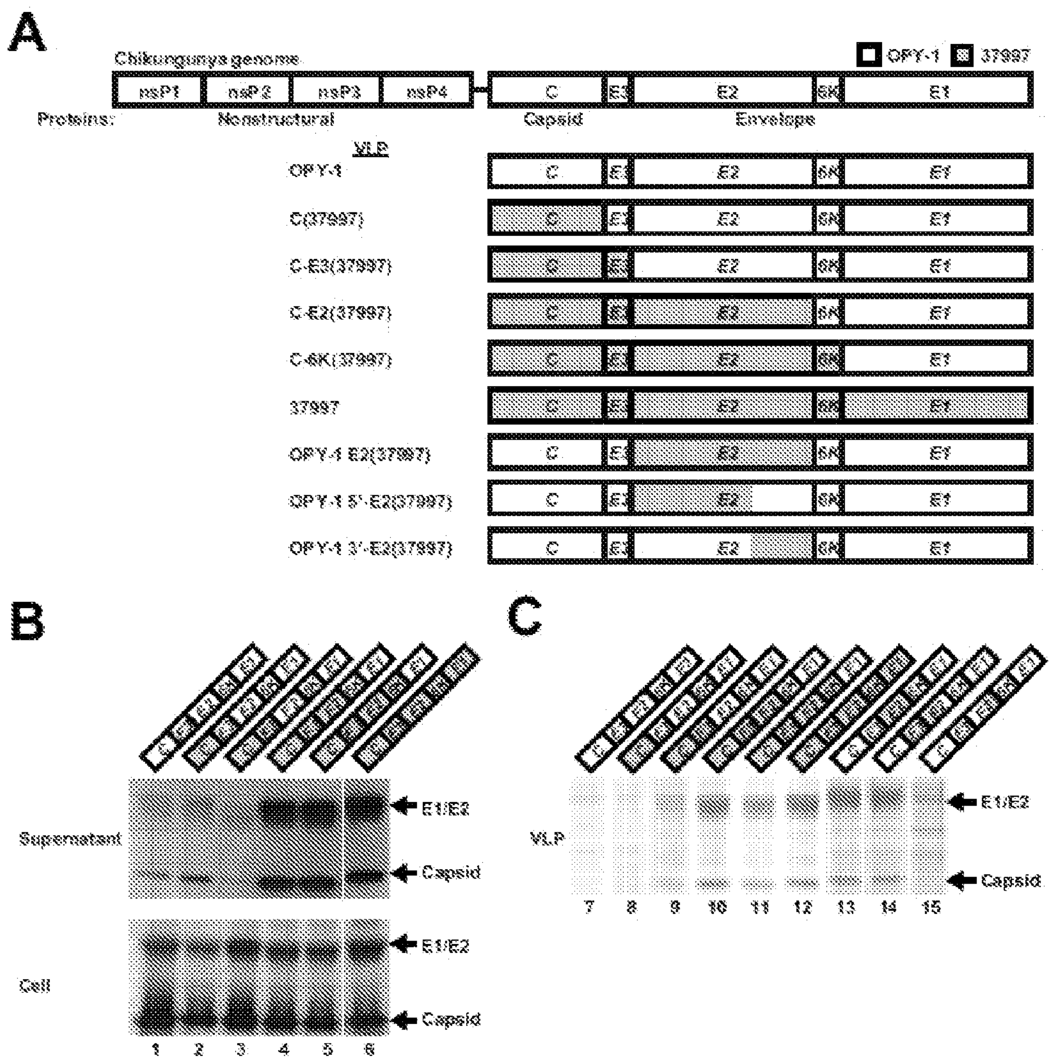
A
Chikungunya genome
OPY-1
37997
nsP1
nsP2
nsP3
nsP4
C
E3
E2
6K
E1
Proteins:
Nonstructural
Capsid
Envelope
VLP
OPY-1
C(37997)
C-E3(37997)
C-E2(37997)
C-6K(37997)
37997
OPY-1 E2(37997)
OPY-1 5'-E2(37997)
OPY-1 3'-E2(37997)
B
Supernatant
E1/E2
Capsid
Cell
E1/E2
Capsid
1
2
3
4
5
6
C
VLP
E1/E2
Capsid
7
8
9
10
11
12
13
14
15

Figure 7

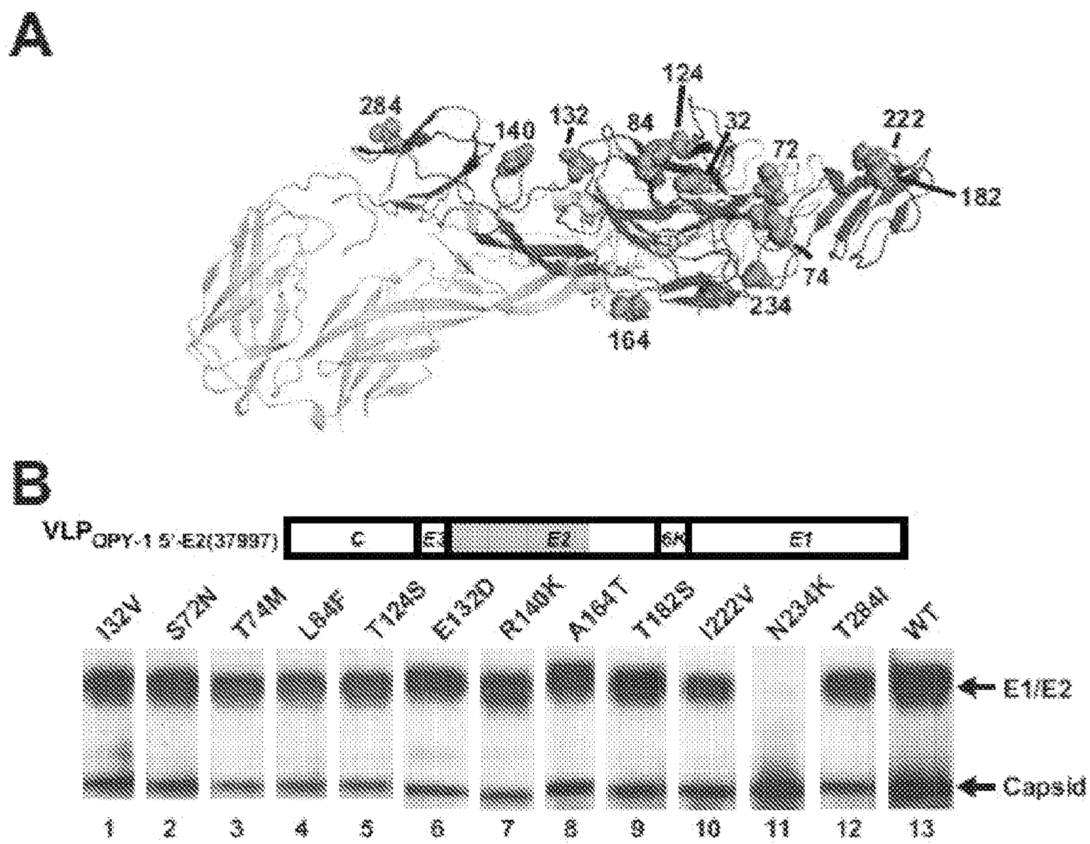
A
284
140
132
84
124
32
222
72
182
74
234
164
B
C
E2
E1
I32V
S72N
T74M
L84F
T124S
E132D
R140K
A164T
T182S
I222V
N234K
T284I
WT
E1/E2
Capsid
1
2
3
4
5
6
7
8
9
10
11
12
13

Figure 8

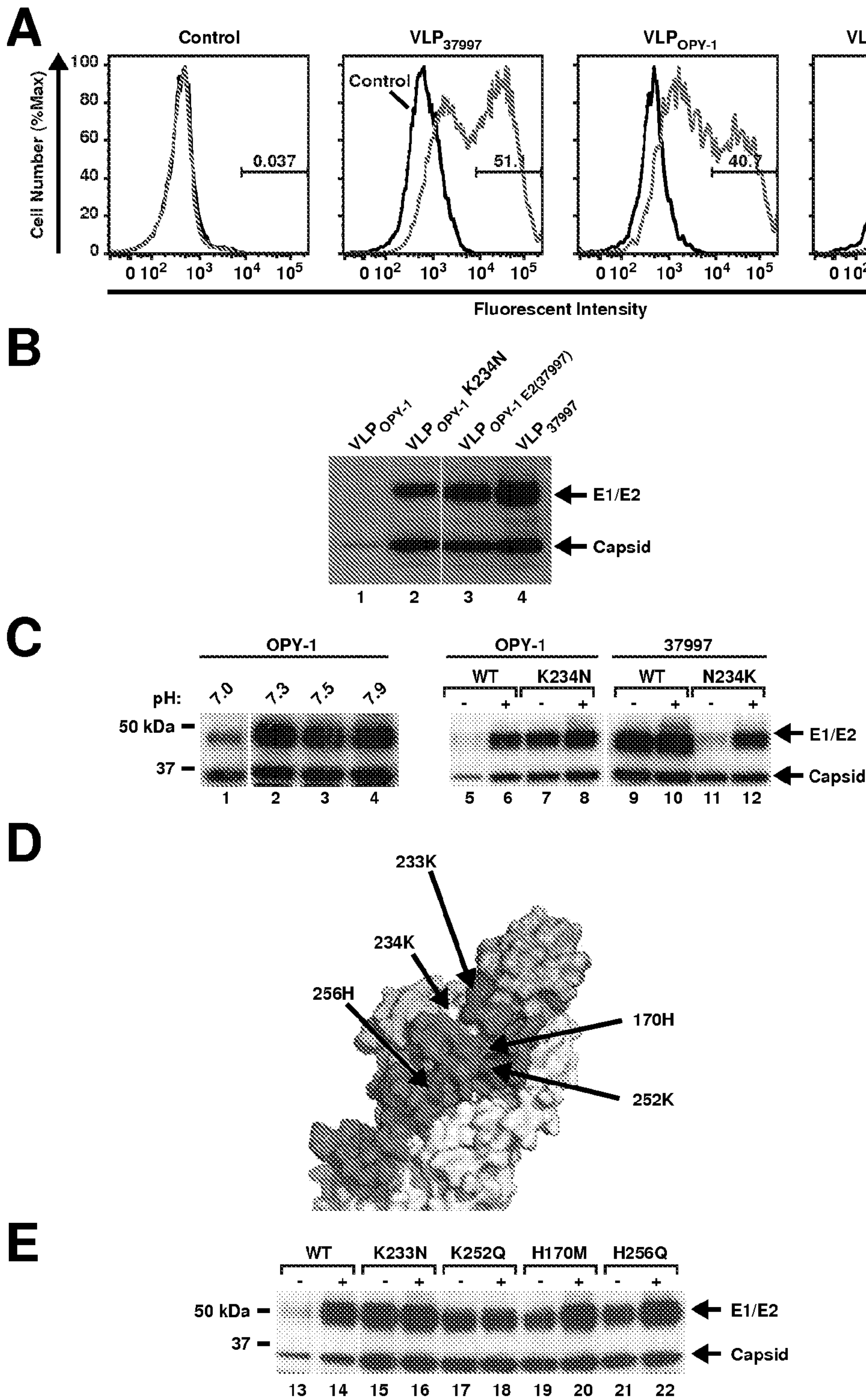
A
Control
VLP37997
VLPOPY-1
VLPOPY-1 K234N
Control
Cell Number (%Max)
100
80
60
40
20
0
0.037
51.
40.7
56.5
0 10²
10³
10⁴
10⁵
Fluorescent Intensity
B
VLP OPY-1
VLP OPY-1 K234N
VLP OPY-1 E2(37997)
VLP 37997
E1/E2
Capsid
1 2 3 4
C
OPY-1
pH:
7.0
7.3
7.5
7.9
50 kDa
37
1 2 3 4
OPY-1
37997
WT
K234N
WT
N234K
E1/E2
Capsid
5 6 7 8 9 10 11 12
D
233K
234K
256H
170H
252K
E
WT
K233N
K252Q
H170M
H256Q
50 kDa
37
E1/E2
Capsid
13 14 15 16 17 18 19 20 21 22

Figure 9A

```
37997         KDNFNVYKATRPYLAHCPDCGEGHSCHSPIALERIRNEATDGTLKIQVSLQIGIKTDDSH
OPY-1         KDNFNVYKATRPYLAHCPDCGEGHSCHSPVALERIRNEATDGTLKIQVSLQIGIKTDDSH
Ross River    TEHFNVYKATRPYLAYCADCGDGYFCYSPVAIEKIRDEASDGMLKIQVSAQIGLDKAGTH
Sindbis       IDDFT---LTSPYLGTCSYCHHTVPCFSPVKIEQVWDEADDNTIRIQTSAQFGYDQSGAA
WEEV          TDDFT---LTSPYLGFCPYCRHSAPCFSPIKIENVWDESDDGSIRIQVSAQFGYNQAGTA
EEEV          DTHFTQYKLARPYIADCPNCGHSR-CDSPIAIEEVRGDAHAGVIRIQTSAMFGLK----T
VEEV          EELFKEYKLTRPYMARCIRCAVG-SCHSPIAIEAVKSDGHDGYVRLQTSSQYGLDSSGNL
                 *       **   *  *     * **   *             * *   *

37997         DWTKLRYMD---SHTPADAERAGLLVRTSAPCTITGTMGHFILARCPKGETLTVGFTDSR
OPY-1         DWTKLRYMD---NHMPADAERAGLFVRTSAPCTITGTMGHFILARCPKGETLTVGFTDSR
Ross River    AHTKIRYMA---GHDVQESKRDSLRVYTSAACSIHGTMGHFIVAHCPPGDYLKVSFEDAD
Sindbis       SANKYRYMSLKQDHTVKEGTMDDIKISTSGPCRRLSYKGYFLLAKCPPGDSVTVSIVSSN
WEEV          DVTKFRYMSYDHDHDIKEDSMEKLAISTSGPCRRLGHKGYFLLAQCPPGDSVTVSITSGA
EEEV          DGVDLAYMSFMNGKTQKSIKIDNLHVRTSAPCSLVSHHGYYILAQCPPGDTVTVGFHDGP
VEEV          ---KGRTMRYDMHGTIEEIPLHQVSLHTSRPCHIVDGHGYFLLARCPAGDSITMEFKKGS
                     *                   **  *      *    * ** *

37997         KISHTCTHPFHHEPPVIGRERFHSRPQHGKELPCSTYVQSTAATAEEIEVHMPPDTPDRT
OPY-1         KISHSCTHPFHHDPPVIGREKFHSRPQHGKELPCSTYVQSTAATTEEIEVHMPPDTPDRT
Ross River    SHVKACKVQYKHDPLPVGREKFVVRPHFGVELPCTSYQLTTAPTDEEIDMHTPPDIPDRT
Sindbis       S-ATSCTLARKIKPKFVGREKYDLPPVHGKKIPCTVYDRLKETTAGYITMHRPRPHAYTS
WEEV          S-ENSCTVEKKIRRKFVGREEYLFPPVHGKLVKCHVYDHLKETSAGYITMHRPGPHAYKS
EEEV          N-RHTCTVAHKVEFRPVGREKYRHPPEHGVELPCNRYTHKRADQGHYVEMHQPGLVADHS
VEEV          V-THSCSVPYEVKFNPVGRELYTHPPEHGAEQACQVYAHDAQNRGAYVEMHLPGSEVDSS
                   *          ***     *  *    *  *             **
233
234
37997         LMTQQSGNVKITV-NGQTVRYKCNCGG-SNEGLTTTDKVINNC-KIDQCHAAVTNHKKWQ
OPY-1         LMSQQSGNVKITV-NGQTVRYKCNCGG-SNEGLTTTDKVINNC-KVDQCHAAVTNHKKWQ
Ross River    LLSQTAGNVKITA-GGRTIRYNCTCGR-DNVGTTSTDKTINTC-KIDQCHAAVTSHLKWQ
Sindbis       YLEESSGKVYAKPPSGKNITYECKCGD-YKTGTVSTRTEITGCTAIKQCVAYKSDQTKWV
WEEV          YLEEASGEVYIKPPSGKNVTYECKCGD-YSTGIVSTRTKMNGCTKAKQCIAYKRDQTKWV
EEEV          LLSIHSAKVKITVPSGAQVKYYCKCPD-VREGTTSS-DYTTTCTDVKQCRAYLIDNKKWV
VEEV          LISLSGSSVTVTPPVGTSALVKCKCGGTKISETINKAKQFSQCTKKEQCRAYRLQNDKWV
                      *      *      * *                 *    ** *       *
251
37997         YNSPLVPRNAELGDRKGKIHIPFPLANVTCRVPKARNPTVTYGKNQVTMLLYPDHPTLLS
OPY-1         YNSPLVPRNAELGDRKGKIHIPFPLANVTCRVPKARNPTVTYGKNQVIMLLYPDHPTLLS
Ross River    FTSPFVPR-ADQTARRGKVHVPFPLTNVTCRVPLARAPDVTYGKKEVTLRLHPDHPTLFS
Sindbis       FNSPDLIR-HDDHTAQGKLHLPFKLIPSTCMVPVAHAPNVIHGFKHISLQLDTDHLTLLT
WEEV          FNSPDLIR-HTDHSVQGKLHIPFRLTPTVCPVPLAHTPTVTKWFKGITLHLTATRPTLLT
EEEV          YNSGRLPR-GEGDTFKGKLHVPFVPVKAKCIATLAPEPLVEHKHRTLILHLYPDHPTLLT
VEEV          YNSDKLPK-AAGATLKGKLHVPFLLADGKCTVPLAPEPMITFGFRSVSLKLHPKNPTYLT
                *            ** *  **      *    *  *           *     *
```

Figure 9B

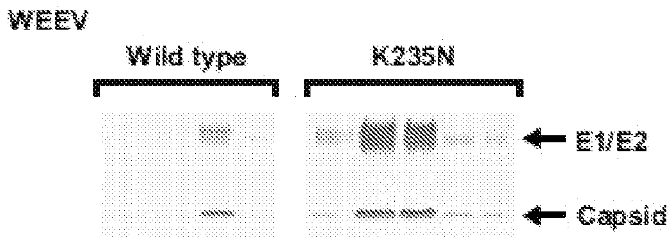

Figure 9C

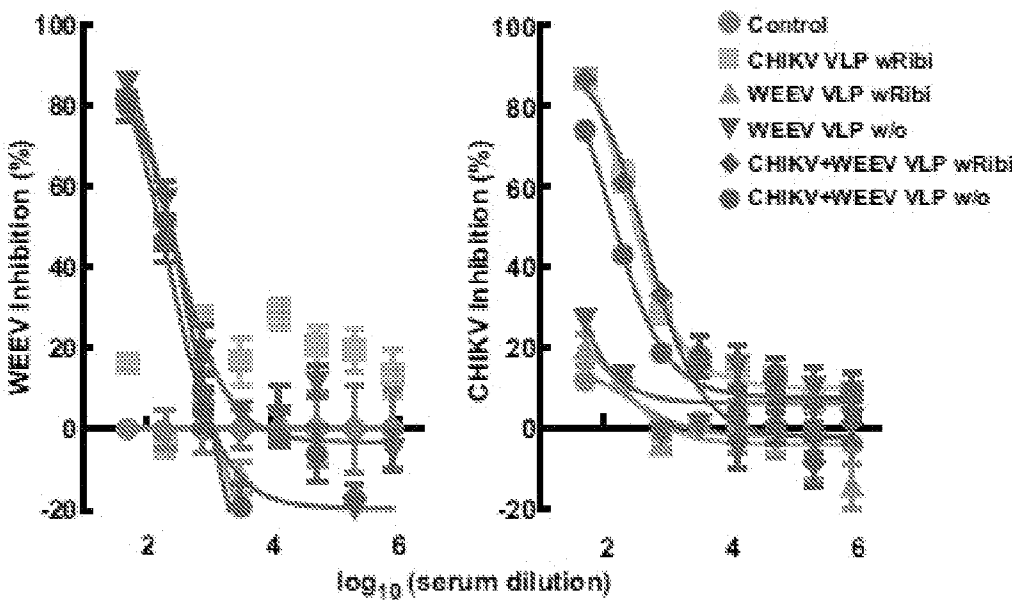

Figure 10A

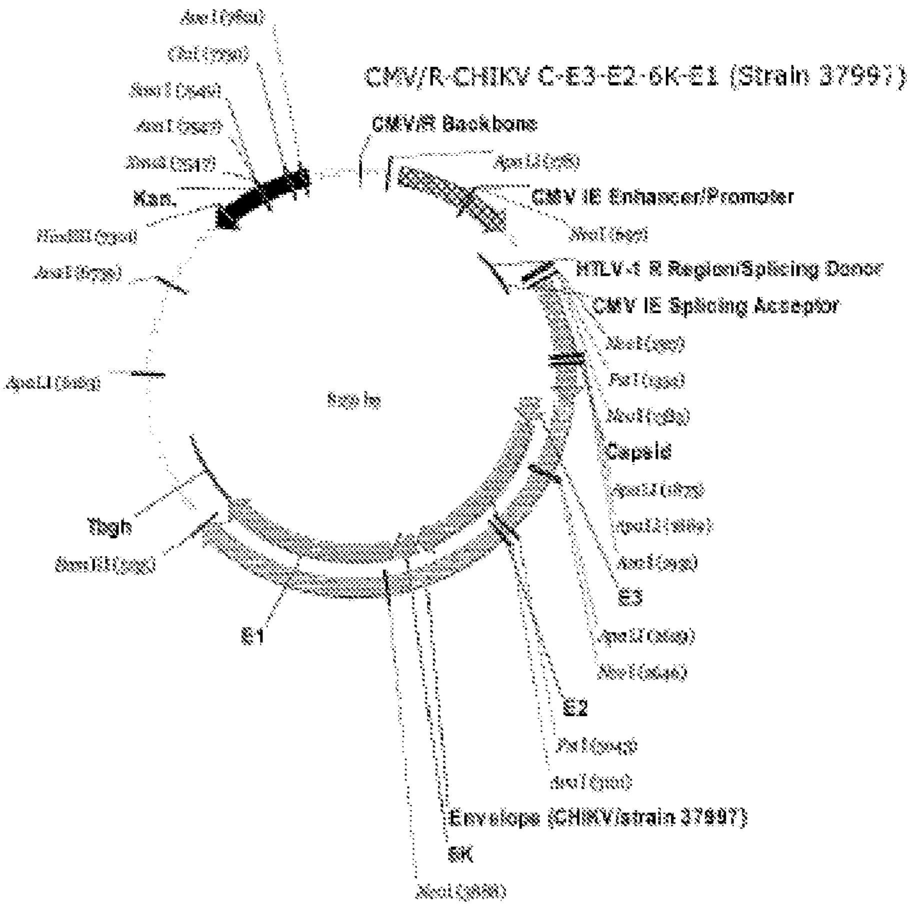
CMV/R-CHIKV C-E3-E2-6K-E1 (Strain 37997)
CMV/R Backbone
CMV IE Enhancer/Promoter
HTLV-1 R Region/Splicing Donor
CMV IE Splicing Acceptor
Capsid
E3
E2
Envelope (CHIKV/strain 37997)
6K
E1
Tbgh
Kan.

Figure 10B

Insert C-E3-E2-6K-E1 (strain 37997)

atggagttcatcccgacgcaaactttctataacagaaggtaccaaccccgaccctgggccccacgccctacaatt
caagtaattagacctagaccacgtccacagaggcaggctgggcaactcgcccagctgatctccgcagtcaacaaa
ttgaccatgcgcgcggtacctcaacagaagcctcgcagaaatcggaaaaacaagaagcaaaggcagaagaagcag
gcgccgcaaaacgacccaaagcaaaagaagcaaccaccacaaaagaagccggctcaaaagaagaagaaaccaggc
cgtagggagagaatgtgcatgaaaattgaaaatgattgcatcttcgaagtcaagcatgaaggcaaagtgatgggc
tacgcatgcctggtgggggataaagtaatgaaaccagcacatgtgaagggaactatcgacaatgccgatctggct
aaactggcctttaagcggtcgtctaaatacgatcttgaatgtgcacagataccggtgcacatgaagtctgatgcc
tcgaagtttacccacgagaaacccgaggggtactataactggcatcacggagcagtgcagtattcaggaggccgg
ttcactatcccgacgggtgcaggcaagccgggagacagcggcagaccgatcttcgacaacaaaggacgggtggtg
gccatcgtcctaggaggggccaacgaaggtgcccgcacggccctctccgtggtgacgtggaacaaagacatcgtc
acaaaaattaccccctgagggagccgaagagtggagcctcgccctcccggtcttgtgcctgttggcaaacactaca
ttcccctgctctcagccgccttgcacaccctgctgctacgaaaaggaaccggaaagcaccttgcgcatgcttgag
gacaacgtgatgagacccggatactaccagctactaaaagcatcgctgacttgctctccccaccgccaaagacgc
agtactaaggacaattttaatgtctataaagccacaagaccatatctagctcattgtcctgactgcggagaaggg
cattcgtgccacagccctatcgcattggagcgcatcagaaatgaagcaacggacggaacgctgaaaatccaggtc
tctttgcagatcgggataaagacagatgacagccacgattggaccaagctgcgctatatggatagccatacgcca
gcggacgcggagcgagccggattgcttgtaaggacttcagcaccgtgcacgatcaccgggaccatgggacacttt
attctcgcccgatgcccgaaaggagagacgctgacagtgggatttacggacagcagaaagatcagccacacatgc
acacacccgttccatcatgaaccacctgtgataggtagggagaggttccactctcgaccacaacatggtaaagag
ttaccttgcagcacgtacgtgcagagcaccgctgccactgctgaggagatagaggtgcatatgcccccagatact
cctgaccgcacgctgatgacgcagcagtctggcaacgtgaagatcacagttaatgggcagacggtgcggtacaag
tgcaactgcggtggctcaaacgagggactgacaaccacagacaaagtgatcaataactgcaaaattgatcagtgc
catgctgcagtcactaatcacaagaattggcaatacaactcccctttagtcccgcgcaacgctgaactcggggac
cgtaaaggaaagatccacatcccattcccattggcaaacgtgacttgcagagtgccaaaagcaagaaaccctaca
gtaacttacggaaaaaaccaagtcaccatgctgctgtatcctgaccatccgacactcttgtcttaccgtaacatg
ggacaggaaccaaattaccacgaggagtgggtgacacacaagaaggaggttaccttgaccgtgcctactgagggt
ctggaggtcacttggggcaacaacgaaccatacaagtactggccgcagatgtctacgaacggtactgctcatggt
cacccacatgagataatcttgtactattatgagctgtaccccactatgactgtagtcattgtgtcggtggcctcg
ttcgtgcttctgtcgatggtgggcacagcagtgggaatgtgtgtgtgcgcacggcgcagatgcattacaccatat
gaattaacaccaggagccactgttcccttcctgctcagcctgctatgctgcgtcagaacgaccaaggcggccaca
tattacgaggctgcggcatatctatggaacgaacagcagcccctgttctggttgcaggctcttatcccgctggcc
gccttgatcgtcctgtgcaactgtctgaaactcttgccatgctgctgtaagaccctggctttttttagccgtaatg
agcatcggtgcccacactgtgagcgcgtacgaacacgtaacagtgatcccgaacacggtgggagtaccgtataag
actcttgtcaacagaccgggttacagccccatggtgttggagatggagctacaatcagtcaccttggaaccaaca
ctgtcacttgactacatcacgtgcgagtacaaaactgtcatcccctccccgtacgtgaagtgctgtggtacagca
gagtgcaaggacaagagcctaccagactacagctgcaaggtctttactggagtctacccatttatgtggggcggc
gcctactgcttttgcgacgccgaaaatacgcaattgagcgaggcacatgtagagaaatctgaatcttgcaaaaca
gagtttgcatcggcctacagagcccacaccgcatcggcgtcggcgaagctccgcgtcctttaccaaggaaacaac
attaccgtagctgcctacgctaacggtgaccatgccgtcacagtaaaggacgccaagtttgtcgtgggcccaatg
tcctccgcctggacaccttttgacaacaaaatcgtggtgtacaaaggcgacgtctacaacatggactacccacct
tttggcgcaggaagaccaggacaatttggtgacattcaaagtcgtacaccggaaagtaaagacgtttatgccaac
actcagttggtactacagaggccagcagcaggcacggtacatgtaccatactctcaggcaccatctggcttcaag
tattggctgaaggaacgaggagcatcgctacagcacacggcaccgttcggttgccagattgcgacaaacccggta
agagctgtaaattgcgctgtggggaacataccaatttccatcgacataccggatgcggcctttactagggttgtc
gatgcaccctctgtaacggacatgtcatgcgaagtaccagcctgcactcactcctccgactttgggggcgtcgcc
atcatcaaatacacagctagcaagaaaggtaaatgtgcagtacattcgatgaccaacgccgttaccattcgagaa
gccgacgtagaagtagaggggaactcccagctgcaaatatccttctcaacagccctggcaagcgccgagtttcgc
gtgcaagtgtgctccacacaagtacactgcgcagccgcatgccaccctccaaaggaccacatagtcaattaccca
gcatcacacaccacccttggggtccaggatatatccacaacggcaatgtcttgggtgcagaagattacggagga
gtaggattaattgttgctgttgctgccttaattttaattgtggtgctatgcgtgtcgtttagcaggcactaa

Figure 10C

CMV/R 37997 C-E3-E2-6K-E1

tcgcgcgtttcggtgatgacggtgaaaacctctgacacatgcagctcccggagacggtcacagcttgtctgtaag
cggatgccgggagcagacaagcccgtcagggcgcgtcagcgggtgttggcgggtgtcggggctggcttaactatg
cggcatcagagcagattgtactgagagtgcaccatatgcggtgtgaaataccgcacagatgcgtaaggagaaaat
accgcatcagattggctattggccattgcatacgttgtatccatatcataatatgtacatttatattggctcatg
tccaacattaccgccatgttgacattgattattgactagttattaatagtaatcaattacggggtcattagttca
tagcccatatatggagttccgcgttacataacttacggtaaatggcccgcctggctgaccgcccaacgacccccg
cccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacgtcaatgggtgga
gtatttacggtaaactgcccacttggcagtacatcaagtgtatcatatgccaagtacgccccctattgacgtcaa
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Figure 10C continued

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Figure 11 A

A

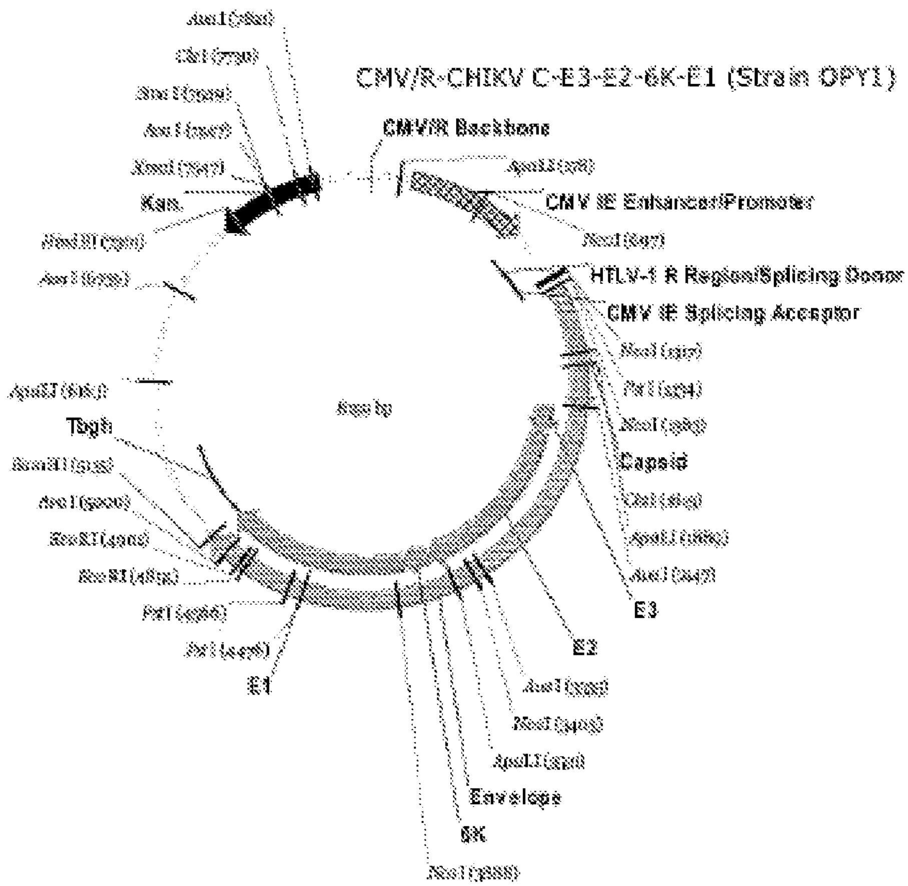
CMV/R-CHIKV C-E3-E2-6K-E1 (Strain OPY1)
CMV/R Backbone
CMV IE Enhancer/Promoter
HTLV-1 R Region/Splicing Donor
CMV IE Splicing Acceptor
Capsid
E3
E2
Envelope
6K
E1
Tbgh
Kan.

Figure 11 B

Insert C-E3-E2-6K-E1 (strain OPY-1)

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Figure 11C

CMV/R C-E3-E2-6K-E1 strain OPY-1

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Figure 11C continued

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Figure 12A

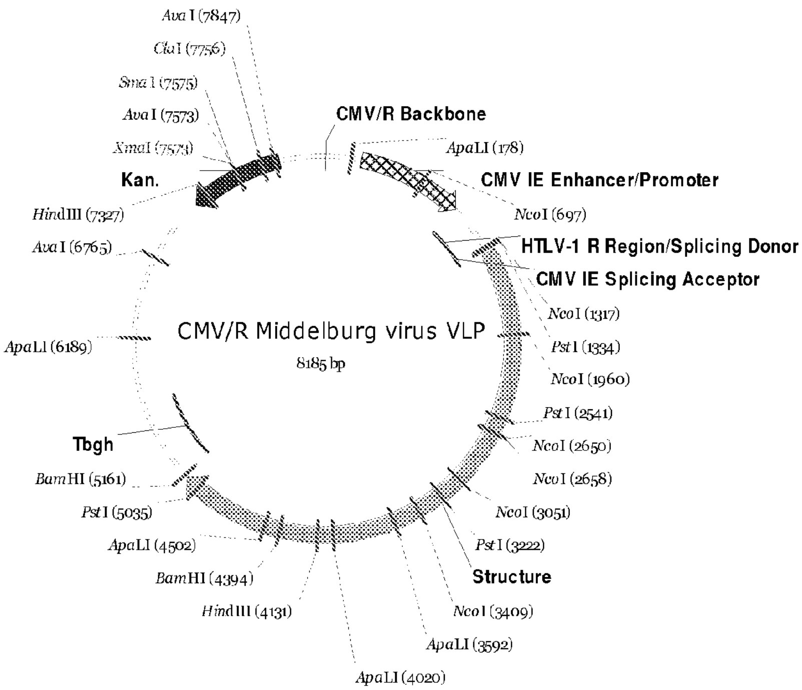

Figure 12B

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Figure 12B continued

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Figure 12B continued

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Figure 13A

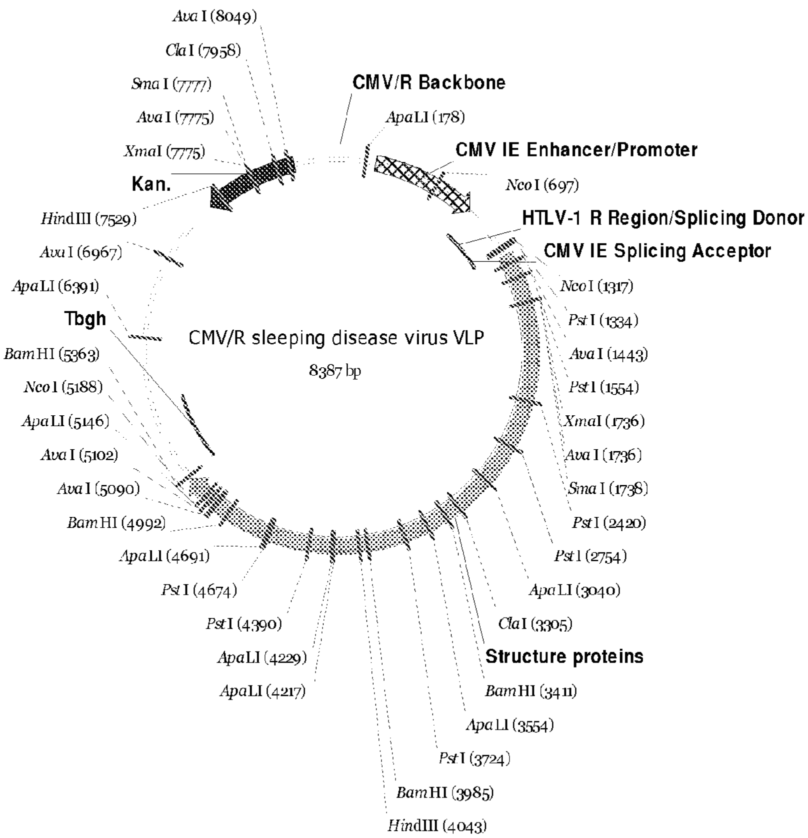

Figure 13B

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Figure 13B continued

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Figure 13B continued

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Figure 14A

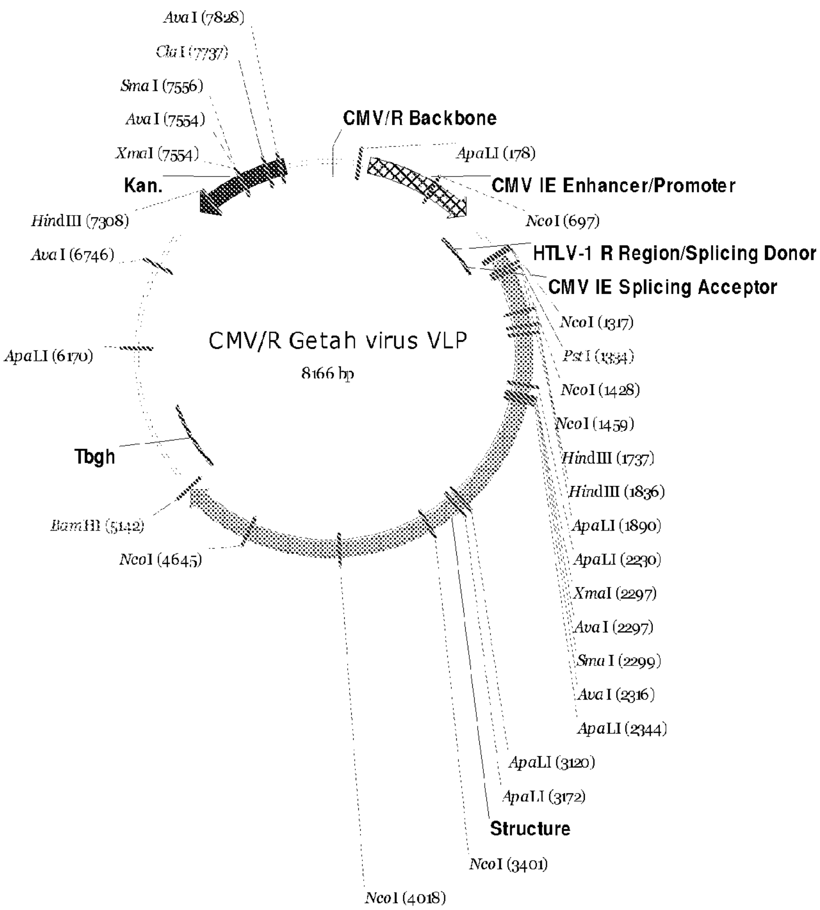

Figure 14B

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Figure 14B continued

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Figure 14B continued

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Figure 15A

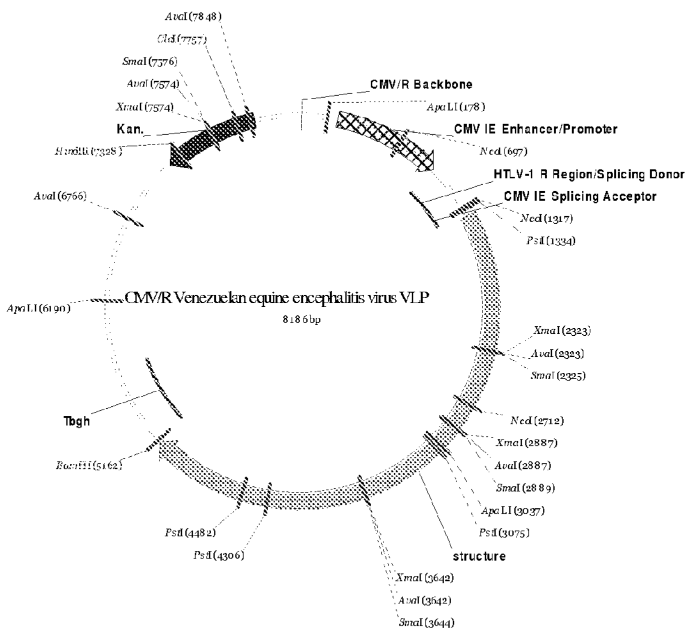

Figure 15B

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Figure 15B continued

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Figure 15B continued

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Figure 16A

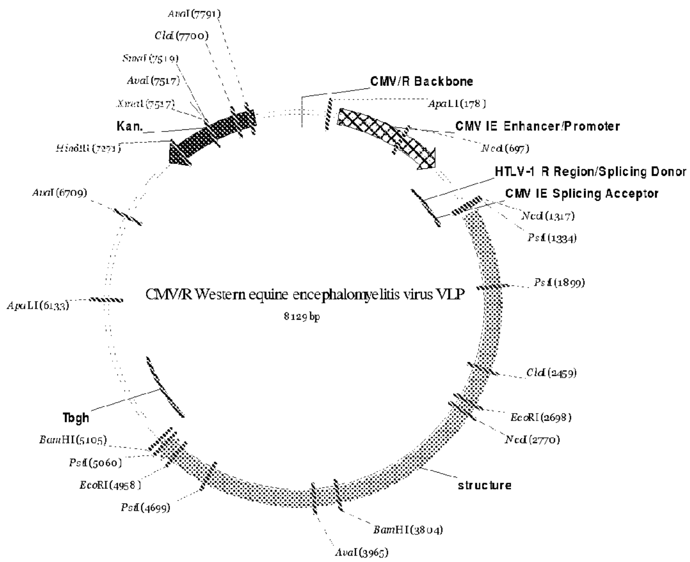

Figure 16B

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Figure 16B continued

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Figure 16B continued

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Figure 17A

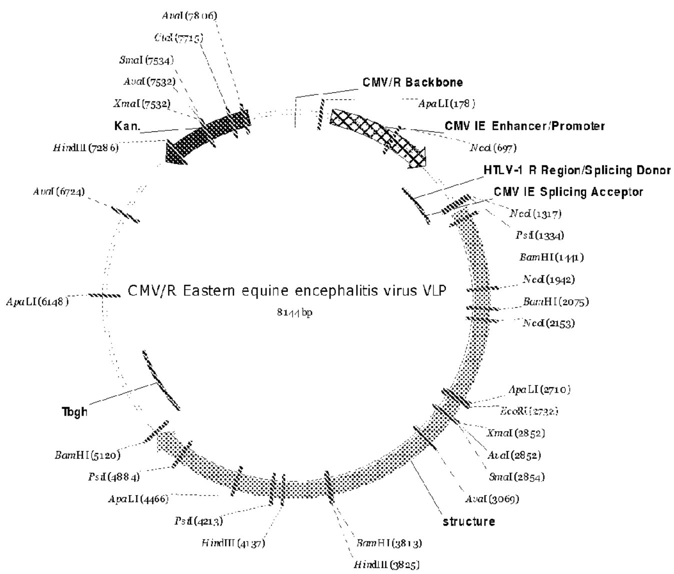

Figure 17B

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Figure 17B continued

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Figure 17B continued

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Figure 18A

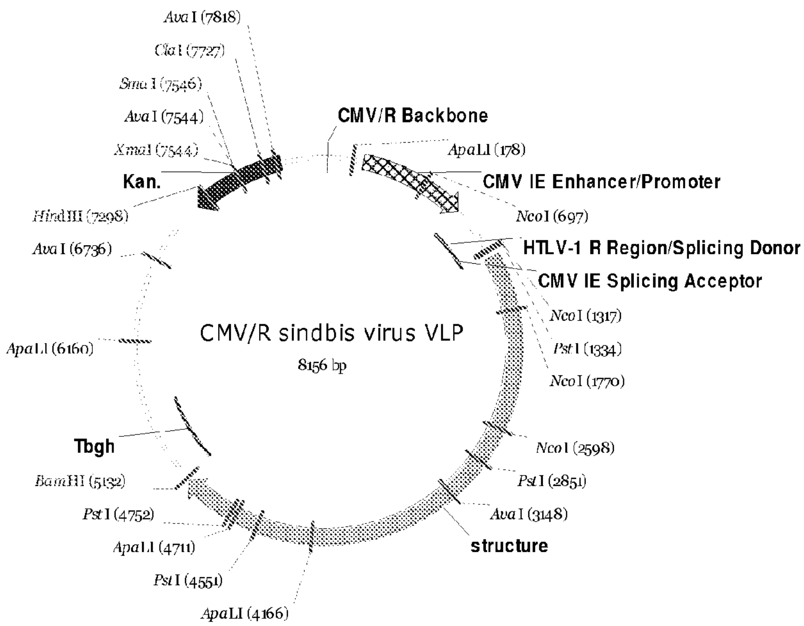

Figure 18B

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Figure 18B continued

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Figure 18B continued

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Figure 19A

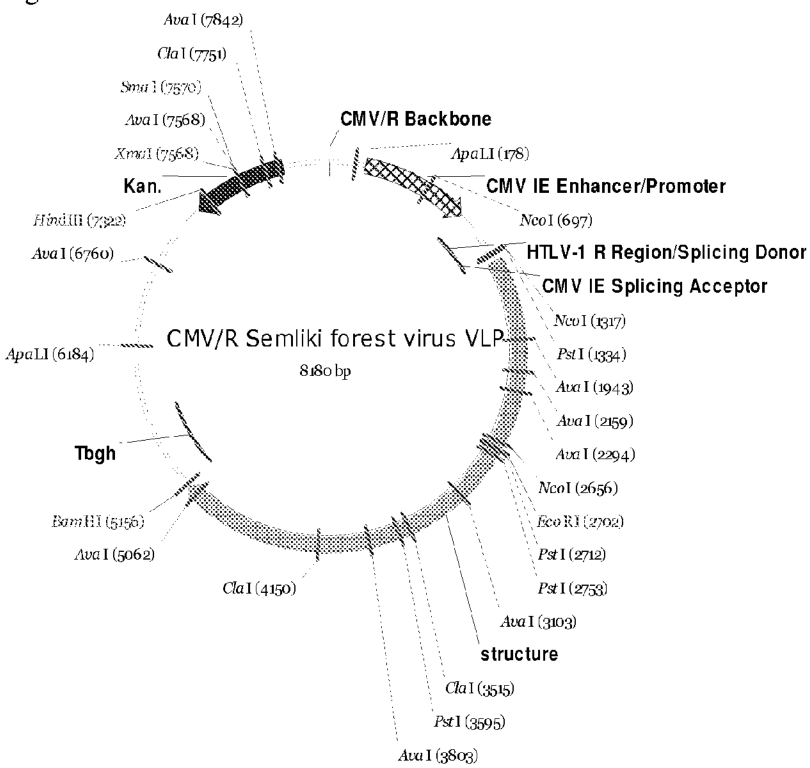

Figure 19B

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Figure 19B continued

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Figure 19B continued

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Figure 20A

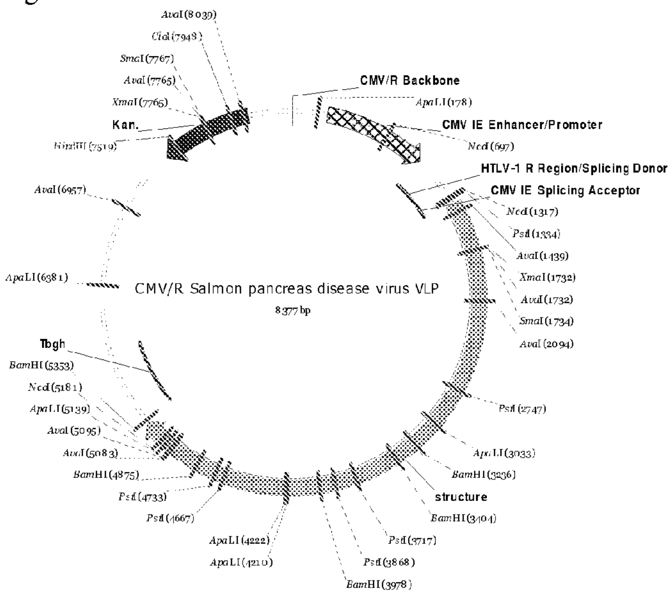

Figure 20B

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Figure 20B continued

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Figure 20B continued

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Figure 21A

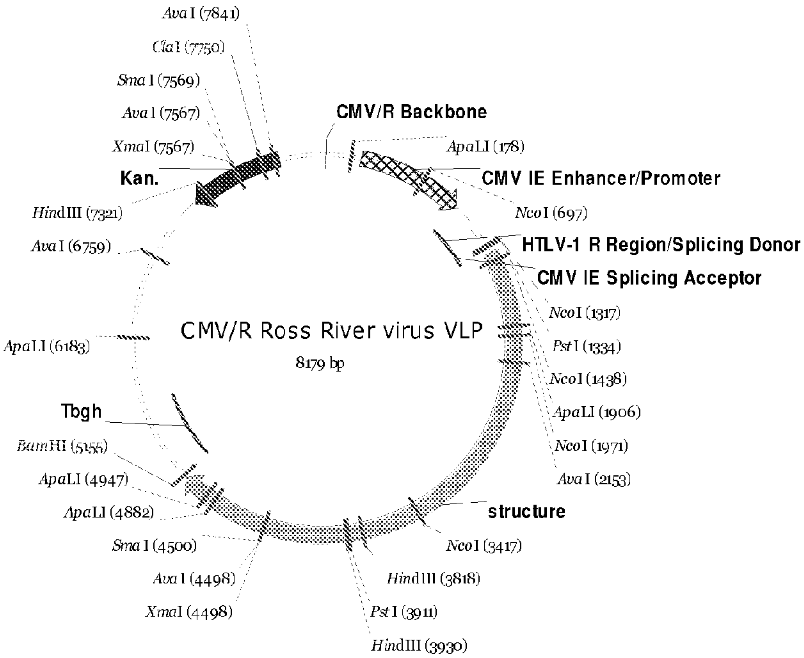

Figure 21B

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Figure 21B continued

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Figure 21B continued

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Figure 22A

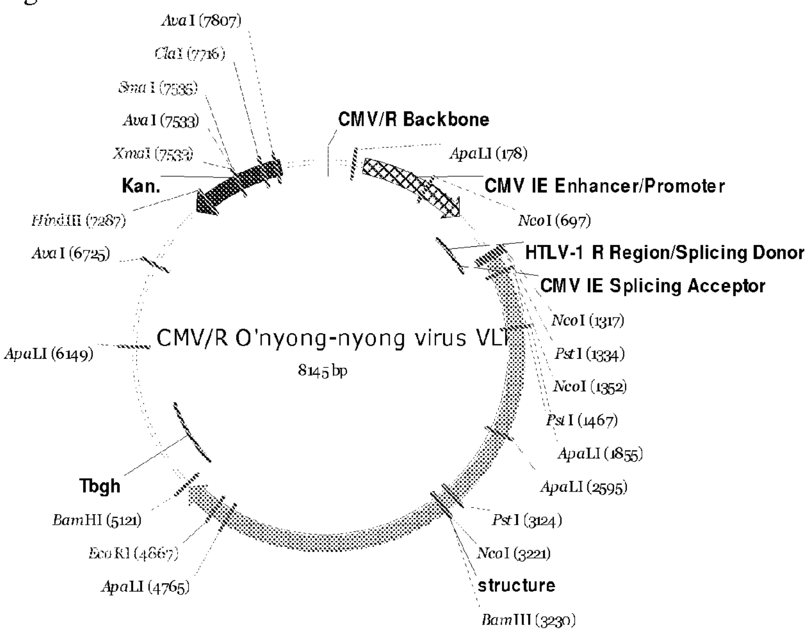

Figure 22B

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Figure 22B continued

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Figure 22B continued

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Figure 23A

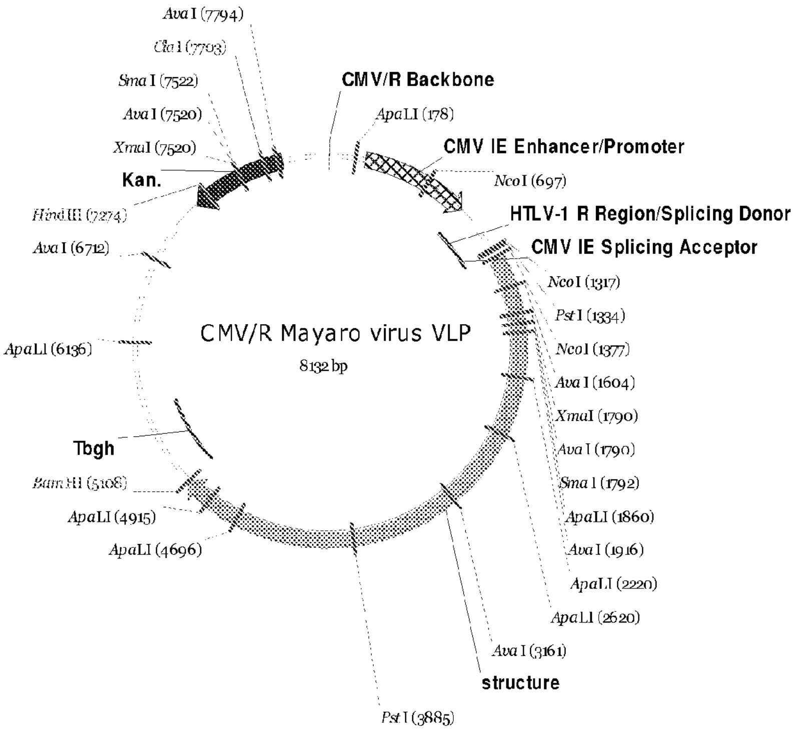

Figure 23B

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Figure 23B continued

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Figure 23B continued

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Figure 24A

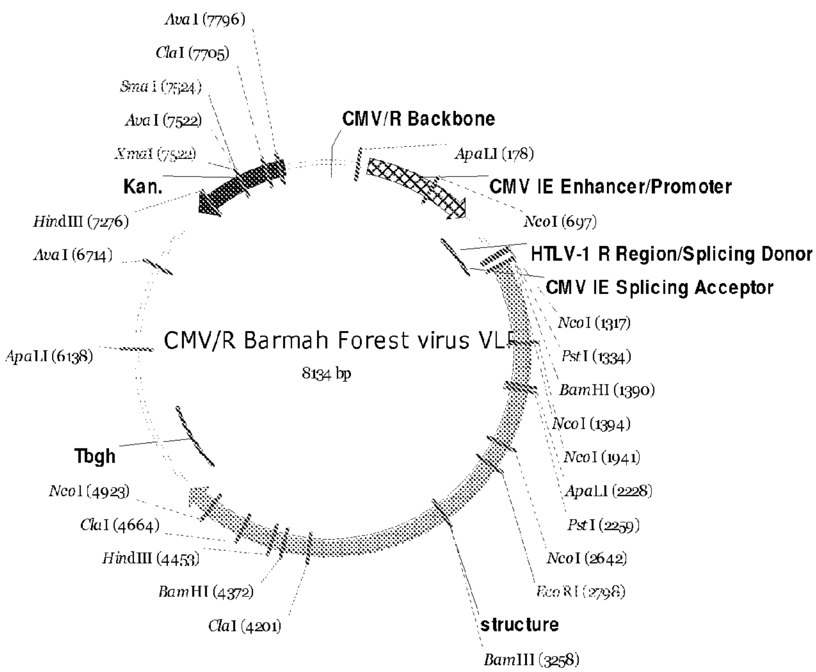

Figure 24B

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Figure 24B continued

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Figure 24B continued

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Figure 25A

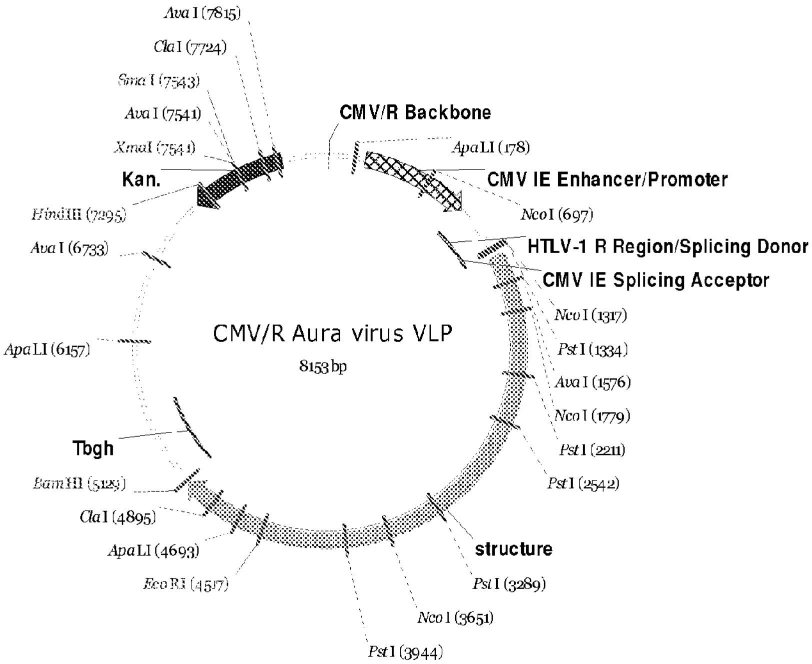

Figure 25B

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Figure 25B continued

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Figure 25B continued

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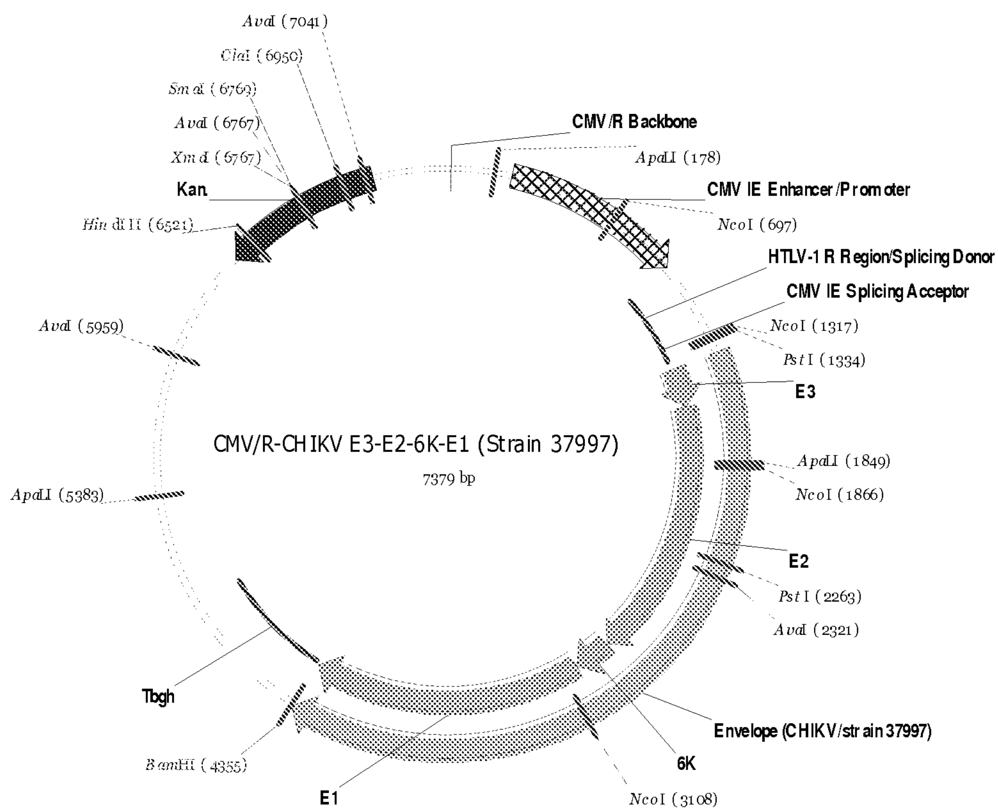
Figure 26A



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Kan
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AvaI (5959)
ApaLI (5383)
Tbgh
BamHI (4355)
E1
CMV/R Backbone
ApaLI (178)
CMV IE Enhancer/Promoter
NcoI (697)
HTLV-1 R Region/Splicing Donor
CMV IE Splicing Acceptor
NcoI (1317)
PstI (1334)
E3
ApaLI (1849)
NcoI (1866)
E2
PstI (2263)
AvaI (2321)
Envelope (CHIKV/strain 37997)
6K
NcoI (3108)
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7379 bp

Figure 26B

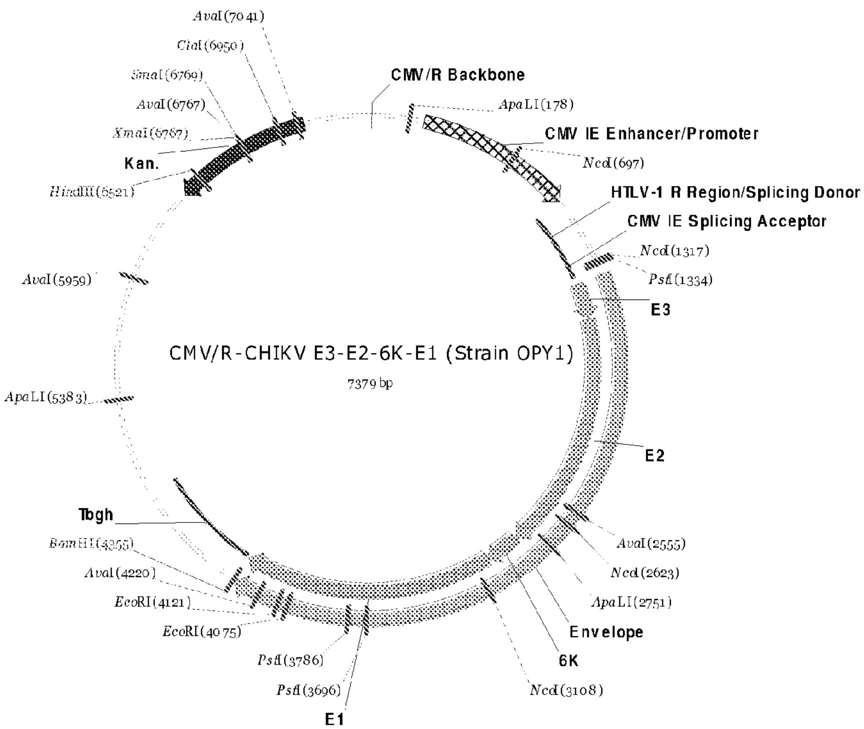

Figure 26C

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Figure 26C continued

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Figure 26C continued

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Figure 27

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Figure 27 continued

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Figure 27 continued

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Figure 27 continued

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Figure 28

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Figure 28 continued

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Figure 28 continued

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Figure 28 continued

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Figure 29A

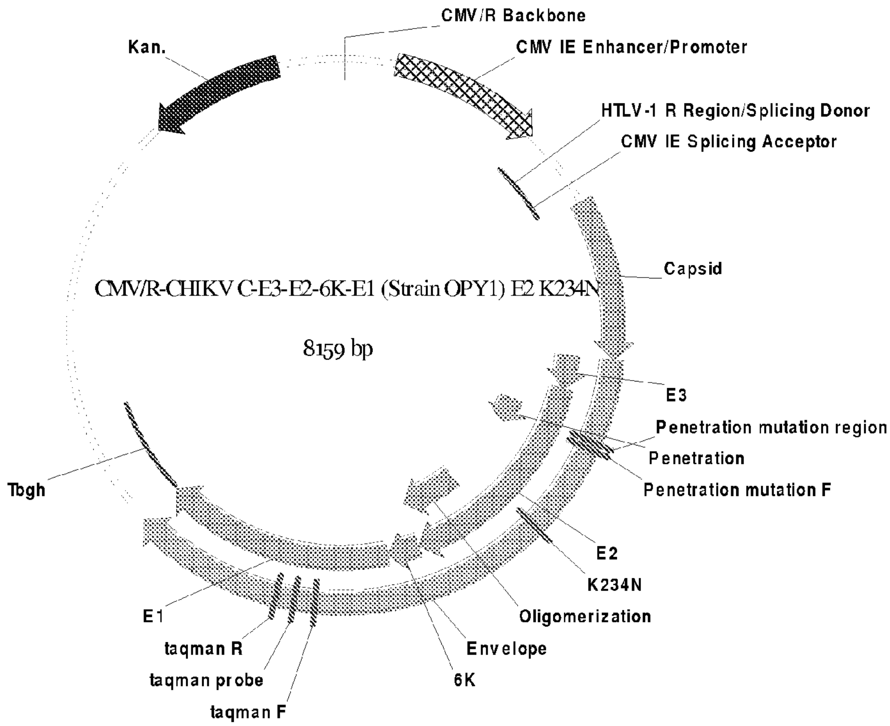

Figure 29B

Sequence of insert

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Figure 29B continued

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Figure 29B continued

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Figure 29B continued

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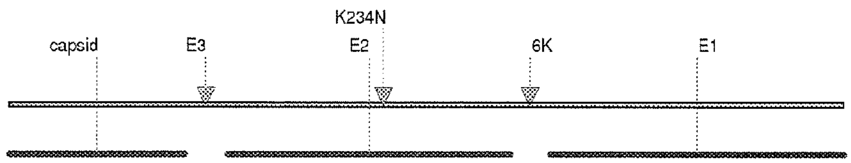
Figure 29C



Translation of CMV/R-CHIKV C-E3-E2-6K-E1 (Strain OPY1) E2 K234N

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Figure 30A

CMV/R Western equine encephalomyelitis virus (71V-1658) E2 K235N VLP

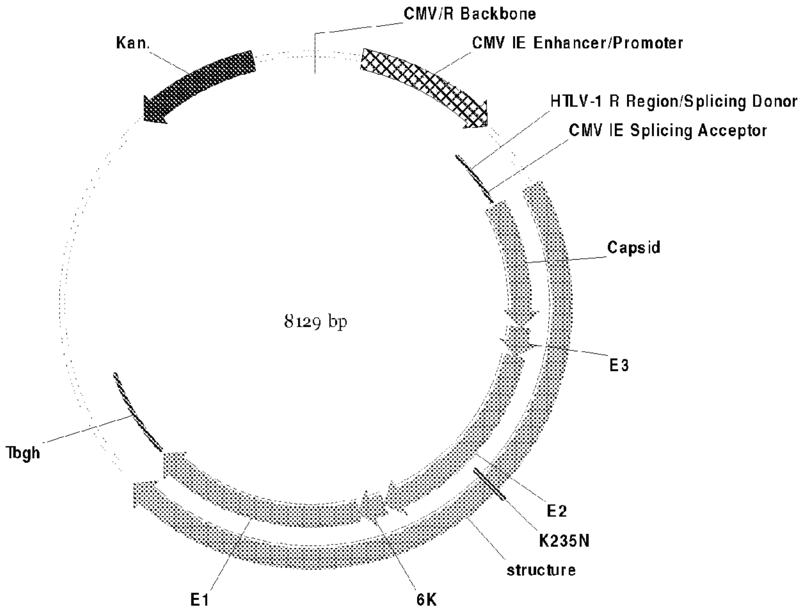

Figure 30B

Insert sequence

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Figure 30B continued

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Figure 30B continued

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Figure 30B continued

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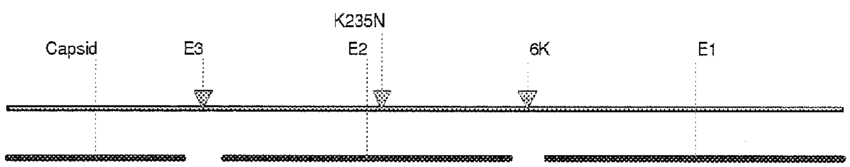
Figure 30C



Translation of CMV/R Western equine encephalomyelitis virus (71V-1658) E2 K235N VLP

1236 aa

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Figure 31A

CMV/R-CHIKV C-E3-E2(37997)-6K-E1 (Strain OPY1)

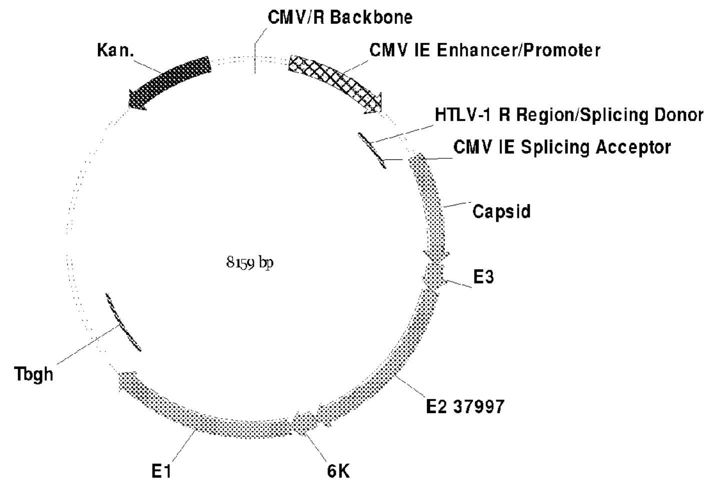

Figure 31B

Insert sequence

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Figure 31B continued

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Full sequence

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Figure 31B continued

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Figure 31B continued

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aaccattattatcatgacattaacctataaaaataggcgtatcacgaggccctttcgtc

Figure 31C

Translation of CMV/R-CHIKV C E3 E2(37997)-6K-E1 (OPY1)

1248 aa

MEFIPTQTFYNRRYQPRPWAPRPTIQVIRPRPRPQRQAGQLAQLISAVNKLTMRAVPQQKPRRNRKNKKQRQKKQ
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VTYGKNQVTMLLYPDHPTLLSYRNMGQEPNYHEEWVTHKKEVTLTVPTEGLEVTWGNNEPYKYWPQMSTNGTAHG
HPHEIILYYYELYPTMTVVIVSVASFVLLSMVGTAVGMCVCARRRCITPYELTPGATVPFLLSLLCCVRTTKAAT
YQEAAIYLWNEQQPLFWLQALIPLAALIVLCNCLRLLPCCCKTLAFLAVMSVGAHTVSAYEHVTVIPNTVGVPYK
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IIKYAASKKGKCAVHSMTNAVTIREAEIEVEGNSQLQISFSTALASAEFRVQVCSTQVHCAAECHPPKDHIVNYP
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Figure 32A

CMV/R-CHIKV C E3 E2 6K(37997)-E1 (OPY1)

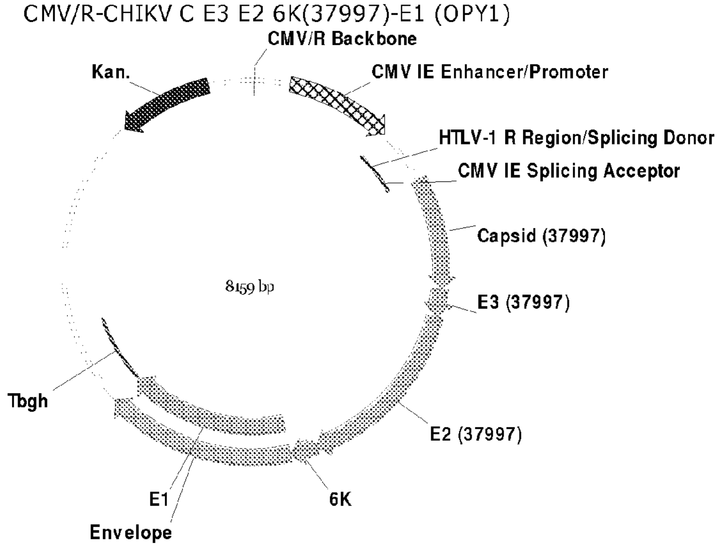

Figure 32B

Insert sequence

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Figure 32B continued

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Full sequence

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Figure 32B continued

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Figure 32B continued

```
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```

Figure 32C

Translation of CMV/R-CHIKV C E3 E2 6K(37997)-E1 (OPY1)

1248 aa

```
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ASHTTLGVQDISATAMSWVQKITGGVGLVVAVAALILIVVLCVSFSRH
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Figure 33A

CMV/R-CHIKV C-E3-6K-E1 (Strain OPY1)-E2(strain 37997)

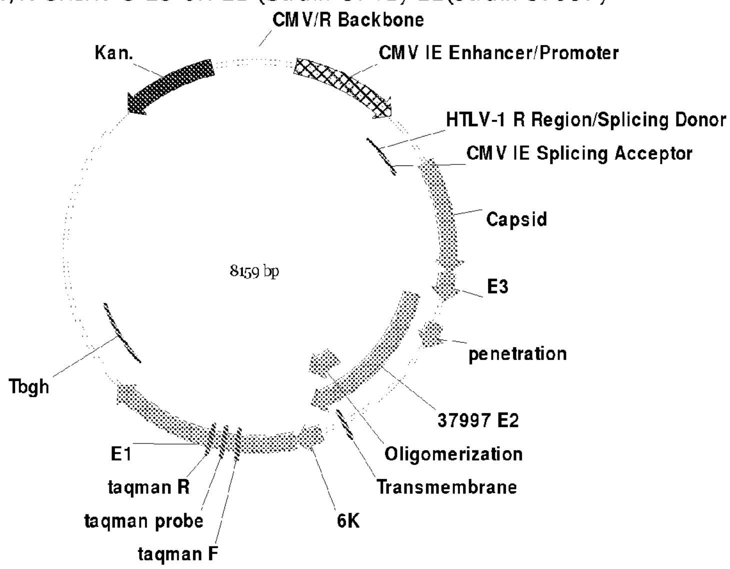

Figure 33B

Insert sequence

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Figure 33B continued

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Full sequence

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Figure 33B continued

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Figure 33B continued

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Figure 33C

Translation of CMV/R-CHIKV C-E3-6K-E1 (Strain OPY1)-E2(strain 37997)

1248 aa

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Figure 34A

CMV/R-CHIKV C-E3-6K-E2-E1 (Strain OPY1) 5'E2(strain 37997)

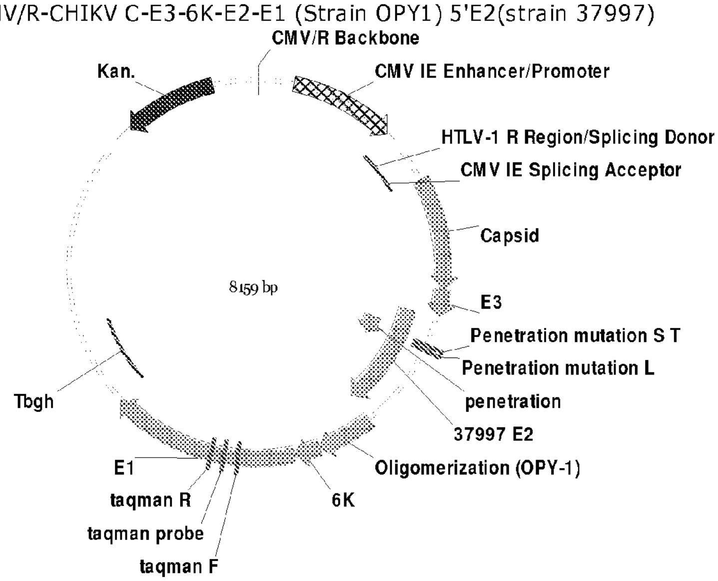

Figure 34B

Insert sequence

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Figure 34B continued

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Full sequence

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Figure 34B continued

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Figure 34B continued

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Figure 34C

Translation of CMV/R-CHIKV C-E3-6K-E2-E1 (Strain OPY1) 5'E2(strain 37997)

1248 aa

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Figure 35

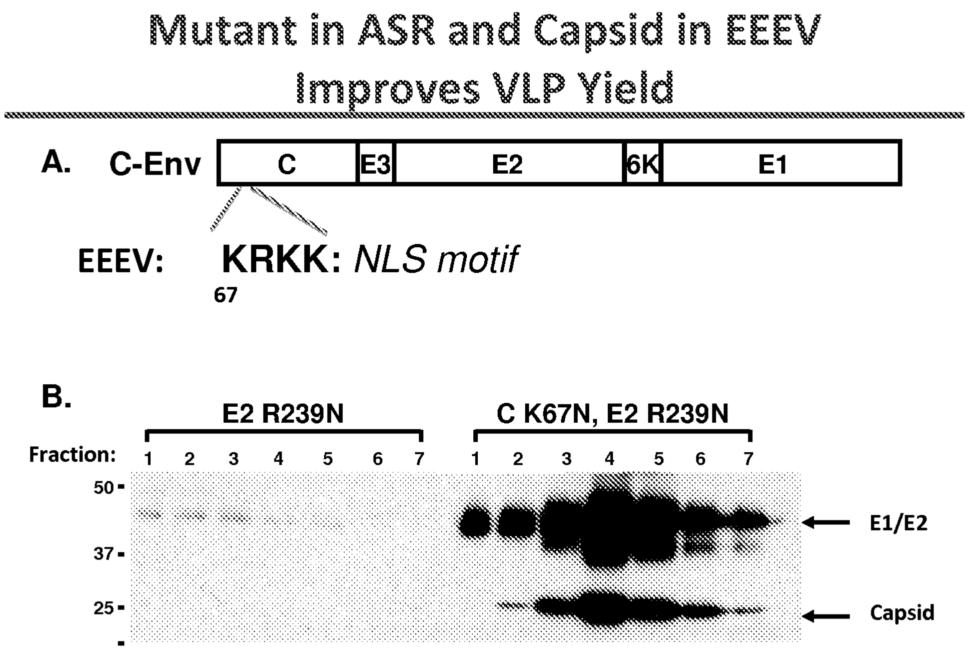
Mutant in ASR and Capsid in EEEV Improves VLP Yield
A.
C-Env
C
E3
E2
6K
E1
EEEV:
KRKK: NLS motif
67
B.
E2 R239N
C K67N, E2 R239N
Fraction:
1 2 3 4 5 6 7
1 2 3 4 5 6 7
50
37
25
E1/E2
Capsid

Figure 36

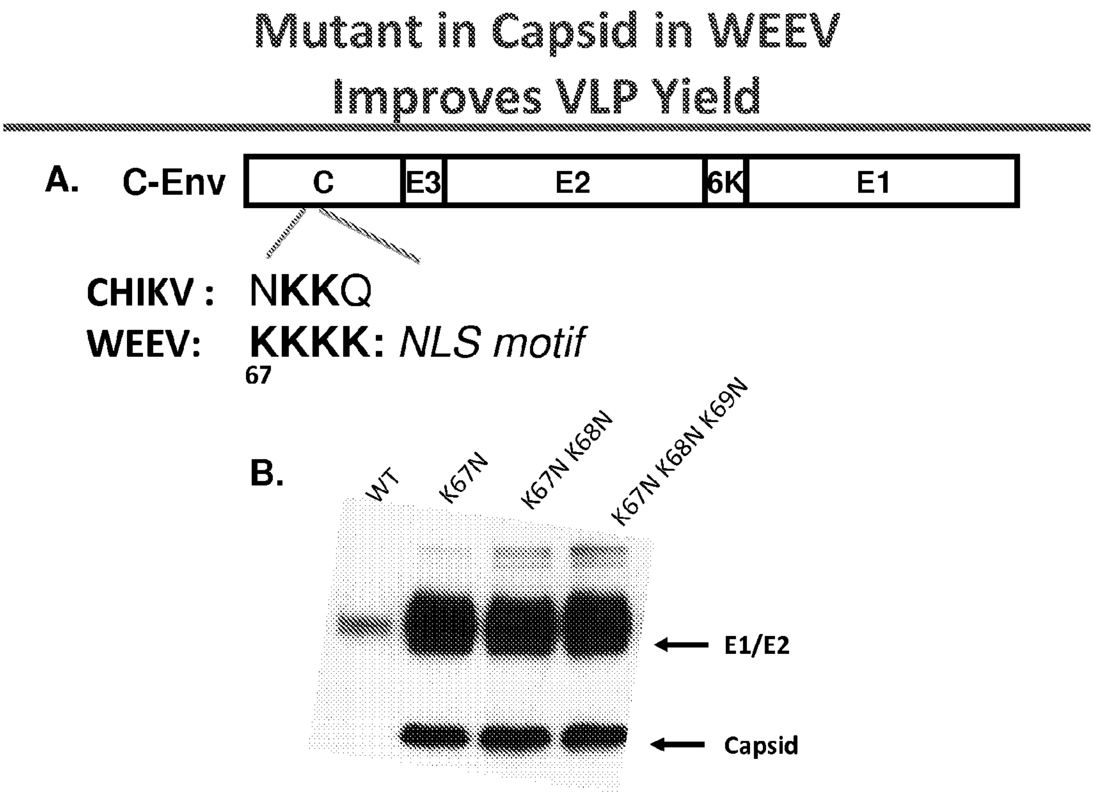
Mutant in Capsid in WEEV
Improves VLP Yield
A. C-Env
C
E3
E2
6K
E1
CHIKV : NKKQ
WEEV: KKKK: NLS motif
67
B.
WT
K67N
K67N K68N
K67N K68N K69N
E1/E2
Capsid

Figure 37

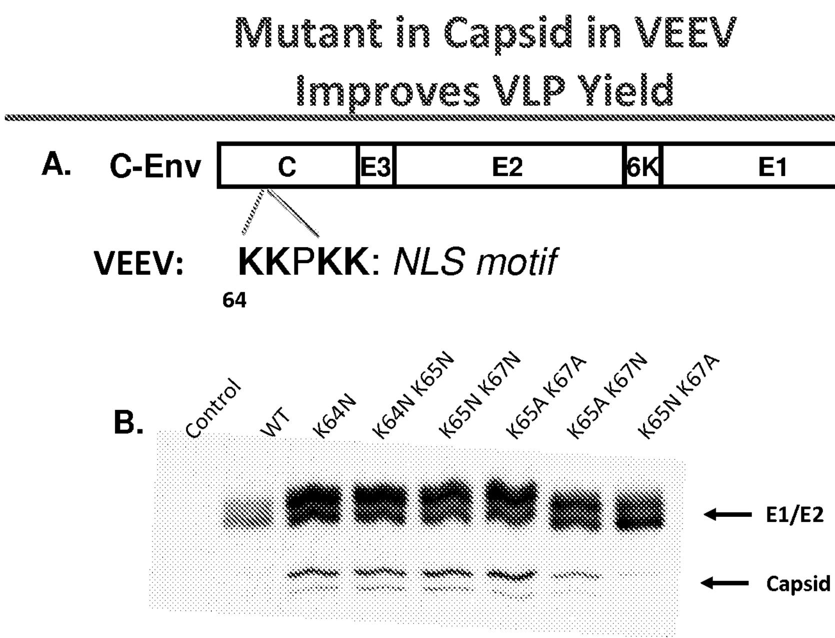
Mutant in Capsid in VEEV
Improves VLP Yield
A.
C-Env
C
E3
E2
6K
E1
VEEV:
KKPKK: NLS motif
64
B.
Control
WT
K64N
K64N K65N
K65N K67N
K65A K67A
K65A K67N
K65N K67A
E1/E2
Capsid

Figure 38

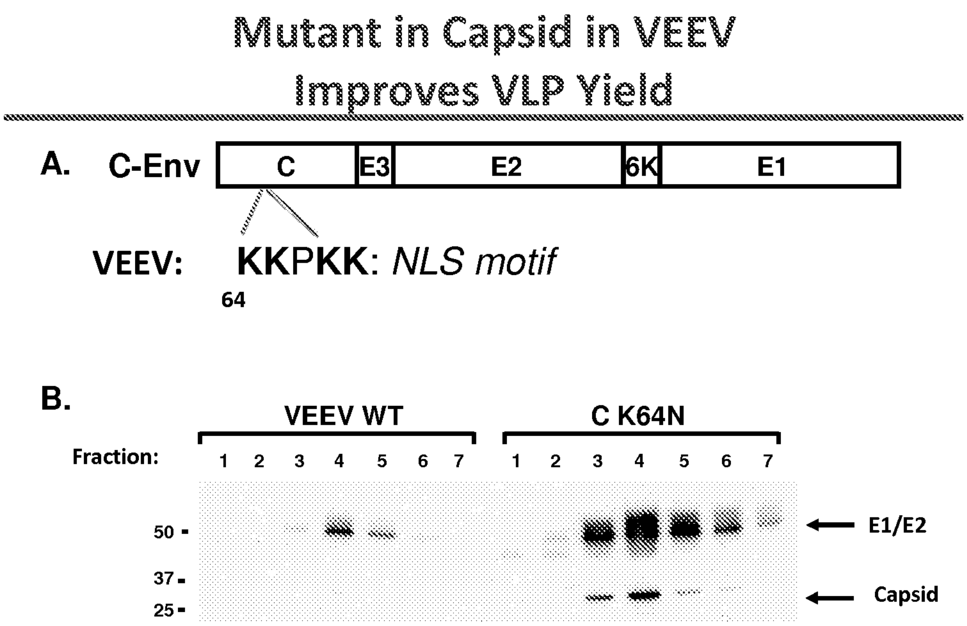
Mutant in Capsid in VEEV
Improves VLP Yield
A.
C-Env
C
E3
E2
6K
E1
VEEV:
KKPKK: NLS motif
64
B.
VEEV WT
C K64N
Fraction:
1 2 3 4 5 6 7
1 2 3 4 5 6 7
50
37
25
E1/E2
Capsid

Figure 39

Mutant in Capsid in CHIKV (37997)
To Knockout potential NLS

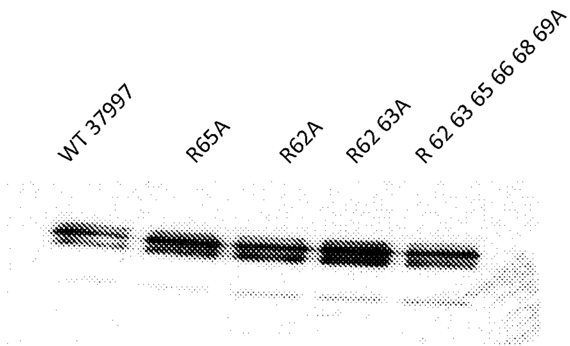

Figure 40A

CMV/R WEEV CBA87 strain capsid K67N VLP

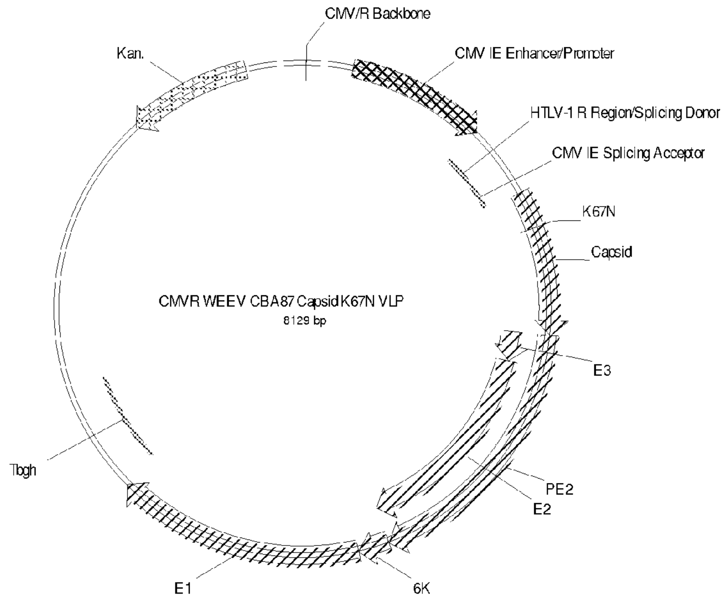

Figure 40B

Capsid insert sequence

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Figure 40C

Full sequence

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Figure 40C continued

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Figure 40C continued

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Figure 40D

AA sequence

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Figure 41A

CMV/R WEEV CBA87 strain capsid K67N K68N VLP

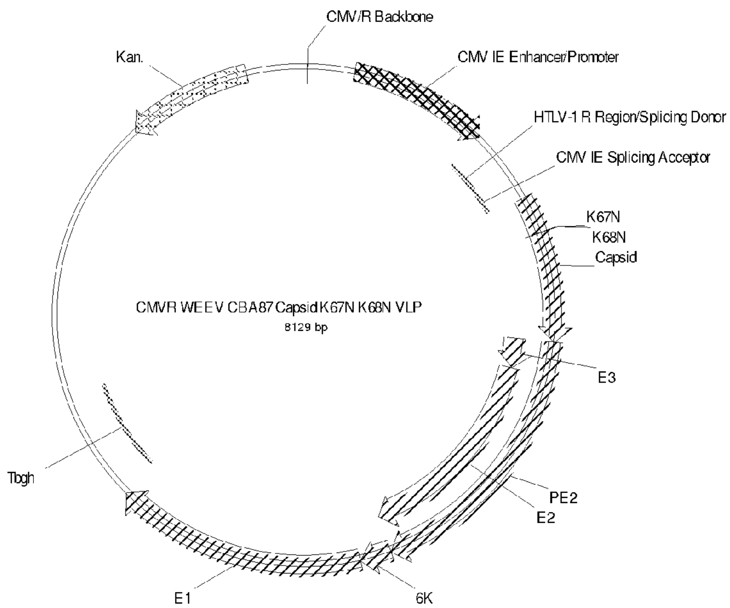

Figure 41B
Capsid insert

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Figure 41C
Full sequence

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Figure 41C continued

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Figure 41C continued

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Figure 41D
AA sequence of capsid

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Figure 42A

CMV/R WEEV CBA87 strain capsid K67N K68N K69N VLP

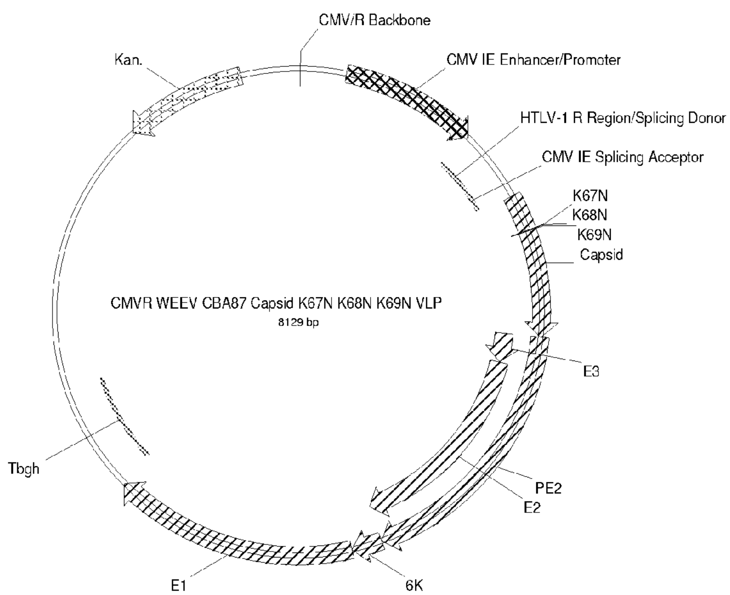

Figure 42B
Capsid insert

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Figure 42C
Full sequence

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Figure 42C continued

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Figure 42C continued

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Figure 42D

AA sequence of capsid

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Figure 42D continued

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pvhshsttavlkeatthvtatgsitlhfstsspqanfivslcgkkttcnaeckppadhiigephkvdqefqaavs
ktswnwllalfggasslivvglivlvcssmlintrr

Figure 43A

CMV/R VEEV TC83 strain K64N VLP

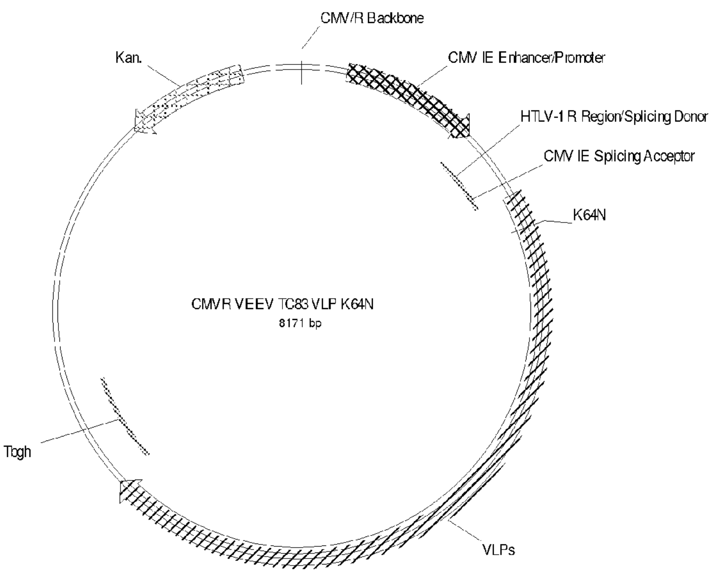

Figure 43B
Insert sequence

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Figure 43B continued

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Figure 43C
Full sequence

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Figure 43C continued

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Figure 43C continued

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Figure 43D
Capsid AA sequence

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Figure 44A

CMV/R VEEV TC83 strain K64N K65N VLP

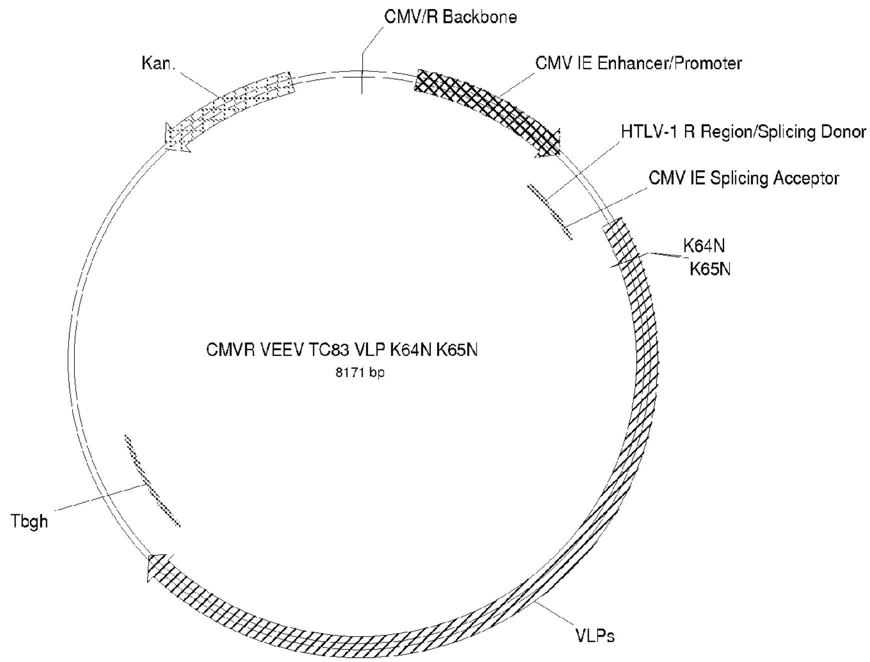

Figure 44B
Insert sequence

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Figure 44B continued

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aaccagaaacataattaag

Figure 44C

Full sequence

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Figure 44C continued

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Figure 44C continued

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Figure 44D
Capsid AA sequence

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Figure 45A

CMV/R VEEV TC83 strain K65N K67N VLP

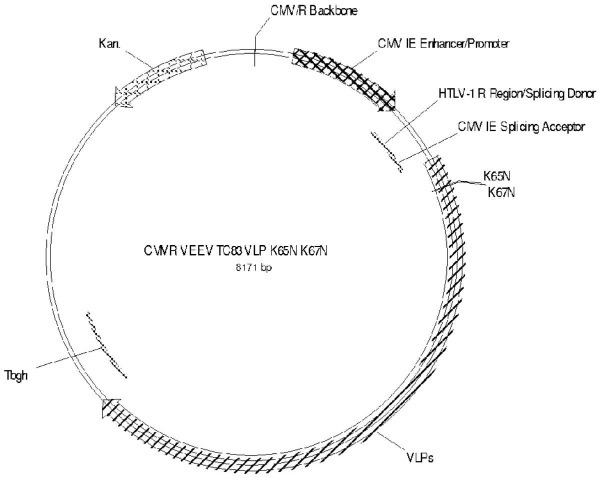

Figure 45B
Insert sequence

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Figure 45B continued

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gattttcctgagtatggggcaggacaaccaggagcatttggagatatacaatccagaacagtctcaagctctgat
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tcgggttttgagcaatggaagaaagataaagctccatcattgaaatttaccgcccctttcggatgcgaaatatat
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accagggtgtcagaaacaccgacactttcagcggccgaatgcactcttaacgagtgcgtgtattcttccgacttt
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gtgacacaccctcagtatcacgcccaaacatttacagccgcggtgtcaaaaaccgcgtggacgtggttaacatcc
ctgctgggaggatcagccgtaattattataattggcttggtgctggctactattgtggccatgtacgtgctgacc
aaccagaaacataattaag

Figure 45C
Full sequence

tcgcgcgtttcggtgatgacggtgaaaacctctgacacatgcagctcccggagacggtcacagcttgtctgtaag
cggatgccgggagcagacaagcccgtcagggcgcgtcagcgggtgttggcgggtgtcggggctggcttaactatg
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Figure 45C continued

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Figure 45C continued

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Figure 45D
Capsid AA sequence

mfpfqpmypmqpmpyrnpfaaprrpwfprtdpflamqvqeltrsmanltfkqrrdappegpsaknpnkeasqkqk
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sgrpildnqgrvvaivlggvnegsrtalsvvmwnekgvtvkytpenceqwslvttmcllanvtfpcaqppicydr
kpaetlamlsvnvdnpgydelleaavkcpgrkrrsteelfneykltrpymarcircavgschspiaieavksdgh
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tvplapepmitfgfrsvslklhpknptylitrqladephythelisepavrnftvtekgwefvwgnhppkrfwaq
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cartaraettwesldhlwnnnqqmfwiqlliplaalivvtrllrcvccvvpflvmagaagagayehattmpsqag
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sgfeqwkkdkapslkftapfgceiytnpiraencavgsiplafdipdalftrvsetptlsaaectlnecvyssdf
ggiatvkysasksgkcavhvpsgtatlkeaaveltegqsatihfstanihpefrlqictsyvtckgdchppkdhi
vthpqyhaqtftaavsktawtwltsllggsaviiiiglvlativamyvltnqkhn*

Figure 46A

CMV/R VEEV TC83 strain K65A K67A VLP

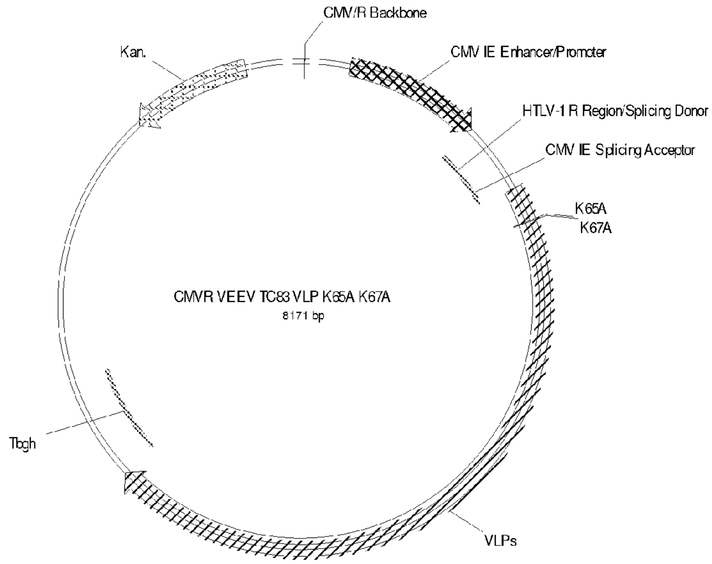

Figure 46B
Insert sequence

atgttcccgttccagccaatgtatccgatgcagccaatgccctatcgcaacccgttcgcggccccgcgcaggccc
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aagcaacgccgggacgcgccacctgaggggccatccgctaaggcaccggcgaaggaggcctcgcaaaaacagaaa
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Figure 46B continued

cctttttagtcatggccggcgccgcaggcgccggcgcctacgagcacgcgaccacgatgccgagccaagcggga
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aaccagaaacataattaag

Figure 46C
Full sequence

tcgcgcgtttcggtgatgacggtgaaaacctctgacacatgcagctcccggagacggtcacagcttgtctgtaag
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Figure 46C continued

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Figure 46C continued

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Figure 46D
Capsid AA sequence

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Figure 47A

CMV/R EEEV PE-6 strain capsid K67N VLP

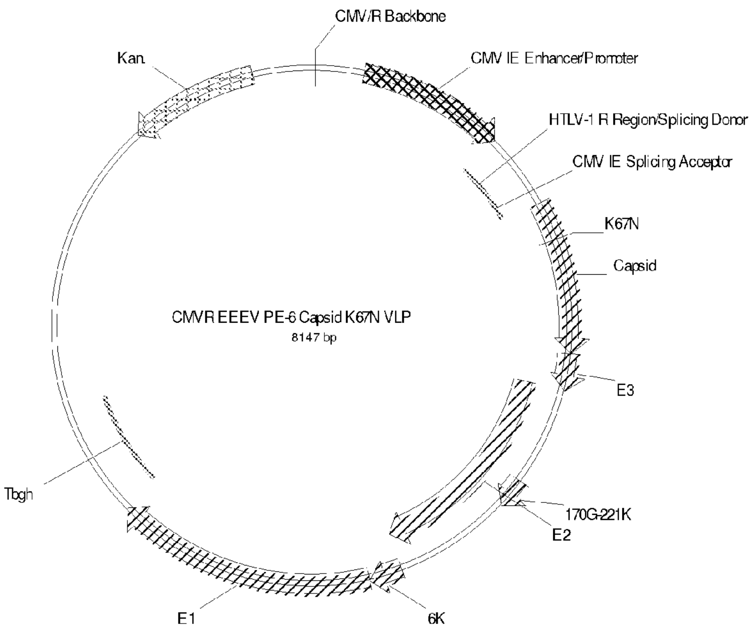

Figure 47B
Capsid insert sequence

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Figure 47C
Full sequence

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Figure 47C continued

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Figure 47C continued

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Figure 47D
Capsid AA sequence

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Figure 48A
CMV/R EEEV PE-6 strain capsid K67N E2 R239N VLP

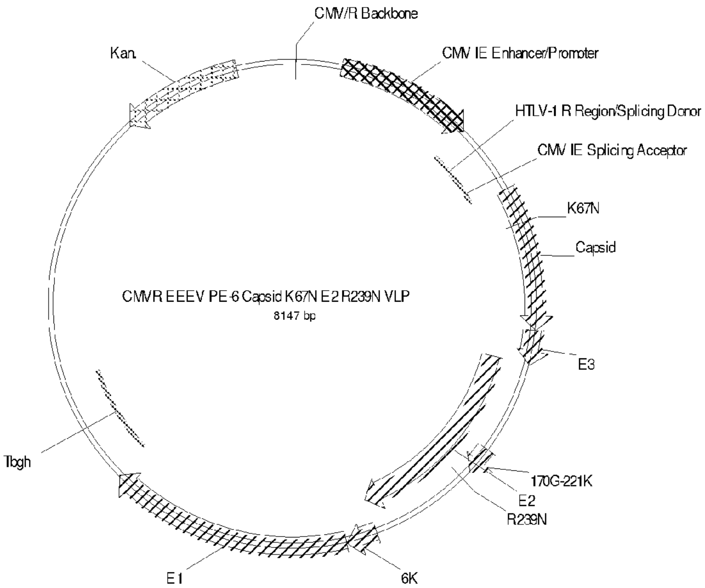

Figure 48B
Capsid sequence

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Figure 48C
Full sequence

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Figure 48C continued

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Figure 48C continued

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Figure 48D
Capsid AA sequence

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Figure 49A

CMV/R CHIKV(Strain 37997) Capsid R62A

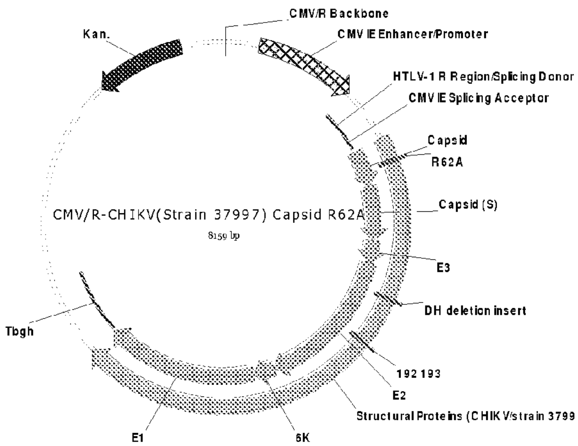

Figure 49B
Insert sequence

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Figure 49B continued

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Figure 49C

Full sequence

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Figure 49C continued

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Figure 49C continued

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Figure 49D

Amino acid sequence

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Figure 50A

CMV/R CHIKV(Strain 37997) Capsid R62A R63A

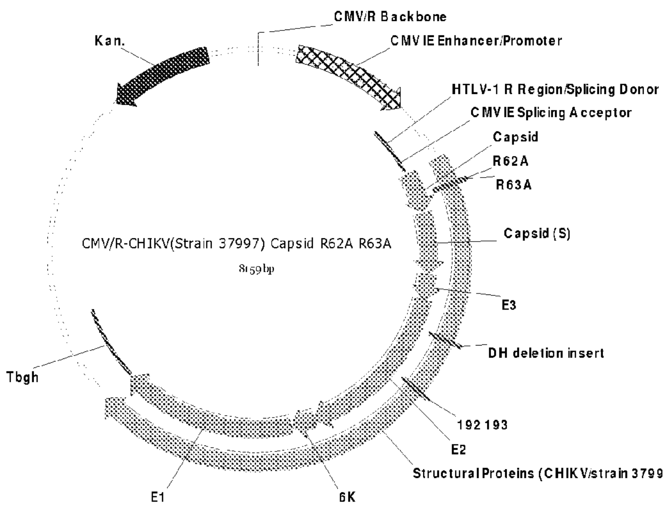

Figure 50B
Insert sequence

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Figure 50B continued

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Figure 50C

Full sequence

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Figure 50C continued

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Figure 50C continued

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Figure 50D

Amino acid sequence

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ashttlgvqdisttamswvqkitggvglivavaalilivvlcvsfsrh

Figure 51A

CMV/R CHIKV(Strain 37997) Capsid R62A R63A R65A K66A K68A K69A

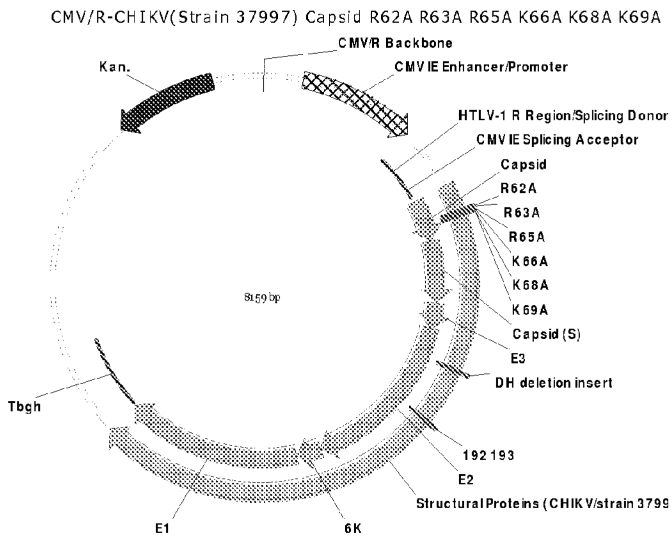

Figure 51B
Insert sequence

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Figure 51B continued

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Figure 51C
Full sequence

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Figure 51C continued

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Figure 51C continued

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Figure 51D
Amino Acid sequence

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Figure 52A

CMV/R CHIKV(Strain 37997) Capsid R65A

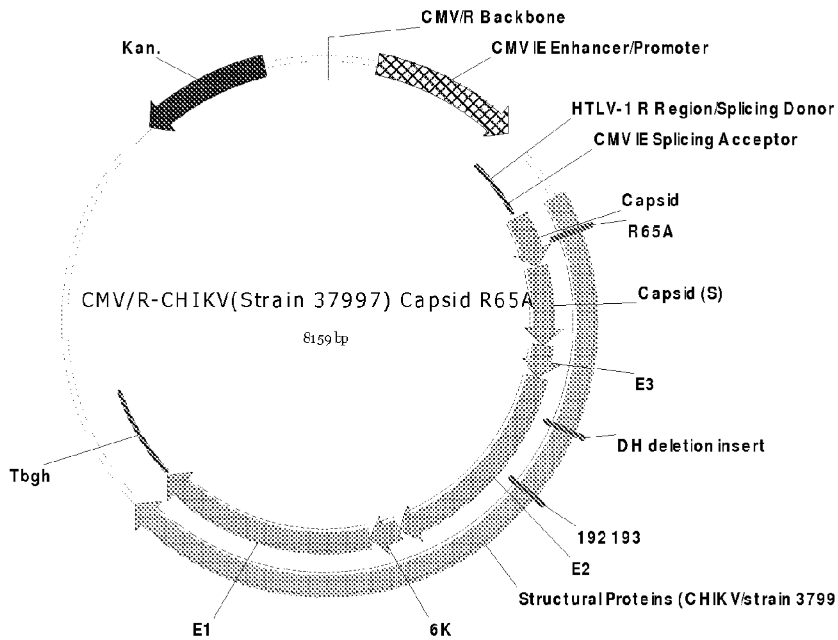

Figure 52B
Insert sequence

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Figure 52B continued

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Figure 52C

Full sequence

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Figure 52C continued

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Figure 52C continued

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Figure 52D

AA sequence

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Figure 53A

CMV/R Ross River Virus T48 capsid R71N

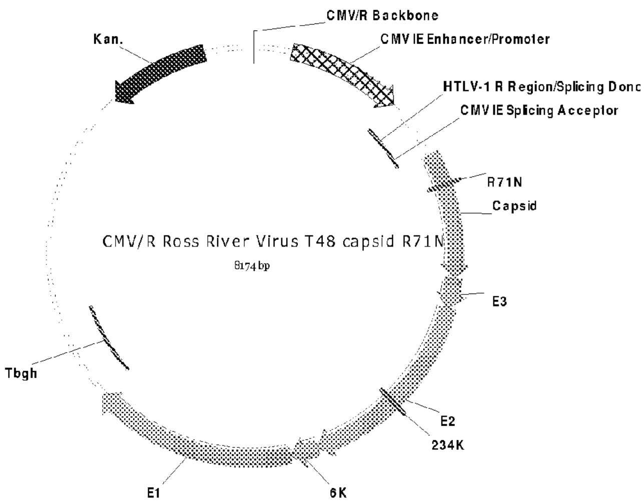

Figure 53B
Insert sequence of capsid

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Figure 53C
Full sequence

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Figure 53C continued

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Figure 53C continued

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gagcggatacatatttgaatgtatttagaaaaataaacaaataggggttccgcgcacatttccccgaaaagtgcc
acctgacgtctaagaaaccattattatcatgacattaacctataaaaataggcgtatcacgaggccctttcgtc

Figure 53D

Amino acid sequence

mnyiptqtfygrrwrprpafrpwqvpmqptptmvtpmlqapdlqaqqmqqlisavsalttkqnvkapkgqnkkkq
qkpkekkenqkkkptqkkkqqqkpkpqakkkkpgrrermcmkiendcifevkldgkvtgyaclvgdkvmkpahvk
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sdgmlkiqvsaqigldkagthahtkirymaghdvqeskrdslrvytsaacsihgtmghfivahcppgdylkvsfe
dadshvkackvqykhdplpvgrekfvvrphfgvelpctsyqlttaptdeeidmhtppdipdrtllsqtagnvkit
aggrtirynctcgrdnvgttstdktintckidqchaavtshdkwqftspfvpradqtarrgkvhvpfpltnvtcr
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sdfggvatlsyktdkpgkcavhshsnvatlqeatvdvkedgkvtvhfstasaspafkvsvcdakttctaaceppk
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Figure 54A

CMV/R Ross River Virus T48 capsid R71N K72N

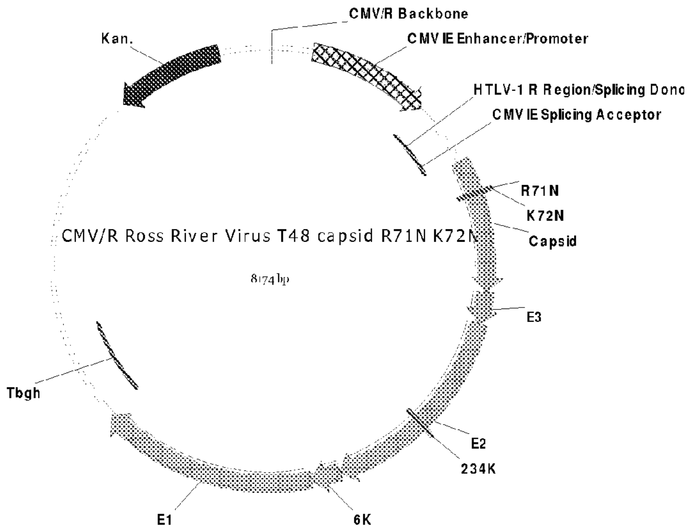

Figure 54B
Insert sequence of capsid

atgaattacataccaacccagactttttacggacgccgttggcggcctcgcccggcgttccgtccatggcaggtg
ccgatgcagccgacacctactatggttacacccatgctgcaagcaccagacctacaggcccaacagatgcaacaa
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cagaaaccaaaggaaaagaaggaaaaccagaagaaaaagccgacgcaaaagaagaagcagcagcagaaaccaaaa
ccacaggctaagaagaagaaaccagggagaagagaaagaatgtgcatgaagatcgagaatgactgcatattcgag
gtcaaactggatggcaaggttaccggttatgcgtgcctagtcggagacaaggtcatgaagccggctcacgttaaa
ggcacaattgataacccagaccttgcgaagctgacttacaagaaatccagtaagtatgacctcgaatgcgcccag
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ggagcagtgcagtacagcggaggaaggtttaccatccccacaggcgccggcaaaccgggagatagcggtaggcct
atttttgacaacaaagggcgagtagtggccatcgtgttaggcggggccaacgaaggtgctcgcactgcgctgtct
gtggtgacgtggacaaaagacatggtcactcgggtaacgccagaaggaactgaagagtgg

Figure 54C
Full sequence

tcgcgcgtttcggtgatgacggtgaaaacctctgacacatgcagctcccggagacggtcacagcttgtctgtaag
cggatgccgggagcagacaagcccgtcagggcgcgtcagcgggtgttggcgggtgtcggggctggcttaactatg
cggcatcagagcagattgtactgagagtgcaccatatgcggtgtgaaataccgcacagatgcgtaaggagaaaat
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tccaacattaccgccatgttgacattgattattgactagttattaatagtaatcaattacggggtcattagttca
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Figure 54C continued

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Figure 54C continued

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Figure 54D

Amino acid sequence

mnyiptqtfygrrwrprpafrpwqvpmqptptmvtpmlqapdlqaqqmqqlisavsalttkqnvkapkgqnnkkq
qkpkekkenqkkkptqkkkqqqkpkpqakkkkpgrrermcmkiendcifevkldgkvtgyaclvgdkvmkpahvk
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dhivpygashnnqvfpdmsgtamtwvqrlasglgglaliavvvlvlvtcitmrr

Figure 55A

CMV/R Ross River Virus T48 capsid R71N K72N K73N

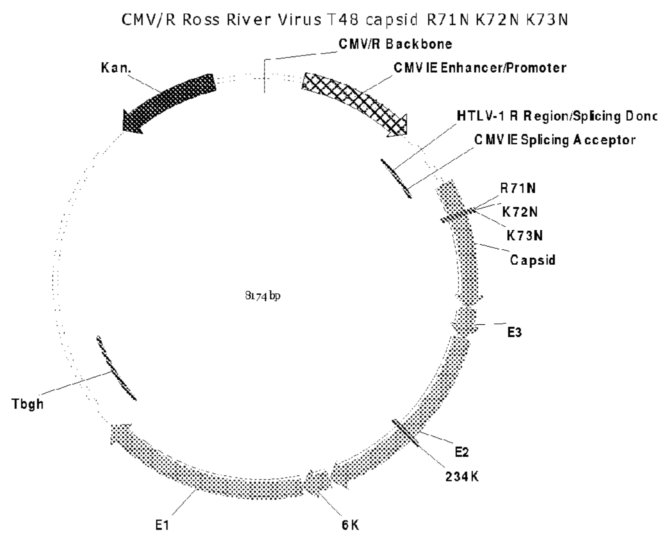

Figure 55B
Insert sequence of capsid

atgaattacataccaacccagacttttttacggacgccgttggcggcctcgcccggcgttccgtccatggcaggtg
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cagaaaccaaaggaaaagaaggaaaaccagaagaaaaagccgacgcaaaagaagaagcagcagcagaaaccaaaa
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Figure 55C
Full sequence

tcgcgcgtttcggtgatgacggtgaaaacctctgacacatgcagctcccggagacggtcacagcttgtctgtaag
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Figure 55C continued

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Figure 55C continued

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Figure 55D

Amino acid sequence

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Figure 56A

CMV/R Ross River Virus T48 capsid R71N K72N K73N K74N

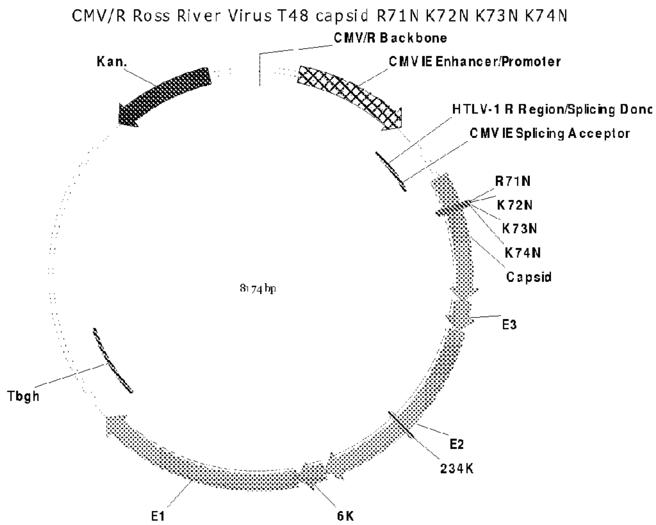

Figure 56B
Insert sequence of capsid

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Figure 56C
Full sequence

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Figure 56C continued

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Figure 56C continued

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Figure 56D
Amino Acid sequence

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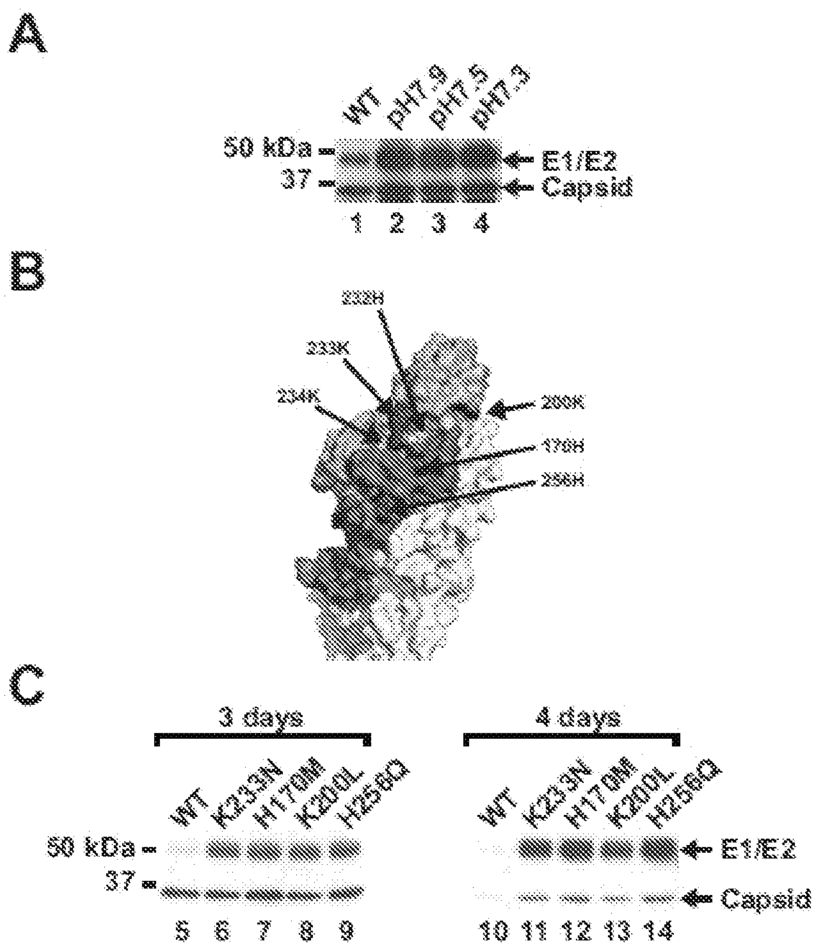
Figure 57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WT
pH7.9
pH7.5
pH7.3
50 kDa
37
E1/E2
Capsid
1 2 3 4
B
232H
233K
234K
200K
170H
256H
C
3 days
4 days
WT
K233N
H170M
K200L
H256Q
50 kDa
37
E1/E2
Capsid
5 6 7 8 9
10 11 12 13 14

Figure 58A

Wild type Barmah Forest Virus (BFV) VLP

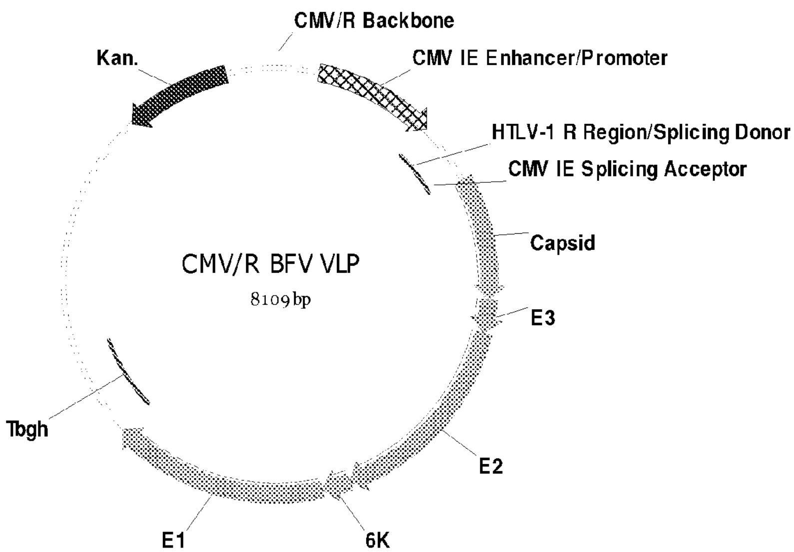

Figure 58B

Sequence of capsid region

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gtggagtgg

Figure 58C

Sequence of entire insert

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Figure 58C continued

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Figure 58D

Full vector sequence

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Figure 58D continued

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Figure 58D continued

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Figure 58E

AA sequence of capsid

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VEW

Figure 59A

Barmah Forest Virus (BFV) with capsid mutation VLP

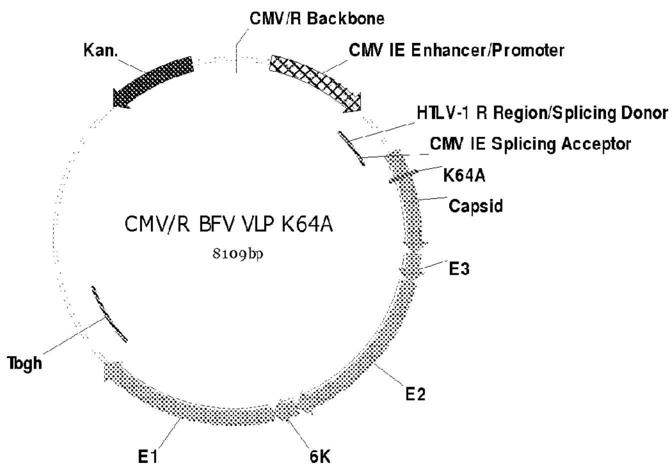

Figure 59B
Sequence of capsid region

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Figure 59C
Sequence of entire insert

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Figure 59C continued

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Figure 59D

Sequence of entire vector

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Figure 59D continued

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Figure 59D continued

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Figure 59E
AA sequence of capsid

MDFIPTQTFYGRRWRPAPVQRYIPQPQPPAPPRRRRGPSQLQQLVAALGA
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VEW

Figure 60

EEEV VLPs were purified at pH7.9 and yield compared to yields at pH 7.1

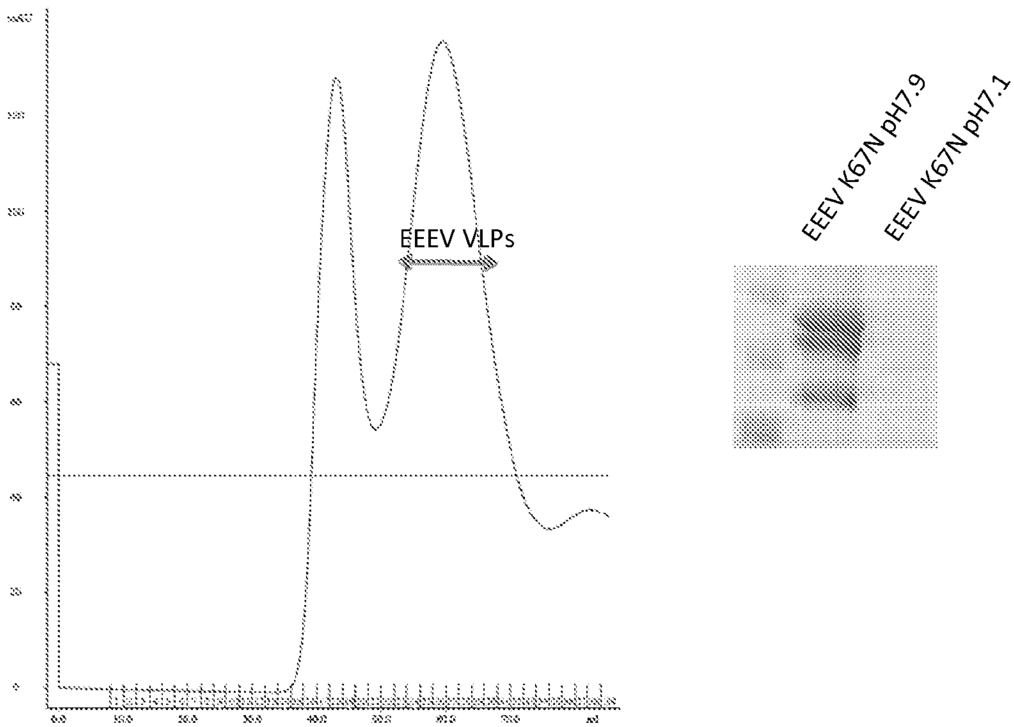
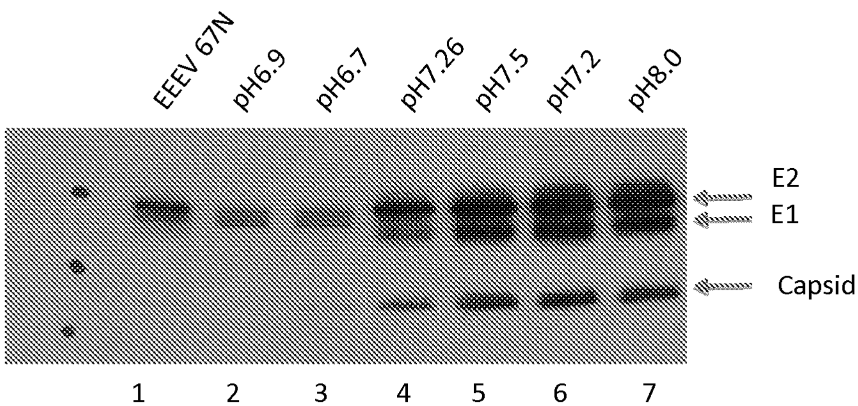


Figure 61

Yield of EEEV VLPs is pH dependent



1. EEEV Capsid K67N without changing medium (final pH7.1)
2. Adding Sodium Acetate buffer pH5.5 (1M, final 20mM and pH6.9)
3. Adding KH_2PO_4 buffer pH6.2 (1M, final 20mM and pH6.7)
4. Adding HEPES buffer pH7.5 (1M, final 20mM and pH7.26)
5. Adding Tris-HCl buffer pH8.0 (1M, final 20mM and pH7.5)
6. Adding Tris-HCl buffer pH7.5 (1M, final 20mM and pH7.2)
7. Adding Tris-HCl buffer pH8.8 (2M, final 20mM and pH8.0)

Figure 62

Addition of Tris-HCl buffer 24 after transfection of EEEV 67N

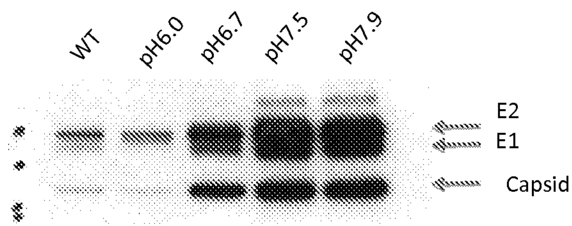
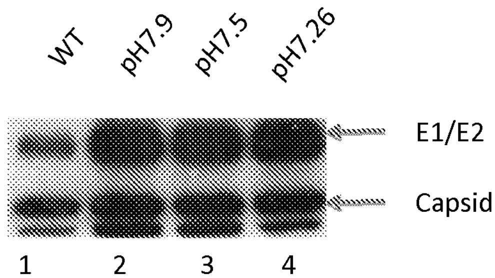


Figure 63

A

Adding Tris-HCl buffer to maintain higher pH
24h after transfection of CHIKV OPY-1



1. CHIKV OPY-1 without changing medium (final pH7.1)
2. Adding Tris-HCl buffer pH8.8 (2M, final 20mM and pH7.9)
3. Adding Tris-HCl buffer pH8.0 (1M, final 20mM and pH7.5)
4. Adding Tris-HCl buffer pH7.5 (1M, final 20mM and pH7.2)

B

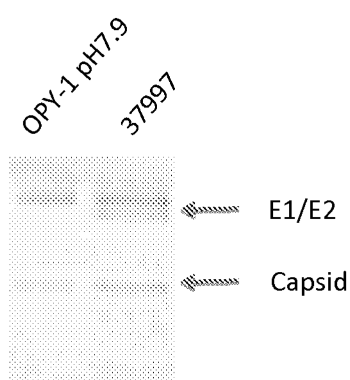
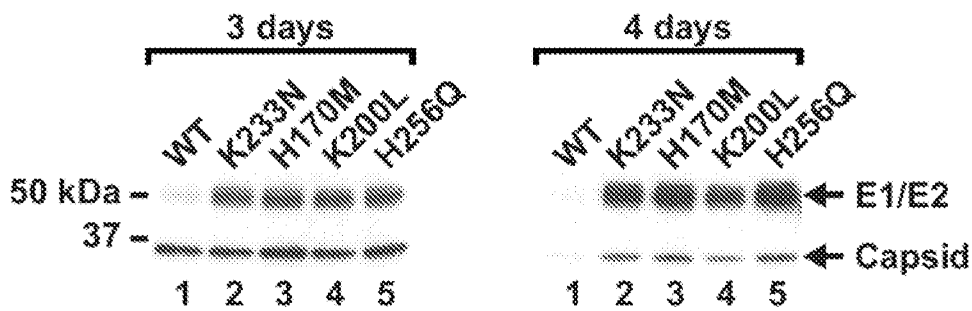


Figure 64

Adding mutation to the pH sensitive ASR region of CHIKV OPY-1



3 and 4 days after transfection mutations are expressed at similar levels but WT CHIKV OPY-1 is almost gone

Figure 65

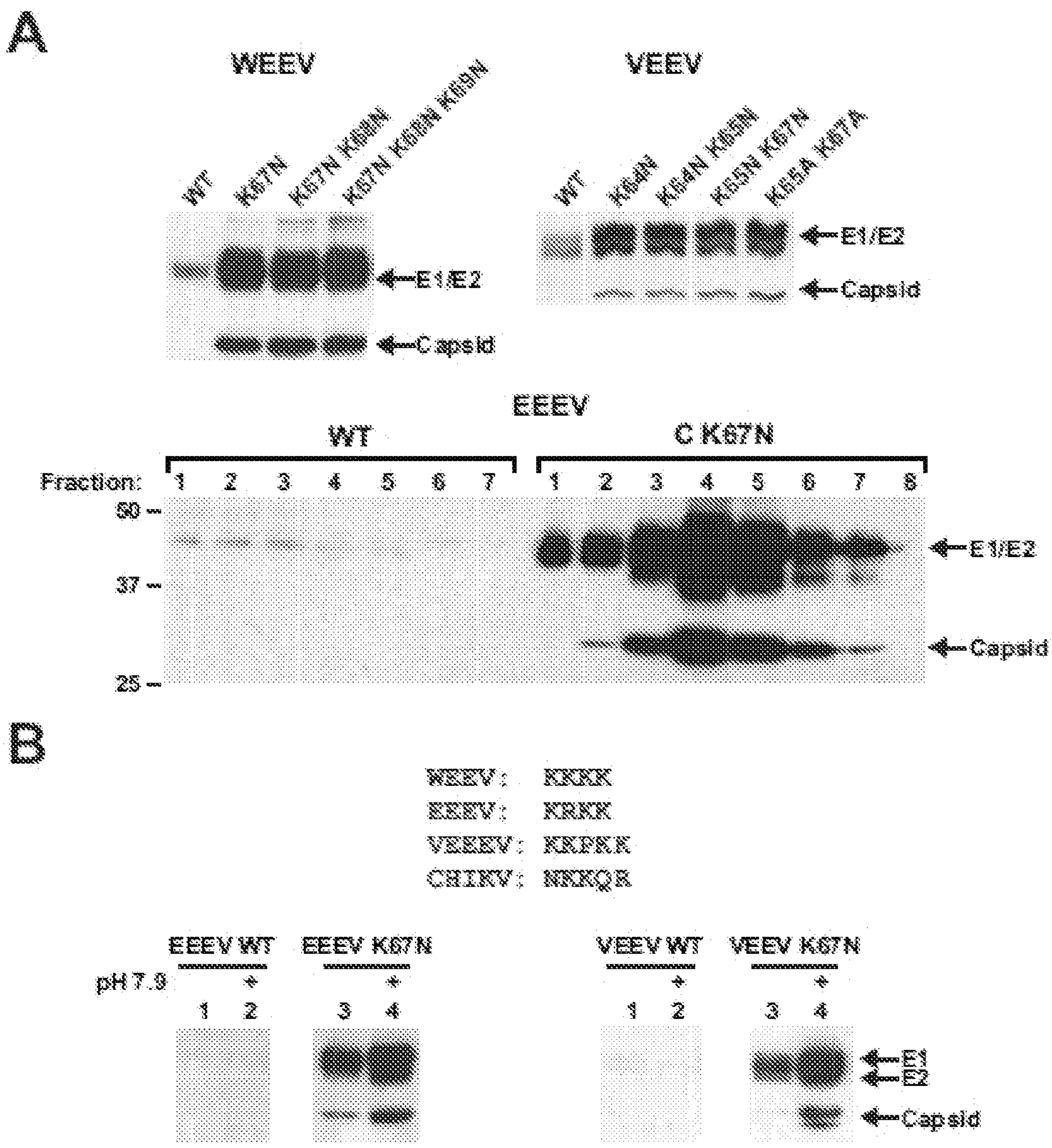
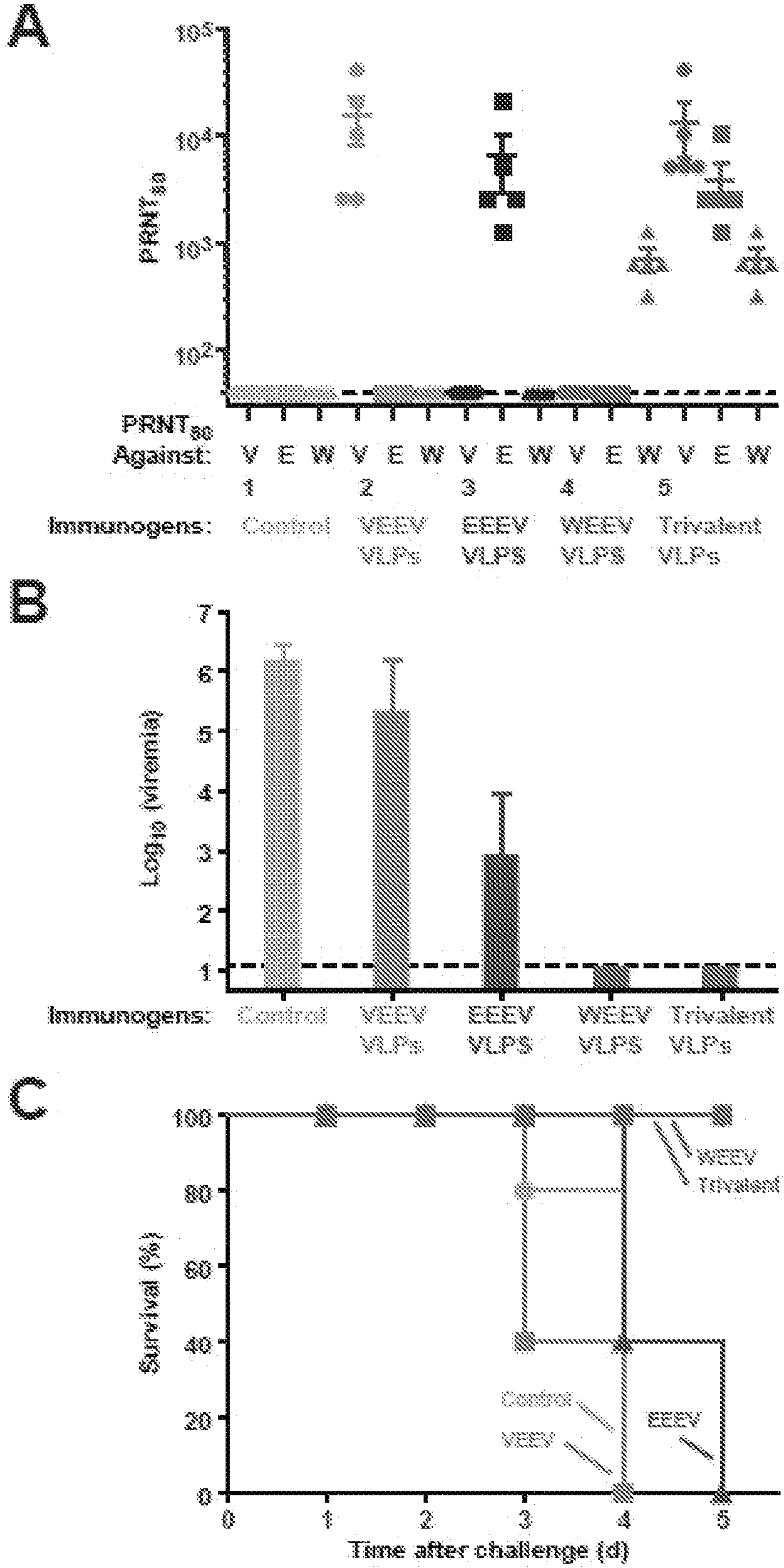

A
WEEV
WT
K67N
K67N K68N
K67N K68N K69N
E1/E2
Capsid
VEEV
WT
K64N
K64N K65N
K65N K67N
K65A K67A
E1/E2
Capsid
EEEV
WT
C K67N
Fraction: 1 2 3 4 5 6 7 1 2 3 4 5 6 7 8
50
37
25
E1/E2
Capsid
B
WEEV: KKKK
EEEV: KRKK
VEEEV: KKPKK
CHIKV: NKKQR
EEEV WT
EEEV K67N
VEEV WT
VEEV K67N
pH 7.9
+
1 2 3 4
E1
E2
Capsid

Figure 66


A
10^5
10^4
10^3
10^2
$PRNT_{80}$
$PRNT_{80}$ Against: V E W V E W V E W V E W V E W
1 2 3 4 5
Immunogens: Control VEEV VLPs EEEV VLPS WEEV VLPS Trivalent VLPs
B
7
6
5
4
3
2
1
Log_{10} (viremia)
Immunogens: Control VEEV VLPs EEEV VLPS WEEV VLPS Trivalent VLPs
C
100
80
60
40
20
0
Survival (%)
0 1 2 3 4 5
Time after challenge (d)
WEEV
Trivalent
Control
VEEV
EEEV

VIRUS-LIKE PARTICLES AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a national stage application under 35 U.S.C. 371 of PCT Patent Application No. PCT/US12/23361 having an international filing date of 31 Jan. 2012, which designated the United States; which PCT application claims the benefit of the following U.S. Provisional Application Nos. 61/438,236, filed Jan. 31, 2011, and 61/501,012, filed Jun. 24, 2011, each of which is incorporated herein by reference in its entirety.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

Research supporting this application was carried out by the United States of America as represented by the Secretary, Department of Health and Human Services. This research was supported by the Intramural Research Program, Vaccine Research Center, NIAID of the National Institute of Health. The Government has certain rights in this invention.

REFERENCE TO SEQUENCE LISTING

This application contains a Sequence Listing submitted as an electronic text file named "6137NIAID-31-PUS_sequence_listing_ST25.txt", having a size of 860 KB and created on Feb. 24, 2014. The information contained in this electronic file is hereby incorporated by reference in its entirety pursuant to 37 CFR §1.52(e)(5).

BACKGROUND OF THE INVENTION

Alphaviruses comprise a set of genetically, structurally, and serologically related mosquito-borne viruses of the Togaviridae family. Twenty-seven known viruses and virus subtypes have been classified within the alphavirus genus, eleven of which are recognized to be pathogenic to humans. Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV), and Semliki Forest virus (SFV) are known to produce encephalitis, and infection by Chikungunya virus (CHIKV), O'nyong-nyong virus, Sindbis virus, Mayaro virus, Ross River virus, Barmah Forest virus, and Ockelbo virus result in acute onset of flu-like fever, followed by the development of a rash and arthritis. The evolution and spread of alphaviruses into new geographic areas, and the disease severity resulting from alphavirus infection present a serious public health issue in the absence of a vaccines or anti-viral therapies.

Flaviviruses comprise a set of genetically, structurally, and serologically related mosquito-borne or tick-borne viruses of the Flaviviridae family that also pose current or potential threats to global public health. Yellow Fever Virus (YFV), Dengue Virus (DENV), Japanese Encephalitis Virus (JEV), Tick-Borne Encephalitis Virus (TBEV), and West Nile Virus (WNV) result in a range of symptoms ranging from flu-like symptoms such as fever, chills, and vomiting to severe symptoms such as muscular rigidity, photophobia, hyperexcitability, abnormal tremors and movements, incoordination, paralysis, sensory loss, convulsions, respiratory dysfunction, and severe hemorrhages. Like other arthropod-borne viruses, the evolution and spread of flaviruses into

new geographic areas, and the disease severity resulting from flavivirus infection present a serious public health issue in the absence of a vaccines or anti-viral therapies.

SUMMARY OF THE INVENTION

The present invention features compositions and methods for the prevention or treatment of a disease or disorder mediated by an alphavirus or a flavivirus (e.g., Chikungunya virus, WEEV, EEEV, VEEV, Ross River virus, or Barmah Forest virus).

Expression of alphavirus structural proteins, such as CHIKV, WEEV, EEEV, VEEV, Ross River virus, or Barmah Forest virus structural proteins, gives rise to virus-like particles (VLPs) that resemble replication-competent alphavirus. In some cases, expression of wild-type alphavirus proteins does not produce VLPs, such as EEEV and WEEV CBA, and one or more alterations in one or more of an alphavirus E2 protein or an alphavirus capsid protein Nuclear Localization Signal (NLS) allows or enhances VLP production. As reported in detail below, an alphavirus VLP-based vaccine efficiently induced high-titer neutralizing antibodies against homologous and heterologous alphavirus strains in monkeys, and the immunized animals showed complete protection against a high titer of a heterologous alphavirus strain in a challenge study. Because VLP vaccines are known to have advantages such as safety and high immunogenicity, it is desirable to use VLPs and a VLP vaccine strategy against pathogenic alphaviruses. In addition, based on the ability of VLPs to bind and deliver agents to a cell, it is desirable to produce alphavirus VLPs for delivering target agents to cells.

Similarly, expression of flavivirus structural proteins gives rise to VLPs that resemble replication-competent flavivirus. Therefore, it is desirable to use VLPs and a VLP vaccine strategy against pathogenic flaviviruses. In addition, based on the ability of VLPs to bind and deliver agents to a cell, it is desirable to produce flavivirus VLPs for delivering target agents to cells.

Accordingly, the invention provides virus-like particles (VLP) having one or more alterations that enhance or allow VLP production, where the alteration is in one or more of an E2 protein or an alphavirus capsid protein Nuclear Localization Signal (NLS).

In one aspect, the invention generally provides a virus-like particle (VLP) containing an alphavirus E2 protein containing at least one alteration that enhances VLP production, where the alteration is at an amino acid position corresponding to amino acid 234 or amino acid 251 in a Chikungunya virus (CHIKV) E2 protein.

In another aspect, the invention provides a VLP containing a Chikungunya virus (CHIKV) E2 protein containing an alteration at amino acid 234 and/or at amino acid 251.

In yet another aspect, the invention provides a VLP containing a WEEV E2 protein, where the WEEV E2 protein has an alteration at amino acid position 235.

In still another aspect, the invention provides a virus-like particle (VLP) containing a flavivirus envelop protein containing an alteration at an amino acid position corresponding to amino acid 234 or amino acid 251 in a Chikungunya virus (CHIKV) E2 protein.

In one aspect, the invention provides a virus-like particle (VLP) having one or more alterations that enhance or allow VLP production, wherein the alteration is in an alphavirus capsid protein Nuclear Localization Signal (NLS).

In still another aspect, the invention provides an isolated polynucleotide encoding a VLP of a previous aspect or that is delineated herein.

In still another aspect, the invention provides an expression vector containing an isolated polynucleotide encoding a VLP of a previous aspect or that is delineated herein. In one embodiment, the expression vector is capable of expression in a prokaryotic or eukaryotic cell. In another embodiment, the vector contains the CMV/R promoter.

In still another aspect, the invention provides a prokaryotic or eukaryotic cell containing the expression vector of any previous aspect or a vector that is described herein.

In still another aspect, the invention provides an immunogenic composition containing an effective amount of the VLP of any previous aspect or that is delineated herein or an effective amount of the expression vector of any previous aspect, where administration of the immunogenic composition to a subject results in production of a VLP. In one embodiment, the VLP induces an immune response in the subject. In another embodiment, the immune response treats or prevents a virus infection in the subject. In yet another embodiment, the VLP induces antibodies against homologous or heterologous strains of alphavirus or flavivirus. In one embodiment, the immunogenic composition further contains an adjuvant.

In another aspect, the invention provides an immunogenic composition or pan-alphavirus immunogenic composition containing at least two VLPs that are any one or more of Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV), Chikungunya virus (CHIKV), Ross River virus, Barmah Forest virus (BFV), Semliki Forest virus (SFV), O'nyong-nyong virus, Sindbis virus, Mayaro virus, or Ockelbo virus. In various embodiments of the above aspects or any other aspect of the invention delineated herein, VLPs have one or more alterations that enhance VLP production, where the alteration is in an E2 protein or an alphavirus capsid protein Nuclear Localization Signal (NLS).

In another aspect, the invention provides an immunogenic composition containing at least two VLPs that are any one or more of a Yellow Fever Virus (YFV), Dengue Virus (DENV), Japanese Encephalitis Virus (JEV), Tick-Borne Encephalitis Virus (TBEV), or West Nile Virus (WNV) protein.

In another aspect, the invention provides an method of inducing an immune response against a virus, including one or more of EEEV, WEEV, VEEV, CHIKV Ross River virus, or Barmah Forest virus, in a subject, involving administering to the subject an effective amount of the immunogenic composition of any previous aspect or any other aspect of the invention delineated herein. In one embodiment, the method induces neutralizing antibodies in the subject. In one embodiment, the method protects the subject against infection by the alphavirus and/or protects the subject against viremia or an inflammatory consequence of infection with said virus.

In another aspect, the invention provides a vaccine containing an effective amount of a VLP of any previous aspect or any other aspect of the invention delineated herein.

In another aspect, the invention provides a vaccine containing a polynucleotide encoding the VLP of any previous aspect or any other VLP delineated herein. In one embodiment, the vaccine is a DNA vaccine.

In another aspect, the invention provides a method for treating or preventing a virus infection in a subject, involving administering to the subject an effective amount of the immunogenic composition of any previous aspect. In one embodiment, the vaccine or immunogenic composition is administered in one or more doses. In another embodiment, the vaccine or immunogenic composition is administered in one or more priming immunizations and one or more boosting immunizations. In yet another embodiment, the priming immunizations are administered at one, two, three, four, five, six, seven, or eight week intervals. In still another embodiment, the boosting immunizations are administered two weeks, one month, two months, or three months after the priming immunization. In yet another embodiment, the administration of the vaccine or immunogenic composition protects the subject against viremia or the inflammatory consequences of an alphavirus or flavivirus infection. In one embodiment, the administration of the vaccine or immunogenic composition protects the subject from lethality.

In another aspect, the invention provides a method for producing a VLP, the method involving expressing one or more of an alphavirus E2 protein having an alteration or an alphavirus capsid protein having an alteration in an NLS in a eukaryotic cell, and isolating said VLP.

In another aspect, the invention provides a method for producing a VLP, the method involving expressing an alphavirus structural protein in a cell, thereby resulting in self-assembly of the VLP, where the cell expresses an alphavirus E2 protein that has i) an asparagine residue at the amino acid position corresponding to amino acid 234 in a CHIKV E2 protein, or ii) a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein, and where the modification destabilizes the alphavirus E2 protein during VLP budding. In one embodiment, the cell further expresses an alphavirus capsid (C) or an alphavirus envelope protein that is any one or more of E3, 6K, and E1. In another embodiment, the cell expresses an alphavirus polyprotein containing C-E3-E2-6K-E1. In yet another embodiment, the alphavirus envelope protein(s) or the alphavirus capsid protein is derived from EEEV, WEEV, VEEV, CHIKV, Ross River virus, Barmah Forest virus, SFV, O'nyong-nyong virus, Sindbis virus, Mayaro virus, or Ockelbo virus.

In another aspect, the invention provides a method for producing a VLP involving expressing an alphavirus capsid protein comprising an alteration in an NLS in a eukaryotic cell, and isolating said VLP.

In another aspect, the invention provides a method for enhancing VLP production, involving expressing an alphavirus structural protein of any previous aspect or any other aspect of the invention delineated herein in a cell under conditions that provide for self-assembly of the VLP.

In another aspect, the invention provides a method for producing a VLP, where the method involves expressing a flavivirus structural protein in a cell, thereby resulting in self-assembly of the VLP, where the cell expresses a flavivirus envelope protein that has i) an asparagine residue at the amino acid position corresponding to amino acid 234 in a CHIKV E2 protein, or ii) a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein, and where the modification destabilizes the flavivirus envelope protein during VLP budding. In one embodiment, the cell further expresses a flavivirus capsid protein. In another embodiment, the flavivirus envelope protein or flavivirus capsid protein is derived from YFV, DENV, JEV, TBEV, or WNV.

In another aspect, the invention provides a method for enhancing VLP production in a cell involving altering an amino acid residue in an alphavirus E2 protein that corresponds to amino acid 234 and/or amino acid 251 in a

CHIKV E2 protein; and expressing the alphavirus E2 protein in a cell; thereby resulting in self-assembly of the VLP. In one embodiment, the method further involves expressing an alphavirus capsid (C) or an alphavirus envelope protein that is any one or more of E3, 6K, and E1 in the cell. In another embodiment, the method involves expressing an alphavirus polyprotein containing C-E3-E2-6K-E1 in the cell. In another embodiment, the alphavirus E2 protein contains an asparagine residue at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein. In another embodiment, the alphavirus E2 protein is altered at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein such that the alteration destabilizes the alphavirus E2 protein during VLP budding.

In another aspect, the invention provides a method for enhancing VLP production in a cell involving altering an amino acid residue in a flavivirus envelope protein that corresponds to amino acid 234 and/or amino acid 251 in a CHIKV E2 protein; and expressing the flavivirus envelope protein in a cell; thereby resulting in self-assembly of the VLP. In one embodiment, the method further contains expressing an flavivirus capsid protein in the cell. In another embodiment, the flavivirus envelope protein is altered such that it has an asparagine residue at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein. In still another embodiment, the flavivirus envelope protein is altered at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein such that the alteration destabilizes the flavivirus envelope protein during VLP budding. In another embodiment, the method further involves isolating the VLP.

In another aspect, the invention provides a VLP produced by the method of any above aspect or any other method described herein.

In another aspect, the invention provides a kit containing the VLP of any previous aspect, and directions for the use of said VLP or expression vector to generate an immune response in a subject.

In yet another aspect, the invention provides a method for introducing an agent into a cell involving packaging the agent into the VLP of any previous aspect, contacting a cell with the packed VLP; and allowing the packed VLP to enter the cell, thereby introducing the agent into the cell. In one embodiment, the agent is any one or more of a small molecule chemical compound, an antibody, a nucleic acid molecule, a polypeptide, or fragments thereof.

In various embodiments of the above aspects or any other aspect of the invention delineated herein, the VLP further contains an alphavirus (e.g., Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV), Semliki Forest virus (SFV), Chikungunya virus (CHIKV), O'nyong-nyong virus, Sindbis virus, Mayaro virus, Ross River virus, Barmah Forest virus, or Ockelbo virus) or flavivirus (e.g., Yellow Fever Virus (YFV), Dengue Virus (DENV), Japanese Encephalitis Virus (JEV), Tick-Borne Encephalitis Virus (TBEV), or West Nile Virus (WNV) protein) capsid (C) protein or an alphavirus envelop protein that is any one or more of E3, 6K, and E1. In other embodiments, the VLP contains an alphavirus polyprotein containing C-E3-E2-6K-E1. In other embodiments, the alphavirus E2 protein or the alphavirus capsid protein is a CHIKV or WEEV protein. In other embodiments, the alphavirus E2 protein(s) or the alphavirus capsid protein is derived from CHIKV strain 37997. In other embodiments, the alphavirus E2 protein(s) or the alphavirus capsid protein is derived from WEEV strain 71V-1658. In other embodiments, the alphavirus E2 protein has an asparagine residue at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein. In still other embodiments, the alphavirus E2 protein has a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during VLP budding. In other embodiments, the alphavirus E2 protein contains alterations at an amino acid corresponding to amino acid 234 and at amino acid 251 in a Chikungunya virus (CHIKV) E2 protein.

In various embodiments of the above aspects or any other aspect of the invention delineated herein, the alphavirus capsid protein is an EEEV, WEEV, VEEV, CHIKV Ross River virus, or Barmah Forest virus capsid protein. In various embodiments of the above aspects or any other aspect of the invention delineated herein, the one or more alterations is in an NLS at amino acids 67-70 of an EEEV capsid protein; at amino acids 67-70 of an WEEV capsid protein; at amino acids 64-68 of an VEEV capsid protein; at amino acids 62-69 of a CHIKV capsid protein; at amino acids 71-74 of a Ross River virus capsid protein; or at amino acids 64-68 of a Barmah Forest virus capsid protein.

In various embodiments of the above aspects or any other aspect of the invention delineated herein, the alteration is a substitution in a charged amino acid of the NLS or basic charged amino acid of the NLS. In some embodiments, the charged amino acid or basic charged amino acid is lysine or arginine. In certain embodiments, the lysine or arginine is substituted with a non-lysine or non-arginine amino acids. In specific embodiments, the lysine or arginine is substituted with asparagine or alanine.

In various embodiments of the above aspects or any other aspect of the invention delineated herein, the EEEV virus capsid protein NLS is altered at amino acid 67. In particular embodiments, the EEEV virus capsid protein NLS has a substitution K67N.

In various embodiments of the above aspects or any other aspect of the invention delineated herein, the WEEV virus capsid protein NLS is altered at one or more of amino acids 67, 68, and 69. In particular embodiments, the WEEV capsid protein NLS comprises K67N, K68N, and/or K69N.

In various embodiments of the above aspects or any other aspect of the invention delineated herein, the VEEV virus capsid protein NLS is altered at one or more of amino acids 64, 65, and 67. In particular embodiments, the VEEV virus capsid protein NLS comprises K64N, K65A or K65N, and/or K67A or K67N.

In various embodiments, the Chikungunya virus capsid protein NLS is altered at one or more of amino acids 62, 63, 65, 66, 68, and 69. In particular embodiments, the Chikungunya virus capsid protein NLS comprises R62A, R63A, R65A, R66A, R68A and/or R69A.

In various embodiments of the above aspects or any other aspect of the invention delineated herein, the Ross River virus capsid protein NLS is altered at one or more of amino acids 71, 72, 73, and 74. In particular embodiments, the Ross River virus capsid protein NLS comprises R71N, R72N, R73N, and/or R74N.

In various embodiments of the above aspects or any other aspect of the invention delineated herein, the Barmah Forest virus capsid protein NLS is altered at one or more of amino acids 64, 65, 67, and 68. In particular embodiments, the Barmah Forest virus capsid protein NLS comprises K64A, K65A or K65N, K67A, K67N, K68A and/or K68N.

In various embodiments of the above aspects or any other aspect of the invention delineated herein, the VLP contains a protein or polynucleotide of interest for delivery to a cell.

In another embodiment, the protein or polynucleotide of interest is derived from a pathogen, including a virus, bacteria, or fungus.

In various embodiments of the above aspects or any other aspect of the invention delineated herein, the expression vector is E37997, EOPY-1, CMV/R WEEV CBA87 strain capsid K67N VLP, CMV/R WEEV CBA87 strain capsid K67N K68N VLP, CMV/R WEEV CBA87 strain capsid K67N K68N K69N VLP, CMV/R VEEV TC83 strain K64N VLP, CMV/R VEEV TC83 strain K64N K65N VLP, CMV/R VEEV TC83 strain K65N K67N VLP, CMV/R VEEV TC83 strain K65A K67A VLP, CMV/R EEEV PE-6 strain capsid K67N VLP, CMV/R EEEV PE-6 strain capsid K67N E2 R239N VLP, CMV/R CHIKV (Strain 37997) Capsid R62A, CMV/R CHIKV (Strain 37997) Capsid R62A R63A, CMV/R CHIKV (Strain 37997) Capsid R62A R63A R65A K66A K68A K69A, CMV/R CHIKV (Strain 37997) Capsid R65A, CMV/R Ross River Virus T48 capsid R71N, CMV/R Ross River Virus T48 capsid R71N K72N, CMV/R Ross River Virus T48 capsid R71N K72N K73N, CMV/R Ross River Virus T48 capsid R71N K72N K73N K74N, or CMV/R BFV VLP K64A.

In various embodiments of the above aspects or any other aspect of the invention delineated herein, the virus-like particle (VLP) or VLP expressing cell is exposed to a high pH condition at least about pH 7.2 (pH 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, or higher). In various embodiments, exposing VLP to high pH conditions during VLP production (in cell culture, during purification) increases VLP yield.

The invention provides immunogenic compositions featuring VLPs comprising polypeptides (e.g., CHIKV polypeptides, WEEV polypeptides) or polynucleotides for delivery to a mammalian cell. In certain embodiments, the invention provides compositions and methods for the prevention or treatment of CHIKV or WEEV viral disease. The invention also provides immunogenic compositions featuring VLPs comprising flavivirus polypeptides for the prevention or treatment of flavivirus viral disease. Compositions and articles defined by the invention were isolated or otherwise manufactured in connection with the examples provided below. Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein. The recitation of an embodiment for any aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

DEFINITIONS

By "alphavirus structural protein" is meant a polypeptide or fragment thereof having at least about 80% amino acid sequence identity to a naturally occurring viral capsid or envelope protein and having immunogenic activity in a mammal. In one embodiment, the alphavirus structural protein has at least about 85%, 90%, 95% or greater amino acid sequence identity with a CHIKV, EEEV, WEEV, VEEV, Ross River virus, or Barmah Forest virus structural protein or immunogenic fragment thereof. In one embodiment, the protein exemplary alphaviruses include, but are not limited to, EEEV, WEEV, VEEV, SFV, CHIKV, O'nyong-nyong virus, Sindbis virus, Mayaro virus, Ross River virus, Barmah Forest virus, and Ockelbo virus.

By "flavivirus structural protein" is meant a polypeptide or fragment thereof having at least about 80% amino acid sequence identity to a naturally occurring viral capsid or envelope protein and having immunogenic activity in a mammal. In one embodiment, the flavivirus structural protein has at least about 85%, 90%, 95% or greater amino acid sequence identity with a YFV, DENV, JEV, or TBEV structural protein or immunogenic fragment thereof.

By "agent" is meant any small molecule chemical compound, antibody, nucleic acid molecule, or polypeptide, or fragments thereof.

As used herein, the term "adjuvant" is meant to refer to a compound that, when used in combination with a specific immunogen in a formulation, will augment, alter or modify the resultant immune response. In certain embodiments, the adjuvant is used in combination with a VLP. In other embodiments, the adjuvant is used in combination with a DNA vaccine. Modification of the immune response includes intensification or broadening the specificity of either or both antibody and cellular immune responses. Modification of the immune response can also mean decreasing or suppressing certain antigen-specific immune responses. In one embodiment, the adjuvant is Ribi adjuvant.

As used herein "alphavirus" is meant to refer to RNA-containing viruses that belong to the Flaviviridae family of viruses. Exemplary flaviviruses include but are not limited to EEEV, WEEV, VEEV, SFV, CHIKV, O'nyong-nyong virus, Sindbis virus, Mayaro virus, Ross River virus, Barmah Forest virus, and Ockelbo virus.

As used herein "flavivirus" is meant to refer to RNA-containing viruses that belong to the group Flaviviridae family of viruses. Exemplary flaviviruses include but are not limited to YFV, DENV, JEV, and TBEV.

As used herein "inducing immunity" is meant to refer to any immune response generated against an antigen. In one embodiment, immunity is mediated by antibodies against an infectious agent, which is exhibited by a vertebrate (e.g., a human), that prevents or ameliorates an infection or reduces at least one symptom thereof. VLPs or DNA vaccines of the invention can stimulate the production of antibodies that, for example, neutralize infectious agents, block infectious agents from entering cells, block replication of infectious agents, and/or protect host cells from infection and destruction. The term can also refer to an immune response that is mediated by T-lymphocytes and/or other white blood cells against an infectious agent, exhibited by a vertebrate (e.g., a human), that prevents or ameliorates an infection (e.g., alphavirus or flavivirus) or reduces at least one symptom thereof.

By "ameliorate" is meant decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease or a symptom thereof.

By "alteration" is meant a change in an amino acid or nucleotide at a specified position with reference to a polypeptide sequence or polynucleotide sequence. As used herein, an alteration includes a substitution, deletion, or insertion of an amino acid or nucleotide at a specified position of a polypeptide or polynucleotide. In some embodiments, an alteration in an alphavirus capsid protein nuclear localization signal includes substitution of a charged amino acid (e.g., lysine or arginine) with an uncharged amino acid (e.g., alanine or asparagine, or any amino acid except a basic charged amino acid such as lysine or arginine).

By "alteration" is meant a change (increase or decrease) with reference to the expression levels or activity of a gene or polypeptide as detected by standard art known methods, such as those described herein. As used herein, an alteration includes a 10%, 25%, 50%, 75%, 100% or greater change in expression levels. An alteration includes a 10-, 20-, 50-, 70-, 80-, 90-, 100-, 200-, 500-, 1000-fold or greater change in expression levels.

By "analog" is meant a molecule that is not identical, but has analogous functional or structural features. For example, a polypeptide analog retains the biological activity of a corresponding naturally-occurring polypeptide, while having certain biochemical modifications that enhance the analog's function relative to a naturally occurring polypeptide. Such biochemical modifications could increase the analog's protease resistance, membrane permeability, or half-life, without altering, for example, ligand binding. An analog may include an unnatural amino acid.

In this disclosure, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean "includes," "including," and the like; "consisting essentially of" or "consists essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

"Detect" refers to identifying the presence, absence or amount of the analyte to be detected.

By "disease" is meant any condition or disorder that damages or interferes with the normal function of a cell, tissue, or organ. Examples of diseases include viral infections including but not limited to EEEV, WEEV, VEEV, SFV, CHIKV, O'nyong-nyong virus, Sindbis virus, Mayaro virus, Ross River virus, Barmah Forest virus, Ockelbo virus, YFV, DENV, JEV, and TBEV.

By "effective amount" is meant the amount of an agent required to ameliorate the symptoms of a disease relative to an untreated patient. The effective amount of active compound(s) used to practice the present invention for prevention or treatment of a disease varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.

The invention provides a number of targets that are useful for the development of highly specific drugs to treat or prevent a diseases delineated herein. In addition, the methods of the invention provide a facile means to identify therapies that are safe for use in subjects. In addition, the methods of the invention provide a route for analyzing virtually any number of compounds for effects on a disease described herein with high-volume throughput, high sensitivity, and low complexity.

By "fragment" is meant a portion of a polypeptide or nucleic acid molecule. This portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or amino acids.

By "high pH" is meant a pH at least about 7.2 or greater (pH 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, or higher), including basic, alkaline, or non-acidic conditions. In various embodiments, exposing VLP to high pH conditions during VLP production (e.g., in cell culture, during purification) increases VLP yield.

"Hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleobases. For example, adenine and thymine are complementary nucleobases that pair through the formation of hydrogen bonds.

By "isolated polynucleotide" is meant a nucleic acid molecule (e.g., a DNA) that is free of the genes which, in the naturally-occurring genome of the organism from which the nucleic acid molecule of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. In addition, the term includes an RNA molecule that is transcribed from a DNA molecule, as well as a recombinant DNA that is part of a hybrid gene encoding additional polypeptide sequence.

By an "isolated polypeptide" is meant a polypeptide of the invention that has been separated from components that naturally accompany it. Typically, the polypeptide is isolated when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, a polypeptide of the invention. An isolated polypeptide of the invention may be obtained, for example, by extraction from a natural source, by expression of a recombinant nucleic acid encoding such a polypeptide; or by chemically synthesizing the protein. Purity can be measured by any appropriate method, for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

By "marker" is meant any protein or polynucleotide having an alteration in expression level or activity that is associated with a disease or disorder.

As used herein, "nuclear localization signal" or "NLS" is an amino acid sequence that, when present on the surface of a polypeptide, targets the polypeptide to the nucleus of the cell. NLS sequences are known in the art. See, for example, Goldfarb, D., and N. Michaud (1991) *Trends Cell Biol.* 1, 20-24; Gorlich, D., and I. W. Mattaj (1996) *Science* 271, 1513-1518). In one embodiment, an NLS includes one or more short sequences of positively charged amino acids, such as lysines or arginines. Consensus sequences for NLS include K-K/R-X-K/R (Schneider, J. et al. (1988) *Cell* 54, 117-125) and two clusters of basic amino acids, separated by a spacer of about 10 amino acids, e.g., KR[PAATKKAGQA]KKKK (SEQ ID NO:1) (Dingwall et al., *J Cell Biol.* 107 (3): 841-9). With reference to the alphavirus amino acid sequences of the invention, NLS are present at amino acids 67-70 of an EEEV capsid protein (KRKK) (SEQ ID NO:2); at amino acids 67-70 of an WEEV capsid protein (KKKK) (SEQ ID NO:3); at amino acids 64-68 of a VEEV capsid protein (KKPKK) (SEQ ID NO:4); at amino acids 62-69 of a CHIKV capsid protein (RRNRKNKK) (SEQ ID NO:5); at amino acids 71-74 of a Ross River virus capsid protein (RKKK) (SEQ ID NO:6); and at amino acids 64-68 of a Barmah Forest virus capsid protein (KKPKK) (SEQ ID NO:7).

As used herein, "obtaining" as in "obtaining an agent" includes synthesizing, purchasing, or otherwise acquiring the agent.

By "reduces" is meant a negative alteration of at least 10%, 25%, 50%, 75%, or 100%.

By "reference" is meant a standard or control condition.

A "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset of or the entirety of a specified sequence; for example, a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence. For polypeptides, the length of the reference polypeptide sequence will generally be at least about 16 amino acids, preferably at least about 20 amino acids, more preferably at least about 25 amino acids, and even more preferably about 35 amino acids, about 50 amino acids, or about 100 amino acids. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least about 50 nucleotides, preferably at least about 60 nucleotides, more preferably at least about 75 nucleotides, and even more preferably about 100 nucleotides or about 300 nucleotides or any integer thereabout or therebetween.

By "specifically binds" is meant a compound or antibody that recognizes and binds a polypeptide of the invention, but which does not substantially recognize and bind other molecules in a sample, for example, a biological sample, which naturally includes a polypeptide of the invention.

Nucleic acid molecules useful in the methods of the invention include any nucleic acid molecule that encodes a polypeptide of the invention or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. Nucleic acid molecules useful in the methods of the invention include any nucleic acid molecule that encodes a polypeptide of the invention or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. By "hybridize" is meant pair to form a double-stranded molecule between complementary polynucleotide sequences (e.g., a gene described herein), or portions thereof, under various conditions of stringency. (See, e.g., Wahl, G. M. and S. L. Berger (1987) Methods Enzymol. 152:399; Kimmel, A. R. (1987) Methods Enzymol. 152:507).

For example, stringent salt concentration will ordinarily be less than about 750 mM NaCl and 75 mM trisodium citrate, preferably less than about 500 mM NaCl and 50 mM trisodium citrate, and more preferably less than about 250 mM NaCl and 25 mM trisodium citrate. Low stringency hybridization can be obtained in the absence of organic solvent, e.g., formamide, while high stringency hybridization can be obtained in the presence of at least about 35% formamide, and more preferably at least about 50% formamide. Stringent temperature conditions will ordinarily include temperatures of at least about 30° C., more preferably of at least about 37° C., and most preferably of at least about 42° C. Varying additional parameters, such as hybridization time, the concentration of detergent, e.g., sodium dodecyl sulfate (SDS), and the inclusion or exclusion of carrier DNA, are well known to those skilled in the art. Various levels of stringency are accomplished by combining these various conditions as needed. In a preferred: embodiment, hybridization will occur at 30° C. in 750 mM NaCl, 75 mM trisodium citrate, and 1% SDS. In a more preferred embodiment, hybridization will occur at 37° C. in 500 mM NaCl, 50 mM trisodium citrate, 1% SDS, 35% formamide, and 100 μg/ml denatured salmon sperm DNA (ssDNA). In a most preferred embodiment, hybridization will occur at 42° C. in 250 mM NaCl, 25 mM trisodium citrate, 1% SDS, 50% formamide, and 200 μg/ml ssDNA. Useful variations on these conditions will be readily apparent to those skilled in the art.

For most applications, washing steps that follow hybridization will also vary in stringency. Wash stringency conditions can be defined by salt concentration and by temperature. As above, wash stringency can be increased by decreasing salt concentration or by increasing temperature. For example, stringent salt concentration for the wash steps will preferably be less than about 30 mM NaCl and 3 mM trisodium citrate, and most preferably less than about 15 mM NaCl and 1.5 mM trisodium citrate. Stringent temperature conditions for the wash steps will ordinarily include a temperature of at least about 25° C., more preferably of at least about 42° C., and even more preferably of at least about 68° C. In a preferred embodiment, wash steps will occur at 25° C. in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 42 C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 68° C. in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. Additional variations on these conditions will be readily apparent to those skilled in the art. Hybridization techniques are well known to those skilled in the art and are described, for example, in Benton and Davis (Science 196: 180, 1977); Grunstein and Hogness (Proc. Natl. Acad. Sci., USA 72:3961, 1975); Ausubel et al. (Current Protocols in Molecular Biology, Wiley Interscience, New York, 2001); Berger and Kimmel (Guide to Molecular Cloning Techniques, 1987, Academic Press, New York); and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 50% identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). Preferably, such a sequence is at least 60%, more preferably 80% or 85%, and more preferably 90%, 95% or even 99% identical at the amino acid level or nucleic acid to the sequence used for comparison.

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e^{-3} and e^{-100} indicating a closely related sequence.

By "structural polyprotein" is meant a composite amino acid molecule comprising at least two separable polypeptides that contribute to a viral capsid or envelope. In one embodiment, the polypeptides are susceptible to cleavage with a viral enzyme (e.g., capsid autoproteinase and signalases).

By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline.

Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

As used herein, the terms "treat," treating," "treatment," and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.

As used herein, the term "vaccine" refers to a composition to be used in generating an immune response. In particular embodiments, a vaccine of the invention contains VLPs, DNAs, or other gene-based vaccine vectors in a form that is capable of being administered to a subject and which induces a protective immune response sufficient to induce immunity to prevent and/or ameliorate an infection and/or to reduce at least one symptom of an infection and/or to enhance the efficacy of another dose of VLPs or DNA vaccines. Typically, the vaccine comprises a pharmaceutically acceptable excipient, such as conventional saline or buffered aqueous solution medium in which the composition of the present invention is suspended or dissolved. In this form, the composition of the present invention can be used conveniently to prevent, ameliorate, or otherwise treat an infection. Upon introduction into a host, the vaccine induces an immune response including, but not limited to, the production of antibodies and/or cytokines and/or the activation of cytotoxic T cells, antigen presenting cells, helper T cells, dendritic cells and/or other cellular responses. In certain embodiments, a vaccine can also be a protein. For example, recombinant proteins have been produced by genetically engineering cells to produce one or more foreign genes, which in turn produce proteins that serve as the immunogen.

As used herein, the term "virus-like particle" (VLP) refers to a structure that in at least one attribute resembles a virus, but which has not been demonstrated to be infectious. Virus-like particles in accordance with the invention do not carry genetic information encoding the proteins of the virus-like particles. In general, virus-like particles lack a viral genome and, therefore, are noninfectious. In addition, virus-like particles can often be produced in large quantities by heterologous expression and can be easily purified.

As used herein, the term "virus budding," "virus-like particle budding," or "VLP budding" refers to the process of virion or VLP release from a host cell. This process includes steps associated with maturation, fusion, and cleavage.

Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive. Unless specifically stated or obvious from context, as used herein, the terms "a", "an", and "the" are understood to be singular or plural.

Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1**A-**1**D show the characterization of CHIKV E pseudotyped lentiviral vectors. FIG. **1**A is a schematic representation of the CHIKV genome and CHIKV E expression vector used for incorporation of CHIKV E from strains 37997 and LR2006 OPY-1 into pseudotyped lentiviral vectors. The CHIKV genome consists of nonstructural polyproteins NS1, NS2, NS3 and NS4 and structural polyproteins capsid (C) and envelope (E: E3, E2, 6K and E1) (top). The polypeptide E genes from strains 37997 and LR2006 OPY-1 were inserted into an expression vector (bottom). FIG. **1**B includes two graphs. The graph on the left shows the infectivity of the indicated pseudotyped lentiviral vectors in several CHIKV-permissive cell lines, including 293A human renal epithelial, HeLa cervical epithelial, Vero renal epithelial, A549 squamous epithelial and baby hamster kidney (BHK) cells. The pseudotyped vectors were standardized by HIV-1 Gag p24 (left) or the indicated concentration of p24 and used to infect 293A cells (right). After incubation with pseudotyped vectors for 24 hours, cells were lysed and luciferase activity was measured. The experiment was performed in triplicate. FIG. **1**C includes two graphs that show the pH-dependent entry of CHIKV pseudotyped lentiviral vectors. Pseudotyped lentiviral vectors were incubated in the presence of the indicated amounts of ammonium chloride (left) and chloroquine (right). The experiment was performed in triplicate. Data are presented as the percentage of activity at the indicated dose relative to activity with no treatment. FIG. **1**D is a graph showing neutralization measured with pseudotyped lentiviral vectors in sera from mice injected with CHIKV (strain S-27). Sera were incubated at the indicated dilutions with VSV-G, CHIKV strain 37997 or LR2006 OPY-1 E-pseudotyped lentiviral vectors and the mixture infected to 293A cells. Luciferase activity was analyzed 24 hours after infection. The experiment was performed in triplicate. No inhibition was observed with control non-immune antisera.

FIGS. **2**A-**2**C show the schematic representation of plasmid expression vectors and characterization of CHIKV VLPs. FIG. **2**A provides a schematic representation of CHIKV C-E or E expression vectors used for DNA vaccine and VLP production. The CHIKV structural polyproteins capsid plus envelope (C-E) or E alone from strains 37997 and LR2006 OPY-1 were inserted into an expression vector. 293T cells were transfected with each of the indicated plasmids. Expression was measured 48 hours after transfection by Western blotting with antisera reactive with CHIKV. FIG. **2**B includes a graph, Western blot, and electron micrograph. VLPs were purified from the supernatants of 293F cells transfected with C-E expression vector (C-E37997) (left). The supernatants were harvested 72 hours after transfection followed by OptiPrep density gradient centrifugation. Each fraction was characterized for its buoyant density (left upper panel) and protein content (left lower panel) by Western blot analysis with antisera to CHIKV. The fractionated VLPs were observed by transmission electron microscopy with magnification 20,000× (left, bar 100 nm) (right). FIG. **2**C provides a comparison of cryo-EM reconstructions of CHIKV VLP with Sindbis virus showing that CHIKV VLP is structurally similar to alphaviruses. Shaded-surface representation of the 3D density map of CHIKV VLP (left upper panel) and Sindbis virus (right upper panel) viewed along an icosahedral 2-fold axis. The white triangle marks the boundary of an icosahedral asymmetric unit. The numbers show the positions of the icosahedral 2-, 3-, and 5-fold axes limiting an asymmetric unit. The central cross-section through the cryo-EM maps of CHIKV VLP (left lower panel) and Sindbis virus (right lower panel). The orientations of the icosahedral (2-, 3-, and 5-fold) axes as well as the quasi-threefold (q3) axis are shown with white lines. Maps are calculated to 1 8 Å resolution.

FIGS. **3**A-**3**D are graphs showing the neutralization of CHIKV strains 37997 and LR2006 OPY-1 after DNA or VLP vaccination in mice and monkeys. Sera from immunized mice 10 days after the final immunization were tested with CHIKV strain 37997 (FIG. **3**A) or LR2006 OPY-1 (FIG. **3**B) E pseudotyped lentiviral vectors. Mice were immunized with the indicated DNA or VLP_{37997}. Each C-E or E (strain 37997 and LR2006 OPY-1, respectively) plasmid was injected at 0, 3 and 6 weeks. VLP_{37997} with or without Ribi adjuvant was injected at 2 and 6 weeks. The experiment was performed in triplicate. The symbols show the average of the five mice and bars show the standard error of the mean. The curve fit was calculated by Prism software. FIG. **3**C shows results from monkeys immunized with VLP_{37997} or PBS (control) at 0, 4, and 24 weeks. A neutralizing assay was performed with CHIKV strain 37997 (left panel) or LR2006 OPY-1 (right panel) E pseudotyped lentiviral vectors in sera collected from immunized monkeys at 10 days after each immunization. The symbols show the average of the six monkeys and bars show the standard error of the mean. FIG. **3**D shows the neutralizing activity against CHIKV LR2006 OPY-1 in immunized monkeys' sera after the 2nd and 3rd immunizations was confirmed by a standard plaque reduction neutralization test (PRNT). The symbols show the average of the six monkeys and bars show the standard error of the mean.

FIGS. **4**A-**4**D are graphs showing protection against CHIKV LR2006 OPY-1 challenge in monkeys immunized with VLPs and in a CHIKV mouse model after passive transfer of purified IgG. FIG. **4**A quantitates results obtained in monkeys injected with PBS (Control) or immunized with VLP_{37997}. Monkeys were challenged with 10^{10} PFU of the CHIKV strain LR2006 OPY-1 15 weeks after the final boost. The peak viremia at 24 hours after challenge was measured by plaque assay. The serum dilutions started from 1:200 (limit of detection=1000 PFU/ml). Error bars represent the standard error of the mean. FIG. **4**B is a graph showing the percentage of monocytes in the monkeys' white blood cells. Monocyte percentage was measured using a hematology analyzer before and 7 days after challenge with CHIKV. Error bars represent the standard error of the mean. A non-parametric two t-test was used for statistical analysis (Control vs. VLPs at 7 days, P=0.0036; Control at 0 days vs. 7 days, P=0.0015; VLPs at 0 days vs. 7 days, P>0.5). FIG. **4**C shows the number of viral RNA copies present following passive transfer of purified IgG from a monkey immunized with VLPs (Immune) or a control monkey (Control IgG) into mice (2 mg of total IgG per mouse, n=5 per group). Recipient mice were challenged 24 hours after IgG transfer with a lethal LR2006 OPY-1 challenge (30 PFU) by intradermal injection. The viremia in the mice after challenge was measured by quantitative RT-PCR (limit of detection=40 RNA copies/ml). Error bars represent the standard error of the mean. FIG. **4**D shows a survival curve of mice passively transferred with control IgG or CHIKV immunized IgG against lethal LR2006 OPY-1 challenge.

FIG. **5** shows the characterization of CHIKV E pseudotyped lentiviral vectors by buoyant density sedimentation and Western blot analysis. Plasmids encoding the indicated CHIKV Env strains were cotransfected with lentiviral expression vectors into 293T cells. Forty-eight hours after transfection, supernatants were harvested and run on sedimentation gradients as described previously. Quantification of gradient fractions is shown with the indicated strains, showing colocalization of Env with the Gag fraction of the expected buoyant density for lentiviral particles (1.08-1.1 g/ml) (upper panel). Western blot analysis of gradient fractions for CHIKV E1/E2 and Gag are shown (lower panel).

FIG. **6**A-**6**C show the schematic representation of plasmid expression vectors and characterization of chimeric CHIKV and WEEV VLPs. FIG. **6**A shows the schematic representation of the CHIKV genome and the chimeric CHIKV C-E expression vector used for VLP production from strains 37997 (blue) and OPY-1 (white). The CHIKV genome consists of the nonstructural polyproteins nsP1, nsP2, nsP3, and nsP4 and the structural polyproteins capsid (C) and envelope (E3, E2, 6K and E1) (top). The schematic representation of chimeric genes from strains 37997 and OPY-1 are shown, 1: $VLP_{OPY\text{-}1}$, 2: $VLP_{C(37997)}$, 3: $VLP_{C\text{-}E3(37997)}$, 4: $VLP_{C\text{-}E2(37997)}$, 5: $VLP_{C\text{-}6K(37997)}$, 6: VLP_{37997}, 7: $VLP_{OPY\text{-}1\ E2(37997)}$, 8: $VLP_{OPY\text{-}1\ 5'\text{-}E2(37997)}$ and 9: $VLP_{OPY\text{-}1\ 3'\text{-}E2(37997)}$. FIG. **6**B includes a Western blot. 293F cells (Invitrogen, Carlsbad, Calif.) were transfected with each of the indicated plasmids. Expression was measured 72 hours after transfection in the supernatant (top) and cell lysate (bottom) by Western blotting using antisera reactive with CHIKV as a primary antibody and goat anti-mouse immunoglobulins linked to horseradish peroxidase as a secondary antibody. FIG. **6**C includes a stain for total protein. VLPs were purified from the supernatants of 293F cells transfected with the indicated plasmids. The supernatants were harvested 72 hours after transfection followed by OptiPrep buoyant density gradient centrifugation to purify VLPs. Coomassie staining analysis was used to characterize VLP fractions.

FIGS. **7**A and **7**B show structural models for CHIKV OPY-1 E1/E2 compared to the CHIKV E2 37997 sequence, and the effect of the single amino acid mutation, N234K on CHIKV VLP production. FIG. **7**A depicts the structure of the CHIKV E1/E2 complex (OPY-1 strain). The CHIKV E1/E2 (OPY-1 strain) was modeled from PDB accession number 3N42 and displayed using Pymol. E2 is shown in red and E1 is shown in light blue. The green sphere is the E2 234 position; dark green spheres indicate the differences in amino acids between OPY-1 and 37997 in E2 amino acids 1-290. FIG. **7**B identifies the amino acids from the OPY-1 strain that were swapped into the NH_2-terminal E2 domain of chimeric $VLP_{OPY\text{-}1\ 5'\text{-}E2\ (37997)}$. The following mutations were created: 1: I32V, 2: S72N, 3: T74M, 4: L84F, 5: T124S, 6: E132D, 7: R140K, 8: A164T, 9: T182S, 10: I222V, 11: N234K, and 12: T284I. 13 is VLPOPY-1 5'-E2 (37997) (wild type). Each of the mutant VLPs were purified from supernatants of 293F cells transfected with the indicated plasmids. The supernatants were harvested 72 hours after transfection followed by OptiPrep density gradient centrifugation. Expression was measured by Western blot using antisera reactive with CHIKV as a primary antibody and goat anti-mouse immunoglobulins linked to horseradish peroxidase as a secondary antibody.

FIGS. **8**A-**8**E show the effect of pH and amino acid mutations in the E2 ASR on VLP production. FIG. **8**A includes flow cytometry results. Envelope expression on transfected cell membranes was measured by flow cytometry with a CHIKV E1/E2 monoclonal antibody (red line) or a control mouse monoclonal antibody (black line) as a primary antibody and goat anti-mouse immunoglobulins linked to Phycoerythrin as a secondary antibody. FIG. **8**B includes a Western blot. The indicated amino acid sequence from the 37997 strain was swapped into the E2 region of $VLP_{OPY\text{-}1}$. The following mutations were transfected into 293F cells. 1: $VLP_{OPY\text{-}1}$, 2: $VLP_{OPY\text{-}1}$ K234N, 3: $VLP_{OPY\text{-}1\ E2(37997)}$, and 4: VLP_{37997}. The supernatants were harvested 72 hours after transfection followed by OptiPrep density gradient centrifugation. Expression was measured by Western blot using antisera reactive with CHIKV as a primary antibody and goat anti-mouse immunoglobulins linked to horseradish peroxidase as a secondary antibody. FIG. **8**C includes a Western blot. 24 hr after transfection of OPY-1, Tris-HCl buffer was added to change the pH to that indicated (left). 24 hr after transfection of the indicated plasmids, Tris-HCl buffer was added (+) (right). Expression of VLPs in the supernatant 48 hr after transfection was measured by Western blotting, with antisera reactive with CHIKV as a primary antibody and goat anti-mouse immunoglobulins linked to horseradish peroxidase as a secondary antibody. FIG. **8**D include structural models showing the form of CHIKV Envelope modified form PDB code 3N42. The E2 a.a. 170, 233, 252 and 256 positions in CHIKV OPY-1 are shown in blue. The E2 a.a. 234 position in CHIKV OPY-1 is shown in white. The E2 domain B is shown in green, the E2 domain A is shown in cyan, the E2 domain C is shown in pink, the E2 β-ribbon connector is shown in purple and the E2 ASR domain in the E2 β-ribbon connector is shown in red. The E1 is shown in yellow. FIG. **8**E includes a Western blot. 24 hr after transfection of the indicated plasmids, Tris-HCl buffer was added to change the pH to 7.9 (+). Expression in the supernatant 48 hr after transfection was measured by Western blotting, with antisera reactive with CHIKV as a primary antibody and goat anti-mouse immunoglobulins linked to horseradish peroxidase as a secondary antibody.

FIGS. **9**A-**9**C show the ability of K234N WEEV VLPs on neutralizing antibody production. FIG. **9**A includes an amino acid alignment of CHIKV 37997, CHIKV OPY-1, Ross River, Sindbis, WEEV, EEEV, and WEEV E2 regions. The WEEV E2 235 amino acid position corresponding to E2 234 in CHIKV is highlighted in the box. The mutation K235N was swapped into WEEV. FIG. **9**A discloses SEQ ID NOS 65-71, respectively, in order of appearance. FIG. **9**B includes a Western blot. The wild type VLP_{WEEV} and the mutated plasmid, $VLP_{WEEV\ K235N}$, were transfected into 293F cells. Supernatants were harvested 72 hours after transfection followed by OptiPrep density gradient centrifugation. Expression was measured by Western blot using anti-WEEV antisera as a primary antibody and goat anti-mouse immunoglobulins linked to horseradish peroxidase as a secondary antibody. FIG. **9**C includes graphs showing the neutralization titers of sera from BALB/c mice immunized with WEEV 71V-1658 strain (left) or CHIKV LR2006 OPY-1 strain (right) E-pseudotyped lentiviral vectors. Mice were immunized intramuscularly with the indicated amount of VLPs. The experiment was performed in triplicate. The symbols show the average of the five mice, and error bars show the s.e.m. The curve fit was calculated by Prism software.

FIG. **10**A shows the CMV/R-CHIKV C-E3-E2-6K-E1 plasmid (Strain 37997). FIG. **10**B shows the sequence of the insert (SEQ ID NO:74). FIG. **10**C shows the sequence of the entire plasmid sequence (SEQ ID NO:75).

FIG. **11**A shows the CMV/R-CHIKV C-E3-E2-6K-E1 plasmid (Strain OPY1). FIG. **11**B shows the sequence of the insert (SEQ ID NO:76). FIG. **11**C shows the entire plasmid sequence (SEQ ID NO:77).

FIG. **12**A shows the CMV/R-Middleburg virus VLP plasmid. FIG. **12**B shows the entire plasmid sequence (SEQ ID NO:78).

FIG. **13**A shows the CMV/R-Sleeping disease virus VLP plasmid. FIG. **13**B shows the entire plasmid sequence (SEQ ID NO:79).

FIG. **14**A shows the CMV/R-Getah virus VLP plasmid. FIG. **14**B shows the entire plasmid sequence (SEQ ID NO:80).

FIG. **15**A shows the CMV/R-Venezuelan equine encephalitis virus VLP plasmid. FIG. **15**B shows the entire plasmid sequence (SEQ ID NO:81).

FIG. **16**A shows the CMV/R-Western equine encephalitis virus VLP plasmid. FIG. **16**B shows the entire plasmid sequence (SEQ ID NO:82).

FIG. **17**A shows the CMV/R-Eastern equine encephalitis virus VLP plasmid. FIG. **17**B shows the entire plasmid sequence (SEQ ID NO:83).

FIG. **18**A shows the CMV/R-Sindbis virus VLP plasmid. FIG. **18**B shows the entire plasmid sequence (SEQ ID NO:84).

FIG. **19**A shows the CMV/R-Semliki forest virus VLP plasmid. FIG. **19**B shows the entire plasmid sequence (SEQ ID NO:85).

FIG. **20**A shows the CMV/R-Salmon pancreas disease virus VLP plasmid. FIG. **20**B shows the entire plasmid sequence (SEQ ID NO:86).

FIG. **21**A shows the CMV/R-Ross River virus VLP plasmid. FIG. **21**B shows the entire plasmid sequence (SEQ ID NO:87).

FIG. **22**A shows the CMV/R—O'nyong-nyong virus VLP plasmid. FIG. **22**B shows the entire plasmid sequence (SEQ ID NO:88).

FIG. **23**A shows the CMV/R-Mayaro virus VLP plasmid. FIG. **23**B shows the entire plasmid sequence (SEQ ID NO:89).

FIG. **24**A shows the CMV/R-Barmah Forest virus VLP plasmid. FIG. **24**B shows the entire plasmid sequence (SEQ ID NO:90).

FIG. **25**A shows the CMV/R-Aura virus VLP plasmid. FIG. **25**B shows the entire plasmid sequence (SEQ ID NO:91).

FIG. **26**A shows the CMV/R-CHIKV E3-E2-6K-E1 plasmid (Strain 37997). FIG. **26**B shows the CMV/R-CHIKV E3-E2-6K-E1 plasmid (Strain OPY1). FIG. **26**C shows the sequence of the insert without the capsid (SEQ ID NO:92).

FIG. **27** shows the sequence of Genbank Accession No. EU224268, which is a Cloning vector pCHIKV-LR ic, complete sequence (SEQ ID NO:93). See, Tsetsarkin, K., Higgs, S., McGee, C. E., De Lamballerie, X., Charrel, R. N. and Vanlandingham, D. L. Infectious clones of Chikungunya virus (La Reunion isolate) for vector competence studies, Vector Borne Zoonotic Dis. 6 (4), 325-337 (2006).

FIG. **28** shows the sequence of Genbank Accession No. EU224270, which is the complete sequence of the Cloning vector pCHIK-37997ic (SEQ ID NO:94).

FIG. **29**A shows the CMV/R CHIKV C-E3-E2-6K-E1 (strain OPY1) E2 K234N plasmid. This plasmid contains a CMV/R mammalian expression backbone expressing the following CHIKV OPY1 strain of proteins: capsid, E3, E2, 6K, and E1, with a swap mutation to change expression from amino acid K (lysine) to amino acid N (asparagine) in amino acid 234 of the E2 protein. FIG. **29**B shows the entire plasmid sequence (SEQ ID NO:96). FIG. **29**B also shows the sequence of the insert (SEQ ID NO:95). FIG. **29**C shows the amino acid sequence and map of the CMV/R CHIKV C-E3-E2-6K-E1 (strain OPY1) E2 K234N plasmid (SEQ ID NO:97).

FIG. **30**A shows the CMV/R WEEV C-E3-E2-6K-E1 (strain 71V-1658) E2 K235N plasmid. This plasmid contains a CMV/R mammalian expression backbone expressing the following WEEV 71V-1658 strain of proteins: capsid, E3, E2, 6K, and E1, with a swap mutation to change expression from amino acid K (lysine) to amino acid N (asparagine) in amino acid 235 of the E2 protein. FIG. **30**B shows the entire plasmid sequence (SEQ ID NO:99). FIG. **30**B also shows the sequence of the insert (SEQ ID NO:98). FIG. **30**C shows the amino acid sequence and map of the CMV/R WEEV C-E3-E2-6K-E1 (strain 71V-1658) E2 K235N plasmid (SEQ ID NO:100).

FIG. **31**A shows the CMV/R-CHIKV C-E3-E2(37997)-6K-E1 (OPY1) plasmid, also known as VLPC-E2(37997). This plasmid contains a CMV/R mammalian expression backbone expressing the 6K and E1 proteins from the CHIKV OPY1 strain, and the capsid, E3, and E2 proteins from the CHIKV 37997 strain. FIG. **31**B shows the entire plasmid sequence (SEQ ID NO:102). FIG. **31**B also shows the sequence of the insert (SEQ ID NO:101). FIG. **31**C shows the amino acid sequence and map of the CMV/R-CHIKV C-E3-E2(37997)-6K-E1 (OPY1) plasmid (SEQ ID NO:103).

FIG. **32**A shows the CMV/R-CHIKV C E3 E2 6K(37997)-E1 (OPY1) plasmid, also known as VLPC-6K (37997). This plasmid contains a CMV/R mammalian expression backbone expressing the E1 protein from the CHIKV OPY1 strain, and the capsid, E3, E2, and 6K proteins from the CHIKV 37997 strain. FIG. **32**B shows the entire plasmid sequence (SEQ ID NO:105). FIG. **32**B also shows the sequence of the insert (SEQ ID NO:104). FIG. **32**C shows the amino acid sequence and map of the CMV/R-CHIKV C E3 E2 6K(37997)-E1 (OPY1) plasmid (SEQ ID NO:106).

FIG. **33**A shows the CMV/R-CHIKV C-E3-6K-E1 (Strain OPY1)-E2 (strain 37997) plasmid, also known as VLPOPY-1 E2(37997). This plasmid contains a CMV/R mammalian expression backbone expressing the capsid, E3, E1, and 6K proteins from the CHIKV OPY1 strain, and the E2 protein from the CHIKV 37997 strain. FIG. **33**B shows the entire plasmid sequence (SEQ ID NO:108). FIG. **33**B also shows the sequence of the insert (SEQ ID NO:107). FIG. **33**C shows the amino acid sequence and map of the CMV/R-CHIKV C-E3-6K-E1 (Strain OPY1)-E2 (strain 37997) plasmid (SEQ ID NO:109).

FIG. **34**A shows the CMV/R-CHIKV C-E3-6K-E2-E1 (Strain OPY1) 5'E2 (strain 37997) plasmid, also known as VLPOPY-1 5'-E2(37997). This plasmid contains a CMV/R mammalian expression backbone expressing the capsid, E3, E2, E1, and 6K proteins from the CHIKV OPY1 strain, and the 5' region of the E2 protein from the CHIKV 37997 strain. FIG. **34**B shows the entire plasmid sequence (SEQ ID NO:111). FIG. **34**B also shows the sequence of the insert (SEQ ID NO:110). FIG. **34**C shows the amino acid sequence and map of the CMV/R-CHIKV C-E3-6K-E2-E1 (Strain OPY1) 5'E2 (strain 37997) plasmid (SEQ ID NO:112).

FIGS. **35**A and **35**B show that alterations in an EEEV capsid protein NLS and an E2 envelope protein generate expression of VLPs and increase VLP yield when expressed in mammalian cells. FIG. **35**A depicts a schematic representation of the Eastern Equine Encephalitis (EEEV) C-E expression vector used for VLP production from the PE6 strain. The EEEV capsid protein has a predicted nuclear localization signal (NLS) at amino acid (a.a.) positions 67-70 ("KRKK" disclosed as SEQ ID NO:2). FIG. **35**B depicts a Western blot of fractions collected from density gradient centrifugation of EEEV VLPs containing R239N variant EEEV E2 envelope protein and wild-type EEEV capsid protein or R239N variant EEEV E2 envelope protein and K67N variant EEEV capsid protein.

FIGS. **36**A and **36**B show that an alteration in a WEEV capsid protein NLS generates expression of VLPs and increases VLP yield when expressed in mammalian cells. FIG. **36**A depicts a schematic representation of the Western Equine Encephalitis Virus (WEEV) capsid-envelope (C-E) expression vector used for VLP production from a CBA strain. The WEEV capsid protein has a predicted nuclear localization signal (NLS) at amino acid (a.a.) positions 67-70 ("KKKK" disclosed as SEQ ID NO:3). FIG. **36**A discloses "NKKQ" as SEQ ID NO:72. FIG. **36**B depicts a Western blot showing the expression of WEEV VLPs containing wild-type WEEV capsid protein; K67N variant WEEV capsid protein; K67N, K68N variant capsid protein; and K67N, K68N, K69N variant WEEV capsid protein.

FIGS. **37**A and **37**B show that an alteration in a VEEV capsid protein NLS increases VLP yield when expressed in mammalian cells. FIG. **37**A depicts a schematic representation of the Venezuelan Equine Encephalitis Virus (VEEV) C-E expression vector used for VLP production from the T-83 strain. The VEEV capsid protein has a predicted NLS at amino acid positions 64-68 ("KKPKK" disclosed as SEQ ID NO:4). FIG. **37**B depicts a Western blot showing the expression of VEEV VLPs containing wild-type VEEV capsid protein; K64N variant VEEV capsid protein; K64N, K65N variant VEEV capsid protein; K65N, K67N variant VEEV capsid protein; K65A, K67A variant VEEV capsid protein; K65A, K67N variant VEEV capsid protein; and K65N, K67A variant VEEV capsid protein.

FIGS. **38**A and **38**B show that an alteration in a VEEV capsid protein NLS increases VLP yield when expressed in mammalian cells. FIG. **38**A depicts a schematic representation of the Venezuelan Equine Encephalitis Virus (VEEV) C-E expression vector used for VLP production from the T-83 strain. The VEEV capsid protein has a predicted NLS at amino acid positions 64-68 ("KKPKK" disclosed as SEQ ID NO:4). FIG. **38**B depicts a Western blot of fractions collected from density gradient centrifugation of VEEV VLPs containing wild-type VEEV capsid protein or K64N variant VEEV capsid protein.

FIG. **39** depicts a Western blot of alterations in CHIKV (37997) capsid protein NLS to knock out the nuclear localization sequence.

FIG. **40**A depicts a plasmid map of the CMV/R WEEV CBA87 strain capsid K67N VLP plasmid. FIG. **40**B shows the sequence of the insert (SEQ ID NO:113). FIG. **40**C shows the sequence of the plasmid (SEQ ID NO:114). FIG. **40**D shows the amino acid sequence of the CMV/R WEEV CBA87 strain capsid protein K67N (SEQ ID NO:115).

FIG. **41**A depicts a plasmid map of the CMV/R WEEV CBA87 strain capsid K67N K68N VLP plasmid. FIG. **41**B shows the sequence of the insert (SEQ ID NO:116). FIG.

41C shows the sequence of the plasmid (SEQ ID NO:117). FIG. **41**D shows the amino acid sequence of the CMV/R WEEV CBA87 strain capsid protein K67N K68N (SEQ ID NO:118).

FIG. **42**A depicts a plasmid map of the CMV/R WEEV CBA87 strain capsid K67N K68N K69N VLP plasmid. FIG. **42**B shows the sequence of the insert (SEQ ID NO:119). FIG. **42**C shows the sequence of the plasmid (SEQ ID NO:120). FIG. **42**D shows the amino acid sequence of the CMV/R WEEV CBA87 strain capsid protein K67N K68N K69N (SEQ ID NO:121).

FIG. **43**A depicts a plasmid map of the CMV/R VEEV TC83 strain K64N VLP plasmid. FIG. **43**B shows the sequence of the insert (SEQ ID NO:122). FIG. **43**C shows the sequence of the plasmid (SEQ ID NO:123). FIG. **43**D shows the amino acid sequence of the VEEV TC83 strain capsid protein K64N (SEQ ID NO:124).

FIG. **44**A depicts a plasmid map of the CMV/R VEEV TC83 strain K64N K65N VLP plasmid. FIG. **44**B shows the sequence of the insert (SEQ ID NO:125). FIG. **44**C shows the sequence of the plasmid (SEQ ID NO:126). FIG. **44**D shows the amino acid sequence of the VEEV TC83 strain capsid protein K64N K65N (SEQ ID NO:127).

FIG. **45**A depicts a plasmid map of the CMV/R VEEV TC83 strain K65N K67N VLP plasmid. FIG. **45**B shows the sequence of the insert (SEQ ID NO:128). FIG. **45**C shows the sequence of the plasmid (SEQ ID NO:129). FIG. **45**D shows the amino acid sequence of the VEEV TC83 strain capsid protein K65N K67N (SEQ ID NO:130).

FIG. **46**A depicts a plasmid map of the CMV/R VEEV TC83 strain K65A K67A VLP plasmid. FIG. **46**B shows the sequence of the insert (SEQ ID NO:131). FIG. **46**C shows the sequence of the plasmid (SEQ ID NO:132). FIG. **46**D shows the amino acid sequence of the VEEV TC83 strain capsid protein K65A K67A (SEQ ID NO:133).

FIG. **47**A depicts a plasmid map of the CMV/R EEEV PE-6 strain capsid K67N VLP plasmid. FIG. **47**B shows the sequence of the insert (SEQ ID NO:134). FIG. **47**C shows the sequence of the plasmid (SEQ ID NO:135). FIG. **47**D shows the amino acid sequence of the EEEV PE-6 strain capsid protein K67N (SEQ ID NO:136).

FIG. **48**A depicts a plasmid map of the CMV/R EEEV PE-6 strain capsid K67N E2 R239N VLP plasmid. FIG. **48**B shows the sequence of the insert (SEQ ID NO:137). FIG. **48**C shows the sequence of the plasmid (SEQ ID NO:138). FIG. **48**D shows the amino acid sequence of the EEEV PE-6 strain capsid protein K67N (SEQ ID NO:139).

FIG. **49**A depicts a plasmid map of the CMV/R CHIKV (Strain 37997) Capsid R62A plasmid. FIG. **49**B shows the sequence of the insert (SEQ ID NO:140). FIG. **49**C shows the sequence of the plasmid (SEQ ID NO:141). FIG. **49**D shows the amino acid sequence of the CHIKV(Strain 37997) Capsid protein R62A (SEQ ID NO:142).

FIG. **50**A depicts a plasmid map of the CMV/R CHIKV (Strain 37997) Capsid R62A R63A plasmid. FIG. **50**B shows the sequence of the insert (SEQ ID NO:143). FIG. **50**C shows the sequence of the plasmid (SEQ ID NO:144). FIG. **50**D shows the amino acid sequence of the CHIKV (Strain 37997) Capsid protein R62A R63A (SEQ ID NO:145).

FIG. **51**A depicts a plasmid map of the CMV/R CHIKV (Strain 37997) Capsid R62A R63A R65A K66A K68A K69A plasmid. FIG. **51**B shows the sequence of the insert (SEQ ID NO:146). FIG. **51**C shows the sequence of the plasmid (SEQ ID NO:147). FIG. **51**D shows the amino acid sequence of the CHIKV(Strain 37997) Capsid protein R62A R63A R65A K66A K68A K69A (SEQ ID NO:148).

FIG. **52**A depicts a plasmid map of the CMV/R CHIKV (Strain 37997) Capsid R65A plasmid. FIG. **52**B shows the sequence of the insert (SEQ ID NO:149). FIG. **52**C shows the sequence of the plasmid (SEQ ID NO:150). FIG. **52**D shows the amino acid sequence of the CHIKV(Strain 37997) Capsid protein R65A (SEQ ID NO:151).

FIG. **53**A depicts a plasmid map of the CMV/R Ross River Virus T48 capsid R71N K72N plasmid. FIG. **53**B shows the sequence of the insert (SEQ ID NO:152). FIG. **53**C shows the sequence of the plasmid (SEQ ID NO:153). FIG. **53**D shows the amino acid sequence of the Ross River Virus T48 capsid protein R71N K72N (SEQ ID NO:154).

FIG. **54**A depicts a plasmid map of the CMV/R Ross River Virus T48 capsid R71N K72N plasmid. FIG. **54**B shows the sequence of the insert (SEQ ID NO:155). FIG. **54**C shows the sequence of the plasmid (SEQ ID NO:156). FIG. **54**D shows the amino acid sequence of the Ross River Virus T48 capsid protein R71N K72N (SEQ ID NO:157).

FIG. **55**A depicts a plasmid map of the CMV/R Ross River Virus T48 capsid R71N K72N K73N plasmid. FIG. **55**B shows the sequence of the insert (SEQ ID NO:158). FIG. **55**C shows the sequence of the plasmid (SEQ ID NO:159). FIG. **55**D shows the amino acid sequence of the Ross River Virus T48 capsid protein R71N K72N K73N (SEQ ID NO:160).

FIG. **56**A depicts a plasmid map of the CMV/R Ross River Virus T48 capsid R71N K72N K73N K74N plasmid. FIG. **56**B shows the sequence of the insert (SEQ ID NO:161). FIG. **56**C shows the sequence of the plasmid (SEQ ID NO:162). FIG. **56**D shows the amino acid sequence of the Ross River Virus T48 capsid protein R71N K72N K73N K74N (SEQ ID NO:163).

FIGS. **57**A-**57**C show that VLP yield was significantly increased in high pH conditions. FIG. **57**A shows that VLP yield was significantly increased in high pH buffer. FIG. **57**B is a structural determination of the acid sensitive region. FIG. **57**C is a Western Blot analysis showing that mutations to the pH sensitive ASR region of CHIKV OPY-1 increase stability of VLPs over 3-4 days, compared to wild-type VLPs.

FIG. **58**A shows the CMV/R BFV virus VLP plasmid. FIG. **58**B shows the sequence of the capsid region (SEQ ID NO:164). FIG. **58**C shows the sequence of the entire insert (SEQ ID NO:165). FIG. **58**D shows the sequence of the entire vector (SEQ ID NO:166). FIG. **58**E shows the amino acid sequence of the CMV/R BFV capsid protein (SEQ ID NO:167).

FIG. **59**A shows the CMV/R BFV capsid K64A VLP plasmid. FIG. **59**B shows the sequence of the BFV K64A capsid region (SEQ ID NO:168). FIG. **59**C shows the sequence of the entire insert (SEQ ID NO:169). FIG. **59**D shows the sequence of the entire vector (SEQ ID NO:170). FIG. **59**E shows the amino acid sequence of the BFV capsid K64A capsid protein (SEQ ID NO:171).

FIG. **60** shows that EEEV VLPs purified at pH 7.9 have higher yield compared to those purified at pH 7.1.

FIG. **61** is a Western Blot analysis showing that yield of EEEV VLPs is pH dependent.

FIG. **62** is a Western Blot analysis showing that addition of Tris-HCl buffer 24 hr after transfection increased EEEV 67N VLP yield.

FIGS. **63**A and **63**B show that addition of Tris-HCl buffer 24 hr after transfection increased CHIKV OPY-1 yield by Western Blot analysis and SDS-PAGE analysis, respectively.

FIG. **64** is a Western Blot analysis showing that mutations to the pH sensitive ASR region of CHIKV OPY-1 increase stability of VLPs over 3-4 days, compared to wild-type VLPs.

FIGS. **65**A and **65**B are Western Blots showing the expression and purification of WEEV, EEEV, and VEEV VLPs containing NLS signal mutations. FIG. **65**A are Western Blots showing the expression of WEEV and VEEV VLPs containing NLS signal mutations and the purification of EEEV VLP with the K67N mutation compared to that of wild-type EEEV VLP. FIG. **65**B are Western Blots showing that NLS signal mutations in combination with high pH conditions increase EEEV and VEEV production. FIG. **65**B discloses SEQ ID NOS 3, 2, 4, and 73, respectively, in order of appearance.

FIGS. **66**A-**66**C are graphs showing that multivalent Virus-Like Particle vaccine against Eastern, Western and Venezuelan Equine Encephalitis Virus protected mice against infection. FIG. **66**A is a graph showing that mice vaccinated with multivalent VLPs showed high levels of neutralizing antibodies against all the viruses. FIG. **66**B is a graph showing that high viremia was observed in the control, VEEV VLP and EEEV VLP groups but not in the WEEV VLPs and the trivalent groups. FIG. **66**C is a graph showing that mice immunized with WEEV VLPs controlled the challenge virus, while all control mice developed severe infections and died.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides methods for enhancing alphavirus or flavivirus VLP production involving making one or more alterations in an E2 protein and/or a alphavirus capsid protein Nuclear Localization Signal (NLS).

The invention is based, at least in part, on the discovery that amino acid 234 in the CHIKV E2 protein plays an important role in allowing VLPs to bud efficiently from the cell membrane. Almost all alphaviruses have a lysine residue at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein. When this residue is converted from a lysine to an asparagine, use of the modified E2 protein in the alphavirus VLP expression system results in increased yield of VLPs. In addition, amino acid 251 in the CHIKV E2 protein has been shown to be important in stabilizing the E2 protein during viral budding. Modification of this residue to destabilize the E2 protein results also results in increased VLP synthesis. Therefore, use of an alphavirus E2 protein or a flavivirus envelope protein having a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein is a strategy for improving alphavirus VLP production, which will reduce the cost of making alphavirus VLP vaccines and delivery vehicles. Without being bound to a particular theory, it is believed that amino acid 234 resides within an acid sensitive (ASR) of the molecule. As shown herein, exposure of VLP to high pH conditions (pH>7.2, e.g., pH 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, or higher), also improves VLP production.

The invention is also based, at least in part, on the discovery that amino acid 251 in the CHIKV E2 protein plays an important role in allowing VLPs to bud efficiently from the cell membrane. Amino acid 251 in the CHIKV E2 protein has been shown to be important in stabilizing the E2 protein during viral budding. Modification of this residue destabilizes the E2 protein during viral maturation, which results in increased VLP synthesis and VLP yield. Therefore, use of an alphavirus E2 protein having a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein, which destabilizes the alphavirus E2 protein during viral budding, is another strategy for improving alphavirus VLP production.

The invention is also based, at least in part, on the discovery that alterations in the charged residues in the alphavirus capsid protein nuclear localization sequences provided or increased the expression of alphavirus VLPs and increased alphavirus VLP yields. Without being bound to a particular theory, the nuclear localization signal motif of the alphavirus capsid protein accumulates alphavirus capsid protein into the nucleus and prevents the secretion of alphavirus VLPs. It has been found that altering lysine and arginine residues in an alphavirus capsid protein NLS (e.g., to uncharged amino acids alanine or asparagine) improves alphavirus VLP production. Improvement in yields of alphavirus VLPs allows for their use as immunogenic compositions or vaccines, including a pan-alphavirus vaccine.

Accordingly, the invention provides nucleic acid molecules encoding alphavirus (e.g., CHIKV and WEEV) structural polypeptides, including an alphavirus E2 protein having a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein, expression vectors comprising these coding sequences, and methods of using these nucleic acid molecules for the preparation of virus-like particles. The invention also provides nucleic acid molecules encoding alphavirus (e.g., CHIKV and WEEV) structural polypeptides, including an alphavirus E2 protein having a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding, expression vectors comprising these coding sequences, and methods of using these nucleic acid molecules for the preparation of virus-like particles. The invention further provides immunogenic compositions containing one or more alphavirus (e.g., CHIKV and WEEV) structural polypeptides, including an E2 protein having a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein and/or a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding. In particular, the immunogenic composition (e.g., vaccine) contains envelope or capsid polypeptides sufficient to form a virus-like particle. In other embodiments, the invention provides DNA vaccines that provide for the expression of one or more viral polypeptides in the cell of a subject, including an alphavirus E2 protein having a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein and/or a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding.

The invention also provides nucleic acid molecules encoding flavivirus structural polypeptides, including an envelope protein having a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein, expression vectors comprising these coding sequences, and methods of using these nucleic acid molecules for the preparation of virus-like particles. The invention further provides immunogenic compositions containing one or more flavivirus structural polypeptides, including an envelope protein having a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein. In particular, the immunogenic composition (e.g., vaccine) contains envelope or capsid polypeptides sufficient to form a virus-like particle. In other embodiments, the invention provides DNA vaccines that provide for the expression of one or more viral polypeptides in the cell of a subject, including a flavivirus envelope protein having an asparagine residue at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein.

Alphavirus and Flavivirus Polynucleotides

In general, the invention includes any nucleic acid sequence encoding a VLP having an alteration in a structural protein that enhances VLP expression in a mammalian cell. In one embodiment, the alphavirus polypeptide(s) includes at least an alphavirus E2 protein or capsid protein NLS comprising an alteration that increases VLP expression in a mammalian cell. In one embodiment, the alphavirus E2 protein has a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein and/or a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding. In another embodiment, the alphavirus polypeptide(s) includes at least an alphavirus capsid protein having a non-lysine residue (e.g., alanine or asparagine) at an amino acid position corresponding to a lysine residue in an alphavirus capsid protein NLS and/or a non-arginine residue (e.g., alanine or asparagine) at an amino acid position corresponding to a arginine residue in an alphavirus capsid protein NLS. In specific embodiments, the alphavirus capsid protein is a WEEV CBA87 strain capsid protein having one or more of the alterations K67N, K68N, and/or K69N. In certain embodiments, the alphavirus capsid protein is a VEEV TC83 strain capsid protein having one or more of the alterations K64N, K65A, K65N, K67A, and/or K67N. In some embodiments, the alphavirus capsid protein is a EEEV PE-6 strain capsid protein having an alteration K67N. In particular embodiments, the alphavirus capsid protein is a CHIKV(Strain 37997) strain capsid protein having one or more of the alterations R62A, R63A, R65A, K66A, K68A, and/or K69A. In specific embodiments, the alphavirus capsid protein is a Ross River Virus capsid protein having one or more of the alterations R71N, K72N, K73N, and/or K74N. In specific embodiments, the alphavirus capsid protein is a Barmah Forest Virus capsid protein having one or more of the alterations K64A, K64N, K65A, K65N, K67A, K67N, K68A and/or K68N. An isolated nucleic acid molecule can be manipulated by recombinant DNA techniques well known in the art. Thus, a nucleotide sequence contained in a vector in which 5' and 3' restriction sites are known, or for which polymerase chain reaction (PCR) primer sequences have been disclosed, is considered isolated, but a nucleic acid sequence existing in its native state in its natural host is not. In certain exemplary embodiments, the vector comprises CHIKV 37997 or WEEV 71V-1658 nucleic acid segments, or fragments thereof. The vector may further comprise a CMV/R promoter. The vector may also comprise the capsid protein, or a fragment thereof.

In other exemplary embodiments, in addition to the E2 protein, the vector comprises another envelope protein selected from the group consisting of E3, 6K, and E1. In certain examples, the vaccine may comprise capsid, E3, E2, 6K and E1. In other examples, the vaccine may comprise E3, E2, 6K and E1.

The invention also includes any nucleic acid sequence encoding a VLP comprising one or more flavivirus polypeptides or a fragment thereof, where the fragment induces an immune response. The flavivirus polypeptide(s) includes at least a flavivirus envelope protein having a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein. The flavivirus polypeptide(s) includes at least a flavivirus capsid protein having a non-lysine residue (e.g., alanine or asparagine) at an amino acid position corresponding to a lysine residue in a flavivirus capsid protein NLS and/or a non-arginine residue (e.g., alanine or asparagine) at an amino acid position corresponding to a arginine residue in a flavivirus capsid protein NLS. An isolated nucleic acid molecule can be manipulated by recombinant DNA techniques well known in the art.

In a particular embodiment, a nucleic acid molecule set forth in the sequences disclosed herein includes a nucleotide sequence encoding a polypeptide having at least about 50%, 60%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or more identity (e.g., when compared to the overall length of the amino acid sequence) to a polypeptide encoding a protein selected from alphavirus capsid, E3, E2, 6K and E1, including CHIKV or WEEV capsid, E3, E2, 6K and E1; and flavivirus envelope and capsid.

In some embodiments of the invention, proteins may comprise mutations containing alterations which produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded protein or how the proteins are made. Nucleotide variants can be produced for a variety of reasons, e.g., to optimize codon expression for a particular host, see U.S. patent publication 2005/0118191, herein incorporated by reference in its entirety for all purposes.

In addition, the nucleotides can be sequenced to ensure that the correct coding regions were cloned and do not contain any unwanted mutations. The nucleotides can be subcloned into an expression vector (e.g., baculovirus) for expression in any cell. A person with skill in the art understands that various subcloning methods are available and are possible.

An isolated nucleic acid may be substantially purified, but need not be. For example, a nucleic acid that is isolated within a cloning or expression vector is not pure in that it may comprise only a tiny percentage of the material in the cell in which it resides. Such a nucleic acid is isolated, as the term is used herein, because it is readily manipulatable by standard techniques known to those of ordinary skill in the art.

Polypeptide Expression

In general, VLPs comprising one or more alphavirus polypeptides of the invention may be produced by transformation of a suitable host cell with all or part of a polypeptide-encoding nucleic acid molecule or fragment thereof in a suitable expression vehicle.

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant protein. The precise host cell used is not critical to the invention. A polypeptide of the invention may be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *Saccharomyces cerevisiae*, insect cells, e.g., Sf21 cells, or mammalian cells, e.g., NIH 3T3, HeLa, COS cells). Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, Md.; also, see, e.g., Ausubel et al., supra). Non limiting examples of insect cells are, *Spodoptera frugiperda* (Sf) cells, e.g., Sf9, Sf21, *Trichoplusia ni* cells, e.g., High Five cells, and *Drosophila* S2 cells. Examples of fungi (including yeast) host cells are *S. cerevisiae, Kluyveromyces lactis* (*K lactis*), species of *Candida* including *C. albicans* and *C. glabrata, Aspergillus nidulans, Schizosaccharomyces pombe* (*S. pombe*), *Pichia pastoris*, and *Yarrowia lipolytica*. Examples of mammalian cells are COS cells, baby hamster kidney cells, mouse L cells, LNCaP cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) cells, African green monkey cells, CV1 cells, HeLa cells, MDCK cells, Vero and Hep-2 cells. *Xenopus laevis* oocytes, or other cells of amphibian origin, may also be used. Prokaryotic host cells include bacterial cells, for example, *E. coli, B. subtilis*, and mycobacteria.

Methods of cloning said proteins are known in the art. For example, the gene encoding a specific alphavirus protein, e.g., a CHIKV, WEEV, EEEV, VEEV, Ross River virus, or Barmah Forest virus structural protein, or a specific flavivirus protein can be isolated by RT-PCR from polyadenylated mRNA extracted from cells which had been infected with said virus. The resulting product gene can be cloned as a DNA insert into a vector. The term "vector" refers to the means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include plasmids, viruses, bacteriophages, pro-viruses, phagemids, transposons, artificial chromosomes, and the like, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that is not autonomously replicating. In many, but not all, common embodiments, the vectors of the present invention are plasmids or bacmids.

The invention further provides nucleotides that encode proteins, including chimeric molecules, cloned into an expression vector that can be expressed in a cell that provides for the formation of VLPs. An "expression vector" is a vector, such as a plasmid, that is capable of promoting expression, as well as replication of a nucleic acid incorporated therein. Typically, the nucleic acid molecule to be expressed is "operably linked" to a promoter and/or enhancer, and is subject to transcription regulatory control by the promoter and/or enhancer.

In one aspect, the invention provides an expression vector for expressing an alphavirus VLP having one or more alterations in an E2 protein and/or an alphavirus capsid protein Nuclear Localization Signal (NLS). In one embodiment, the VLP comprises one or more alphavirus envelope proteins, and in particular a CHIKV or WEEV E2 envelope protein having a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein and/or a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding. In another embodiment, the VLP further comprises any one or more of alphavirus envelope proteins E3, 6K, and E1. In another embodiment, the VLP further comprises an alphavirus capsid protein. In related embodiments, the CHIKV or WEEV capsid protein is used. In another embodiment, the VLP comprises of capsid, E3, E2, 6K, and E1. In still another embodiment, the VLP comprises one or more flavivirus envelope proteins. In related embodiments, the VLP further comprises one or more flavirus capsid proteins. In another embodiment, the expression vector is a mammalian expression vector or baculovirus vector.

In various embodiments, one or more charged residues in the nuclear localization sequence of an alphavirus (CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus) capsid protein are altered. In particular embodiments, the charged residues in the alphavirus capsid protein NLS are lysine and arginine. In specific embodiments, lysine and arginine in the alphavirus capsid protein NLS are replaced with an alanine or asparagine. In related embodiments, one or more alterations in an alphavirus capsid protein Nuclear Localization Signal (NLS) provides or increases the expression of alphavirus VLPs and increased alphavirus VLP yields. In specific embodiments, the alphavirus capsid protein is a WEEV CBA87 strain capsid protein having one or more of the alterations K67N, K68N, and/or K69N. In certain embodiments, the alphavirus capsid protein is a VEEV TC83 strain capsid protein having one or more of the alterations K64N, K65A, K65N, K67A, and/or K67N. In some embodiments, the alphavirus capsid protein is a EEEV PE-6 strain capsid protein having an alteration K67N. In particular embodiments, the alphavirus capsid protein is a CHIKV(Strain 37997) strain capsid protein having one or more of the alterations R62A, R63A, R65A, K66A, K68A, and/or K69A. In specific embodiments, the alphavirus capsid protein is a Ross River Virus capsid protein having one or more of the alterations R71N, K72N, K73N, and/or K74N. In specific embodiments, the alphavirus capsid protein is a Barmah Forest Virus capsid protein having one or more of the alterations K64A, K64N, K65A, K65N, K67A, K67N, K68A and/or K68N.

The method of transformation or transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al. (supra); expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (P. H. Pouwels et al., 1985, Supp. 1987).

A variety of expression systems exist for the production of the polypeptides of the invention. Expression vectors useful for producing such polypeptides include, without limitation, chromosomal, episomal, and virus-derived vectors, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof.

Constructs and/or vectors provided herein comprise alphavirus or flavivirus polynucleotides that encode structural polypeptides, including envelope proteins or capsid proteins or portions thereof as described herein. The vector may be, for example, a phage, plasmid, viral, or retroviral vector. The constructs and/or vectors that comprise the nucleotides should be operatively linked to an appropriate promoter, such as the CMV promoter, phage lambda PL promoter, the *E. coli* lac, phoA and tac promoters, the SV40 early and late promoters, and promoters of retroviral LTRs are non-limiting examples. Other suitable promoters will be known to the skilled artisan depending on the host cell and/or the rate of expression desired. The expression constructs will further contain sites for transcription initiation, termination, and, in the transcribed region, a ribosome-binding site for translation. The coding portion of the transcripts expressed by the constructs will preferably include a translation initiating codon at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

Expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Among vectors preferred are virus vectors, such as baculovirus, poxvirus (e.g., vaccinia virus, avipox virus, canarypox virus, fowlpox virus, raccoonpox virus, swinepox virus, etc.), adenovirus (e.g., canine adenovirus), herpesvirus, and retrovirus. Other vectors that can be used with the invention comprise vectors for use in bacteria, which comprise pQE70, pQE60 and pQE-9, pBluescript vectors, Phagescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5. Among preferred eukaryotic vectors are pFastBac1 pWINEO, pSV2CAT, pOG44, pXT1 and pSG, pSVK3, pBPV, pMSG, and pSVL. Other suitable vectors will be readily apparent to the skilled artisan.

Recombinant constructs can be prepared and used to transfect, infect, or transform and can express viral proteins, including those described herein, into eukaryotic cells and/or prokaryotic cells. Thus, the invention provides for host cells which comprise a vector (or vectors) that contain nucleic acids which code for alphavirus structural genes, including capsid, E3, E2, 6K, and E1 or portions thereof, and/or any chimeric molecule described above, and permit the expression of alphavirus structural genes, including capsid E3, E2, 6K, and E1, or portions thereof, and/or any chimeric molecule described above in said host cell under conditions which allow the formation of VLPs. The invention also provides for host cells which comprise a vector (or vectors) that contain nucleic acids which code for flavivirus structural genes, including capsid, envelope, or portions thereof, and/or any chimeric molecule described above, and permit the expression of flavivirus structural genes, including capsid, envelope, or portions thereof, and/or any chimeric molecule described above in said host cell under conditions which allow the formation of VLPs

In one embodiment, said vector is a recombinant baculovirus. In another embodiment, said recombinant baculovirus is transfected into an insect cell. In a preferred embodiment, said cell is an insect cell. In another embodiment, said insect cell is a Sf9 cell.

In another embodiment, said vector and/or host cell comprises nucleotides that encode alphavirus genes, including capsid, E3, E2, 6K, and E1, or portions thereof as described herein. In another embodiment, said vector and/or host cell consists essentially of alphavirus capsid, E3, E2, 6K, and E1, or portions thereof as described herein. In a further embodiment, said vector and/or host cell consists of alphavirus protein comprising capsid, E3, E2, 6K, and E1, or portions thereof, as described herein. These vector and/or host cell contain alphavirus core, E3, E2, 6K, and E1, or portions thereof, as described herein, and may contain additional cellular constituents such as cellular proteins, baculovirus proteins, lipids, carbohydrates etc.

In another embodiment, said vector and/or host cell comprises nucleotides that encode flavivirus genes, including capsid, envelope, or portions thereof as described herein. In another embodiment, said vector and/or host cell consists essentially of alphavirus flavivirus capsid, envelope, or portions thereof as described herein. These vector and/or host cell may contain additional cellular constituents such as cellular proteins, baculovirus proteins, lipids, carbohydrates etc.

One particular bacterial expression system for polypeptide production is the *E. coli* pET expression system (Novagen, Inc., Madison, Wis.). According to this expression system, DNA encoding a polypeptide is inserted into a pET vector in an orientation designed to allow expression. Since the gene encoding such a polypeptide is under the control of the T7 regulatory signals, expression of the polypeptide is achieved by inducing the expression of T7 RNA polymerase in the host cell. This is typically achieved using host strains that express T7 RNA polymerase in response to IPTG induction. Once produced, a recombinant polypeptide is then isolated according to standard methods known in the art, for example, those described herein.

Another bacterial expression system for polypeptide production is the pGEX expression system (Pharmacia). This system employs a GST gene fusion system that is designed for high-level expression of genes or gene fragments as fusion proteins with rapid purification and recovery of functional gene products. The protein of interest is fused to the carboxyl terminus of the glutathione S-transferase protein from *Schistosoma japonicum* and is readily purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B. Fusion proteins can be recovered under mild conditions by elution with glutathione. Cleavage of the glutathione S-transferase domain from the fusion protein is facilitated by the presence of recognition sites for site-specific proteases upstream of this domain. For example, proteins expressed in pGEX-2T plasmids may be cleaved with thrombin; those expressed in pGEX-3X may be cleaved with factor Xa.

Once a recombinant polypeptide of the invention is expressed, it is isolated, e.g., using affinity chromatography. In one example, an antibody (e.g., produced as described herein) raised against a polypeptide of the invention may be attached to a column and used to isolate the recombinant polypeptide. Lysis and fractionation of polypeptide-harboring cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., supra).

Once isolated, the recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher, Laboratory Techniques In Biochemistry and Molecular Biology, eds., Work and Burdon, Elsevier, 1980). Polypeptides of the invention, particularly short peptide fragments, can also be produced by chemical synthesis (e.g., by the methods described in Solid Phase Peptide Synthesis, 2nd ed., 1984 The Pierce Chemical Co., Rockford, Ill.). These general techniques of polypeptide expression and purification can also be used to produce and isolate useful peptide fragments or analogs (described herein).

Alphavirus and Flavivirus Polypeptides and Analogs

The invention provides VLPs comprising one or more alphavirus, CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus, or flavivirus polypeptides. Also included in the invention are VLPs comprising one or more alphavirus, CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus, or flavivirus polypeptides or fragments thereof that are modified in ways that enhance or do not inhibit their ability to modulate an immune response. In one embodiment, the invention provides methods for optimizing an alphavirus, CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus, or flavivirus amino acid sequence or nucleic acid sequence by producing an alteration. Such alterations may include certain mutations, deletions, insertions, or post-translational modifications. The invention further includes analogs of any naturally-occurring polypeptide of the invention. Analogs can differ from the naturally-occurring the polypeptide of the invention by amino acid sequence differences, by post-translational modifications, or by both. Analogs of the invention will generally exhibit at least 85%, more preferably 90%, and most preferably 95% or even 99% identity with all or part of a naturally-occurring amino, acid sequence of the invention.

The length of sequence comparison is at least 10, 13, 15 amino acid residues, preferably at least 25 amino acid residues, and more preferably more than 35 amino acid residues.

Alterations of an alphavirus, CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus, or flavivirus polypeptide include but are not limited to site-directed, random point mutagenesis, homologous recombination (DNA shuffling), mutagenesis using uracil containing templates, oligonucleotide-directed mutagenesis, phosphorothioate-modified DNA mutagenesis, mutagenesis using gapped duplex DNA or the like. Additional suitable methods include point mismatch repair, mutagenesis using repair-deficient host strains, restriction-selection and restriction-purification, deletion mutagenesis, mutagenesis by total gene synthesis, double-strand break repair, and the like. Mutagenesis, e.g., involving chimeric constructs, is also included in the present invention. In one embodiment, mutagenesis can be guided by known information of the naturally occurring molecule or altered or mutated naturally occurring molecule, e.g., sequence, sequence comparisons, physical properties, crystal structure or the like.

In one embodiment, the invention provides polypeptide variants that differ from a reference polypeptide. The term "variant" refers to an amino acid sequence that is altered by one or more amino acids with respect to a reference sequence. The variant can have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, e.g., replacement of leucine with isoleucine. Alternatively, a variant can have "nonconservative" changes, e.g., replacement of a glycine with a tryptophan. Analogous minor variations can also include amino acid deletion or insertion, or both. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without eliminating biological or immunological activity can be found using computer programs well known in the art, for example, DNASTAR software. Desirably, variants show substantial biological activity. In one embodiment, a protein variant forms a VLP and elicits an antibody response when administered to a subject.

Natural variants can occur due to mutations in the proteins. These mutations may lead to antigenic variability within individual groups of infectious agents. Thus, a person infected with a particular strain develops antibody against that virus, as newer virus strains appear, the antibodies against the older strains no longer recognize the newer virus and reinfection can occur. The invention encompasses all antigenic and genetic variability of proteins from infectious agents for making VLPs.

Again, in an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e^{-3} and e^{-100} indicating a closely related sequence. Modifications include in vivo and in vitro chemical derivatization of polypeptides, e.g., acetylation, carboxylation, phosphorylation, or glycosylation; such modifications may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes. Analogs can also differ from the naturally-occurring polypeptides of the invention by alterations in primary sequence. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis by irradiation or exposure to ethanemethylsulfate or by site-specific mutagenesis as described in Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press, 1989, or Ausubel et al., supra). Also included are cyclized peptides, molecules, and analogs which contain residues other than L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., .beta. or .gamma. amino acids.

In addition to full-length polypeptides, the invention also includes fragments of any one of the polypeptides of the invention. As used herein, the term "a fragment" means at least 5, 10, 13, or 15. In other embodiments a fragment is at least 20 contiguous amino acids, at least 30 contiguous amino acids, or at least 50 contiguous amino acids, and in other embodiments at least 60 to 80 or more contiguous amino acids. Fragments of the invention can be generated by methods known to those skilled in the art or may result from normal protein processing (e.g., removal of amino acids from the nascent polypeptide that are not required for biological activity or removal of amino acids by alternative mRNA splicing or alternative protein processing events).

Non-protein analogs having a chemical structure designed to mimic alphavirus, CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus, or flavivirus VLPs or one or more alphavirus, CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus, or flavivirus polypeptides functional activity can be administered according to methods of the invention. Alphavirus, CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus, or flavivirus analogs may exceed the physiological activity of native alphavirus, CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus, or flavivirus. Methods of analog design are well known in the art, and synthesis of analogs can be carried out according to such methods by modifying the chemical structures such that the resultant analogs exhibit the immunomodulatory activity of a native alphavirus, CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus, or flavivirus polypeptide. These chemical modifications include, but are not limited to, substituting alternative R groups and varying the degree of saturation at specific carbon atoms of the native alphavirus, CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus, or flavivirus molecule. Preferably, the analogs are relatively resistant to in vivo degradation, resulting in a more prolonged therapeutic effect upon administration. Assays for measuring functional activity include, but are not limited to, those described in the Examples below.

Alphavirus and Flavivirus VLP Production

The invention also provides constructs and methods for producing a VLP comprising alphavirus or flavivirus polypeptides, or fragments thereof, as well as compositions and methods that increase the efficiency of VLP production. In embodiments, inclusion of an alphavirus E2 protein or a flavivirus envelope protein having a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein results in enhanced VLP production. In embodiments, inclusion of an alphavirus E2 protein having a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding results in enhanced VLP production. In a related embodiment, VLP are exposed to high pH, basic, or non-acidic conditions during VLP production (e.g., in cell culture, during purification). In specific embodiments, the pH is at least about 7.2 (pH 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, or higher). In certain embodiments, the addition of leader sequences to the alphavirus capsid, E3, E2, 6K, E1, or portions thereof, or to flavivirus capsid, envelope, or portions thereof, can improve the efficiency of protein transporting within the cell. In another example, a heterologous signal sequence can be fused to the alphavirus capsid, E3, E2, 6K, E1, or portions thereof, or to flavivirus capsid, envelope, or portions thereof. In one embodiment, the signal sequence can be derived from the gene of an insect cell. Another method to increase efficiency of VLP production is to codon optimize the nucleotides that encode alphavirus capsid, E3, E2, 6K, E1, or portions thereof, or flavivirus capsid, envelope, or portions thereof for a specific cell type.

In various embodiments, one or more charged residues in the nuclear localization sequence of an alphavirus (CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus) capsid protein are altered. In particular embodiments, the charged residues in the alphavirus capsid protein NLS are lysine and arginine. In specific embodiments, lysine and arginine in the alphavirus capsid protein NLS are replaced with an alanine or asparagine. In related embodiments, one or more alterations in an alphavirus capsid protein Nuclear Localization Signal (NLS) provides or increases the expression of alphavirus VLPs and increased alphavirus VLP yields. In specific embodiments, the alphavirus capsid protein is a WEEV CBA87 strain capsid protein having one or more of the alterations K67N, K68N, and/or K69N. In certain embodiments, the alphavirus capsid protein is a VEEV TC83 strain capsid protein having one or more of the alterations K64N, K65A, K65N, K67A, and/or K67N. In some embodiments, the alphavirus capsid protein is a EEEV PE-6 strain capsid protein having an alteration K67N. In particular embodiments, the alphavirus capsid protein is a CHIKV(Strain 37997) strain capsid protein having one or more of the alterations R62A, R63A, R65A, K66A, K68A, and/or K69A. In specific embodiments, the alphavirus capsid protein is a Ross River Virus capsid protein having one or more of the alterations R71N, K72N, K73N, and/or K74N. In specific embodiments, the alphavirus capsid protein is a Barmah Forest Virus capsid protein having one or more of the alterations K64A, K64N, K65A, K65N, K67A, K67N, K68A, and/or K68N.

Methods of cloning said proteins are known in the art. For example, the gene encoding a specific alphavirus protein can be isolated by RT-PCR from polyadenylated mRNA extracted from cells which had been infected with said virus. The resulting gene can be cloned as a DNA insert into a vector. The term "vector" refers to the means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include plasmids, viruses, bacteriophages, pro-viruses, phagemids, transposons, artificial chromosomes, and the like, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that is not autonomously replicating. In many, but not all, common embodiments, the vectors of the present invention are plasmids or bacmids.

Thus, the invention comprises nucleotides that encode proteins, including chimeric molecules, cloned into an expression vector that can be expressed in a cell that induces the formation of VLPs of the invention. An "expression vector" is a vector, such as a plasmid that is capable of promoting expression, as well as replication of a nucleic acid incorporated therein. Typically, the nucleic acid to be expressed is "operably linked" to a promoter and/or enhancer, and is subject to transcription regulatory control by the promoter and/or enhancer. In embodiments, the VLP comprises an alphavirus E2 envelope protein having a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein, and in particular a CHIKV or WEEV E2 protein. In embodiments, the VLP comprises an alphavirus E2 envelope protein having a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding. In another embodiment, the VLP further comprises one or more additional envelope proteins selected from the group consisting of E3, 6K and E1. In another embodiment, the VLP comprises an alphavirus capsid protein. In related embodiments, the CHIKV or WEEV capsid protein is used. In another embodiment, the VLPs are comprised of E3, E2, 6K and E1. In still another embodiment, the VLPs are comprised of capsid, E3, E2, 6K and E1. In embodiments, the VLP comprises a flavivirus envelope protein having a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein. In another embodiment, the VLP comprises a flavivirus capsid protein. In another embodiment, the expression vector is a baculovirus vector.

The invention also provides methods of producing a VLP comprising alphavirus or flavivirus polypeptides, or fragments thereof, including an alphavirus E2 polypeptide or a flavivirus envelope polypeptide that has a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein. In embodiments, the alphavirus E2 polypeptide has a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding. In one example, the method involves expressing in a cell a polynucleotide encoding an alphavirus polypeptide, such as an CHIKV or WEEV polypeptide, or a flavivirus polypeptide and culturing said cell, thereby producing VLPs. In one embodiment, a cell (e.g., human cell) is infected with a DNA vaccine, where the DNA vaccine is a DNA vector, comprising a nucleic acid segment encoding an alphavirus capsid protein or one or more alphavirus envelope proteins, or fragments thereof to produce an alphavirus VLP. In particular, the alphavirus is CHIKV or WEEV. In another embodiment, a cell (e.g., human cell) is infected with a DNA vaccine, where the DNA vaccine is a DNA vector, comprising a nucleic acid segment encoding an flavivirus capsid protein or one or more flavivirus envelope proteins, or fragments thereof to produce a flavivirus VLP.

Depending on the expression system and host cell selected, the VLPs are produced by growing host cells transformed by an expression vector under conditions whereby the recombinant proteins are expressed and VLPs are formed. In one embodiment, the invention comprises a method of producing a VLP, that involves transfecting vectors encoding at least one alphavirus protein into a suitable host cell and expressing said alphavirus protein under conditions that allow VLP formation. In another embodiment, the eukaryotic cell is selected from the group consisting of, yeast, insect, amphibian, avian or mammalian cells. The selection of the appropriate growth conditions is within the skill or a person with skill of one of ordinary skill in the art.

Methods to grow cells that produce VLPs of the invention include, but are not limited to, batch, batch-fed, continuous and perfusion cell culture techniques. In one embodiment, a cell comprising an alphavirus polynucleotide, such as a CHIKV or WEEV polynucleotide, or a flavivirus polynucleotide, is grown in a bioreactor or fermentation chamber where cells propagate and express protein (e.g., recombinant proteins) for purification and isolation. Typically, cell culture is performed under sterile, controlled temperature and atmospheric conditions. A bioreactor is a chamber used to culture cells in which environmental conditions such as temperature, atmosphere, agitation and/or pH can be monitored. In one embodiment, the bioreactor is a stainless steel chamber. In another embodiment, said bioreactor is a pre-sterilized plastic bag (e.g., Cellbag®, Wave Biotech, Bridgewater, N.J.). In other embodiment, said pre-sterilized plastic bags are about 50 L to 1000 L bags.

The VLPs are isolated using methods that preserve the integrity thereof, such as by gradient centrifugation, e.g., cesium chloride, sucrose and iodixanol, as well as standard purification techniques including, e.g., ion exchange and gel filtration chromatography.

The following is an example of how VLPs of the invention can be made, isolated and purified. A person of skill in the art appreciates that there are additional methods that can be used to make and purify VLPs. Accordingly, the invention is not limited to the methods described herein.

In general, production of VLPs of the invention is accomplished by seeding a mammalian cell (e.g., human embryonic kidney (293T) cells) or Sf9 cells (non-infected) into shaker flasks, allowing the cells to expand and scaling up as the cells grow and multiply (for example from a 125-ml flask to a 50 L Wave bag). The medium used to grow the cells is formulated for the appropriate cell line (preferably serum free media, e.g., insect medium ExCell-420, JRH). Next, the cells are transfected or infected with an appropriate vector (e.g., mammalian expression vector or for SF (cells recombinant baculovirus at the most efficient multiplicity of infection (e.g., from about 1 to about 3 plaque forming units per cell). The polynucleotides, or portions thereof, are expressed in the cells where they self assemble into VLPs and are secreted from the cells approximately 24 to 72 hours post infection. Usually, transfection or infection is most efficient when the cells are in mid-log phase of growth (4-8.$\times 10^6$ cells/ml) and are at least about 90% viable. Additionally, the transfected cells may be exposed to high pH conditions in cell culture (pH>7.2, e.g., pH 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, or higher) to increase VLP production.

VLPs of the invention are harvested approximately 48 to 120 hours post infection, when the levels of VLPs in the cell culture medium are near the maximum but before extensive cell lysis. The cell density and viability at the time of harvest can be about 0.5×10^6 cells/ml to about 1.5×10^6 cells/ml with at least 20% viability, as shown by dye exclusion assay. Next, the medium is removed and clarified. NaCl can be added to the medium to a concentration of about 0.4 to about 1.0 M, preferably to about 0.5 M, to avoid VLP aggregation. The removal of cell and cellular debris from the cell culture medium containing VLPs of the invention can be accomplished by tangential flow filtration (TFF) with a single use, pre-sterilized hollow fiber 0.5 or 1.00 μm filter cartridge or a similar device. Additionally, the VLPs may be exposed to high pH conditions during purification (pH>7.2, e.g., pH 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, or higher) to increase VLP production.

Next, VLPs in the clarified culture medium are concentrated by ultrafiltration using a disposable, pre-sterilized 500,000 molecular weight cut off hollow fiber cartridge. The concentrated VLPs can be diafiltrated against 10 volumes pH 7.0 to 8.0 phosphate-buffered saline (PBS) containing 0.5 M NaCl to remove residual medium components.

The concentrated, diafiltered VLPs can be furthered purified on a 20% to 60% discontinuous sucrose gradient in pH 7.2 PBS buffer with 0.5 M NaCl by centrifugation at 6,500×g for 18 hours at about 4 C to about 10 C. Usually VLPs will form a distinctive visible band between about 30% to about 40% sucrose or at the interface (in a 20% and 60% step gradient) that can be collected from the gradient and stored. This product can be diluted to comprise 200 mM of NaCl in preparation for the next step in the purification process. This product contains VLPs and may contain intact baculovirus particles.

Further purification of VLPs can be achieved by anion exchange chromatography, or 44% isopycnic sucrose cushion centrifugation. In anion exchange chromatography, the sample from the sucrose gradient (see above) is loaded into column containing a medium with an anion (e.g., Matrix Fractogel EMD TMAE) and eluded via a salt gradient (from about 0.2 M to about 1.0 M of NaCl) that can separate the VLP from other contaminates (e.g., baculovirus and DNA/RNA). In the sucrose cushion method, the sample comprising the VLPs is added to a 44% sucrose cushion and centrifuged for about 18 hours at 30,000 g. VLPs form a band at the top of 44% sucrose, while baculovirus precipitates at the bottom and other contaminating proteins stay in the 0% sucrose layer at the top. The VLP peak or band is collected.

The intact baculovirus can be inactivated, if desired. Inactivation can be accomplished by chemical methods, for example, formalin or .beta.-propiolactone (BPL). Removal and/or inactivation of intact baculovirus can also be largely accomplished by using selective precipitation and chromatographic methods known in the art, as exemplified above. Methods of inactivation comprise incubating the sample containing the VLPs in 0.2% of BPL for 3 hours at about 25° C. to about 27° C. The baculovirus can also be inactivated by incubating the sample containing the VLPs at 0.05% BPL at 4° C. for 3 days, then at 37° C. for one hour.

After the inactivation/removal step, the product comprising VLPs can be run through another diafiltration step to remove any reagent from the inactivation step and/or any residual sucrose, and to place the VLPs into the desired buffer (e.g., PBS). The solution comprising VLPs can be sterilized by methods known in the art (e.g., sterile filtration) and stored in the refrigerator or freezer.

The above techniques can be practiced across a variety of scales. For example, T-flasks, shake-flasks, spinner bottles, up to industrial sized bioreactors. The bioreactors can comprise either a stainless steel tank or a pre-sterilized plastic bag (for example, the system sold by Wave Biotech, Bridgewater, N.J.). A person with skill in the art will know what is most desirable for their purposes.

As described herein, upon administration to a desired host, the VLPs of the present invention are taken up by cells normally infected by the particular virus. When the VLP contains/packages a target agent, the agent is internalized into the cell upon VLP entry. This property facilitates the use of the VLPs described herein as delivery vehicles because they enable the delivery of a target agent(s) into a desired cell.

Thus, in certain embodiments, a DNA vaccine or VLP comprises an agent(s), such as a therapeutic or diagnostic agent(s) that needs to be delivered to a subject, e.g., imaging agent, nucleic acid sequence (including siRNA and microRNA), radionuclide, hormone, peptide, antiviral agent, antitumor/chemotherapeutic agent, cell growth modulating agent, cell growth inhibitor, cytokine, antigen, adjuvant, toxin, etc. The agent encapsulated should not adversely affect the VLP, or VLP stability. This may be determined by producing VLP containing the desired agent and assessing its effects, if any, on VLP stability.

Accordingly, the present invention provides methods for introducing an agent into a cell. In embodiments, the agent is packaged into a VLP as described herein, producing a packed VLP. In related embodiments, the packed VLP is contacted with a cell. In related embodiments, the packed VLP is allowed to enter the cell, thereby resulting in delivery of the agent into the cell.

In embodiments, the invention provides methods of treating viral diseases and/or disorders or symptoms thereof which comprise administering a therapeutically effective amount of a pharmaceutical composition comprising a VLP or DNA of the formulae herein to a subject (e.g., a mammal such as a human). Thus, one embodiment is a method of treating a subject suffering from or susceptible to a viral infection, viral disease or disorder or symptom thereof. The method includes the step of administering to the mammal a therapeutic or prophylactic amount of an amount of a compound herein sufficient to treat the disease or disorder or symptom thereof, under conditions such that the disease or disorder is prevented or treated.

The methods herein include administering to the subject (including a subject identified as in need of such treatment) an effective amount of a compound described herein, or a composition described herein to produce such effect. Identifying a subject in need of such treatment can be in the judgment of a subject or a health care professional and can be subjective (e.g., opinion) or objective (e.g., measurable by a test or diagnostic method).

As used herein, the terms "treat," treating," "treatment," and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.

As used herein, the terms "prevent," "preventing," "prevention," "prophylactic treatment" and the like refer to reducing the probability of developing a disorder or condition in a subject, who does not have, but is at risk of or susceptible to developing a disorder or condition.

The therapeutic methods of the invention (which include prophylactic treatment) in general comprise administration of a therapeutically effective amount of the agents herein, such as a VLP or DNA of a formulae herein to a subject (e.g., animal, human) in need thereof, including a mammal, particularly a human. Such treatment will be suitably administered to subjects, particularly humans, suffering from, having, susceptible to, or at risk for a disease, disorder, or symptom thereof. Determination of those subjects "at risk" can be made by any objective or subjective determination by a diagnostic test or opinion of a subject or health care provider (e.g., genetic test, enzyme or protein marker, Marker (as defined herein), family history, and the like). The agents herein may be also used in the treatment of any other disorders in which an alphavirus may be implicated.

In one embodiment, the invention provides a method of monitoring treatment progress. The method includes the step of determining a level of diagnostic marker (Marker) (e.g., any target delineated herein modulated by a compound herein, a protein or indicator thereof, etc.) or diagnostic measurement (e.g., screen, assay) in a subject suffering from or susceptible to a disorder or symptoms thereof associated with an alphavirus, in which the subject has been administered a therapeutic amount of a compound herein sufficient to treat the disease or symptoms thereof. The level of Marker determined in the method can be compared to known levels of Marker in either healthy normal controls or in other afflicted patients to establish the subject's disease status. In preferred embodiments, a second level of Marker in the subject is determined at a time point later than the determination of the first level, and the two levels are compared to monitor the course of disease or the efficacy of the therapy. In certain preferred embodiments, a pre-treatment level of Marker in the subject is determined prior to beginning treatment according to this invention; this pre-treatment level of Marker can then be compared to the level of Marker in the subject after the treatment commences, to determine the efficacy of the treatment.

Immunogenic Compositions

The invention provides compositions and methods for inducing an immunological response in a subject, particularly a human, which involves inoculating the subject with a VLP as described herein in a suitable carrier for the purpose of inducing or enhancing an immune response. In one embodiment, an immune response protects the subject from an alphavirus infection, such as a CHIKV, WEEV, EEEV, VEEV, Ross River virus, or Barmah Forest virus infection, a flavivirus infection, or inflammatory consequences thereof (e.g., arthritis). The administration of this immunological composition may be used either therapeutically in subjects already experiencing an alphavirus infection, such as a CHIKV or WEEV infection, or may be used prophylactically to prevent an alphavirus infection. The administration of this immunological composition may also be used either therapeutically in subjects already experiencing a flavivirus infection or prophylactically to prevent a flavivirus infection.

In certain embodiments, the alphavirus candidate vaccines were developed by comparing the immunogenicity of gene products derived from two disparate strains, the 37997 strain from West Africa and the latest outbreak strain, OPY-1, of the East/Central/South African genotype. These strains share ~95% amino acid sequence similarity but have distinct biological differences, particularly related to their host range. In particular, the presence of a non-lysine residue (e.g., asparagine) at amino acid 234 in the CHIKV E2 protein resulted in enhanced VLP production. The introduction of a non-lysine residue (e.g., asparagine) into other alphaviruses at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein also resulted in enhanced VLP production of other alphaviruses. In addition, modification of the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein further destabilized the E2 protein during viral budding, resulting in enhanced VLP production.

VLPs of the invention are useful for preparing vaccines and immunogenic compositions. One important feature of VLPs is the ability to express surface proteins so that the immune system of a vertebrate induces an immune response against said protein. However, not all proteins can be expressed on the surface of VLPs. There may be many reasons why certain proteins are not expressed, or be poorly expressed, on the surface of VLPs. One reason is that said protein is not directed to the membrane of a host cell or that said protein does not have a transmembrane domain.

The preparation of immunogenic compositions and vaccines is known to one skilled in the art. The immunogenic composition or vaccine includes a VLP comprising one or more alphavirus polypeptides, one or more flavivirus polypeptides, or fragments thereof, where the VLP has one or more alterations in an alphavirus E2 protein and/or an alphavirus capsid protein Nuclear Localization Signal (NLS). The immunogenic composition or vaccine may include a VLP comprising an alphavirus E2 polypeptide or a flavivirus envelope polypeptide that has a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein. The alphavirus E2 polypeptide may have a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding. The immunogenic composition or vaccine may include a VLP comprising an alphavirus (CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus) capsid protein in which the nuclear localization sequence is altered. The alphavirus capsid protein nuclear localization sequence is altered in a charged residue (e.g., lysine or arginine), which is replaced with a non-charged residue (e.g., alanine or asparagine). In specific embodiments, the alphavirus capsid protein is a WEEV CBA87 strain capsid protein having one or more of the alterations K67N, K68N, and/or K69N. In certain embodiments, the alphavirus capsid protein is a VEEV TC83 strain capsid protein having one or more of the alterations K64N, K65A, K65N, K67A, and/or K67N. In some embodiments, the alphavirus capsid protein is a EEEV PE-6 strain capsid protein having an alteration K67N. In particular embodiments, the alphavirus capsid protein is a CHIKV(Strain 37997) strain capsid protein having one or more of the alterations R62A, R63A, R65A, K66A, K68A, and/or K69A. In specific embodiments, the alphavirus capsid protein is a Ross River Virus capsid protein having one or more of the alterations R71N, K72N, K73N, and/or K74N. In specific embodiments, the alphavirus capsid protein is a Barmah Forest Virus capsid protein having one or more of the alterations K64A, K64N, K65A, K65N, K67A, K67N, K68A and/or K68N.

The invention also provides expression vectors encoding one or more alphavirus polypeptides, flavivirus polypeptides, or fragments thereof or variants thereof, including an alphavirus E2 polypeptide or a flavivirus envelope polypeptide that has a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein. The alphavirus E2 polypeptide may have a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding. Such an immunogenic composition is delivered in vivo in order to induce or enhance an immunological response in a subject, such as a humoral response.

For example, a VLP comprising one or more CHIKV or WEEV polypeptides or fragments or variants thereof, including a CHIKV or WEEV E2 polypeptide that has a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein and/or a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding, are delivered in vivo in order to induce an immune response.

Typically vaccines are prepared in an injectable form, either as a liquid solution or as a suspension. Solid forms suitable for injection may also be prepared as emulsions, or with the polypeptides encapsulated in liposomes. Vaccine antigens are usually combined with a pharmaceutically acceptable carrier, which includes any carrier that does not induce the production of antibodies harmful to the subject receiving the carrier. Suitable carriers typically comprise large macromolecules that are slowly metabolized, such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates, and inactive virus particles. Such carriers are well known to those skilled in the art. These carriers may also function as adjuvants.

The VLPs described herein may be administered in combination with an adjuvant (e.g., Ribi). Adjuvants are immunostimulating agents that enhance vaccine effectiveness. If desired, the VLP comprising one or more alphavirus polypeptides or fragments or variants thereof are administered in combination with an adjuvant that enhances the effectiveness of the immune response generated against the antigen of interest. Effective adjuvants include, but are not limited to, aluminum salts such as aluminum hydroxide and aluminum phosphate, muramyl peptides, bacterial cell wall components, saponin adjuvants, and other substances that act as immunostimulating agents to enhance the effectiveness of the composition.

Immunogenic compositions, i.e., the VLPs described herein, pharmaceutically acceptable carrier and adjuvant, also typically contain diluents, such as water, saline, glycerol, ethanol. Auxiliary substances may also be present, such as wetting or emulsifying agents, pH buffering substances, and the like. Proteins may be formulated into the vaccine as neutral or salt forms. The immunogenic compositions are typically administered parenterally, by injection; such injection may be either subcutaneously or intramuscularly. Additional formulations are suitable for other forms of administration, such as by suppository or orally. Oral compositions may be administered as a solution, suspension, tablet, pill, capsule, or sustained release formulation.

Immunogenic compositions are administered in a manner compatible with the dose formulation. The immunogenic composition comprises an immunologically effective amount of the VLP described herein and other previously mentioned components. By an immunologically effective amount is meant a single dose, or a composition administered in a multiple dose schedule, that is effective for the treatment or prevention of an infection. The dose administered will vary, depending on the subject to be treated, the subject's health and physical condition, the capacity of the subject's immune system to produce antibodies, the degree of protection desired, and other relevant factors. Precise amounts of the active ingredient required will depend on the judgement of the practitioner, but typically range between 5 μg to 250 μg of antigen per dose.

The invention provides a VLP for use in treating or preventing an alphavirus infection (e.g., CHIKV or WEEV infection). The invention also provides a VLP for use in treating or preventing a flavivirus infection.

Pharmaceutical Compositions and Administration

The invention features pharmaceutical compositions that comprise VLPs as described herein. The pharmaceutical compositions useful herein contain a pharmaceutically acceptable carrier, including any suitable diluent or excipient, which includes any pharmaceutical agent that does not itself induce the production of an immune response harmful to the vertebrate receiving the composition, and which may be administered without undue toxicity and a VLP of the invention. As used herein, the term "pharmaceutically acceptable" means being approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopia, European Pharmacopia or other generally recognized pharmacopia for use in mammals, and more particularly in humans. These compositions can be useful as a vaccine and/or antigenic compositions for inducing a protective immune response in a vertebrate.

In particular embodiments, the invention encompasses an antigenic formulation comprising VLPs which comprises at least one viral protein, for example one alphavirus protein or one flavivirus protein. The alphavirus may be selected from the group consisting of, but not limited to, EEEV, WEEV, VEEV, SFV, CHIKV, Ross River virus, Barmah Forest virus, O'nyong-nyong virus, Sindbis virus, Mayaro virus, and Ockelbo virus.

In certain embodiments, the pharmaceutical compositions comprise alphavirus or flavivirus VLPs and a pharmaceutically acceptable carrier. In certain preferred embodiments, the pharmaceutical composition comprises VLPs of CHIKV, WEEV, EEEV, VEEV, Ross River virus, or Barmah Forest virus, and a pharmaceutically acceptable carrier. In other certain preferred embodiments, the pharmaceutical composition comprises VLPs of CHIKV, WEEV, EEEV, VEEV, Ross River virus, or Barmah Forest virus, an adjuvant, and a pharmaceutically acceptable carrier.

In embodiments, the VLPs comprise an alphavirus (CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus) VLP having one or more alterations in an E2 protein and/or an alphavirus capsid protein Nuclear Localization Signal (NLS). In embodiments, the VLPs are comprised of CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus envelope proteins, including a CHIKV or WEEV E2 envelope protein having a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein and/or a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding. In embodiments, the VLPs comprise additional CHIKV or WEEV envelope proteins selected from the group consisting of CHIKV or WEEV E3, 6K, and E1 envelope proteins. In another embodiment, the pharmaceutical composition further comprises a CHIKV or WEEV capsid protein. In certain examples, the VLPs are comprised of E3, E2, 6K and E1. In other examples, the VLPs are comprised of capsid, E3, E2, 6K and E1. In various embodiments, the VLP comprises an alphavirus (CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus) capsid protein in which the nuclear localization sequence is altered. The alphavirus capsid protein nuclear localization sequence is altered in a charged residue (e.g., lysine or arginine), which is replaced with a non-charged residue (e.g., alanine or asparagine). In specific embodiments, the alphavirus capsid protein is a WEEV CBA87 strain capsid protein having one or more of the alterations K67N, K68N, and/or K69N. In certain embodiments, the alphavirus capsid protein is a VEEV TC83 strain capsid protein having one or more of the alterations K64N, K65A, K65N, K67A, and/or K67N. In some embodiments, the alphavirus capsid protein is a EEEV PE-6 strain capsid protein having an alteration K67N. In particular embodiments, the alphavirus capsid protein is a CHIKV(Strain 37997) strain capsid protein having one or more of the alterations R62A, R63A, R65A, K66A, K68A, and/or K69A. In specific embodiments, the alphavirus capsid protein is a Ross River Virus capsid protein having one or more of the alterations R71N, K72N, K73N, and/or K74N. In specific embodiments, the alphavirus capsid protein is a Barmah Forest Virus capsid protein having one or more of the alterations K64A, K64N, K65A, K65N, K67A, K67N, K68A and/or K68N.

The invention also encompasses a vaccine formulation comprising VLPs that comprise at least one viral protein, an alphavirus E2 protein or a flavivirus envelope protein having a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein. The alphavirus E2 protein may have a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding. The alphavirus may be selected from the group consisting of, but not limited to, EEEV, WEEV, VEEV, SFV, CHIKV, O'nyong-nyong virus, Sindbis virus, Mayaro virus, Ross River virus, Barmah Forest virus, and Ockelbo virus.

In certain preferred embodiments, the vaccine composition comprises VLPs of CHIKV or WEEV, and a pharmaceutically acceptable carrier. In other certain preferred embodiments, the vaccine composition comprises VLPs of CHIKV or WEEV, an adjuvant, and a pharmaceutically acceptable carrier. In one embodiment, the vaccine comprises VLPs that contain a CHIKV or WEEV E2 alphavirus that has a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein and/or a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding. In another embodiment, the vaccine composition comprises VLPs containing additional CHIKV or WEEV envelope proteins, for example, the envelope proteins can be selected from the group consisting of E3, 6K, and E1. In another embodiment, the vaccine composition further comprises a CHIKV or WEEV capsid protein and a pharmaceutically acceptable carrier or excipient. In certain examples, the VLPs are comprised of E3, E2, 6K and E1. In other examples, the VLPs are comprised of capsid, E3, E2, 6K and E1.

Pharmaceutically acceptable carriers include but are not limited to saline, buffered saline, dextrose, water, glycerol, sterile isotonic aqueous buffer, and combinations thereof. A thorough discussion of pharmaceutically acceptable carriers, diluents, and other excipients is presented in Remington's Pharmaceutical Sciences (Mack Pub. Co. N.J. current edition). The formulation should suit the mode of administration. In a preferred embodiment, the formulation is suitable for administration to humans, preferably is sterile, non-particulate and/or non-pyrogenic.

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a solid form, such as a lyophilized powder suitable for reconstitution, a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

In certain embodiments, the VLP composition is supplied in liquid form, for example in a sealed container indicating the quantity and concentration of the VLP composition. Preferably, the liquid form of the VLP composition is supplied in a hermetically sealed container at least about 50 μg/ml, more preferably at least about 100 μg/ml, at least about 200 μg/ml, at least 500 μg/ml, or at least 1 mg/ml.

Generally, VLPs or DNA vaccines of the invention are administered in an effective amount or quantity (as described herein) sufficient to stimulate an immune response against one or more strains of a virus a described here, for example an alphavirus, e.g., CHIKV or WEEV, or a flavivirus. Preferably, administration of the VLP of the invention elicits immunity against a virus, for example an alphavirus, in particular example CHIKV or WEEV, or a flavivirus. Typically, the dose can be adjusted within this range based on, e.g., age, physical condition, body weight, sex, diet, time of administration, and other clinical factors. The prophylactic vaccine formulation is systemically administered, e.g., by subcutaneous or intramuscular injection using a needle and syringe, or a needle-less injection device. Alternatively, the vaccine formulation is administered intranasally, either by drops, large particle aerosol (greater than about 10 microns), or spray into the upper respiratory tract or small particle aerosol (less than 10 microns) or spray into the lower respiratory tract. While any of the above routes of delivery results in an immune response, intranasal administration confers the added benefit of eliciting mucosal immunity at the site of entry of many viruses, including alphaviruses, for example CHIKV or WEEV, or flaviviruses.

Thus, the invention also comprises a method of formulating a vaccine or antigenic composition that induces immunity to an infection or at least one symptom thereof to a mammal, comprising adding to said formulation an effective dose of VLPs, e.g., alphavirus (e.g., CHIKV or WEEV) or flavivirus VLPs. In one embodiment, the infection is an alphavirus infection, for example, but not limited to, EEEV, WEEV, VEEV, SFV, CHIKV, O'nyong-nyong virus, Sindbis virus, Mayaro virus, Ross River virus, Barmah Forest virus, and Ockelbo virus. In another embodiment, the infection is a flavivirus infection.

In certain cases, stimulation of immunity with a single dose is preferred, however additional dosages can be also be administered, by the same or different route, to achieve the desired effect. In neonates and infants, for example, multiple administrations may be required to elicit sufficient levels of immunity. Administration can continue at intervals throughout childhood, as necessary to maintain sufficient levels of protection against infections. Similarly, adults who are particularly susceptible to repeated or serious infections, such as, for example, health care workers, day care workers, family members of young children, the elderly, and individuals with compromised cardiopulmonary function or immune systems may require multiple immunizations to establish and/or maintain protective immune responses. Levels of induced immunity can be monitored, for example, by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to elicit and maintain desired levels of protection.

Prime Boost

The present methods also include a variety of prime-boost regimens. In these methods, one or more priming immunizations is followed by one or more boosting immunizations. The actual immunogenic composition can be the same or different for each immunization and the type of immunogenic composition (e.g., containing protein or expression vector), the route, and formulation of the immunogens can also be varied.

For example, in one embodiment, the prime comprises administering a DNA or gene-based vaccine as described herein and the boost comprises administering a VLP as described herein. In another embodiment, the prime comprises administering a VLP as described herein and the boost comprises administering a DNA or other gene-based vaccine as described herein.

One useful prime-boost regimen provides for two priming immunizations, four weeks apart, followed by two boosting immunizations at 4 and 8 weeks after the last priming immunization. It should also be readily apparent to one of skill in the art that there are several permutations and combinations that are encompassed using the DNA, bacterial and viral expression vectors of the invention to provide priming and boosting regimens.

Methods of administering a composition comprising VLPs and/or DNA vaccines (vaccine and/or antigenic formulations) include, but are not limited to, parenteral administration (e.g., intradermal, intramuscular, intravenous and subcutaneous), epidural, and mucosal (e.g., intranasal and oral or pulmonary routes or by suppositories). In a specific embodiment, compositions of the present invention are administered intramuscularly, intravenously, subcutaneously, transdermally or intradermally. The compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucous, colon, conjunctiva, nasopharynx, oropharynx, vagina, urethra, urinary bladder and intestinal mucosa, etc.) and may be administered together with other biologically active agents. In some embodiments, intranasal or other mucosal routes of administration of a composition comprising VLPs of the invention may induce an antibody or other immune response that is substantially higher than other routes of administration. In another embodiment, intranasal or other mucosal routes of administration of a composition comprising VLPs of the invention may induce an antibody or other immune response that will induce cross protection against other strains of the virus. Administration can be intramuscular, subdermal, intraperitoneal. In one preferred embodiment, the administration is intramuscular.

In yet another embodiment, the vaccine and/or antigenic formulation is administered in such a manner as to target mucosal tissues in order to elicit an immune response at the site of immunization. For example, mucosal tissues such as gut associated lymphoid tissue (GALT) can be targeted for immunization by using oral administration of compositions which contain adjuvants with particular mucosal targeting properties. Additional mucosal tissues can also be targeted, such as nasopharyngeal lymphoid tissue (NALT) and bronchial-associated lymphoid tissue (BALT).

Vaccines and/or antigenic formulations of the invention may also be administered on a dosage schedule, for example, an initial administration of the vaccine composition with subsequent booster administrations. In particular embodiments, a second dose of the composition is administered anywhere from two weeks to one year, preferably from about 1, about 2, about 3, about 4, about 5 to about 6 months, after the initial administration. Additionally, a third dose may be administered after the second dose and from about three months to about two years, or even longer, preferably about 4, about 5, or about 6 months, or about 7 months to about one year after the initial administration. The third dose may be optionally administered when no or low levels of specific immunoglobulins are detected in the serum and/or urine or mucosal secretions of the subject after the second dose. In a preferred embodiment, a second dose is administered about one month after the first administration and a third dose is administered about six months after the first administration. In another embodiment, the second dose is administered about six months after the first administration. In another embodiment, said VLPs of the invention can be administered as part of a combination therapy. For example, VLPs of the invention can be formulated with other immunogenic compositions, antivirals and/or antibiotics. A VLP may be administered concurrently, subsequent to, or sequentially with another immunogenic composition, antiviral, antibiotic, or any other agent that prevents or treats an alphavirus infection (e.g., CHIKV or WEEV infection).

The dosage of the pharmaceutical formulation can be determined readily by the skilled artisan, for example, by first identifying doses effective to elicit a prophylactic or therapeutic immune response, e.g., by measuring the serum titer of virus specific immunoglobulins or by measuring the inhibitory ratio of antibodies in serum samples, or urine samples, or mucosal secretions. Said dosages can be determined from animal studies. A non-limiting list of animals used to study the efficacy of vaccines include the guinea pig, hamster, ferrets, chinchilla, mouse and cotton rat, and non-human primates. Most animals are not natural hosts to infectious agents but can still serve in studies of various aspects of the disease. For example, any of the above animals can be dosed with a vaccine candidate, e.g., VLPs of the invention, to partially characterize the immune response induced, and/or to determine if any neutralizing antibodies have been produced. For example, many studies have been conducted in the mouse model because mice are small size and their low cost allows researchers to conduct studies on a larger scale.

In addition, human clinical studies can be performed to determine the preferred effective dose for humans by a skilled artisan. Such clinical studies are routine and well known in the art. The precise dose to be employed will also depend on the route of administration. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal test systems.

As also well known in the art, the immunogenicity of a particular composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Adjuvants have been used experimentally to promote a generalized increase in immunity against unknown antigens (e.g., U.S. Pat. No. 4,877,611). Immunization protocols have used adjuvants to stimulate responses for many years, and as such, adjuvants are well known to one of ordinary skill in the art. Some adjuvants affect the way in which antigens are presented. For example, the immune response is increased when protein antigens are precipitated by alum. Emulsification of antigens also prolongs the duration of antigen presentation. The inclusion of any adjuvant described in Vogel et al., "A Compendium of Vaccine Adjuvants and Excipients (2nd Edition)," herein incorporated by reference in its entirety for all purposes, is envisioned within the scope of this invention.

Exemplary adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*), incomplete Freund's adjuvants and aluminum hydroxide adjuvant. Other adjuvants comprise GMCSP, BCG, aluminum hydroxide, MDP compounds, such as thur-MDP and nor-MDP, CGP (MTP-PE), lipid A, and monophosphoryl lipid A (MPL). RIBI, which contains three components extracted from bacteria, MPL, trehalose dimycolate (TDM) and cell wall skeleton (CWS) in a 2% squalene/Tween-80 emulsion also is contemplated. MF-59, Novasomes®, MHC antigens may also be used.

The VLPs of the invention can also be formulated with "immune stimulators." These are the body's own chemical messengers (cytokines) to increase the immune system's response. Immune stimulators include, but not limited to, various cytokines, lymphokines and chemokines with immunostimulatory, immunopotentiating, and pro-inflammatory activities, such as interleukins (e.g., IL-1, IL-2, IL-3, IL-4, IL-12, IL-13); growth factors (e.g., granulocyte-macrophage (GM)-colony stimulating factor (CSF)); and other immunostimulatory molecules, such as macrophage inflammatory factor, Flt3 ligand, B7.1; B7.2, etc. The immunostimulatory molecules can be administered in the same formulation as the VLPs, or can be administered separately. Either the protein or an expression vector encoding the protein can be administered to produce an immunostimulatory effect. Thus in one embodiment, the invention comprises antigenic and vaccine formulations comprising an adjuvant and/or an immune stimulator.

Methods of Delivery

The VLPs of the invention are useful for preparing compositions that stimulate an immune response. Such compositions are useful for the treatment or prevention or an alphavirus infection (e.g., a CHIKV, WEEV, EEEV, VEEV, Ross River virus, or Barmah Forest virus infection) or a flavivirus. Both mucosal and cellular immunity may contribute to immunity to infectious agents and disease. In one embodiment, the invention encompasses a method of inducing immunity to an alphavirus infection, for example CHIKV, WEEV, EEEV, VEEV, Ross River virus, or Barmah Forest virus infection in a subject, by administering to the subject a CHIKV, WEEV, EEEV, VEEV, Ross River virus, or Barmah Forest virus VLP or a DNA vaccine. In another embodiment, the invention encompasses a method of inducing immunity to a flavivirus infection by administering to the subject a flavivirus VLP or a DNA vaccine.

The invention also provides a method to induce immunity to viral infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of a VLP or DNA vaccine as described herein, for example a VLP comprising one or more viral proteins, for example one or more alphavirus or flavivirus virus envelope proteins, or a DNA vaccine comprising a nucleic acid segment encoding one or more alphavirus or flavivirus envelope proteins, or fragments thereof. In certain cases, the VLP further comprises an alphavirus or flavivirus capsid protein. In another embodiment, the method comprises inducing immunity to a viral infection, e.g., alphavirus or flavivirus infection, or at least one symptom thereof by administering said formulation in multiple doses.

VLPs of the invention can induce substantial immunity in a vertebrate (e.g., a human) when administered to said vertebrate. The substantial immunity results from an immune response against VLPs of the invention that protects or ameliorates infection or at least reduces a symptom of infection in said vertebrate. In some instances, if the said vertebrate is infected, said infection will be asymptomatic. The response may not be a fully protective response. In this case, if said vertebrate is infected with an infectious agent, the vertebrate will experience reduced symptoms or a shorter duration of symptoms compared to a non-immunized vertebrate.

In one embodiment, the invention comprises a method of inducing substantial immunity to alphavirus infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of a VLP and/or a DNA vaccine comprising a nucleic acid segment encoding an alphavirus or flavivirus capsid protein or one or more alphavirus or flavivirus envelope proteins or fragments thereof, where the VLP has one or more alterations in an E2 protein and/or a alphavirus capsid protein Nuclear Localization Signal (NLS). In various embodiments, the VLP includes an envelope protein having a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein and/or a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding. In particular embodiments, the infection is CHIKV or WEEV and the VLP comprises one or more CHIKV or WEEV envelope proteins as described herein. In another embodiment, the invention comprises a method of vaccinating a mammal against an alphavirus comprising administering to said mammal a protection-inducing amount of VLPs or DNA vaccines comprising at least one alphavirus protein. In one embodiment, said method comprises administering DNA vaccines comprising alphavirus capsid, E3, E2, 6K and E1. In another embodiment, said method comprises administering DNA vaccines comprising alphavirus E3, E2, 6K and E1. In another embodiment, said method comprises administering DNA vaccines comprising C-Env_{37997}. In another embodiment, said method comprises administering DNA vaccines comprising Env_{37997}. In another embodiment, said method comprises administering DNA vaccines comprising CMV/R CHIKV C-E3-E2-6K-E1 (strain OPY1) E2 K234N. In another embodiment, said method comprises administering DNA vaccines comprising CMV/R WEEV C-E3-E2-6K-E1 (strain 71V-1658) E2 K235N. In another embodiment, said method comprises administering DNA vaccines comprising VLPC-E2(37997). In another embodiment, said method comprises administering DNA vaccines comprising VLPOPY-1 E2(37997). In one embodiment, said method comprises administering VLPs comprising alphavirus capsid, E3, E2, 6K, and E1. In another embodiment, said method comprises administering VLPs comprising alphavirus E3, E2, 6K, and E1. In one embodiment, said method comprises administering VLPs comprised of CHIKV or WEEV envelope proteins. In various embodiments, the VLP comprises an alphavirus (CHIKV, WEEV, EEEV, VEEV, Ross River virus, Barmah Forest virus) capsid protein in which the nuclear localization sequence is altered. The alphavirus capsid protein nuclear localization sequence is altered in a charged residue (e.g., lysine or arginine), which is replaced with a non-charged residue (e.g., alanine or asparagine). In specific embodiments, the alphavirus capsid protein is a WEEV CBA87 strain capsid protein having one or more of the alterations K67N, K68N, and/or K69N. In certain embodiments, the alphavirus capsid protein is a VEEV TC83 strain capsid protein having one or more of the alterations K64N, K65A, K65N, K67A, and/or K67N. In some embodiments, the alphavirus capsid protein is a EEEV PE-6 strain capsid protein having an alteration K67N. In particular embodiments, the alphavirus capsid protein is a CHIKV(Strain 37997) strain capsid protein having one or more of the alterations R62A, R63A, R65A, K66A, K68A, and/or K69A. In specific embodiments, the alphavirus capsid protein is a Ross River Virus capsid protein having one or more of the alterations R71N, K72N, K73N, and/or K74N. In specific embodiments, the alphavirus capsid protein is a Barmah Forest Virus capsid protein having one or more of the alterations K64A, K64N, K65A, K65N, K67A, K67N, K68A and/or K68N.

In another embodiment, the invention comprises a method of vaccinating a mammal against a flavivirus comprising administering to said mammal a protection-inducing amount of VLPs or DNA vaccines comprising at least one flavivirus protein. In another embodiment, said method comprises administering VLPs comprising flavivirus envelope protein. In another embodiment, said method comprises administering VLPs comprising flavivirus capsid protein.

In another embodiment, the invention comprises a method of inducing a protective cellular response to a viral infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of a DNA vaccine or a VLP.

As mentioned above, the VLPs of the invention prevent or reduce at least one symptom of an infection in a subject. A reduction in a symptom may be determined subjectively or objectively, e.g., self assessment by a subject, by a clinician's assessment or by conducting an appropriate assay or measurement (e.g., body temperature), including, e.g., a quality of life assessment, a slowed progression of viral infection or additional symptoms, a reduced severity of viral symptoms or a suitable assays (e.g., antibody titer and/or T-cell activation assay). The objective assessment comprises both animal and human assessments.

The invention also provides assays to identify inhibitors of viral entry comprising, in at least one embodiment, genetically modified target cells expressing at least one alphavirus (e.g., CHIKV or WEEV) or flavivirus viral receptor, together with any co-receptors which might be required for infection or entry. These cells are genetically modified in the sense that they express a reporter gene, such as an affinity tag, a fluorogenic protein or an enzyme able to convert substrates into fluorogenic, chromogenic or luminometric products. Coupling this type of reporter signal to an inhibition of viral infection is accomplished by arranging the expression of the reporter gene to be strongly decreased (downregulated) upon infection with the virus of interest. In principle, this can be ensured by any suitable means, but especially preferred are:

The reporter gene product itself is fused to a cellular protein which, upon infection with the virus of interest is itself downregulated. For example, the reporter gene product can be fused to the corresponding viral receptor, which in many cases is downregulated upon infection.

Thus in one aspect a compound library may be screened for the ability to inhibit the infection of cells with alphavirus (e.g., CHIKV, WEEV, EEEV, VEEV, Ross River virus, or Barmah Forest virus) or flavivirus. An appropriate indicator cell line is generated that stably expresses a reporter gene. In one example, these cells are seeded in microtiter plates and incubated with alphavirus (e.g., CHIKV, WEEV, EEEV, VEEV, Ross River virus, or Barmah Forest virus) or flavivirus particles in the presence of different compounds, e.g., antibodies, in each well. Upon infection, the fusion protein is downregulated due to the expression of the viral genes. Consequently, only cells that have not been infected with CHIKV will express the reporter gene. Thus, wells that exhibit a positive reporter signal contain compounds that inhibit infection. Variations and modifications of these assays will be apparent from the relevant sections of the description which explain individual parts of the assay in more detail. Specifically, in one embodiment, the reporter gene can be expressed when infection occurs rather than the reporter gene being downregulated upon infection. In further embodiments, the viral particles are pseudotyped viral particles comprising one or more envelope protein and, optionally, the capsid protein from alphavirus (e.g., CHIKV, WEEV, EEEV, VEEV, Ross River virus, or Barmah Forest virus) or flavivirus.

In another embodiment, the invention provides methods for identifying inhibitors of viral entry using a reporter gene system as exemplified herein. Briefly, the invention provides recombinant lentiviral vectors expressing a reporter gene. Cells are incubated and co-transfected with an expression vector, e.g., $Env_{OPY\text{-}1}$, and a reporter plasmid using a standard techniques.

Cells are plated into one day prior to infection. Alphavirus or flavivirus Env-pseudotyped lentiviral vectors encoding the reporter gene are first titrated by serial dilution. Similar amounts of pseudotyped vectors are then incubated with the candidate inhibitors prior to adding the virus. Cells are then lysed using cell lysis buffer and the reporter gene activity is measured Inhibitors of viral entry are identified based on the expression of the reporter gene.

Kits

The invention also provides for a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the vaccine formulations of the invention. In a preferred embodiment, the kit comprises two containers, one containing VLPs and the other containing an adjuvant. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

The invention also provides that the VLP formulation be packaged in a hermetically sealed container such as an ampoule or sachette indicating the quantity of composition. In one embodiment, the VLP composition is supplied as a liquid, in another embodiment, as a dry sterilized lyophilized powder or water free concentrate in a hermetically sealed container and can be reconstituted, e.g., with water or saline to the appropriate concentration for administration to a subject.

The invention also features a kit comprising a VLP as described herein. The invention also features kits comprising a DNA vaccine as described herein and instructions for use.

The invention also features a kit comprising a VLP in a first container and a DNA vaccine in a second container, and instructions for use in a prime boost immunization.

The following examples are offered by way of illustration, not by way of limitation. While specific examples have been provided, the above description is illustrative and not restrictive. Any one or more of the features of the previously described embodiments can be combined in any manner with one or more features of any other embodiments in the present invention. Furthermore, many variations of the invention will become apparent to those skilled in the art upon review of the specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook, 1989); "Oligonucleotide Synthesis" (Gait, 1984); "Animal Cell Culture" (Freshney, 1987); "Methods in Enzymology" "Handbook of Experimental Immunology" (Weir, 1996); "Gene Transfer Vectors for Mammalian Cells" (Miller and Calos, 1987); "Current Protocols in Molecular Biology" (Ausubel, 1987); "PCR: The Polymerase Chain Reaction", (Mullis, 1994); "Current Protocols in Immunology" (Coligan, 1991). These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the assay, screening, and therapeutic methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention.

EXAMPLES

Example 1

Lentiviral Vectors Pseudotyped with CHIKV Envelope Mediated Entry Through the Same Mechanism as Wild Type Virus

To examine the mechanism and specificity of CHIKV cell entry, lentiviral vector reporters were pseudotyped with glycoproteins from different CHIKV strains that mediate entry into permissive cells. The CHIKV spike on the virion surface is formed by three E1-E2 heterodimers, where E1 glycoproteins mediate fusion and E2 glycoproteins interact with the host receptor. CHIKV E genes expressing the native polypeptide, E3-E2-6K-E1 polyprotein, for the 37997 and for LR2006 OPY-1 strains were inserted into an expression vector (E37997 and EOPY-1) (FIG. **1**A, FIGS. **6**, **7**A, **7**B, and **8**A-**8**C). The incorporation of the two CHIKV Es into the pseudotyped lentiviral vectors was verified by buoyant density gradient sedimentation of the virus. Both CHIKV E and HIV-1 Gag had the same buoyant density as lentivirus particles (FIG. **5**). The 37997 and LR2006 OPY-1 CHIKV pseudotyped lentiviral vectors infected several permissive cell lines (Sourisseau et al., *PLoS. Pathog.* 3, e89 (2007)) as measured by luciferase reporter activity, while a control devoid of CHIKV envelope proteins did not infect these cell lines (FIG. **1**B, left), and infectivity was dose-dependent (FIG. **1**B, right).

To determine whether entry occurred through the same mechanism as native virus, the pH and endosome dependence of entry was analyzed as described previously (Yang et al., *J. Virol.* 78, 5642 (2004)). CHIKV infects cells through a process of pH-dependent cell fusion. Thus, addition of ammonium chloride or chloroquine, which prevents acidification of the endosome, caused a dose-dependent reduction in CHIKV pseudotyped vector entry (FIG. **1**C). Similar inhibition of entry was observed with VSV-G, known to enter in this fashion, but not with amphotropic murine leukemia virus (MuLV) glycoprotein 70, which enters in a pH-independent fashion. These findings demonstrated that lentiviral vectors pseudotyped with CHIKV envelope mediated entry through the same mechanism as wild type virus. Sera from mice injected with a CHIKV strain were next examined. Incubation of immune sera with the CHIKV pseudotyped lentiviral vector but not VSV-G pseudotyped vector inhibited entry (FIG. **1**D). The specificity and potency of neutralizing antibodies could therefore be quantified without exposure to infectious virus.

Example 2

VLPs have Morphology of Wild Type Virus

CHIKV encodes 4 nonstructural proteins, NS1, NS2, NS3 and NS4, which are involved in virus replication, and 5 structural proteins, which consist of capsid (C) and envelope proteins (E; E1, E2, E3 and 6K) that are synthesized as polyproteins and are cleaved by capsid autoproteinase and signalases (Strauss, *Microbiol. Rev.* 58, 491 (1994)). Eukaryotic expression vectors encoding C-E3-E2-6K-E1 from strains 37997 and LR2006 OPY-1 (C-E37997 and C-EOPY-1) were analyzed for their ability to give rise to VLP. The plasmids C-E37997 or C-EOPY-1 or the expression vectors described above, E37997 or EOPY-1 (FIG. **2**A, upper panel), were transfected into human embryonic kidney (293T) cells, and expression was confirmed by Western blotting (FIG. 2A, lower panel). C and E1/E2 proteins were detected in the supernatant after transfection of the C-E37997 or C-EOPY-1 vector, suggesting that CHIKV VLPs had been generated. VLPs were purified by buoyant density gradient sedimentation. The yield of VLPs from strain 37797 was 10-20 mg/L, approximately 100 times higher than that from strain LR2006 OPY-1; strain 37997 was therefore chosen for further VLP characterization and development. Fractionation of clarified supernatant showed peak incorporation of E1/E2 at a density of 1.2 g/ml (FIG. 2B, left), comparable to the density of wild type CHIKV. Examination of the purified fraction from strain 37997 by electron microscopy revealed VLPs with the same morphologic appearance as wild type virus (FIG. 2B, right).

Cryoelectron microscopy and three dimensional image reconstruction assuming icosahedral symmetry showed that the VLPs had an external diameter of 65 nm and a core diameter of 40 nm (FIG. 2C, left). The potent immunogenic E1/E2 glycoproteins are organized into 240 heterodimers, assembled into 80 glycoprotein spikes arranged with T=4 quasi symmetry on the surface of the VLPs (FIG. 2C, left), closely similar to the structure of Sindbis virus (FIG. 2C, right). In addition, the organization of the nucleocapsid core is also remarkably similar to that of other alphaviruses.

Example 3

VLPs Induced a More Potent Neutralizing Antibody Response to CHIKV than DNA Vaccines

The immunogenicity of DNA and VLP vaccines was determined in mice immunized with DNA vaccines encoding C-E or E (strains 37997 and LR2006 OPY-1) or VLPs from strain 37997 (VLP37997) in the presence or absence of Ribi adjuvant. Mice injected with VLPs with adjuvant generated the highest titer neutralizing responses against both the homologous strain 37997 (FIG. 3A, right panel; IC50, 1:10,703) and the heterologous strain LR2006 OPY-1 (FIG. 3B, right panel; IC50, 1:54,600). While immunization with the plasmids encoding C-E and E from both strains elicited neutralizing responses, these responses were 100-fold lower than the VLP-immunized mice (FIG. 3A, B; left panel). These results indicate that VLPs elicited a more potent neutralizing antibody response than DNA vaccines.

To characterize VLP-induced immune responses in a model with strong predictive value for humans, rhesus macaques were immunized with VLPs. Monkeys were injected with VLP37997 or PBS alone as a control. Sera from immunized and control monkeys were tested against CHIKV strain 37997 and LR2006 OPY-1 pseudotyped lentiviral vectors. All non-human primates (NHP) immunized with VLPs developed substantial neutralizing activity to both homologous and heterologous strains after primary immunization that increased after boosting (FIG. 3C; left panel: strain 37997, right panel: strain LR2006 OPY-1). To confirm that these antibodies neutralized infectious virus, a plaque reduction neutralization test (PRNT) was performed against the CHIKV LR2006 OPY-1. The antisera from the immunized monkeys elicited neutralizing antibody responses against LR2006 OPY-1 at titers that exceeded 1:40,000 (FIG. 3D). These data suggested that neutralizing antibodies using pseudotyped lentiviral vectors correlated with the PRNT assay, and that all immunized monkeys generated potent neutralizing antibody responses against CHIKV.

Example 4

Primate VLP Immunization Protected Against Viremia and Inflammatory Consequences of CHIKV Infection

The ability of the VLP vaccine to protect against infection was determined by intravenous challenge of monkeys immunized with VLPs or controls using a high titer LR2006 OPY-1 virus stock 15 weeks after the final immunization. Similar to humans, infection in the NHP resulted in non-lethal viremia and a pro-inflammatory response as measured by an increase in monocyte counts. The control monkeys showed viremia beginning at 6 hours and lasting until 72 hours after challenge, while all of the immunized monkeys controlled the challenge virus completely (FIG. 4A). Similarly, the monocyte counts in control monkeys increased markedly relative to vaccinated monkeys by 4 days after challenge (FIG. 4B, Control vs. VLPs; p=0.0036). These data indicated that immunization protected against viremia as well as the inflammatory consequences of infection. To define the mechanism of protection in these animals, the question of whether immune IgG could protect against lethal challenge was examined using an adoptive transfer model.

Example 5

Humoral Immune Responses Induced by CHIKV VLPs Conferred Protection Against CHIKV Infection

Previous studies have shown that immunodeficient mice with defective type-I IFN signaling developed severe infection, displaying symptoms and tissue tropism analogous to humans, and providing a model to evaluate immune mechanisms of protection. Purified total IgG from immune or control monkeys was passively transferred into these mice. The recipient mice were challenged intradermally 24 hours after IgG transfer with a lethal dose of LR2006 OPY-1. Recipients of purified CHIKV immune IgG demonstrated no detectable viremia after infection and were completely protected from lethality (FIGS. 4C, D). In contrast, all mice that received purified IgG from control monkeys showed severe infection and viremia, and all died. These results indicate that humoral immune responses induced by CHIKV VLPs conferred protection against CHIKV infection.

Example 6

Importance of Amino Acid 234 in the E2 Protein of CHIKV in VLP Production

Although the two strains are highly related, with 95% amino acid similarity between the sequences of the structural genes, VLPs are produced efficiently by the CHIKV 37997 strain, but yields of VLPs from the OPY-1 strain are very low. In order to understand the mechanism underlying the difference in VLP production between the CHIKV strains, genes between the 37997 and the OPY-1 strains were swapped and VLP production was assessed. Specifically, using the C-E3-E2-6K-E1 expression vector, either capsid (C) alone, C-E3, C-E3-E2, or C-E3-E2-6K regions from $CHIKV_{37997}$ was inserted into the expression vector using an overlap extension PCR method.

The chimeric genes between 37997 and OPY-1 strains were amplified using the primers shown in Table 1.

TABLE 1

Chimeric VLP expression vector primers (SEQ ID NOS 8-57, respectively, in order of appearance)	
CHIKV 37997 F	CTCTAGACACCATGGAGTTCATCCC
CHIKV 37997 R	TGGATCCTCATTAGTGCCTGCTAAACGACA
CHIKV OPY-1 F	ATATCGCGGCCGCTCTAGAC
CHIKV OPY-1 R	TGGATCCTCATTAGTGCCTGCTGAACGACA
CHIKV VLP$_{C(37997)}$ F	TACCCCTGAGGGAGCCGAAGAGTGGAGTCTTGCCAT CCCAGTTATGTGCC
CHIKV VLP$_{C(37997)}$ R	CCACTCTTCGGCTCCCTCAGGGGTAA
CHIKV VLP$_{C-E3(37997)}$ F	TTGCTCTCCCCACCGCCAAAGACGCAGCACCAAGGA CAACTTCAATGTCT
CHIKV VLP$_{C-E3(37997)}$ R	GCGTCTTTGGCGGTGGGGAGAGCAA
CHIKV VLP$_{C-E2(37997)}$ F	ATGCTGCGTCAGAACGACCAAGGCGGCCACATACCA AGAGGCTGCGATAT
CHIKV VLP$_{C-E2(37997)}$ R	CGCCTTGGTCGTTCTGACGCAGCAT
CHIKV VLP$_{C-6K(37997)}$ F	CATCGGTGCCCACACTGTGAGCGCGTACGAACACGT AACAGTGATCCCGA
CHIKV VLP$_{C-6K(37997)}$ R	CGCGCTCACAGTGTGGGCACCGATG
CHIKV VLP$_{OPY-1\ E2(37997)}$ F	ATATCGCGGCCGCTCTAGAC
CHIKV VLP$_{OPY-1\ E2(37997)}$ R	GCGTCGCTGGCGGTGGGGAGAACAT
CHIKV VLP$_{OPY-1\ E2(37997)}$ F	ATGTTCTCCCCACCGCCAGCGACGCAGTACTAAGGA CAATTTTAATGTCT
CHIKV VLP$_{OPY-1\ E2(37997)}$ R	TGGATCCTCATTAGTGCCTGCTGAA
CHIKV VLP$_{OPY-1\ 5'-E2(37997)}$ F	CCATGCTGCTGTATCCTGACCACCCAACACTCCTGTC CTA
CHIKV VLP$_{OPY-1\ 5'-E2(37997)}$ R	TAGGACAGGAGTGTTGGGTGGTCAGGATACAGCAGC ATGG
CHIKV VLP$_{OPY-1\ 3'-E2(37997)}$ F	TCATGCTACTGTATCCTGACCATCCGACACTCTTGTC TTA
CHIKV VLP$_{OPY-1\ 3'-E2(37997)}$ R	TAAGACAAGAGTGTCGGATGGTCAGGATACAGTAGC ATGA
Mutagenesis primers	
CHIKV I32V	CATTCGTGCCACAGCCCTGTCGCATTGGAGC
CHIKV I32V_antisense	GCTCCAATGCGACAGGGCTGTGGCACGAATG
CHIKV S72N	ACCAAGCTGCGCTATATGGATAACCATACGCCAGC
CHIKV S72N_antisense	GCTGGCGTATGGTTATCCATATAGCGCAGCTTGGT
CHIKV T74M	TGCGCTATATGGATAGCCATATGCCAGCGGACG
CHIKV T74M_antisense	CGTCCGCTGGCATATGGCTATCCATATAGCGCA
CHIKV L84F	GGAGCGAGCCGGATTGTTTGTAAGGACTTCAGC
CHIKV L84F_antisense	GCTGAAGTCCTTACAAACAATCCGGCTCGCTCC
CHIKV T124S	CAGAAAGATCAGCCACTCATGCACACACCCGTT
CHIKV T124S_antisense	AACGGGTGTGTGCATGAGTGGCTGATCTTTCTG
CHIKV E132D	CACACACCCGTTCCATCATGATCCACCTGTGATA
CHIKV E132D_antisense	TATCACAGGTGGATCATGATGGAACGGGTGTGTG
CHIKV R140K	GTGATAGGTAGGGAGAAGTTCCACTCTCGACCA

TABLE 1-continued

Chimeric VLP expression vector primers (SEQ ID NOS 8-57, respectively, in order of appearance)	
CHIKV R140K_antisense	TGGTCGAGAGTGGAACTTCTCCCTACCTATCAC
CHIKV A164T	GCACCGCTGCCACTACTGAGGAGATAGAG
CHIKV A164T_antisense	CTCTATCTCCTCAGTAGTGGCAGCGGTGC
CHIKV T182S	CCGCACGCTGATGTCGCAGCAGTCTGG
CHIKV T182S_antisense	CCAGACTGCTGCGACATCAGCGTGCGG
CHIKV I222V	AAGTGATCAATAACTGCAAAGTTGATCAGTGCCATG CTGC
CHIKV I222V_antisense	GCAGCATGGCACTGATCAACTTTGCAGTTATTGATC ACTT
CHIKV N234K	GCTGCAGTCACTAATCACAAGAAGTGGCAATACAAC TC
CHIKV N234K_antisense	GAGTTGTATTGCCACTTCTTGTGATTAGTGACTGCAG C
CHIKV T284I	CGGAAAAAACCAAGTCATCATGCTGCTGTATCCTG
CHIKV T284I_antisense	CAGGATACAGCAGCATGATGACTTGGTTTTTTCCG
CHIKV OPY-1 K234N	CCGCGGTCACCAATCACAAAAATTGGCAGTATAAC
CHIKV OPY-1 K234N_antisense	GTTATACTGCCAATTTTTGTGATTGGTGACCGCGG
WEEV K235N	CTACAAGAGCGACCAAACGAATTGGGTCTTCAACTC
WEEV K235N_antisense	GAGTTGAAGACCCAATTCGTTTGGTCGCTCTTGTAG
CHIKV OPY-1 K233N	GCCGCGGTCACCAATCACAATAAGTGGCAGTA
CHIKV OPY-1 K233N_anti	TACTGCCACTTATTGTGATTGGTGACCGCGGC

Briefly, two fragments of the chimeric genes were amplified with 40 to 51 base pairs of primers that overlapped by 20 oligonucleotides and either of CHIKV 37997 F, CHIKV 37997 R, CHIKV OPY-1 F, or CHIKV OPY-1 R primers. These two fragments were assembled in the overlapping region and amplified again with CHIKV 37997 F, CHIKV 37997 R, CHIKV OPY-1 F, or CHIKV OPY-1 R primers. The PCR products were cloned into the C-EOPY-1 expression vector after confirming the sequence. Chimeric CHIKV were made using the PCR-based Quickchange (Stratagene, La Jolla, Calif.) method according to the manufacturer's instructions with sense and anti-sense primers shown in Table 1. The OPY-1 genes of either the capsid alone, the capsid to E3, the capsid to E2 or the capsid to 6K were replaced with the corresponding genes from 37997 ($VLP_{C(37997)}$, $VLP_{C-E3(37997)}$, $VLP_{C-E2(37997)}$ or $VLP_{C-6K(37997)}$). Each mutant was confirmed by sequencing, and the chimeric CHIKV expression vectors are depicted in FIG. **6**A.

293F cells were transfected with these plasmids and the expression of CHIKV structural proteins and yield of VLPs in the supernatant and cell lysate were determined using Western blot analysis (FIG. **6**B). Expression of capsid and E1/E2 in cell lysates was similar for all of the plasmids, but VLP release in supernatants was significantly different (FIG. **6**B). Notably, the yield of OPY-1 VLP increased when the 37997 strain E2 region was included in the vector (FIG. **6**B, lane 4, upper panel). To determine which region was responsible for this increase, the different polypeptide regions of 37997 were inserted into the OPY-1 expression vector. This analysis revealed that the E2 region alone was responsible for increased VLP production. Replacement of E2 enhanced VLP as determined by Commassie blue staining of sucrose density sedimentation purified particles (FIG. **6**C, lane 13). To map the subregion responsible for this effect, chimeras that further subdivided E2 were prepared. The NH_2-terminal E2 domain (E2 1-290 a.a.) or the COOH-terminal E2 domain (E2 291-423 a.a.) was replaced in the VLP_{OPY-1} expression vector. VLP production in transfected cells revealed that the NH_2-terminal region (1-290 a.a.) was necessary and sufficient for efficient VLP synthesis (FIG. **6**C, lane 14).

The sequences of 37997 and OPY-1 differed in this region by twelve amino acids (FIG. **7**A). To determine which amino acids were critical for VLP generation, site-specific mutations were introduced individually at these sites. Eleven of the twelve mutants synthesized VLPs at levels similar to the NH_2-terminal chimeric E2 expression vector (WT) (FIG. **7**B). In contrast, the N234K mutation from OPY-1 abolished VLP release (FIG. **7**B, lane 11), indicating that this amino acid residue played a critical role in the regulation of VLP synthesis.

To determine whether modification of amino acid residue 234 from strain 37997 could improve VLP yield, this site was mutated from K to N in the OPY-1 expression vector (VLP_{OPY-1} K234N). Though expression of envelope from VLP_{OPY-1} K234N on the cell surface was similar to that of VLP_{OPY-1} (FIG. **8**A), VLP release was increased by 86-fold compared to the parental OPY-1 expression vector (FIG. **8**B). It was hypothesized that VLP_{OPY-1} has a role in pH-dependent VLP yield, and that the VLP_{OPY-1} K234N mutant is insensitive to pH. To test this hypothesis, the yield of VLP mutants was compared at pH 7.0 (wt), 7.3, 7.5, and 7.8. VLP_{OPY-1} yield increased at pH 7.3, 7.5, and 7.8, while the VLP_{OPY-1} K234N mutant yield did not increase at high pH (FIG. **8**C).

That alphaviruses conserve lysine (K) at position 234 (Voss et al., *Nature* 468:709-712 (2010)), indicates that 234K plays important roles for the virus life cycle. Based on recent structure determination of CHIKV OPY-1 E2 protein, E2 234K is in an acid sensitive region (ASR) that was disordered in the alphavirus E2-E1 structure at low pH (Voss et al., Nature 468:709-712; Li L, 2010 nature p 705). This region initiates a conformational change in E1/E2 virus spikes in acidic pH conditions. The conformational changes allow the hydrophobic fusion peptide loop of E1 to interact with the cellular membrane and initiate fusion. The exposure of different pH conditions to Semliki Forest virus (SFV) particle changed the conformation of E1/E2 (Wu. SR, 2007, J of Biological chemistry, p 6752). The pH condition is also important for the budding process. The budding of SFV became efficient when the infected cells were incubated at a pH above 7.5 than at a pH below 7.0 (J of Virology. 2001, p 8329-8339, Lu et al.).

Therefore, it was examined whether pH conditions in the medium increase the yield of VLP_{OPY-1}. The VLP yield was significantly increased in higher pH conditions (FIG. **57**A). GFP expression as an expression indicator was similar level in different pH condition, suggesting that there was no protein expression effect in pH condition. Without being bound to a particular theory, it was hypothesized that K234N mutant might decrease the activity of the pH sensor amino acids and result in the increase of VLP yield. Histidine amino acids in E1/E2 play important roles for the pH dependent conformational change, as the pK is in the same range as the pH of the conformational transition (Voss et al., Nature 468:709-712). Without being bound to a particular theory, it was hypothesized that 232H might play an important role as a pH sensor because 234K and 232H are in close proximity with one another based on the structure model (FIG. **57**B). To test this hypothesis, it was examined whether additional alternation of this specific region of E2 also affected VLP production (FIG. **57**C). Although several point mutations in 232H were introduced to be insensitive to pH, none of the mutants produced the VLPs, possibly due to destabilization by the mutations. It was found that K233N or H256Q in ASR region mutants increased the yield of VLPs (FIG. **57**C, lane 6, 9, 11 and 14). Because K233N mutant is next to 232H, it might have the same effect as the K234N mutant. In addition, mutation E2 H170M was also introduced into the OPY-1 strain, as these amino acids interact strongly with a salt bridge and one hydrogen bond between E2 and E1 (Voss et al., Nature 468:709-712). The mutant E2 H170M increased the yield of OPY-1 VLPs (FIG. **57**C, lane 7 and 12). These mutants suggesting that inactivation of amino acids that play important roles in E1/E2 conformational change increased VLP synthesis.

Thus, specific sequences in the E2 region responsible for robust CHIKV VLP generation were identified, and this mechanism was related to pH dependent E1/E2 conformational change. It has been shown that alphavirus assembly and budding efficiency is related to several factors, such as pH condition (J of Virology. 2001, p 8329-8339, Lu et al), the COOH-terminal of E1 and E2 palmitylation (Ivanova et al., J. Virol. 67:2546-2551; Ryan et al., Virology 249:62-67), interactions between E1 and E2 (Yao et al., J. Virol. 72:1418-1423; Yao et al., J. Virol. 70:7910-7920), interactions between the cytoplasmic domain of E2 and capsid proteins (Kong et al., J Virol 77:12764-12772; Wilkinson et al., Biochemistry 44:2800-2810; Zhao et al., EMBO J. 13:4204-4211) or the requirement for cholesterol in the cell membrane (Lu et al., J. Virol. 73:4272-4278; Marquardt et al., J. Cell. Biol. 123:57-65; Vashishtha et al. J. Cell. Biol. 140:91-99); however, no studies have shown a correlation of budding efficiency with the E2 ASR. This observation suggests that correct conformation of the E1/E2 is important for budding efficiency and several amino acids in ASR play an important role for E1/E2 conformational change for budding and entry. Although further studies are needed to develop the vaccine against alphaviruses, since VLP vaccines are known to have advantages, such as a good safety profile and their ability to induce high levels of immunogenicity (Bachmann et al., Science 262:1448-1451; Chackerian, Vaccines 6:381-390). (Akahata 2010), VLP vaccine strategy may prove to be optimal and this results described here furthers developments of alphavirus vaccines.

Example 7

Importance of Amino Acid 234 in the E2 Protein of Other Alphaviruses in VLP Production

To determine the role of the amino acid corresponding to amino acid 234 of CHIKV in other alphaviruses, the sequences of the E2 protein for Aura virus, Una virus, Mayaro virus, Middelburg virus, O'nyong-nyong virus (strain SG650), Ndumu virus, Barmah Forest virus, Seal louse virus, Salmon pancreas disease virus (SPDV), Whataroa virus, Sindbis virus, Western equine encephalomyelitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV), Ross river virus (strain NB5092), Bebaru virus, Semliki forest virus, Alphavirus M1, Fort Morgan virus, and Eastern equine encephalitis virus (EEEV) were aligned using the NCBI database. Examination of these sequences revealed that the K at the position corresponding to amino acid 234 of CHIKV was highly conserved. Similarly, the generation of VLP from expression vectors encoding the structural genes of these viruses is typically low. A representative sequence alignment showing the sequences of the E2 protein from CHIKV 37997, CHIKV OPY-1, Ross River, Sindbis, WEEV, EEEV, and WEEV is shown in FIG. **9**A.

To evaluate the effect of this residue in WEEV, an expression vector encoding WEEV E2 with the K235N mutation was prepared. Mutation of K235N in the WEEV expression vector increased VLP yield by >7-fold in transfected 293F cells compared to the wild type WEEV expression vector (FIG. **9**B). These VLPs were immunogenic in mice. Neutralizing antibody responses in BALB/c mice were measured using a pseudotyped lentiviral vector system (see Akahata et al., *Nat. Med.* 16:334-338 (2010)) after immunization with WEEV VLPs, alone, or in an equal mixture with CHIKV VLPs. Mice immunized with WEEV or WEEV and CHIKV VLPs generated similarly high titers of neutralizing antibodies to WEEV (FIG. **9**C). This result indicated that the WEEV VLPs were effective immunogens and that combination with CHIKV did not diminish their immunogenicity. To determine whether these immune responses are protective, immunized mice can be challenged with a lethal dose of a heterologous WEEV, the Fleming strain, a highly virulent strain isolated from a human patient. If the immune responses are protective, mice immunized with WEEV VLPs will be able to control the challenge virus.

Example 8

Alterations in Alphavirus Capsid Protein NLS Increase VLP Yield

The ability to produce large quantities of VLPs is important to their use as vaccines. The yield of VLPs from CHIKV strain 37997 was 100 times greater than that of the OPY-1 strain, although the amino acid profiles of the two strains are more than 95% identical. Other alphaviruses showed varying but lower yields of VLPs or no VLP expression. Based on these observations, an analysis of the structure of CHIKV and other alphaviruses was undertaken to determine which regions are responsible for this difference in VLP yields.

Different mutations were made to the EEEV capsid protein and subsequently tested to determine their effect on cellular toxicity, as well as their ability to improve VLP yields when expressed in mammalian cells. Several mutants were made to the acid sensitive region (ASR) region of EEEV capsid protein, but this did this not improve EEEV VLP expression. Interestingly, there was no capsid expression in the supernatant. For example, in EEEV VLP constructs expressing the EEEV E2 envelope variant R239N EEEV capsid protein was undetectable by Western Blot in fractions collected from density gradient centrifugation (FIG. **35**B). Additionally, EEEV capsid protein was observed in the nucleus at early time points after EEEV infection, although it was exported into the cytoplasm in the late stage of replication. Eastern Equine Encephalitis Virus (EEEV) capsid has also been reported as toxic to cells (Aguilar et al. 2007, Journal of Virology 81(8):3866-76 at page 3866).

The Eastern Equine Encephalitis (EEEV) capsid protein has a predicted nuclear localization signal (NLS) at amino acid (a.a.) positions 67-70 (FIG. **35**A). Targeted mutations to the nuclear localization signal (NLS) motif (KRKK) (SEQ ID NO:2) of EEEV capsid protein were created that changed lysine (K) to asparagine (N) at amino acids 67 (K67N), 68 (K68N) and 69 (K69N). The lysine (K) to asparagine (N) mutation was designed to diminish the inhibition of VLP production by the NLS. The structure and properties of the proposed modifications to the nuclear localization signal (NLS) were analyzed using PredictProtein software. Maps and sequences of exemplary EEEV PE-6 strain capsid protein K67N expression vectors are shown at FIGS. **47**A-**47**D and **48**A-**48**D.

Modification of the EEEV capsid protein NLS resulted in improved yields of EEEV VLPs when expressed in mammalian cells. The K67N mutation was tested alone, in combination with K68N, or in combination with K68N and K69N. All 3 groups of EEEV capsid protein mutations showed a substantially increased level of VLP expression when compared to the wild type version of the EEEV capsid protein. For example, in EEEV VLP constructs expressing an EEEV E2 envelope variant R239N and an EEEV variant capsid protein K67N, abundant EEEV VLPs having were readily detectable by Western blot of density gradient centrifugation fractions (FIG. **35**B). In contrast, in EEEV VLP constructs expressing an EEEV E2 envelope variant R239N and wild-type EEEV capsid protein, EEEV VLPs and EEEV capsid proteins were undetectable by Western Blot in corresponding density gradient centrifugation fractions (FIG. **35**B). Thus, an alteration in a EEEV capsid protein NLS provided EEEV VLP expression and increased EEEV VLP yield.

Alphavirus capsid proteins have amino acid sequences that can act as nuclear localization signals. Additional alphavirus capsid protein NLS sequences were identified as above. The Western Equine Encephalitis Virus (WEEV) capsid protein has a predicted nuclear localization signal (NLS) at amino acid (a.a.) positions 67-70 (FIG. **36**A). The Venezuelan Equine Encephalitis Virus (VEEV) capsid protein has a predicted NLS at amino acid positions 64-68 (FIG. **37**A). The Chikungunya (CHIKV) capsid protein has a predicted nuclear localization signal (NLS) at amino acid (a.a.) positions 62-69. The Ross River virus capsid protein has a predicted nuclear localization signal (NLS) at amino acid (a.a.) positions 71-74. The Barmah Forest virus capsid protein has a predicted nuclear localization signal (NLS) at amino acid (a.a.) positions 64-68.

Mutations to the alphavirus capsid protein have also been applied to Western equine encephalitis virus (WEEV) (FIG. **36**B) and Venezuelan Equine Encephalitis Virus (VEEV) (FIG. **37**B), which are similar to EEEV in sequence, and, like EEEV, are termed "new world" alphaviruses. Mutations to the alphavirus capsid protein have also been applied to Chikungunya (CHIKV) (FIG. **39**) and Ross River viruses, which are termed "old world" alphaviruses. Maps and sequences of exemplary WEEV CBA87 strain capsid protein expression vectors having one or more of the alterations K67N, K68N, and/or K69N are shown at FIGS. **40**A-**40**D, **41**A-**41**D, and **42**A-**42**D. Maps and sequences of exemplary VEEV TC83 strain capsid protein expression vectors having one or more of the alterations K64N, K65A, K65N, K67A, and/or K67N are shown at FIGS. **43**A-**43**D, **44**A-**44**D, **45**A-**45**D, and **46**A-**46**D. Maps and sequences of exemplary CHIKV(Strain 37997) strain capsid protein expression vectors having one or more of the alterations R62A, R63A, R65A, K66A, K68A, and/or K69A are shown at FIGS. **50**A-**50**D, **51**A-**51**D, and **52**A-**52**D. Maps and sequences of exemplary Ross River Virus capsid protein expression vectors having one or more of the alterations R71N, K72N, K73N, and/or K74N are shown at FIGS. **53**A-**53**D, **54**A-**54**D, **55**A-**55**D, and **56**A-**56**D. Maps and sequences of exemplary Barmah Forest Virus capsid protein expression vectors having an alteration at K64A is shown at FIGS. **59**A-**59**E.

As with EEEV VLPs, WEEV VLPs having a wild-type capsid protein were unable to be expressed. The introduction of alterations to the WEEV capsid protein NLS provided WEEV VLP expression and increased WEEV VLP yield (FIG. **36**B). The introduction of alterations to the VEEV capsid protein NLS increased VEEV VLP expression by more than 100-fold compared to the wild type VEEV and increased EEEV VLP yield (FIGS. **37**B and **38**B).

It has been discovered that mutating an NLS in an alphavirus capsid protein improves or allows expression of alphavirus VLPs in mammalian cells. Without being bound to a particular theory, the nuclear localization signal motif of the alphavirus capsid protein accumulates alphavirus capsid protein into the nucleus and prevents the secretion of alphavirus VLPs. Improvement in yields of alphavirus VLPs allows for their use as immunogenic compositions or vaccines, including a pan-alphavirus vaccine.

Example 9

High pH Mediates Efficient Virus-Like Particle Formation

As shown for CHIKV, it was examined whether pH conditions in the medium increase the yield of other VLPs. The VLP yield of EEEV was significantly increased under higher pH conditions, e.g., at pH 7.9 compared to at pH 7.1

(FIG. **60**). The yield of EEV VLP was shown to increase with increasing pH (FIG. **61**). Additionally, the pH dependent effect on yield was also observed when Tris-HCl buffer was added to cells after transfection with expression vectors for EEEV67N VLP (FIG. **62**) or VLP_{OPY-1} (FIGS. **63**A and B).

Additional mutations in the pH sensitive region of CHIKV OPY-1 (K233N, H170M, K200L, and H256Q) were constructed and studied. The mutations in the pH sensitive region resulted in VLPs being stable longer than 3-4 days after transfection compared to wild-type VLP which was present at a lower level at 3 days and nearly undetectable at 4 days (FIG. **64**).

Example 10

Multivalent Virus-Like Particle Vaccine Against Eastern, Western and Venezuelan Equine Encephalitis Virus Protected Mice Against Infection

A multivalent virus-like particle (VLP) vaccine was developed against WEEV, EEEV and VEEV using VLPs that have the mutations that allowed WEEV, EEEV and VEEV to express VLPs. To evaluate the effect of the NLS signals, expression vectors were constructed encoding WEEV, EEEV and VEEV containing NLS signal mutations (FIGS. **65**A and **65**B). These mutations increased VLP yield by >100-fold compared to the wild type expression vector. The resulting VLPs were purified by buoyant density gradient sedimentation. Fractionation of clarified supernatant showed that the greatest incorporation of E1/E2 into the VLPs occurred at a density of 1.2 g/ml, and was comparable to the density of the wild type VLPs. Cryoelectron microscopy and three dimensional image reconstruction, assuming icosahedral symmetry, showed that the VLPs had an external diameter of 65 nm and a core diameter of 40 nm with T=4 quasi symmetry. These specifications were very similar to the structure of CHIKV VLPs and other WT alphaviruses previously described.

To evaluate the immunogenicity of these VLPs, BALB/c mice were injected intramuscularly with VLPs from an individual alphavirus VLPs or all the combinations of WEEV, EEEV and VEEV VLPs. While the immunized mice did not generate cross neutralizing antibodies to different viruses, they all produced high titer neutralizing responses against the virus with which they were immunized (FIG. **2**A; IC_{50}, 1:36586, 1:17284, 1:2263 against VEEV, EEEV and WEEV, respectively). The mice vaccinated with multivalent VLPs showed high levels of neutralizing antibodies against all of the viruses (FIG. **66**A: IC_{50}, 1:23492, 1:10796, 1:1091, against VEEV, EEEV, WEEV, respectively). This result indicated that the multivalent VLP vaccine was effective in eliciting a multivalent immune response and that combination with other alphavirus VLPs did not diminish immunogenicity against the individual viruses.

To determine whether these immune responses were protective, immunized mice were challenged with a lethal dose of a heterologous WEEV, the Fleming strain, which is a highly virulent strain isolated from a human patient. High viremia was observed in the control, VEEV VLP and EEEV VLP groups but not in the WEEV VLPs and the trivalent groups (FIG. **66**B). Mice immunized with WEEV VLPs controlled the challenge virus, while all control mice developed severe infections and died (FIG. **66**C). These data demonstrate that the WEEV VLP vaccine, alone or in combination with VLP of another alphavirus, confers protection.

To characterize VLP-induced immune responses in a model with stronger predictive value for humans, rhesus macaques were immunized with VLPs. Monkeys were injected intramuscularly with WEEV VLPs alone, a multivalent WEEV/EEEV/VEEV VLP vaccine or PBS alone as a control. Sera from immunized and control monkeys are tested using pseudotyped lentiviral vectors and monkeys are challenged to measure protection from disease.

Although there have been several reports of progress in development of alphavirus vaccines, including a DNA vaccine (Nagata, L. P, 2005. Vaccine 23:2280-2283, Dupuy L, 2009, vaccine), a recombinant Ad5 vaccine (Barabe, N. D. 2007. Vaccine 25:6271-6276, Phillpotts 2005, vaccine 1615-1623), a live attenuated vaccine (Pittman P R, 1996; 14(4) p 337-43), and an inactivated vaccine (Coke F, Applied Microbiology, 1974, p 150-, Edelman R, Journal of infectious disease, 1979, Vol 140 p 708-), no candidates have been licensed to date. A live attenuated VEEV vaccine, TC-83, caused fever, headache and malaise in 25% and no response in 20% of vaccinated people (Pittman P R, 1996; 14(4) p 337-43). In addition, sequential administration of live attenuated alphavirus vaccines in human trials revealed an immunologic interference between the viruses and induced poor immunogenicity (McClain, D. J, 1998, J Infect Dis 177:634-641). A formalin-killed VEEV, EEEV and WEEV required multiple injections and even then, resulted in low levels of immunogenicity (Jahrling 1984, 19(3) p 429-31). Global climate, trade, and frequent travel has caused the spread of mosquitoes, the carrier of these viruses, to new geographic areas, which may pose a threat of other alphavirus outbreaks. In this study, an effective multivalent alphavirus VLP vaccine was developed, suggesting the potential for creating a more global pan-alphavirus VLP vaccine using this approach. The safety and efficiency of VLP vaccines combined with their relatively less complicated production requirements make them promising candidates in the future of vaccine field.

Example 9

Additional Embodiments

Additional embodiments are disclosed in the attached appendix, which is hereby incorporated by reference.

In this study, specific sequences in the E2 region were identified as responsible for robust CHIKV VLP generation. This strategy was then adapted to enhance the expression of a different alphavirus, WEEV. CHIKV is an enveloped positive-strand RNA virus, member of the alphavirus genus. Its structural proteins consist of one capsid (C) and an envelope (E) polyprotein that is processed to form E1/E2 heterodimers. The structural proteins are synthesized as polyproteins and are cleaved by capsid autoproteinase between the capsid and envelopes. The capsid protein assembles into a core particle, which is transported to the plasma membrane. After cleavage of the capsid protein, the PE2 (the precursor to the E3-E2 proteins)-6K-E1 envelope polyprotein is recognized by a signal sequence in the E3 protein domain to direct translocation into the endoplasmic reticulum (ER) membrane and transported to the cell surface through the Golgi network. PE2 is cleaved by furin, and E3 dissociates from E2. E1 and E2 then form a heterodimer (E1/E2). Capsid proteins associate with envelope on the cell surface and are encased within a lipid bilayer containing viral envelope proteins that initiate the budding of viral particles.

It has been shown that alphavirus assembly and budding efficiency is related to several factors, such as the COOH-terminal of E1 and E2 palmitylation, interactions between E1 and E2, interactions between the cytoplasmic domain of E2 and capsid proteins or the requirement for cholesterol in the cell membrane; however, no studies have shown a correlation of budding efficiency with the E2 and E3 binding domains. Interestingly, several mutants that block the furin cleavage site between E2 and E3 have been characterized, and most of these rescue mutants contain modifications in residues associated with the E2 and E3 binding domain. This observation suggests that destabilization of the E3/E2 binding domain is important for restoring infectivity, and that conformational changes due to the destabilization might help increase virus replication, including the maturation and fusion processes.

WEEV transmitted by mosquitoes has caused encephalitis in birds, horses and humans in the USA, Canada and South America. Because of these reports of illness and the potential bioterrorism threat through aerosol transmission, WEEV vaccine development is important. Although there have been several reports of progress in WEEV vaccine development, including a DNA vaccine and a recombinant Ad5 vaccine, no candidates have been licensed to date. Since VLP vaccines are known to have advantages, such as a good safety profile and their ability to induce high levels of immunogenicity, this vaccine strategy may prove to be optimal.

Previous human trials revealed immunologic interference from sequential administration of live attenuated vaccines against heterologous alphaviruses using CHIKV and Venezuelan equine encephalitis virus. In this study, the combination of CHIKV and WEEV VLPs in a vaccine did not decrease the immune responses against both viruses, suggesting that a pan-alphavirus VLP vaccine that could include other pathogenic alphaviruses, such as Eastern equine encephalitis virus, Venezuelan equine encephalitis virus Ross River virus, or Barmah Forest virus might be possible. The results described here furthers our understanding of the mechanism of VLP budding and vaccine development in alphaviruses.

VLPs are known to be highly immunogenic and elicit higher titer neutralizing antibody responses than subunit vaccines based on individual proteins. Such VLPs authentically present viral spikes and other surface components in a repetitive array that effectively elicits recognition by B-cells to stimulate antibody secretion. This recognition leads to B cell signaling and MHC class II up-regulation that facilitates the generation of high titer specific antibodies. VLPs from other viruses, including hepatitis B virus (HBV) and human papillomavirus (HPV), elicit high titer neutralizing antibody responses that contribute to protective immunity in humans. Similarly, alphavirus VLPs have been found to confer protection against alphavirus infection in a non-human primate model of infection.

Accordingly, VLPs are a viable vaccine strategy for treating alphavirus and flavivirus infection. As reported herein, alphavirus VLPs can be modified at the amino acid positions corresponding to amino acids 234 and 251 in the CHIKV E2 protein to enhance the production of VLPs. The methods described herein provide an attractive approach to developing and producing vaccines for alphaviruses and flaviviruses.

The results reported herein were obtained using the following methods and materials.

Vector Construction

Plasmids encoding the structural polyproteins C, E1, E2, E3 and 6K (strains 37997 and LR2006 OPY-1, GenBank EU224270) (FIG. **24**) and EU224268 (FIG. **23**), respectively) were synthesized as previously described (Yang et al., *Science* 317, 825 (2007)) (GeneArt, Regensburg, Germany). Plasmids encoding the polyproteins E3, E2, 6K, and E1 were amplified by PCR using the following primers:

```
sense primer
                                        (SEQ ID NO: 58)
5' GCTCTAGACACCATGAGCCTCGCCCTCCCGGTCTTG 3'
and

antisense primer
                                        (SEQ ID NO: 59)
5' TGGATCCTCATTAGTGCCTGCTAAACGACA 3'
(37997);
and

sense primer
                                        (SEQ ID NO: 60)
5' GCTCTAGACACCATGAGTCTTGCCATCCCAGTTATG 3'
and

antisense primer
                                        (SEQ ID NO: 61)
5' TGGATCCTCATTAGTGCCTGCTGAACGACA 3'
(LR2006 OPY-1).
```

XbaI and BamHI sites were inserted for cloning. Each fragment was digested with XbaI/BamHI and inserted into a eukaryotic expression vector under the control of a cytomegalovirus enhancer/promoter, CMV/R (Yang et al., *Science* 317, 825 (2007)) ($C\text{-}E_{37997}$, $C\text{-}E_{OPY\text{-}1}$, E_{37997} *and* $E_{OPY\text{-}1}$). To confirm expression of CHIKV C and E proteins, 293T cells were transfected using a FuGENE™ 6 Transfection Reagent kit (Roche Diagnostics GmbH, Germany) with 3 μg of the plasmid DNAs, following the manufacturer's recommendations.

The chimeric CHIKV and WEEV expression vectors $VLP_{C(37997)}$, $VLP_{C\text{-}E3(37997)}$, $VLP_{C\text{-}E2(37997)}$, $VLP_{C\text{-}6K(37997)}$, $VLP_{OPY\text{-}1\ E2(37997)}$, $VLP_{OPY\text{-}1\ 5'\text{-}E2(37997)}$, and $VLP_{OPY\text{-}1\ 3'\text{-}E2(37997)}$ were constructed using an overlap extension PCR method described previously in Kong et. al., *J. Virol.* 77, 12764 (2003). The chimeric genes between 37997 and OPY-1 strains were amplified using the primers shown in Table 1 (see above). Briefly, two fragments of the chimeric genes were amplified with 40 to 51 base pairs of primers that overlapped by 20 oligonucleotides and either of CHIKV 37997 F, CHIKV 37997 R, CHIKV OPY-1 F or CHIKV OPY-1 R primers. These two fragments were assembled in the overlapping region and amplified again with CHIKV 37997 F, CHIKV 37997 R, CHIKV OPY-1 F or CHIKV OPY-1 R primers. The PCR products were cloned into the $VLP_{OPY\text{-}1}$ expression vector after confirming of sequence. A vector encoding the Western Equine Encephalitis virus (WEEV) structural polyproteins C-E3-E2-6K-E1 (strain 71V-1658, GenBank AF214040) was synthesized as previously described in Akahata et. al., *Nat. Med.* 16, 334 (2010). CHIKV and WEEV mutants were made using the PCR-based Quickchange (Stratagene, La Jolla, Calif.) method according to the manufacturer's instructions with sense and anti-sense primers shown in Table 1 (see above). Each mutant was confirmed by sequencing. Other sequences and maps useful to the invention include those shown at FIGS. **10**A-**10**C, **11**A-**11**C, **12**A, **12**B, **13**A, **13**B, **14**A, **14**B, **15**A, **15**B, **16**A, **16**B, **17**A, **17**B, **18**A, **18**B, **19**A, **19**B, **20**A, **20**B, **21**A, **21**B, **22**A, **22**B, **23**A, **23**B, **24**A, **24**B, **25**A, **25**B, **26**A, **26**B, **27**, **28**, **29**A, **29**B, **30**A-**30**C, **32**A-**32**C, **33**A-**33**C, **34**A and **34**B, **38**A, **58**A-**58**E, and **59**A-**59**E.

Cell Culture

293T and 293A (human embryonic kidney cells), Vero (African green monkey kidney epithelial cells), HeLa (human cervical adenocarcinoma), A549 (human lung carcinoma) and BHK (baby hamster kidney cells) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Carlsbad, Calif.) containing 10% heat-inactivated fetal bovine serum (FBS) (GIBCO BRL, Carlsbad, Calif.).

Production of Pseudotyped Lentiviral Vectors

Lentiviral vectors expressing glycoproteins from different CHIKV strains were created. The recombinant lentiviral vectors expressing a luciferase reporter gene were produced as previously described (Naldini et al., *Proc. Natl. Acad. Sci. USA* 93, 11382 (1996), Yang et al., *Science* 317, 825 (2007)). Briefly, 293T cells were co-transfected with 500 ng CHIKV E plasmid from either strain $_{(E37997}$ ^{or} $E_{OPY\text{-}1)}$, 7 μg of a transducing vector encoding a luciferase reporter gene (pHR'CMV-luciferase plasmid), and 7 μg of a packaging plasmid expressing human immunodeficiency virus-1 (HIV-1) structural proteins (pCMVåR8.2). 2 μg of vesicular stomatitis virus glycoprotein (VSV-G), 2 μg of pNGVL-4070A amphotropic MuLV gp70 expression vector or 500 ng of empty vector served as positive and negative controls for these pseudotyped reporters respectively. After a calcium phosphate transfection (Invitrogen, Carlsbad, Calif.) overnight, the culture media was replenished with fresh media. 48 hours later, supernatants were harvested, filtered through a 0.45 μm syringe filter, stored in aliquots, and frozen at −80° C. The viruses were standardized by the amount of HIV-1 Gag p24. CHIKV pseudotyped lentiviral vectors harvested 72 hours after transfection were normalized according to HIV-1 Gag p24 levels before infection, as previously described (Yang et al., *Science* 317, 825 (2007)).

When the 293-derived suspension cell line 293F (Invitrogen, Carlsbad, Calif.) was used, the 293F cells were cultured in FreeStyle™ 293 Expression medium (Invitrogen, Carlsbad, Calif.). 293F cells (3×10^7) (Invitrogen) were transfected with 293fectin transfection reagent (Invitrogen, Carlsbad, Calif.) and 30 μg of each VLP plasmid following the manufacturer's recommendations

Buoyant Density Gradient Sedimentation Analysis and Purification of VLPs

Buoyant density gradient analysis and purification of VLPs was performed as described previously in Akahata et al., *J. Virol.* 79, 626 (2005) and Akahata et. al., *Nat. Med.* 16, 334 (2010). Briefly, a 293-derived suspension cell line, 293F (2.5×10^8 cells) (Invitrogen, Carlsbad, Calif.) was transfected with 293fectin transfection reagent (Invitrogen, Carlsbad, Calif.) and 125 μg of $C_{-E37997}$ plasmid following the manufacturer's recommendations. The supernatants were harvested 72 hours after transfection and filtered through a 0.45 ȝ m pore size filter, then layered onto a 60% Optiprep (Iodixanol) medium (Invitrogen, Carlsbad, Calif.) and centrifuged at 50,000×g for 1.5 hours with a Surespin 630 rotor (Sorvall). The supernatants were removed to leave 4 ml above the virus band and mixed to a 20% final concentration of OptiPrep. A density gradient was formed by centrifugation at 360,000×g for 3.5 hours with an NVT100 rotor (Beckman). 500 μl of each fraction was collected, weighed, and the densities of the fractions were plotted. 20 μl of each fraction was separated on a 4%-15% SDS-PAGE gel, and then stained using a Coomassie staining kit (InstantBlue, expedeon) following the manufacturer's recommendations or transferred onto an Immobilon-P membrane, and blotted with antisera reactive to the respective alphavirus (EEEV, WEEV, VEEV, CHIKV) as a primary antibody (e.g., sera from mice injected with the CHIKV strain S-27 (ATCC, VR-1241AF), CHIKV VLPs (strain 37997), or WEEV immune ascitic fluid (ATCC, VR-1251AF)) and goat anti-mouse immunoglobulins linked to horseradish peroxidase as a secondary antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.).

Production and Purification of Anti-CHIKV Mouse Monoclonal Antibodies

Monoclonal antibodies against CHIKV E2 were developed based on methods described previously in Yang et al., *Science* 317, 825 (2007). Briefly, female BALB/c mice were immunized with CHIKV VLPs three times. Injections with 20 μg CHIKV VLPs were administered every 4 weeks for a total of 3 injections. Three days after the final boost, spleens from the mice were harvested and used to produce hybridoma cells. Hybrids producing the antibody of interest were screened by ELISA using plates coated with CHIKV VLPs, and also using a neutralizing assay employing CHIKV psuedotyped viral vectors as previously mentioned in Akahata et. al., *Nat. Med.* 16, 334 (2010). Two clones, m10-18 and m242, showed strong neutralization and were purified as described previously in Yang et al., *Science* 317, 825 (2007).

Neutralization of CHIKV E Pseudotyped Lentiviral Vectors by Mouse and Monkey Antisera

The neutralization assay was performed as described previously (Yang et al., *Science* 317, 825 (2007)). A total of 10^4 293A cells were plated into each well of a 96-well dish one day prior to infection. CHIKV E-pseudotyped lentiviral vectors encoding luciferase were first titrated by serial dilution. Similar amounts of pseudotyped lentiviral vectors (with p24 levels of approximately 50 ng/ml) were then incubated with the indicated dilutions of mouse antisera for 60 minutes at room temperature prior to adding the virus: sera solution to 293A cells (10^4 cells/well in a 96-well dish, 50 μl/well, in triplicate). Sera from non-immune mice or monkeys were used as a negative control. After a 24 hour incubation, cells were lysed using cell lysis buffer (Cell Signal) and the luciferase activity was measured using Microbeta® JET (PerkinElmer, Turku, Finland) following incubation with "Luciferase assay reagent" (Promega, Madison, Wis.), according to the manufacturer's protocol Inhibition values were calculated as follows: inhibition (%)=[1−(luciferase activity (cps) in pseudotyped lentiviral vector infected cells incubated with the indicated dilutions of mouse antisera)/(luciferase activity (cps) in pseudotyped lentiviral vector infected cells incubated with the same dilutions of non-immune mouse serum)]×100. The $_{IC50}$ was calculated with Prism software (version 5).

Electron Microscopy

The morphology of the VLPs was examined by the Image Analysis Laboratory at the National Cancer Institute. VLPs were purified by Optiprep density centrifugation and were then fixed in 4% formaldehyde in PBS. Negative-stain electron microscopy for viral diagnosis has been described previously (Palmer and Martin, Electron Microscopy in Viral Diagnosis (CRC Press, Boca Raton, Fla., 1988)). Briefly, 1.0 μl of the sample was placed onto a carbon-coated Formvar-filmed copper grid (Tousimis Research Corp., Rockville, Md.) and VLPs were allowed to attach. The VLPs were negatively stained by addition of 2 μl of 1% PTA solution (phosphotungstic acid, pH 7.0) (Fisher Scientific Co., Fairlawn, N.J.). The grid was then examined by electron microscope (Hitachi H7000, Tokyo, Japan) operated at 75 kV. Digital images were taken by a CCD camera (AMT, Danvers, Mass.).

Cryo-Electron Microscopy and Image Analysis

CHIKV VLPs were flash-frozen on holey grids in liquid ethane. Images were recorded at 47K magnification with a CM300 FEG microscope with electron dose levels of approximately 20 $e^i/Å^2$. All micrographs were digitized at 6.35 μm pixel^{i1} using a Nikon scanner. Individual particle images were boxed using the program e2boxer in the EMAN2 package (Tang et al., *J Struct. Biol.* 157, 38 (2007)). CTF parameters were determined and phases were flipped using the CTFIT program from the EMAN package (Ludtke et al., *J. Struct. Biol.* 128, 82 (1999)). An initial model was constructed in EMAN using assigned 2-, 3-, and 5-fold views and was refined in EMAN assuming icosahedral symmetry. The number of particles incorporated into the final reconstruction was 1489, giving a final resolution of 18 Å based on a 0.5 Fourier shell correlation threshold.

Immunizations and Challenge of Mouse and Monkeys

19 μg of VLPs (equivalent to approximately 10 μg of E1/E2) in 60 μl normal saline were mixed with 60 μl of Ribi solution (Sigma Adjuvant system, Sigma-Aldrich) per mouse following the manufacturer's recommendations. Female 6- to 8-week-old BALB/c mice were injected in the right and left quadriceps muscles with VLPs in normal saline or Ribi in 120 μl total volume, two times at weeks 2 and 6. For DNA vaccination groups, the mice were injected in the right and left quadriceps muscles with a total of 15 μg of purified plasmid C-E_{37997}, E_{37997}, C-E_{OPY-1}, or E_{OPY-1} suspended in 100 μl of normal saline three times at weeks 0, 3, and 6. Five mice/group were injected. 10 days after the last injection, sera and spleen were collected.

In the monkey experiments, rhesus macaques (*Macaca mulatta*) weighing 3-4 kg were injected intramuscularly in the anterior quadriceps with either twenty μg of VLPs in 1 ml PBS (VLP group) or 1 ml PBS alone (control group) at weeks 0, 4 and 24. Six monkeys/group were injected. Blood was collected to measure antibody titers on days −14, 0, 10, 28, 38, 56, 70, 161 and 178. The monkeys (n=3 per group, randomly selected from each group) were challenged with 10^{10} PFU of CHIKV (strain LR2006 OPY-1) by intravenous injection. Blood was collected to measure viremia at 0, 6, 24, 48, 72, 96, 120 and 168 hours. The monkeys were sacrificed at 168 hours after challenge. The whole blood cells were measured using a hematology analyzer (IDEXX Laboratories, Inc., Westbrook, Me.). Bleeds were EDTA-anticoagulated using 20-22 gauge needles and either syringes or vacuum tubes. The maximum blood volume removed did not exceed 20% (12 ml/kg) per month, with no more than 15% (9 ml/kg) removed during any single draw.

All animal experiments were reviewed and approved by the Animal Care and Use Committee, Vaccine Research Center (VRC), National Institute of Allergy and Infectious Diseases and performed in accordance with all relevant federal and National Institutes of Health guidelines and regulations.

Virus Preparation

CHIKV (strain LR2006 OPY-1) was prepared and the virus titers were determined as previously described (Tsetsarkin et al., PLoS. Pathog. 3, e201 (2007) and Pastorino et al., *J. Virol. Methods* 124, 65 (2005)). Briefly, viral RNA transcribed from plasmid CHIK-LR ic was transfected into BHK-21 cells by electroporation. The supernatants from the transfected cells were aliquotted and the stock virus was titrated and tissue culture infectious dose 50% ($TCID_{50}$) endpoint titers were determined using Vero cells. To produce virus for vertebrate challenge, C6/36 (*Aedes albopictus*) cells grown to confluence in T150 flasks were infected with stock virus at a multiplicity of infection of 0.03. Supernatants were harvested at 48 hours post-infection, aliquotted and titrated to determine $TCID_{50}$ endpoint titers on Vero cells.

Plaque Assay

Serum samples were tested for CHIKV neutralizing antibody by a standard plaque reduction neutralization test (PRNT). Briefly, monkey sera were heat inactivated at 56° C. for 30 minutes and diluted in virus diluent (PBS/5% BSA). Diluted serum samples were mixed with an equal volume of 40 PFU CHIKV (strain LR2006 OPY-1) and incubated for 1 hour at 37° C. Six-well plates of confluent Vero cells were inoculated with 200 μl of the serum-virus mixtures in duplicate and incubated at 37° C. for 1 hour. Plates were overlaid with 3 ml of medium containing 0.9% agarose (Lonza Rockland, Rockland, Me.) and incubated at 37° C. in a 5% CO_2 incubator for 2 days. A second overlay medium containing neutral red and 1% agarose was then added and the plates were incubated overnight before plaques were visualized and counted. The viremia in the monkeys after challenge was measured by plaque assay. Six-well plates of confluent Vero cells were inoculated with 200 μl of the serum-PBS mixtures in duplicate. The serum dilutions were 1:200, 1:400, 1:800, 1:1000, 1:10,000 and 1:100,000, since at lower dilutions toxicities were observed in the cells (detection limit 1:200 dilution=1000 PFU/ml).

Passive Transfer of Immunoglobulin and Challenge in IFNα/βR$^{-/-}$ Mice

IFNα/βR$^{-/-}$ mice were kindly given by Robert Seder and Daniel D. Pinschewer. IgG was purified from the serum in monkeys immunized with CHIKV VLPs or injected with PBS (control) using a HiTrap™ Protein G HP column (GE Healthcare) following the manufacturer's recommendations. IgG was further purified using a Melon Gel IgG Purification Kit (Pierce) following the manufacturer's recommendations. Purified IgG was dialyzed 3 times against PBS. 2 mg of purified IgG (from approximately 200 μl of serum) was administered intravenously into each recipient IFNα/βR$^{-/-}$ mouse by tail vein injection 24 hours before challenge. The mice were challenged with 30 PFU of CHIKV (strain LR2006 OPY-1) by intradermal injection.

Detection of CHIKV RNA by Quantitative RT-PCR

For RNA isolation, serum samples were spun down at 10,000×g for 1 hour, liquid poured off and 1 ml of RNA-STAT 60 (Isotex Diagnostics, Friendswood, Tex.) added. Samples were then incubated at RT for 5 min and resuspended in 250 μl of chloroform by vortexing. The samples were spun down at 10,000×g for 1 hour, the aqueous top-layer removed, 0.5 ml isopropanol and 10 μl tRNA (10 μg/ml) added and precipitated overnight at −20° C. Samples were spun down for 1 hour, washed with cold 75% ethanol and spun again for another hour. RNA was resuspended in 30 μl RNAse-free water. For RT-PCR, 10% RNA was added to TaqMan reagents (Applied Biosystems, Foster City, Calif.) along with primers and probe (listed below) and amplified in a 7700 Sequence Detection System (Applied Biosystems). Briefly, the sample was reverse-transcribed at 48° C. for 30 min., held at 95° C. for 10 min, then run for 40 cycles of 95° C. for 30 s and 60° C. for 1 min. The signal was compared to a standard curve of known concentrations of plasmid containing the LR2006 OPY-1 sequence starting at 10^7 down to 1 copy/mL and multiplied by 10, giving a detection range from 40-10^8 copies/mL. All samples were performed in triplicate. The primers and probe were designed to bind to a highly conserved region on the E1 structural protein gene.

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Primer sequences:
                                        (SEQ ID NO: 62)
CHIK-F
5' AAGCTCCGCGTCCTTTACCAAG 3'
and
                                        (SEQ ID NO: 63)
CHIK-R
5' CCAAATTGTCCTGGTCTTCCT3'.

Probe sequence:
                                        (SEQ ID NO: 64)
CHICK-P
FAM-CCAATGTCTTCAGCCTGGACACCTTT-TAMRA
as described previously in Huang
et al., J. Virol. 78, 12557 (2004) and
Pastorino et al., J Virol. Methods 124,
65 (2005)).
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Structural Models for Analysis of CHIKV E1/E2 and Surface Area Calculations

The CHIKV E1/E2 (OPY-1 strain) was modeled from PDB accession number 3N40-44 and displayed using Pymol. The electrostatic potential of the E1/E2 surface was calculated using APBS and visualized with Pymol using blue and red to represent positive and negative charges, respectively.

Alignment of Alphaviruses E2 Protein

The representative alphavirus E2 glycoproteins were aligned in the NCBI database to a panel that included Aura virus, Una virus, Mayaro virus, Middelburg virus, O'nyong-nyong virus (strain SG650), Ndumu virus, Barmah Forest virus, Seal louse virus, Salmon pancreas disease virus (SPDV), Whataroa virus, Sindbis virus, Western equine encephalomyelitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV), Ross river virus (strain NB5092), Barmah Forest virus, Bebaru virus, Semliki forest virus, Alphavirus M1, Fort Morgan virus, and Eastern equine encephalitis virus (EEEV). CHIKV strains were aligned using the NCBI protein blast tool.

Flow Cytometry

CHIKV envelope expression on transfected cell membranes was measured by flow cytometry as described previously in Wu et al., *J. Virol.* 83, 5077 (2009), with the CHIKV E2 monoclonal antibody m10-18 or a control mouse monoclonal antibody as a primary antibody and goat anti-mouse immunoglobulins linked to Phycoerythrin as a secondary antibody (Sigma). The data was analyzed and displayed with FlowJo 8.8.6 software (Tree Star).

Other Embodiments

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

All patents and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent and publication was specifically and individually indicated to be incorporated by reference.

SEQUENCE LISTING

The patent contains a lengthy "Sequence Listing" section. A copy of the "Sequence Listing" is available in electronic form from the USPTO web site (http://seqdata.uspto.gov/?pageRequest=docDetail&DocID=US09487563B2). An electronic copy of the "Sequence Listing" will also be available from the USPTO upon request and payment of the fee set forth in 37 CFR 1.19(b)(3).

What is claimed is:

1. An alphavirus virus-like particle (VLP) comprising at least one altered viral protein selected from the group consisting of:

a. an alphavirus E2 protein comprising one or more alterations, relative to the wild-type amino acid sequence, at one or more amino acid locations corresponding to one or more amino acid locations selected from the group consisting of H170, K200, K233, K234, R251 and H256 of CHKV E2 protein; and

b. an alphavirus capsid protein comprising one or more alterations, relative to the wild-type amino acid sequence, at a charged amino acid residue in the Nuclear Localization Signal (NLS);

wherein the at least one altered viral protein is capable of self-assembling into a VLP; and wherein the one or more alterations enhance VLP production.

2. The VLP of claim **1**, wherein the one or more altered viral protein(s) are derived from a virus selected from the group consisting of Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV), Semliki Forest virus (SFV), Chikungunya virus (CHIKV), O'nyong-nyong virus, Sindbis virus, Mayaro virus, Ross River virus, Barmah Forest virus, and Ockelbo virus.

3. The VLP of claim **1**, wherein the one or more alteration in a charged amino acid residue in the NLS is in at least one amino acid region selected from the group consisting of:

a. amino acids 67-70 of an EEEV capsid protein;

b. amino acids 67-70 of an WEEV capsid protein;

c. amino acids 64-68 of an VEEV capsid protein;

d. amino acids 62-69 of a CHIKV capsid protein;

e. amino acids 71-74 of a Ross River virus capsid protein; and

f. amino acids 64-68 of a Barmah Forest virus capsid protein.

4. The VLP of claim **1**, wherein the VLP comprises an agent to be introduced to a cell.

5. A method of inducing an immune response against an alphavirus in a subject, comprising administering to the subject an effective amount of the VLP of claim **1**.

6. A pan-viral immunogenic composition comprising:

a. the VLP of claim **1**; and

b. a second alphavirus VLP comprising at least one altered viral protein selected from the group consisting of:

i. an alphavirus E2 protein comprising one or more alterations, relative to the wild-type sequence, at one or more amino acid locations corresponding to one or more amino acid locations selected from the group consisting of H170, H200, K233, K234, R251 and H256 of CHKV E2 protein; and

ii. an alphavirus capsid protein comprising one or more alterations, relative to the wild-type sequence, at a charged amino acid residue in the Nuclear Localization Signal (NLS);

wherein the at least one altered viral protein is capable of self-assembling into a VLP;

wherein the one or more alterations enhance VLP production, and wherein the altered viral proteins of the VLP of claim **1** and the second VLP are from different alphaviruses.

7. A kit comprising the VLP of claim **1**.

8. A method of introducing an agent into a cell comprising:

a. packaging the agent into an alphavirus VLP comprising at least one altered viral proteins selected from the group consisting of:

i. an alphavirus E2 protein comprising one or more alterations, relative to the wild-type amino acid sequence, at one or more amino acid location corresponding to one or more amino acid location selected from the group consisting of H170, K200, K233, K234, R251 and H256 of CHKV E2 protein; and

ii. an alphavirus capsid protein comprising one or more alterations, relative to the wild-type amino acid sequence, at a charged amino acid residue in the Nuclear Localization Signal (NLS);

wherein the at least one altered viral protein is capable of self-assembling into a VLP; and wherein the one or more alterations enhance VLP production;

b. contacting a cell with the VLP under conditions that allow the VLP to enter the cell, thereby introducing the agent into the cell.

9. The method of claim **8**, wherein the one or more altered viral protein(s) are derived from a virus selected from the group consisting of Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV), Semliki Forest virus (SFV), Chikungunya virus (CHIKV), O'nyong-nyong virus, Sindbis virus, Mayaro virus, Ross River virus, Barmah Forest virus, and Ockelbo virus.

10. The method of claim **8**, wherein the one or more alteration in a charged amino acid residue in the NLS is in at least one amino acid region selected from the group consisting of:

a. amino acids 67-70 of an EEEV capsid protein;

b. amino acids 67-70 of an WEEV capsid protein;

c. amino acids 64-68 of an VEEV capsid protein;

d. amino acids 62-69 of a CHIKV capsid protein;

e. amino acids 71-74 of a Ross River virus capsid protein; and

f. amino acids 64-68 of a Barmah Forest virus capsid protein.

11. The VLP of claim **1**, wherein the E2 protein comprising one or more alterations comprises an amino acid sequence selected from the group consisting of:

a) an amino acid sequence selected from the group consisting of SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70 and SEQ ID NO:71, wherein the amino acid at a position corresponding to position 170 of SEQ ID NO:65 is not a histidine;

b) an amino acid sequence selected from the group consisting of SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70 and SEQ ID NO:71, wherein the amino acid at a position corresponding to position 200 of SEQ ID NO:65 has been modified;

c) an amino acid sequence selected from the group consisting of SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70 and SEQ ID NO:71, wherein the amino acid at a position corresponding to position 233 of SEQ ID NO:65 has been modified;

d) an amino acid sequence selected from the group consisting of consisting of SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70 and SEQ ID NO:71, wherein the amino acid at a position corresponding to position 234 of SEQ ID NO:65 is not a lysine;

e) an amino acid sequence consisting of SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70 and SEQ ID NO:71, wherein the amino acid at a position corresponding to position 251 of SEQ ID NO:65 has been modified; and,

f) an amino acid sequence selected from the group consisting of SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70 and SEQ ID NO:71, wherein the amino acid at a position corresponding to position 256 of SEQ ID NO:65 is not a histidine;

wherein the E2 protein comprising one or more alterations can form a VLP.

12. The VLP of claim **11**, wherein the wherein the amino acid at a position corresponding to position 234 of SEQ ID NO:65 is an asparagine.

13. The VLP of claim **1**, wherein the capsid protein comprises an amino acid sequence selected from the group consisting of:

a) an amino acid sequence comprising SEQ ID NO:115 (WEEV), wherein the amino acid sequence is altered at one or more amino acid positions selected from the group consisting of amino acid position 67, amino acid position 68 and amino acid position 69;

b) an amino acid sequence comprising SEQ ID NO:124 (VEEV), wherein the amino acid sequence is altered at amino acid position 67;

c) an amino acid sequence comprising SEQ ID NO:142 (CHIKV), wherein the amino acid sequence is altered at one or more amino acid positions selected from the group consisting of amino acid position 62, amino acid position 63, amino acid position 65, amino acid position 66, amino acid position 68 and amino acid position 69;

d) an amino acid sequence comprising SEQ ID NO:154 (Ross River), wherein the amino acid sequence is altered at one or more amino acid positions selected from the group consisting of amino acid position 71, amino acid position 72, amino acid position 73 and amino acid position 74;

e) an amino acid sequence comprising SEQ ID NO:171 (BFV), wherein the amino acid sequence is altered at one or more amino acid position selected from the group consisting of amino acid position 64, amino acid position 65, amino acid position 67 and amino acid position 68.

14. The VLP of claim **1**, wherein the capsid protein comprises an amino acid sequence selected from the group consisting of:

a) an amino acid sequence comprising SEQ ID NO:115 (WEEV), wherein the amino acid sequence comprises one or more alterations selected from the group consisting of K67N, K68N and K69N;

b) an amino acid sequence comprising SEQ ID NO:124 (VEEV), wherein the amino acid sequence comprises one or more alterations selected from the group consisting of K64N, K65A, K65N, K67A and K67N;

c) an amino acid sequence comprising SEQ ID NO:142 (CHIKV), wherein the amino acid sequence comprises one or more alterations selected from the group consisting of R62A, R63A, R65A, K66A, K68A and K69A;

d) an amino acid sequence comprising SEQ ID NO:154 (Ross River), wherein the amino acid sequence comprises one or more alterations selected from the group consisting of R71N, K72N, K73N and K74N; and,

e) an amino acid sequence comprising SEQ ID NO:171 (BFV), wherein the amino acid sequence comprises one or more alterations selected from the group consisting of K64A, K64N, K65A, K65N, K67A, K67N, K68A and K68N.

15. The VLP of claim **1**, wherein the capsid protein comprises an amino acid sequence selected from the group consisting of SEQ ID NO:115, SEQ ID NO:118, SEQ ID NO:121, SEQ ID NO:124, SEQ ID NO:127, SEQ ID NO:130, SEQ ID NO:133, SEQ ID NO:136, SEQ ID NO:139, SEQ ID NO:142, SEQ ID NO:145, SEQ ID NO:148, SEQ ID NO:151, SEQ ID NO:154, SEQ ID NO:157, SEQ ID NO:160, SEQ ID NO:163 and SEQ ID NO:171.

16. The VLP of claim **11**, wherein the wherein the amino acid at a position corresponding to position 170 of SEQ ID NO:65 is a methionine.

17. The VLP of claim **11**, wherein the wherein the amino acid at a position corresponding to position 200 of SEQ ID NO:65 is a lysine.

18. The VLP of claim **11**, wherein the wherein the amino acid at a position corresponding to position 233 of SEQ ID NO:65 is an asparagine.

19. The VLP of claim **11**, wherein the wherein the amino acid at a position corresponding to position 256 of SEQ ID NO:65 is a glutamine.

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