MEMBRANE VIRUS HOST RANGE MUTATIONS AND THEIR USES AS VACCINE SUBSTRATES

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to virology and disease control. Specifically, the present invention relates to mutated arthropod vectored viruses and their uses as vaccines.

Description of the Related Art

Arthropod vectored viruses (Arboviruses) are viral agents which are transmitted in nature by blood sucking insects. Many of these viruses have membrane bilayers with associated integral membrane proteins which make up the protective envelope of the virus particle (Togaviruses) (Schlesinger, S. and M.J. Schlesinger, 1990).

Collectively, the arthropod vectored viruses are second only to malaria as a source of insect-transmitted disease and death in man and animals throughout the world (Berge A. O. 1975). Among these viral agents are Eastern, Western, and Venezuelan Equine Encephalitis Viruses, Dengue Fever, Japanese Encephalititis, San Angelo Fever, West Nile Fever and Yellow Fever. Further, diseases caused by these agents are in resurgence in North America (*NIAID Report of the*

Task Force on Microbiology and Infectious Diseases 1992, NIH Publication No. 92-3320) as a result of the introduction of the mosquito vector Aedes albopictus (Sprenger, and Wuithiranyagool 1985).

By their very nature, Arboviruses must be able to replicate in the tissues of both the invertebrate insect and the mammalian host (Brown, D.T., and L. Condreay, 1986, Bowers et al. 1995). Differences in the genetic and biochemical environment of these two host cell systems provide a basis for the production of viruses which can replicate in one host but not the other (Host Range Mutants).

Currently, Dengue Fever and Eastern Equine Encephalitis and other insect bourne viruses are in resurgence in the United States. The U.S. Army and other government agencies have been trying to make vaccines against these viruses since the 1960s with little success. Thus, the prior art is deficient in a vaccine against most arthropod vectored viruses and other membrane-coated viruses. The present invention fulfills this long-standing need and desire in the art.

SUMMARY OF THE INVENTION

In one embodiment of the present invention, there is provided a genetically engineered, membrane-enveloped virus, wherein the virus codes for a transmembrane protein which has a deletion of one or more amino acids such that the transmembrane protein is able to span or correctly integrate into the viral membrane when the engineered virus replicates in insect cells, but is unable to span or correctly integrate into the viral membrane when the virus replicates in mammalian cells. Preferably, the virus is an Arthropod

vectored virus selected from the group consisting of Togaviruses, Flaviviruses, Bunya viruses and all other enveloped viruses which can replicate naturally in both mammalian and insect cells, as well as other enveloped viruses which can be made to replicate in mammalian and insect cells by genetic engineering of either the virus or the cell. Representative examples of such engineered viruses are ΔK391 virus and TM16 virus.

In another embodiment of the present invention, there is provided a method of producing a viral vaccine from a genetically engineered, membrane-enveloped virus for vaccination of mammals, comprising the steps of producing deletions in the membrane associated domains of the virus which restrict their ability to grow to insect cells, introducing the engineered virus disclosed herein into insect cells and allowing the virus to replicate in the insect cells to produce a viral vaccine. Representative examples of the engineered viruses are ΔK391 virus and TM16 virus.

In still another embodiment of the present invention, there is provided a method for vaccinating an individual in need of such treatment comprising the step of introducing the viral vaccine of the present invention into the individual to produce viral proteins for immune surveillance and stimulate immune system for antibody production.

In still yet another embodiment of the present invention, there is provided a method of producing a viral vaccine from a genetically engineered, membrane-enveloped virus to a disease spread by a wild mosquito population to mammals, comprising the steps of engineering a deletion of one or more amino acids in a viral transmembrane protein to produce an engineered virus, similar to TM16 or delta K391, wherein the transmembrane protein is able to

span the membrane envelope when the virus replicates in mosquito cells, but is unable to span the membrane envelope when the virus replicates in mammalian cells, and wherein the virus remains capable of replicating in wild mosquito cells; introducing the engineered virus into the wild mosquito population; and allowing the engineered virus to replicate in cells of the wild mosquito population to produce a population of mosquitoes which harbor the vaccine strain of the virus and exclude the wild type (pathogenic) virus such that the mosquito bite delivers the vaccine to a mammal bitten. Presence of the mutated virus renders the mosquito incapable of transmitting other membrane containing viruses (Karpf et al 1997)

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of one of the presently preferred embodiments of the invention. These embodiments are given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

The appended drawings have been included herein so that the above-recited features, advantages and objects of the invention will become clear and can be understood in detail. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and should not be considered to limit the scope of the invention.

Figure 1 shows the results of radiolabeled Sindbis virus proteins recovered from transfected tissue-cultured cells. BHK-21 cells mock transfected (1), transfected with mutant Δ391 RNA (2), and Aedes albopictus cells transfected with Δ391 RNA (3), were labeled

with radioactive amino acids as described in Example 3. At 24 hours post-transfection, proteins were precipitated with virus specific anti-serum as described in Example 4. The figure shows that both BHK-21 cells and Aedes albopictus cells transfected with RNA of the deletion mutant produce the three viral structural proteins E1, E2, and C which are not detected in the mock transfected cells.

Figures 2A and 2B are electron micrographs of BHK-21 cells (2A) and Aedes albopictus cells (2B) transfected with RNA of the Sindbis virus deletion mutant Δ391. Cells were transfected as described in Example 2. BHK-21 cells (Figure 2A) show clusters of virus core structures in the cell cytoplasm (A) even though these cells produce very low levels of mature virus (Table 1). Aedes albopictus cells (Figure 2B) also produce clusters of virus cores; however, these cores are found free in the cells' cytoplasm similar to those in BHK-21 cells (A) and are also found associated with cell membranes (B). This latter case is not found in BHK-21 cells, indicating that the glycoproteins E1 and E2, although present, do not function to bind them.

Figure 3 shows the configuration of Sindbis virus glycoproteins after integration into the ER. The protein is a multipass protein with 6 membrane spanning domains (numbered 1-6). 1. The signal sequence for initial integration; 2. The first E2 transmembrane domain (TMD); 3. The second E2 TMD; 4. The first 6k TMD; 5. The second 6k TMD; and 6. The E1 TMD. S = point of cleavage by signal peptidase.

Figure 4 shows the deleted amino acids in the E2 transmembranal domain. The deleted sequence is shown under the appropriate amino acid, ranging from 1 to 16 deletions. Histidine and

Proline sequences beginning at nt 9717 are on the lumenal side of the protein but are used to design the mutagenic primers.

DETAILED DESCRIPTION OF THE INVENTION

It will be apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

As used herein, the term "membrane-bound virus" refers to a virus which contains a lipid membrane bilayer as part of its protective exterior coat.

As used herein the term "viral envelope" refers to the lipid membrane component of the membrane containing virus and its associated proteins.

As used herein, the terms "arthropod vectored virus" or "Arbovirus" refer to viral agents which replicate and produce progeny virus in arthropod (insect) or mammalian cells. This includes Togaviruses, Flaviviruses and Bunyaviruses.

As used herein, the term "Togavirus" refers to a general classification of membrane containing viruses which include the Alphaviruses.

As used herein, the term "membrane bilayer" refers to a structure consisting of opposed amphiphatic phospholipids. The bilayer is organized in cross section from polar head groups to non-polar carbon chains to nonpolar carbon chains to polar head groups.

As used herein, the term "glycoprotein transmembrane region" refers to the amino acid sequence of the region of a membrane-integrated protein which spans the membrane bilayer.

As used herein, the term "viral vaccine" refers to a strain of virus or virus mutant which has the antigenic properties of the virus but cannot produce disease.

As used herein the term "immune surveillance" refers to a process by which blood lymphocytes survey the cells and tissues of a mammal to determine the presence of foreign (virus) proteins and stimulates the production of lymphocytes capable of targeting cells producing the foreign protein for destruction. This process also leads to the production of circulating antibodies against the foreign protein.

As used herein, the term "infectious virus particles" refers to viruses which are capable of entering a cell and producing virus protein, whether or not they are capable of producing progeny virus.

As used herein, the term "non-infectious virus particles" refers to viruses which are not capable of infecting or entering a cell.

As used herein, the term "vertebrate cells" refers to any mammalian cell.

As used herein, the term "invertebrate cells" refers to any insect cell.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. (1985)); "Transcription

and Translation" (B.D. Hames & S.J. Higgins eds. (1984)); "Animal Cell Culture" (R.I. Freshney, ed. (1986)); "Immobilized Cells And Enzymes" (IRL Press, (1986)); B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. A vector is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient animal. Such an agent is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a change in the physiology of a recipient mammal. For example, in the treatment of viral infection, a compound which decreases the extent of infection or of physiologic damage due to infection, would be considered therapeutically effective.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Various promoters may be used to drive vectors.

The term " oligonucleotide" or "probe" as used herein, refers to a molecule comprised of ribonucleotides or deoxyribonucleotides. The exact size of the oligonucleotide or probe will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide. The length of the probe is not critical, but will usually comprise at least about 12 bases, more usually comprising at least about 16 bases, and the probe is substantially complementary to a portion of the bacterial genome; however, the probe need not have perfect complementarity with the genome. The probes may be prepared synthetically, with suitable synthetic techniques, and most likely include a detectable label. Usually, the synthetic sequences are expanded in common, publicly-available cloning vectors and suitable hosts in order to obtain large quantities of the probe. The expanded vectors may themselves be labeled for use as probes, or shorter fragments containing complementary strands may be excised and labeled. Methods for the preparation and utilization of nucleotide probes for diagnostic testing are described in the references listed above, *supra*, and in U.S. Patent No. 4,358,535 to Falkow, et al.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the

presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, the source of primer and the method used. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence or hybridize therewith and thereby form the template for the synthesis of the extension product.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transfecting DNA may or may not be integrated (covalently linked) into the genome of the cell. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth in vitro for many generations.

A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene).

The present invention is directed to a genetically engineered, membrane-enveloped virus, wherein the virus codes for a transmembrane protein which has a deletion of one or more amino acids in the transmembrane region of the protein such that the transmembrane protein is able to span or correctly integrate into the membrane of an infected cell when the engineered virus replicates in insect cells, but is unable to span or integrate into the membrane of an infected cell when the virus replicates in mammalian cells. Preferably, the virus is an Arthropod vectored virus selected from the group consisting of Togaviruses, Flaviviruses, Bunya viruses and all other enveloped viruses that can replicate naturally in both mammalian and insect cells, as well as enveloped viruses that can be made to replicate in mammalian and insect cells by genetic engineering of either the virus or of the cell. Representative examples of such engineered viruses are ΔK391 virus and TM16 virus. Still preferably, the insect cells are mosquito cells, such as Aedes albopictus cells, and the mammalian cells are human cells.

In a preferred embodiment, the genetically engineered, membrane-enveloped virus is Sindbis virus, and the transmembrane

protein is viral glycoprotein E2. However, a person having ordinary skill in this art could readily predict that similar mutations can be successfully installed in the membrane spanning domains of other virus membrane proteins such as E1.

In another preferred embodiment, the genetically engineered, membrane-enveloped virus is selected from the group consisting of HSV, HIV, rabies virus, Hepatitis, and Respiratory Syncycial virus, and the transmembrane protein is selected from the group consisting of glycoprotein E1, glycoprotein E2, and G protein.

In still another preferred embodiment, the genetically engineered, membrane-enveloped virus are RNA tumor viruses, and the transmembrane protein is Env.

The present invention is also drawn to a method of producing a viral vaccine from the genetically engineered, membrane-enveloped virus disclosed herein for vaccination of mammals, comprising the steps of introducing the engineered virus into insect cells and allowing the virus to replicate in the insect cells to produce a viral vaccine. Representative examples of the engineered viruses are ΔK391 virus and TM16 virus.

In addition, the present invention provides a method for vaccination of an individual in need of such treatment, comprising the steps of introducing the viral vaccine of the present invention into the individual and allowing the vaccine to produce viral proteins for immune surveillance and stimulate immune system for antibody production in the individual.

Further, the present invention provides a method of producing a viral vaccine to a disease spread by a wild mosquito population to a mammal, comprising the steps of genetically engineering a deletion of one or more amino acids in a viral

transmembrane protein to produce an engineered virus, wherein the transmembrane protein is able to span the membrane envelope when the virus replicates in mosquito cells, but is unable to span the membrane envelope when the virus replicates in mammalian cells, and wherein the virus remains capable of replicating in mosquito cells; introducing the engineered virus into a wild mosquito population; and allowing the virus to replicate in cells of the wild mosquito population to produce a population of mosquitos which harbor the vaccine strain of the virus and exclude the wild type (pathogenic) virus such that the mosquito bite delivers the vaccine to a mammal bitten.

It is contemplated that pharmaceutical compositions may be prepared using the novel mutated viruses of the present invention. In such a case, the pharmaceutical composition comprises the novel virus of the present invention and a pharmaceutically acceptable carrier. A person having ordinary skill in this art readily would be able to determine, without undue experimentation, the appropriate dosages and routes of administration of this viral vaccination compound. When used *in vivo* for therapy, the vaccine of the present invention is administered to the patient or an animal in therapeutically effective amounts, i.e., amounts that immunize the individual being treated from the disease associated with the particular virus. It will normally be administered parenterally, preferably intravenously or subcutaineusly, but other routes of administration will be used as appropriate. The amount of vaccine administered will typically be in the range of about 10^3 to about 10^6 pfu/kg of patient weight. The schedule will be continued to optimize effectiveness while balancing negative effects of treatment. *See* Remington's Pharmaceutical Science, 17th Ed. (1990) Mark Publishing Co., Easton, Penn.; and *Goodman and Gilman's: The Pharmacological Basis of Therapeutics* 8th

Ed (1990) Pergamon Press; which are incorporated herein by reference. For parenteral administration, the vaccine will be most typically formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are preferably non–toxic and non–therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin.

The vaccines of the present invention are based on deletion mutations in the transmembrane domains of proteins of membrane-enveloped viruses. The strategy for production of these mutations is based on the following information: Unlike mammalian cell membranes, the membranes of insect cells contain no cholesterol (Clayton 1964, Mitsuhashi et al 1983). The presence of cholesterol in membranes in general makes the membrane thicker, with the increase in thickness increasing as the amount of cholesterol increases (Bretscher, 1993). Many membrane-coated viruses have membrane glycoproteins on their surface which are responsible for identifying and infecting target cells (Schlesinger, S. and M.J. Schlesinger, 1990). These membrane glycoproteins have hydrophobic membrane-spanning domains which anchor the proteins in the membrane bilayer (Rice et al 1982).

The membrane-spanning domains of these transmembrane proteins must be long enough to reach from one side of the bilayer to the other in order to hold or anchor the proteins in the membrane. Experiments have shown that if the domains are shortened by the deletion of amino acids within the domain, the proteins do not appropriately associate with the membrane and fall out (Adams and Rose. 1985).

Because insects have no cholesterol in their membranes, the insect-generated viral membrane will be thinner in cross section than the viral membranes generated from mammals. Since the membranes of insects are thinner, the membrane-spanning domains of proteins integrated into insect membranes do not need to be as long as those integrated into the membranes of mammals. It is possible, therefore, to produce deletions in engineered viruses which remove amino acids from the transmembrane domain of the viral glycoprotein. This results in a glycoprotein which can integrate normally into the membrane of a virus replication in an insect cell, but not into the membrane of a virus replicating in a mammal. Thus, the mutated virus is produced in the insect cell replicating as well as the parent, wildtype virus in the insect host. On the other hand, in mammals, the mutant virus can infect the host producing viral proteins; however because the mutated virus glycoprotein cannot span and be anchored in the mammalian membrane, progeny virus cannot be produced in mammalian cells. An example of such a virus is TM16. An additional advantage to the approach of the present invention is that the mutants are engineered as deletion mutants, hence there is absolutely no chance for reversion to wildtype phenotype, a common problem with virus vaccines.

The vaccines envisioned by the present invention work for any membrane-enveloped viruses which grow in vertebrate and invertebrate cells. Indeed, the present invention is applicable to membrane-enveloped viruses which can be either engineered to grow in an insect cell, or to membrane-enveloped viruses which grow in genetically-modified insect cells.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion:

EXAMPLE 1

Site-Directed Mutagenesis of Toto 1101

Using the full length clone of the Alpha virus Sindbis described previously (Liu et al 1996, Rice et al., 1987), a deletion removing 3 bases encoding a lysine at position 391 in the amino acid sequence of the virus glycoprotein E2 has been constructed. This lysine is part of the putative membrane-spanning domain of this protein (Rice et al 1982).

Site-directed mutagenesis was used to generate a deletion mutant (Lys391) in Toto 1101, a plasmid containing the full-length Sindbis cDNA and an SP6 promoter that can be used to transcribe infectious RNA from the clone *in vitro* (Rice et al., 1987 Liu and Brown, 1993a). Using the megaprimer method of PCR mutagenisis (Sarkar and Sommer, 1990) described previously (Liu and Brown, 1993a), three nucleotides were removed in the cDNA clone of Toto 1101, nucleotides (nts) 9801, 9802, 9803, resulting in the removal of the codon AAA (K391).

A 30 base oligonucleotide of the sequence, 5'CTCACGGCGCGCACAGGCACATAACACTGC3' (SEQ ID No.: 1) was used as the mutagenesis primer. This primer, along with the "forward primer" 5'CCATCAAGCAGTGCGTCG3' (SEQ ID No.: 2; 18mer), generated a 518 base "Megaprimer" (nucleotides 9295-9813). The second PCR reaction consisted of 0.5 µg of megaprimer, 100 µg Toto

1101 template and 0.5 µg of the "reverse primer" 5' GGCAGTGTGCACCTTAATCGCCTGC 3' (SEQ ID No.: 3). All PCR reactions employed 30 cycles at 95 degrees for 1 min., 64 degrees for 1 min., 72 degrees for 1 min. and a final incubation at 72 degrees for 8 min. The resulting PCR product (1149 nts) was cleaved with BCL I and SPL and inserted into the corresponding site in Toto 1101, creating the deletion mutant K391. After the deletion was confirmed by dideoxynucleotide sequencing through the entire subcloned region using Sequenase™ (U. S. Biochemical, Cleveland, OH), infectious RNA was transcribed *in vitro* using SP6 polymerase and was introduced into BHK-21 cells.

EXAMPLE 2

In vitro Transcription and RNA Transfection

Plasmid DNA containing the full-length cDNA copy of Sindbis virus K391 or wild type RNA was linearized with XhoI and transcribed *in vitro* with SP6 RNA polymerase as described previously (Rice et. al., 1987). 1 µg of Xho I linearized K391 cDNA or wild type Sindbis virus cDNA was transcribed in buffer consisting of 80 mM Hepes pH 7.5, 12 mM MgCl, 10 mM DTT and 2 mM spermidine and 100 µgm BSA with 3 mM each ATP, UTP, CTP, 1.5 mM GTP and 4.5 mM m^7 GpppG, 20 units SP6 RNA polymerase and 20 units RNase inhibitor in a 20 µl reaction volume. After incubation at 37°C for 2 hours, RNA production was assayed by running 2 µl of the RNA product on a 1% agarose gel.

Baby Hamster Kidney (BHK21) cells and Aedes albopictus (mosquito) cells were transfected with RNA derived from the mutant

or wild type clone. Mosquito cell transfections were carried out using $5x10^6$ cells resuspended in RNase free electroporation buffer consisting of 20 mM Hepes pH 7.05, 137 mM NaCl, 0.7 mM Na_2HPO_4 and 6 mM dextran. Washed cells were resuspended in diethyl pyrocarbonate (depc) treated water (HBS) to a concentration of 5 x 10^7 cells/ml. RNA transcripts in 20 µl were added to 400 µl washed cells and transferred to a 0.2 cm gap length cuvette. Optimal electroporation parameters for these cells was found to be 2 KV 25 µF, 8 resistence. Transfected cells were incubated at 37°C until cytopathic effect was observed (about 24 hours).

After 24 hours of incubation, the media was collected from both infected cell lines as well as non-RNA transfected controls. The media from each cell line was tested for the presence of infectious virus by plaque assay (as described by Renz and Brown 1976) on mosquito and BHK-21 cell monolayers (Table 1).

TABLE 1

Infectious virus produced by transfection of BHK21 or Aedes albopictus (AA) cells with Sindbis virus wild type (wt) or mutant K391

Cell line Transf.	BHK Mock[a] Transf.	BHK with wt RNA	BHK with K391 RNA	AA Mock Transf.	AA with wt RNA	AA with K391 RNA
Media titered on BHK	no virus detected	$1.5x10^9$ virus/ml	3.0×10^3	no virus detected	$5.0x10^8$ virus/ml	1.0×10^8 virus/ml
Media titered on AA	no virus detected	$8x10^7$ virus/ml	8.0×10^4	no virus detected	$1.0x10^9$ virus/ml	2.0×10^9 virus/ml

a. Mock indicates that transfection protocol was carried out without RNA

As shown in Table 1, the mutant K391 produces significant amounts of infectious virus particles only when replicating in the insect cell. BHK cells transfected with K391 produced very low levels of virus, 4 to 5 orders of magnitude lower than the amount produced in insect cells.

EXAMPLE 3

Metabolic Radioactive Labeling of Viral Proteins

Subconfluent monolayers of BHK21 cells in 25 cm^2 flasks were transfected with wild type or K391 mutant RNA as described

above. Monolayers were starved for 30 min in methionine- and cysteine-free medium (MEM-E) containing 1% FCS, 2 mM glutamine and 5% TPB (starvation medium). At 16 hours post-transfection, cells were pulse-labeled with starvation medium containing 50 μCi/ml [^{35}S] Met/Cys protein labeling mix for 20 minutes. Labeling was terminated by washing the monolayers with PBS containing 75 μg/ml cycloheximide. Monolayers were chased for 45 minutes in medium containing 10 times the normal concentration of methionine and cysteine and 75 μg/ml cycloheximide.

EXAMPLE 4

Immunoprecipitation and Polyacrylamide Gel Electrophoresis:

Radiolabeled viral proteins were immunoprecipitated with antisera as described (Knipfer and Brown, 1989). [^{35}S] Met/Cys labeled cells were washed twice in cold PBS and lysed in lysis buffer: 0.5% NP-40, 0.02 M Tris HCl pH 7.4, 0.05 M NaCl, 0.2 mM PMSF, 0.2 mM TPCK and 0.02 mM TLCK. The nuclei were pelleted by centrifugation and discarded. The supernatant was pre-absorbed with 100 μl of protein A/Sepharose beads (Sigma) suspended in lysis buffer for 1 hr, and the beads were pelleted. The pre-absorbed supernatant was treated with 200 μl of protein A/Sepharose beads coupled to rabbit anti-SVHR serum or E2 tail monospecific polyclonal serum and agitated overnight at 4°C. The immunoprecipitated bead-antibody-protein complexes were washed three times with lysis buffer and then solubilized in SDS-PAGE sample buffer consisting of 12% glycerol, 4% SDS, 50 mM Tris pH 6.8, 5% mercaptoethanol and 0.02% bromphenol blue. The samples were heated for 3 min at 95°C and the beads were

removed from the sample by centrifugation. Gel electrophoresis was carried out on a 10.8% SDS-PAGE or 16% Tricine gel as described previously (Liu and Brown, 1993 a,b). Fluorography was performed as described (Bonner and Laskey, 1974) and dried gels were exposed to Kodak XAR-5 film (see Figure 1).

EXAMPLE 5

Transmission Electron Microscopy

BHK-21 cell monolayers infected with K391 produced from transfected mosquito cells or transfected with K391 RNA were lifted from flasks by trypsin treatment at desired time points, and the cells were pelleted by low speed centrifugation. Cell pellets were washed twice in PBS and fixed in 4% glutaraldehyde at 4°C overnight. The cells were then washed three times with 0.2 M cacodylate buffer (pH 7.2), post-fixed with 2% osmium tetroxide for 1 hour at room temperature, and washed three times in cacodylate buffer. The cells were stained en bloc for 1 hr at room temperature with 0.5% uranyl acetate. After three washes, cell pellets were embedded in 1% agarose and dehydrated through a graded ethanol/acetone series. Final embedding was in Mollenhauer's (1964) Epon-Araldite epoxy mixture #1 at 70°C for two days. Ultrathin sections were cut on a Sorvall MT5000 microtome and collected on 150 mesh copper grids. Sections were stained with 1% uranyl acetate and/or lead citrate and were photographed in a Jeol 100CX transmission electron microscope (see Figure 2).

Although BHK cells infected with K391 virus or transfected with K391 RNA produce no virus detectable by the plaque assay, it was

shown by PAGE that they do produce all virus structural proteins (Figure 1). Further, it was shown by electron microscopy that they assemble the intracellular (non infectious) virus cores (Figure 2).

EXAMPLE 6

Uses for the Sindbis Deletion Mutant K391 and Similar Mutations Produced in Other Togaviruses

Delta K391 produces very high titers of mutant Sindbis virus particles when allowed to replicate in mosquito cells. The exposed regions of the proteins (ecto domains) are wild type in sequence. These wild type proteins allow the virus to enter mammalian cells and produce virus proteins (see Figure 1) but new virus is not assembled as shown by electron microscopy in Figure 2.

Delta K391 is a vaccine strain. It is produced in very high concentration in cultured insect cells. However, when the virus is injected into a mammalian host, the virus circulates and infects cells in a mammalian host, these infected cells produce and present virus proteins for immune surveillance, but, because of the truncation in the membrane domain, the infection will be limited primarily to those cells infected initially by the innoculum. Because the vaccine strain is the result of a deletion mutation, reversion to wild type pathogenic phenotype is not possible.

Further, an engineered deletion mutant may be introduced into the wild mosquito population. It has been shown that these viruses are spread from the female parent to the progeny by a process of transovariol transmission (Leakey 1984). When these mosquitoes bite a vertebrate they will provide an immunizing dose (10^6 infectious particles) of the vaccine strain (for example,Delta K391). Karpf et al

(1997) showed that infection of insect cells by one Alpha virus prevents the cells from being infected by another, even distantly-related alpha virus for an indefinite amount of time (over two years in cell culture, where the life of a mosquito is 28 days). Thus, the presence of the vaccine strain (for example Sindbis Delta K391 or TM16) will block the spread of other related and pathogenic viruses by these insects.

EXAMPLE 7

Additional Deletion Mutations

Additional deletion mutations in the membrane-spanning domain of Sindbis virus glycoprotein E2 were prepared. The protocol for production of these deletion mutations is described below. The procedure is described for the model membrane-containing virus Sindbis, however, the procedure can be easily applied to any other virus membrane glycoprotein.

The envelope glycoproteins of Sindbis virus are integrated into the membranes of the endoplasmic reticulum as a multi pass protein with 6 membrane spanning domains. There are, therefore, 6 potential targets for the production of deletion mutations that will prevent the correct integration of a transmembrane domain (TMD) (See Figure 3). Some of these targets are less satisfactory for this procedure than others. TMD #1 (Figure 3) is the signal sequence which is recognized by the Signal Recognition Particle and directs protein synthesis to the membranes of the ER. Truncating this domain would likely disturb targeting in both mammalian and insect cells.

TMD #3 contains the protein sequence of E2 which recognizes and binds capsid protein. It has been shown that this interaction is very specific in nature and requires the sequence that is in the transmembrane domain (Liu *et al.*, 1996; Lopez *et al.*, 1994). TMD#3, therefore, like TMD #1 has a functional as well as a structural component. A significant deletion in this domain would likely eliminate budding in both cell systems. This leaves four transmembrane domains which are targets for the production of deletions which will effect membrane integration (Fig 3., TMD #2, #4, #5, and #6).

The 6k protein is not a component of mature virus and its function in virus assembly is not clear. In the poly protein the proper integration and orientation of 6k in the ER membrane is essential for the correct integration of E1. The transmembrane domains of 6k (TMD #4 and #5) are excellent targets for deletion mutation as failure to integrate one of these domains may cause the poly protein to integrate into the membrane in a wrong configuration or cause the failure to integrate E1. TMD #2 and #6 are the membrane spanning domains of E2 and E1 and are both obvious targets for deletion mutation. Multiple membrane spanning domains in this poly protein suggest that if deletion mutations in a single transmembrane domain do not totally block virus production in mammalian cells, then deletions in additional membrane spanning domains can further reduce maturation to negligible levels.

EXAMPLE 8

Design of Mutagenic Primers for the E2 Hydrophobic Membrane Anchor (TMD#2)

Protocols for testing the requirements placed on the transmembrane domain of E2 (Figure 3, TMD #2) is given. This protocol can be easily replicated for any other of the Sindbis membrane spanning domains or the membrane spanning domains of any other virus glycoprotein. The hydrophobic Sindbis PE2 membrane anchor consists of 26 amino acids. As is common with other membrane spanning domains little amino acid homology is conserved among the alphaviruses, although the length of this hydrophobic region is highly conserved (Strauss and Strauss, 1994). The lack of sequence conservation in this domain suggests that it is the hydrophobic properties of the domain and not its sequence which is critical for integration.

The transmembrane domain of E2 begins at amino acid 365 of the PE2 sequence. This hydrophobic region consists of the sequence: VYTILAVASATVAMMIGVTVAVLCAC (SEQ ID No.: 4). Adams and Rose (1985) demonstrated that a minimum of 14 amino acids in the transmembrane domain of the VSV G protein were necessary for proper anchoring in mammalian cells. Therefore, mutagenic primers have been designed which create a nested set of deletions in the E2 transmembrane domain. Beginning with a deletion of 16 amino acids (which leaves 10 amino acids in the hydrophobic region), a set of deletions were constructed which delete from as many as 16 amino acids, to as few as 1 amino acid from the membrane anchor (Figure 4).

Deletions were constructed using PCR megaprimer mutagenesis to generate deleted fragments containing unique BclI and SplI sites. All resulting constructs were installed into the wt Sindbis

cDNA construct Toto Y420 to generate the mutant plasmids. After linearization with XhoI and transcription using SP6 polymerase, transcripts were transfected into BHK or Aedes albopictus cells by electroporation (as described above). Production of infectious virus from these transfections were titered on both BHK and C710 mosquito cells to determine the host range of these constructs. Table 2 shows the deleted sequences and the primer sequences used in their construction.

For each construct the same primer pair is used to generate the entire BclI to SplI region. The forward primer E1Bcl21 is comprised of the sequence from nucleotide 9306-9327 and reads from 5'–3' GCGTCGCCTATAAGAGCGACC (SEQ ID No.: 5). The reverse primer Splext is comprised of the sequence from nucleotide 10420-10444 which is the complementary sequence reading from 5'-3' CAGTGTGCACCTTAATCGCCTGC (SEQ ID No.: 6).

The virus produced by transfection of insect cells is tested for its ability to produce plaques in BHK and C7-10 mosquito cells as for the mutant E2 ΔK391. Those mutants which do not produce plaques in BHK cells are tested for their ability to infect BHK cell relative to wild type virus by immunofluorescence assay of infected monolayers. This later assay is compared to the total protein in purified preparations of the mutant and wild type virus to establish the relative infectivity of each mutant population. The goal is to truncate the transmembrane domain as much as possible and still obtain reasonable amounts of virus in C7-10 mosquito cell monolayers which can infect but not produce mature virus in BHK cells. If the circumstance arises that truncation of a single transmembrane domain reduces but does not eliminate virus growth in BHK cells a second domain will be truncated and so fourth up to four domains.

TABLE 2

Listing of the deletions in Sindbis E2 and the primers used

Primer-Designated by No. of Transmembranal Amino Acids	Nucleotides Deleted	Oligonucleotide Sequence of Mutagenic Primer (Negative Strand)
E2 TM10	9734-9782	ACATAACACTGCGATGGTGTACAC (SEQ ID No.: 7)
E2 TM12	9740-9782	ACATAACACTGCGGCTAAGATGG (SEQ ID No.: 8)
E2 TM14	9746-9782	ACATAACACTGCTGCGACGGCT (SEQ ID No.: 9)
E2 TM16	9743-9773	GCAACAGTTACGACGGCTAAG (SEQ ID No.: 10)
E2 TM17	9743-9770	ACAGTTACGCCGACGGCTAAG (SEQ ID No.: 11)
E2 TM18	9743-9767	GTTACGCCAATGACGGCTAAG (SEQ ID No.: 12)
E2 TM19	9743-9764	CGCCAATCATGACGGCTAAGA (SEQ ID No.: 13)
E2 TM20	9755-9773	GCAACAGTTACGGTAGCTGA (SEQ ID No.: 14)
E2 TM21	9755-9770	AGTTACGCCGGTAGCTGA (SEQ ID No.: 15)
E2 TM22	9761-9773	TGCAACAGTTACCGCCACGGT (SEQ ID No.: 16)
E2 TM23	9761-9770	ACAGTTACGCCCGCCACGGT (SEQ ID No.: 17)
E2 TM24	9761-9767	GTTACGCCAATCGCCACGGT (SEQ ID No.: 18)
E2 TM25	9761-9764	ACGCCAATCATCGCCACGGT (SEQ ID No.: 19)

This protocol described by the present invention works for any virus which replicates in insects and mammals and has integral membrane proteins as part of its structure, namely, Togaviruses, Flaviviruses and Bunya viruses and all other enveloped viruses which can replicate naturally in both mammalian and insect cells, as well as enveloped viruses which can be made to replicate in mammalian and insect cells by genetic engineering of either the virus or the cell.

Vaccines are made against any membrane-containing virus by removing amino acids from the membrane-spanning domain of a protein in the viral envelope. This is done by removing bases from a cDNA clone of the virus as described. RNA transcribed from the altered clone is transfected into insect cells. The virus produced is amplified by repeated growth in insect cells until large quantities of mutant virus are obtained. This virus is tested for its ability to infect and produce progeny in mammalian cells. Virus which does not produce progeny in mammalian cells are tested for ability to produce immunity in laboratory animals. Those which do produce immunity are candidates for production of human and animal vaccines as is known in the art. This protocol is employed with any arbovirus or other enveloped viruses.

EXAMPLE 9

The Growth of Deletion Mutants

Table 3 shows the growth of the deletion mutants TM12, TM16 and ΔK391 compared to the wild type strain.

TABLE 3

Replication of Sindbis Trans-Membrane Domain Mutants

Growth in Cell Line	Wild Type	TM12	TM16	ΔK391
BHK21	3.5×10^8	3.0×10^5	3.4×10^4	1.5×10^4
A. albopictus	1.8×10^8	4.5×10^4	1.5×10^8	6.0×10^9

Other deletion mutants, other than TM12 and TM16, have been made including utants TM10, TM 14 and TM 17. Infection data is collected. TM 10 may not be as useful as TM12 is already too short to replicate in either cell type. TM 14 may be interesting but it may not work better than TM 16. It will likely not work as well. TM 17 like TM 14 may or may not produce the differential titre seen.

As described in Example 8, TM12 deletes 14 amino acids (nucleotides 9740-9782 deleted, Table 2) from the membrane spanning domain of the glycoprotein. TM16 deletes 10 amino acids (nucleotides 9743-9773 deleted, Table 2) from the membrane spanning domain. The data (Table 3) demonstrate that TM12 grows poorly in both the insect and mammalian cells, while TM16 grows to very high levels in insect cells but poorly (4 orders of magnitude lower) in mammalian cells. In this regard, TM16 is like ΔK391. However, since TM16 has a larger size deletion (10 amino acids deleted) compared to single deletion mutant ΔK391, TM16 should be a better vaccine candidate than ΔK391 as the large size deletion insures that the mutant virus cannot revert to wild type phenotype.

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Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Further, these patents and publications are incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. A genetically engineered, membrane-enveloped virus, wherein said virus codes for a transmembrane protein having a deletion of one or more amino acids, and wherein said deletion provides a transmembrane protein that is able to span or correctly integrate into the membrane envelop resulting in cells which produce infectious virus particles when said virus replicates in insect cells, while wherein said deletion provides a transmembrane protein that is unable to span or correctly integrate into the membrane envelop resulting in cells which produce non-infectious virus particles when said virus replicates in mammalian cells.

2. The genetically engineered, membrane-enveloped virus of claim 1, wherein said virus is an Arthropod vectored virus.

3. The genetically engineered, membrane-enveloped virus of claim 1, wherein said virus is selected from the group consisting of Togaviruses, Flaviviruses, Bunya viruses, enveloped viruses capable of replicating naturally in mammalian and insect cells, and enveloped viruses which replicate in mammalian and insect cells as a result of genetic engineering of either the virus or the cell.

4. The genetically engineered, membrane-enveloped virus of claim 1, wherein said insect cells are mosquito cells.

5. The genetically engineered, membrane-enveloped virus of claim 4, wherein said mosquito cells are Aedes albopictus cells.

6. The genetically engineered, membrane-enveloped virus of claim 1, wherein said mammalian cells are human cells.

7. The genetically engineered, membrane-enveloped virus of claim 1, wherein said virus is Sindbis virus, and said transmembrane protein is viral glycoprotein E2.

8. The genetically engineered, membrane-enveloped virus of claim 7, wherein said virus is selected from the group consisting of ΔK391 virus and TM16 virus.

9. The genetically engineered, membrane-enveloped virus of claim 1, wherein said virus is selected from the group consisting of HSV, HIV, rabies virus, Hepatitis, and Respiratory Syncycial virus, and wherein said transmembrane protein is selected from the group consisting of glycoprotein E1, glycoprotein E2, and G protein.

10. The genetically engineered, membrane-enveloped virus of claim 1, wherein said viruses are RNA tumor viruses, and wherein said transmembrane protein is Env.

11. A method of producing a viral vaccine from the genetically engineered, membrane-enveloped virus of claim 1 for vaccination of mammals, comprising the steps of:

introducing said genetically engineered, membrane-enveloped virus into insect cells; and

allowing said virus to replicate in said insect cells to produce a viral vaccine.

12. The method of claim 11, wherein said genetically engineered, membrane-enveloped virus is an Arthropod vectored virus or any virus capable of replicating.

13. The method of claim 11, wherein said virus is selected from the group consisting of Togaviruses, Flaviviruses, Bunya viruses, enveloped viruses capable of replicating naturally in both mammalian and insect cells, and other enveloped viruses which replicate in mammalian and insect cells as a result of genetic engineering of either the virus or the cell.

14. The method of claim 11, wherein said virus is selected from the group consisting of ΔK391 virus and TM16 virus.

15. A method for vaccinating an individual in need of such treatment, comprising the steps of:

introducing the viral vaccine of claim 11 into said individual; and

allowing said viral vaccine to produce viral proteins for immune surveillance and stimulate immune system for antibody production in said individual.

16. A method of producing a viral vaccine to a disease spread by a mosquito population to a mammal, comprising the steps of:

producing deletions in the membrane associated domains of the virus which restrict their ability to grow to insect cells;

introducing said engineered virus into a wild mosquito population; and

allowing said engineered virus to replicate in cells of said wild mosquito population to produce a mosquito population which harbors the vaccine strain of said engineered virus and excludes the wild type (pathogenic) virus such that the mosquito bite delivers the vaccine to the mammal bitten.

17. The method of claim 16, wherein said deletions in the membrane associated domains of the virus are prodcued by genetically engineering a deletion of one or more amino acids in a viral transmembrane protein to produce an engineered virus, wherein

said transmembrane protein is able to span the membrane envelope when said virus replicates in cells of said mosquito population, but is unable to span the membrane envelope when said virus replicates in cells of said mammal, and wherein said virus remains capable of replicating in the mosquito cells.