(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 February 2006 (09.02.2006)

PCT

(10) International Publication Number
WO 2006/013367 A2

(51) International Patent Classification:
C12N 15/62 (2006.01)

(21) International Application Number:
PCT/GB2005/003053

(22) International Filing Date: 2 August 2005 (02.08.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/598,674	4 August 2004 (04.08.2004)	US
0417390.2	4 August 2004 (04.08.2004)	GB

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2006/013367 A2

(54) Title: CHIMERIC PROTEINS AND USES THEREOF

(57) Abstract: The invention relates to chimeric proteins which comprise an amino acid sequence of a viral protein and which exhibit structural and functional properties of the native parent proteins, as well as nucleic acids and expression vectors encoding such proteins and host cells expressing such proteins. The invention further relates to expression of the chimeric proteins, including in recombinant viruses, as well as uses of the chimeric proteins and recombinant viruses expressing them.

Chimeric Proteins and Uses Thereof

Field of the Invention

The present invention relates to chimeric proteins which exhibit structural and functional properties of the native parent proteins. In particular, the present invention relates to chimeric proteins comprising an amino acid sequence of a viral protein. The present invention further relates to expression of the chimeric proteins, including in recombinant viruses, as well as uses of the chimeric proteins and recombinant viruses expressing them.

Background of the Invention

Attachment and entry of human immunodeficiency virus type-1 (HIV-1) to a host cell is mediated by the envelope (Env) glycoproteins. Env monomers are synthesised as 160 kDa glycoproteins (gp160) in the endoplasmic reticulum, where oligimerisation takes place with trimers being the predominant form. The gp160 precursor is transported to the golgi where it is proteolytically processed by furin and furin-like enzymes into gp120 and gp41 subunits, which are held together non-covalently. The processed Env glycoproteins are then presented on the surface of the infected cell and subsequently, through budding, form the envelopes of progeny virions.

HIV-1 attachment to target cells is mediated by the binding of gp120 to CD4, the primary HIV-1 receptor. This binding exposes a site on gp120 that enables interactions with secondary co-receptors [1]. The two major HIV-1 co-receptors are the chemokine receptors CCR5 and CXCR4; CCR5 is generally considered to be the co-receptor utilised by HIV-1 virions in early, asymptomatic

stages of infection whereas viruses utilising CXCR4 usually emerge during late stages of disease. Binding of gp120 to the appropriate co-receptor triggers a conformational change in gp41. gp41 contains a short N-terminal hydrophobic sequence of amino acids, the fusion peptide, which mediates fusion of viral and host cell membranes, enabling entry of the virus into the host cell. The conformational change in gp41, induced by the gp120-co-receptor interaction, is necessary in order for this fusion to take place. Both the gp120 and gp41 subunits act as major targets for the host immune system.

It is generally accepted that knowledge of the structures of HIV glycoproteins will yield insights into the functions of the proteins, which will assist in the development of vaccines and drugs for anti-HIV therapies. However, an obstacle to the development of treatments for HIV is the difficulty in obtaining the required information about the native structure of HIV glycoproteins.

Indeed, the ability to obtain a detailed understanding of function and involvement in physiological processes is limited for any protein, particularly glycoproteins, for which structural information is incomplete, for example due to structural instability of the protein. This in turn limits the development of therapeutic applications of such proteins.

For HIV, despite intensive effort in a number of laboratories, the current understanding of attachment and fusion mechanisms of HIV-1 Env is incomplete. The structural information available for HIV glycoproteins is relatively poor and relates to severely truncated forms

of monomeric gp120 [1, 2] and trimeric extracellular/extraviral domains of gp41, both of which may represent post-fusion states. The relative lack of success probably relates to the highly glycosylated states of the HIV glycoproteins (many of the added oligosaccharides play a role in determining the correct folding of the protein) and the glycoproteins' inherent instability, which can result in extensive dissociation of gp120 from HIV-1 virus and virus-infected cells [3]. Glycosidases have been used to remove oligosaccharides after glycoprotein synthesis [1, 2] in attempts to overcome the former problem. Domains from other proteins have been introduced into the proteins in attempts to improve stability [4, 5], but with limited success.

At present, the most effective treatment of HIV infection relies on cocktails of drugs which can reduce viral loads to below detectable limits. However, such treatments do not actually clear virus from the host (which would effectively provide a cure) because HIV can persist in a small reservoir of latently-infected memory CD4+ T cells. Further, drug resistance can develop, as can problems related to long term use of drugs.

Gene therapy approaches, largely based on retroviral vectors expressing herpes simplex virus thymidine kinase under the control of an HIV-2 LTR, have been developed to allow selective killing of HIV-infected cells [6]. However, use of retroviral vectors that integrate stably into the host genome in a random manner have resulted in detrimental outcomes, including problems of insertional mutagenesis. Recombinant influenza virus has been used to display epitopes from foreign antigens in which sequences encoding the epitopes were substituted in the

haemagglutinin (HA) gene. Influenza virus haemagglutinin (HA) is a trimeric membrane glycoprotein with receptor-binding and membrane fusion functions [7]. Following initial attachment to the host target cell, via sialic acid containing receptors, and internalisation by host-mediated endocytosis, HA mediates fusion between the viral and endosomal membranes allowing the release of ribonucleoprotein into the infected cell. Since the initial crystallisation of influenza HA [7], its structure and function have been extensively studied and well characterised [8]. HA is initially synthesised as a trimeric precursor (HA0) containing three identical protein chains each of which is proteolytically processed into two subunits, HA1 and HA2, that are held together covalently by a single disulphide bond. The HA1 subunit forms a globular head and contains the receptor-binding sites and the majority of antigenic sites. The HA2 subunit anchors the structure to the membrane, provides stability to the trimeric structure and contains an N-terminal fusion peptide that has a region of homology with the gp41 fusion peptide. Acidic conditions trigger influenza HA to undergo a conformational change that allows fusion of neighbouring membranes. Thus, incorporation of sequences encoding epitopes of foreign antigens into the HA gene results in the expression of the epitopes on the surface of the virus. Recombinant influenza virus displaying foreign epitopes has been used in cancer therapy and as vaccines. Such viruses displaying HIV glycoprotein epitopes have been used as vaccines in mice. Use of larger substitutions in the HA gene have only recently been reported [9, 10]. Attempts have been made to produce HA recombinants between influenza types B and A in an influenza A background. However, success has been limited and the resultant

viruses were attenuated compared to the parental type A virus.

The development of a reverse genetics system that allows efficient recovery of recombinant influenza viruses opens up the use of such viruses for gene therapy [11]. The system is based on the early human type A H1N1 influenza virus, WSN [11, 12]. It has been shown recently that stable recombinant influenza viruses carrying two foreign genes, by replacing the haemagglutinin (HA) and neuraminidase (NA) genes, can be produced [13].

Therefore, generally, there is a need to be able to produce proteins which are stable and retain the structure and function of the native protein in order to further understand their function. In particular there is a need to be able to produce HIV glycoproteins having the structure and function of the native proteins for use in structural studies and to facilitate the development of effective anti-HIV therapeutic strategies.

Summary of the Invention

The present invention aims to address the difficulties of obtaining information about the native structure and function of a protein for which obtaining such information is difficult, for example due to instability of the protein, for example glycoproteins. The present invention is particularly applicable to proteins which are associated with a disease state or condition, in particular, a virus-induced disease. In particular, the present invention aims to address the need to be able to produce stable proteins, such as glycoproteins, for example viral glycoproteins such as HIV glycoproteins, retaining the structure and function of the native

protein. The present invention further aims to address the need to develop therapeutic applications using these proteins, in particular to develop effective therapeutic strategies for diseases such as virus-induced diseases, for example, AIDS caused by HIV.

Generally, the invention lies in providing a chimeric protein which has structural properties of the native proteins of which it is comprised, such that functional properties of the native proteins are retained. The invention further lies in providing a chimeric protein having a stable structure. In particular, the invention lies in providing a chimeric protein which has a stable structure having structural and functional properties of the native proteins.

The invention relates to a chimeric protein comprising influenza haemagglutinin (HA) amino acid sequence. In particular, the invention relates to a chimeric protein comprising amino acid sequence of the HA1 subunit and the HA2 subunit of HA. The invention similarly relates to a chimeric protein comprising a heterologous protein amino acid sequence. In particular, the invention relates to a chimeric protein comprising amino acid sequence of a protein associated with a disease, such as a virus-induced disease, for example a viral glycoprotein amino acid sequence, such as an HIV glycoprotein amino acid sequence.

The invention is concerned with a chimeric protein comprising HA1 and HA2 subunit sequence which has the stable structure of native HA. The invention is further concerned with disulphide bonds between cysteine residues of the chimeric protein. In particular, the invention is

concerned with disulphide bonds between cysteine residues of HA protein in the chimeric protein.

Generally, the invention further lies in the expression of chimeric proteins of the invention. In particular, the invention further lies in providing recombinant virus expressing chimeric proteins of the invention. The invention is particularly concerned with recombinant virus expressing chimeric protein that has a stable structure of the native proteins and functional properties of the native proteins, for example, a chimeric protein comprising HIV glycoprotein that has a stable structure and function of the native glycoprotein.

Furthermore, the invention lies in providing chimeric proteins and recombinant viruses expressing the chimeric proteins for use in medical treatment or therapy. The invention particularly relates to recombinant viruses for use as vaccines or in gene therapy for the treatment of a mammal. In particular, the invention is concerned with recombinant viruses for use as vaccines or for use in gene therapy as an anti-viral therapeutic strategy, for example, an anti-HIV therapeutic strategy for the treatment of HIV-infected individuals.

Accordingly, in a first aspect, the present invention provides a chimeric protein comprising (a) an amino acid sequence of the HA1 subunit of influenza virus haemagglutinin (HA) protein comprising cysteine residues at positions 30, 68 and 293 as numbered from the N-terminal amino acid of the HA1 subunit from influenza virus X31, or corresponding positions in the HA1 subunit from a different influenza virus and (b) an amino acid sequence of the HA2 subunit of influenza virus HA protein

comprising cysteine residues at positions 153, 160 and 164 as numbered from the N-terminal amino acid of the HA2 subunit from influenza virus X31, or corresponding positions in the HA2 subunit from a different influenza virus and (c) an amino acid sequence of a heterologous protein positioned between the cysteine residues at positions 68 and 293, or the corresponding positions, of (a) and having a disulphide bond between the cysteine residues at position 30 of HA1 and position 153 of HA2, at positions 68 and 293 of HA1, and at positions 160 and 164 of HA2, or the corresponding positions, wherein the chimeric protein has the structural properties of the native HA protein such that the structure and function of the native heterologous protein are retained.

In one embodiment of the invention, the amino acid sequence of the HA2 subunit further comprises the membrane anchor region from position 186 to 207, inclusive.

Preferably, the chimeric protein comprises in the direction from the N-terminus to the C-terminus, (i) an amino acid sequence of the HA1 subunit from and including position 1 to and including position 68 as numbered from the N-terminal amino acid of the HA1 subunit from influenza virus X31, or corresponding positions in the HA1 subunit from a different influenza virus; (ii) an amino acid sequence of a heterologous protein; (iii) an amino acid sequence of the HA1 subunit from and including position 293 to and including position 345 as numbered from the N-terminal amino acid of the HA1 subunit from influenza virus X31, or corresponding positions in the HA1 subunit from a different influenza virus and (iv) the amino acid sequence of the HA2 subunit from and including

position 1 to and including position 237 as numbered from the N-terminal amino acid of the HA2 subunit from influenza virus X31, or corresponding positions in the HA2 subunit from a different influenza virus.

In one embodiment of the invention, the chimeric protein further comprises an amino acid linker sequence at either or both of the junctions between the heterologous protein sequence and the HA1 subunit sequence. In particular, the amino acid linker sequence is between sequences (i) and (ii) and/or (ii) and (iii). Preferably, the linker sequence is Gly-Gly-Gly-Ser.

In preferred embodiments, the chimeric protein comprises influenza HA protein from influenza virus X31.

The heterologous protein component of the chimeric protein may be from a protein associated with a disease. In certain embodiments, the heterologous protein component is from a protein associated with a virus-induced disease, for example, from a viral protein. The heterologous protein component may be from a protein that has a trimeric quaternary structure or is functional in a trimeric structure. The heterologous protein component may be from a receptor-binding protein.

In preferred embodiments, the amino acid sequence of the heterologous protein is from a protein from a virus belonging to one of the following virus families:
Orthomyxoviridae, for example, haemagglutinin glycoprotein of influenza virus
Retroviridae, for example, surface glycoprotein SU of Rous Sarcoma virus (RSV), Feline Leukemia virus (FeLV), Human T-cell Leukemia viruses 1 and 2 (HTLV-1/2), Bovine

Leukemia virus (BLV), Human Immunodeficiency viruses (HIV-1/2), Simian Immunodeficiency virus (SIV), Equine Infectious Anemia virus (EIAV), Feline Imunodeficiency virus (FIV), Caprine Arthritis Encephalitis virus (CAEV), Visna/Maedi

Filoviridae, for example, surface glycoprotein GP1 of Ebola, Marburg

Togaviridae, for example, E2 protein of alphaviruses such as Semliki forest virus, Sindbis virus, Ross River, Eastern/Western Equine Encephalitis, and rubiviruses such as Rubella

Coronaviridae, for example, S1 protein of Severe Acquired Respiratory Syndrome (SARS)

Reoviridae, for example, VP2 protein of orbiviruses such as Bluetongue virus, African horse sickness virus

Rhabdoviridae, for example, G proteins of vesiculoviruses such as Vesicular Stomatitis virus (VSV) and Lyssaviruses such as Rabies

Flaviviridae, for example, E (E2) proteins of Tick Borne Encephalitis (TBE), Dengue, Hepatitis C virus (HCV), West Nile, Yellow fever.

Alternatively, amino acid sequence from a protein from a virus of the family *Arenaviridae*, such as GP1 protein of Lassa, Venezuelan hemorrhagic fever, Lymphocytic choriomeningitis may comprise the heterologous protein component.

In particularly preferred embodiments, the amino acid sequence of a heterologous protein is from an HIV glycopotein, preferably an HIV-1 Env glycoprotein. Preferably, the HIV-1 Env glycoprotein is HIV-1 gp120. In preferred embodiments, the HIV-1 gp120 is from HIV-1 strain pNL43 or HIV-1 strain JRFL. More preferably, the

amino acid sequence of HIV-1 gp120 comprises cysteine residues at positions 119, 126, 131, 157, 194, 203, 216, 226, 237, 245, 294, 329, 376, 383, 416 and 443 as numbered from the N-terminal amino acid of gp120 from HIV-1 strain pNL43, or corresponding positions in other strains of HIV. Most preferably, the amino acid sequence of HIV-1 gp120 comprises the amino acid from and including position 89 to the amino acid at and including position 495 as numbered from the N-terminal amino acid of gp120 from HIV-1 strain pNL43, or corresponding positions in other strains of HIV.

In a second aspect, the present invention provides a nucleic acid encoding a chimeric protein of the invention.

In a further aspect, the present invention provides an expression vector comprising a nucleic acid of the invention. The expression vector may be a mammalian expression vector for expression of the chimeric protein in a mammalian cell, preferably for constitutive expression in a mammalian cell. Alternatively, the expression vector may be a viral expression vector for expression of the chimeric protein by a virus infecting a susceptible host cell.

In further aspects, the present invention provides a host cell comprising a nucleic acid of the invention or an expression vector of the invention and a method of producing a host cell of the invention. The host cell may constitutively express the chimeric protein.

Another aspect of the invention is a method of producing a chimeric protein of the invention.

Yet another aspect of the present invention provides a method of producing a recombinant virus capable of expressing a chimeric protein of the invention, comprising contacting a susceptible cell with an expression vector of the invention and optionally isolating the virus from the cell. Preferably, the virus is a vaccinia virus. More preferably, the virus is an influenza virus.

In another aspect, the present invention provides a virus obtained by the method of the invention for producing a recombinant virus. In one embodiment, a virus of the invention is further capable of expressing a conditionally lethal toxic protein. The conditionally lethal toxic protein may be herpes simplex virus thymidine kinase or an apoptosis inducer such as a caspase.

In yet a further aspect, the present invention provides a target cell infected with a virus of the invention.

A virus and/or a chimeric protein of the invention may be used as a treatment or therapy for a mammal, preferably a human. A virus and/or a chimeric protein of the invention may be used as a vaccine for a mammal, preferably as a vaccine for a human. Further, a virus and/or a chimeric protein of the invention may be used for the preparation of a vaccine. A vaccine comprising a virus and/or a chimeric protein of the invention is also provided by the present invention. Further, a virus and/or a chimeric protein of the invention may be used for the preparation of a medicament for the treatment of a mammal.

A virus of the invention which expresses a chimeric protein of the invention comprising an amino acid sequence of a protein associated with a disease state and/or a chimeric protein comprising an amino acid sequence of a protein associated with a disease state may be used as a vaccine for individuals suffering from the disease or those susceptible to developing the disease, or for the preparation of a vaccine or for the preparation of a medicament for the treatment of an individual suffering from the disease. In preferred embodiments, the disease is a virus-induced disease.

In particularly preferred embodiments, a virus of the invention which expresses a chimeric protein of the invention comprising an amino acid sequence of an HIV Env glycoprotein and/or a chimeric protein comprising an amino acid sequence of an HIV Env glycoprotein may be used as an anti-HIV vaccine for HIV-infected individuals or those susceptible to infection, or for the preparation of a vaccine or for the preparation of a medicament for the treatment of an HIV-infected individual.

Furthermore, a virus of the invention may be used in gene therapy for a mammal, preferably a human. A virus of the invention which expresses a chimeric protein of the invention comprising an amino acid sequence of a protein associated with a disease state and/or a chimeric protein comprising an amino acid sequence of a protein associated with a disease state may be used in the treatment of an individual suffering from the disease. Such a virus may also be used for the preparation of a medicament for the treatment of an individual suffering from the disease. In preferred embodiments, the disease is a virus-induced disease. In particularly preferred embodiments, a virus

of the invention which expresses a chimeric protein of the invention comprising an amino acid sequence of an HIV-1 Env glycoprotein and/or a chimeric protein comprising an amino acid sequence of an HIV-1 Env glycoprotein may be used in the treatment of an HIV-infected individual. Such a virus may be used as part of a therapeutic strategy to clear HIV from an infected individual. Such a virus may also be used for the preparation of a medicament for the treatment of an HIV-infected individual.

A virus and/or a chimeric protein of the invention may be used as a treatment or therapy either alone or in combination with other medicaments and/or treatments.

Another aspect of the invention provides a composition comprising a virus and/or a chimeric protein of the invention for the treatment or therapy of a mammal, preferably a human.

Brief Description of the Figures

Embodiments of the invention will now be described in more detail, by way of example only, with reference to the accompanying drawings, in which:

Figure 1 shows a schematic representation of the assembly of the EnvHA chimeric protein of an embodiment of the present invention. (A) The influenza X31 (H3) HA, mRNA sense, with 5′ and 3′ non-coding regions is shown as is the disulphide-bonded structure of the encoded HA in the processed form. HA1/HA2 subunits are covalently linked by a single disulphide bond between residue 30 of HA1 and residue 153 of HA2. The positions of the signal peptide , fusion peptide, and membrane anchor, are shown (residue

numbering is for influenza virus X31 and includes the signal peptide and fusion peptides in HA1 and HA2 respectively) as is the bromelain (Br) cleavage site in HA2. (B) The HIV-1 Env is presented in the processed form of gp120/gp41 with its disulphide bonding pattern. The two subunits are held together non covalently. All numbering refers to the HIV-1 pNL43 Env sequence [14] and the positions of the signal peptide, fusion peptide, and membrane anchor, are shown. (C) The structure of the EnvHA chimeric protein is shown with the Env- and HA-derived domains indicated. Dotted lines represent the sites where linkers (GGGS) are positioned in respective EnvHA constructs (Tables 1 and 2). Note the retention of the HA1 68-293 and HA1/HA2 30-153 disulphide bonds which are believed to bring stability to the EnvHA construct. There is a N to H substitution between HIV-1 NL43 and HIV-1 JRFL at position 92.

Figure 2 shows expression of EnvHA chimeric proteins of embodiments of the present invention. Cell lysates were prepared from recombinant vaccinia virus-infected CV-1 cells expressing the proteins indicated and separated by SDS-PAGE under reducing conditions. Lysates were probed using (a) anti-Env sheep polyclonal serum (ARP401) and (b) anti-HA rabbit polyclonal serum (X31). Neither serum reacted with lysates from parental vaccinia (vRB12) infected and uninfected (NEG) cells. The locations of specific glycoprotein species are indicated.

Figure 3 shows the oligomeric forms of EnvHA chimeric proteins of embodiments of the present invention expressed in CV-1 cells. Cell lysates were prepared in the absence of ß-mercaptoethanol, separated by SDS-PAGE and probed using anti-Env (ARP401) and anti-HA rabbit

polyclonal sera. Results for EnvHA constructs (R3 and Y1), wt Env (JRFL and NL43) and HA (X31) are shown. With both sera, monomeric EnvHA can be seen to run at approx 150 kDa, and two higher molecular weight bands corresponding to dimeric and trimeric forms are observed. Similar forms are observed for Env and HA with their respective anti-sera.

Figure 4 shows detection of EnvHA chimeric protein cell surface expression by immunofluorescence. (a) CV-1 cells infected with recombinant vaccinia viruses were probed with anti-JRFL, anti-NL43 and anti-HA antibodies to detect surface expression of EnvHA protein. Immunofluorescence results for EnvHA constructs R3 (X4 tropic) and Y1 (R5 tropic), wt controls (NL43, JRFL, X31), vaccinia-infected (vRB12) and uninfected-cells (NEG) are shown; all eight EnvHA constructs were detected at the cell surface. (b) CV-1 cells infected with recombinant vaccinia viruses expressing EnvHA constructs (R3 and Y1) and HA (X31) were treated with PBS/2% Triton X-100 (v/v) to permeabilise cell membranes and probed with anti-HA antibody to visualise intracellular staining of the expressed proteins.

Figure 5 shows the effect of trypsin on EnvHA (R3), wt NL43 Env and HA (X31) proteins. CV-1 cells expressing the proteins indicated (R3, NL43, X31) were incubated with increasing concentrations of trypsin at 37°C for 30 min. Cell lysates were prepared and separated by SDS-PAGE under reducing conditions then probed using (a) anti-Env monoclonal antibody (EVA3012) to detect wt EnvHA0 (150 kDa; gp120-HA0) and (b) anti-HA polyclonal serum (X31) to detect EnvHA0, HA0, HA1 and HA2. * The TPCK-trypsin concentration (μg/ml) used is indicated.

Figure 6 shows the identification of the 28kDa protein as HA2. CV-1 cells expressing the proteins indicated (R3, Y1, X31) were incubated in the absence (-) or presence (+) of 5 μg/ml trypsin at 37°C for 30 min. Cell lysates were prepared and separated by SDS-PAGE under reducing conditions. Lysates were probed with R185 polyclonal serum to detect HA2. Cross-reactive bands were present in parental vaccinia (vRB12) infected and uninfected (NEG) cells but HA2 was absent.

Figure 7 shows an assay of membrane fusion under conditions optimal for X31 HA. Ghost cell lines and BHK cells were infected with recombinant vaccinia viruses to express the proteins indicated (R3, Y1, JRFL, NL43, X31) for 16 h, then treated with 5 μg/ml trypsin and briefly exposed to low pH cell buffer (pH 5). Membrane fusion was then monitored as described in materials and methods. Uninfected cell lines (NEG) and those infected with wt vaccinia (vRB12) were treated similarly. Membrane fusion is not observed in NEG and vRB12 cultures, heterokaryon formation is seen in all wt HA (X31) expressing cultures, syncytia are observed in the three Env-NL43 (X4-tropic) expressing Ghost cell cultures and the Env-JRFL (R5 tropic)/Ghost-R5 culture whilst all Ghost cell cultures expressing EnvHA (R3 and Y1) show heterokaryon formation. Similar heterokaryon formation was observed with all eight EnvHA constructs (results not shown). * For wt Envs the photographs shown were taken prior to pH 5 treatment as the treatment caused fused cells to detach from the 24-well plates.

Detailed Description of the Invention

The present invention provides a chimeric protein comprising amino acid sequences of both the HA1 subunit and the HA2 of influenza virus HA protein and of a heterologous protein. The HA1 subunit amino acid sequence comprises cysteine residues at positions 30, 68 and 293 as numbered from the N-terminal amino acid of the HA1 subunit from influenza virus X31, or the corresponding positions in the HA1 subunit from other strains of influenza virus. The amino acid sequence of the HA2 subunit comprises cysteine residues at positions 153, 160 and 164 as numbered from the N-terminal amino acid of the HA2 subunit from influenza virus X31, or the corresponding positions in the HA2 subunit from other strains of influenza virus. The amino acid sequence of the heterologous protein is positioned between the cysteine residues at positions 68 and 293 of the HA1 subunit. The chimeric protein has a disulphide bond between the cysteine residues at position 30 of HA1 and position 153 of HA2, between the cysteine residues at positions 68 and 293 of HA1, and between the cysteine residues at positions 160 and 164 of HA2. The chimeric protein has the structural properties of the native HA protein such that the structure and function of the native heterologous protein are retained.

Influenza virus HA is a trimeric membrane glycoprotein. HA is initially synthesized as a trimeric precursor (HA0) containing three identical protein chains. Each of the chains is proteolytically processed into two subunits, HA1 and HA2, that are held together covalently by a single disulphide bond between the cysteine residue at position 39 of HA1 and the cysteine residue at position 153 of HA2. The HA1 subunit forms a globular head and

contains the receptor-binding sites and the majority of antigenic sites. The HA2 subunit stably anchors the HA protein to the membrane, helping to stabilize the HA trimers. Incorporating part of influenza virus HA into the chimeric protein results in a chimeric protein which is expressed at the cell surface and which has a stable structure having structural properties of the native HA. This helps to ensure that the structural and functional properties of the native heterologous protein are retained in the chimeric protein. Thus, use of influenza virus HA in the chimeric protein allows the expression of a protein with a stable structure and the function of the native protein. Without being bound by any particular theory, it is believed that the incorporation of amino acid sequence of the HA1 subunit and of the HA2 subunit stabilizes the chimeric protein. The presence of disulphide bonds in the HA protein similarly serves to stabilize the structure of the chimeric protein. In particular, the disulphide bond between the HA1 subunit(at cysteine residue 30) and the HA2 subunit(at cysteine residue 153) has a stabilizing effect on the structure. The presence of HA2 subunit and disulphide bonds helps to ensure the expression of a heterologous protein having its native structure. Ensuring the correct, native structure of the heterologous protein in turn ensures the protein retains its native function.

In one embodiment of the invention, the amino acid sequence of the HA2 subunit further comprises the membrane anchor region from position 186 to 207, inclusive. This serves to hold the chimeric protein on the cell surface and allows membrane fusion functional studies to be performed.

In a preferred embodiment, the chimeric protein comprises in the direction from the N-terminus to the C-terminus, (i) an amino acid sequence of the HA1 subunit from and including position 1 to and including position 68 as numbered from the N-terminal amino acid of the HA1 subunit from influenza virus X31, or corresponding positions; (ii) an amino acid sequence of a heterologous protein; (iii) an amino acid sequence of the HA1 subunit from and including position 293 to and including position 345 as numbered from the N-terminal amino acid of the HA1 subunit from influenza virus X31, or corresponding positions and (iv) an amino acid sequence of the HA2 subunit from and including position 1 to and including position 237 as numbered from the N-terminal amino acid of the HA2 subunit from influenza virus X31, or corresponding positions in a different influenza virus.

Thus, a preferred chimeric protein comprises the complete HA1 subunit sequence without residues 69 to 292, inclusive. It also has the complete HA2 subunit sequence. Therefore, such a chimeric protein can be expressed by a recombinant influenza virus infecting a susceptible host cell as the complete HA2 subunit sequence has the components required for this. Thus, the complete HA2 subunit has the membrane anchor which serves to hold the chimeric protein on the virus surface and facilitate membrane fusion, the cytoplasmic tail which is important for trafficking the chimeric protein and assisting virus assembly, and the fusion peptide important for infectivity of the virus. Further, such constructs retain, at the gene level, coding sequences for the N-terminus of HA1 and the C-terminus of HA2 that are important for vRNA packaging and virus assembly [13].

In one embodiment of the invention, the chimeric protein further comprises an amino acid linker sequence at either or both of the junctions between the heterologous protein sequence and the HA1 subunit sequence. In particular, the amino acid linker sequence is between sequences (i) and (ii) and/or (ii) and (iii). The linker sequence preferably comprises between one to four amino acids, most preferably four amino acids. Preferably, the linker sequence is the sequence Gly-Gly-Gly-Ser. Alternatively, the linker sequences may be based on the HA1 subunit sequence directly C-terminal to the cysteine residue at position 68, and/or directly N-terminal to the cysteine residue at position 293, as numbered form the N-terminal amino acid of the HA1 subunit from influenza virus X31, or corresponding positions in the HA1 subunit from a different influenza virus. Still further, the linker sequences may be based on the sequence of the heterologous protein. However, the linker sequence is not limited to any particular amino acid sequence as long as the resultant chimeric protein has the structural properties of the native HA protein such that the structure and function of the native heterologous protein are retained. Thus, the incorporation of linkers at one or both of the junctions should not have an adverse effect on the structure or function of the chimeric protein. For example, the linker sequence above may have the addition, deletion or substitution of one or more amino acids. It is preferable that the amino acid sequence comprises mainly residues with small side-chains. It is also preferable that the amino acids have hydrophilic side-chains.

Thus, a chimeric protein of the invention may have additional amino acid sequences derived from HA1 and/or

the heterologous protein providing the chimeric protein includes the specific cysteine residues described or their equivalents in other influenza HA proteins such that disulphide bonds are present between these residues as specified.

In preferred embodiments of the invention, the chimeric protein comprises influenza HA protein from influenza virus X31. However, the HA protein may be that from any influenza virus. For example, HA protein may be from H1 viruses PR8 or WSN, or from more recent strains such as New Caledonian. For convenience, the HA used is preferably one for which nucleic acid has been isolated and the sequence determined. The skilled person is able to determine the corresponding amino acid positions in HA from other influenza viruses relative to those of X31 HA, for example by sequence homology comparisons. The term "corresponding position" of an amino acid is defined as an amino acid position in HA from influenza virus other than X31 which is equivalent to a given amino acid position in influenza virus X31. Thus, an amino acid at an equivalent position in HA protein from a different influenza virus has the same structural and/or functional role as the amino acid at the given position in X31 HA protein.

The chimeric protein also has a heterologous protein component. "Heterologous protein" is defined as any protein which does not derive from the same source as the HA protein. In preferred embodiments, the heterologous protein is one that is inherently unstable, such that standard techniques for expressing proteins are not effective in producing the protein in a structurally and functionally correct form.

Preferably, the heterologous protein component is from a protein associated with a disease. "A disease" may be any disease for which it is desirable to be able to develop therapeutic strategies, resulting from a greater understanding of a protein associated with that disease. For example, a disease may be an infectious disease or a cancer. In particular, the disease may be induced by a virus such as HIV, or a virus-induced disease, for example, a cancer induced by a virus, more particularly, a cancer induced by Rous Sarcoma virus (RSV), Feline Leukemia virus (FeLV), Human T-cell Leukemia viruses 1 and 2 (HTLV-1/2), Bovine Leukemia virus (BLV) and Equine Infectious Anemia virus (EIAV). Preferably, the heterologous protein component is from a protein associated with a virus-induced disease, that is, from a viral protein.

In an extreme case, the heterologous protein component (amino acid sequence (c) and (ii) in certain embodiments) may be HA derived from a different influenza virus than the source of the HA components (amino acid sequences (a), (b), (i),(iii) and (iv) in certain embodiments) of the chimeric protein. Thus, for example, the technique described in the present invention may be used to produce an attenuated virus strain from a highly pathogenic one which contains a polybasic amino acid sequence at its HA1/HA2 processing site.

The heterologous protein component is preferably from a protein that has a trimeric quaternary structure. A protein with a trimeric structure consists of three polypeptide chains, which may be identical or different to one another, linked together to form a trimer. A trimeric protein will generally only be functionally

active when it is in trimer form. Alternatively, the heterologous protein component may be from any protein that is functional in a trimeric structure, even if it is able to form other quaternary structures.

In preferred embodiments, the heterologous protein component is from a protein associated with a disease, preferably from a viral glycoprotein, and preferably one that has a trimeric structure or is active in trimeric form.

The heterologous protein component may be from a receptor-binding protein. "Receptor-binding protein" is defined as any protein which is capable of interacting with one or more molecules, for example, protein, lipid, carbohydrate or combinations thereof, present on the surface of a cell, preferably a mammalian cell, more preferably a human cell. Thus, a receptor-binding protein is capable of targeting the chimeric protein to a specific cell type that has on its surface the corresponding "receptor" molecule(s).

In preferred embodiments, the heterologous protein component is from a protein associated with a disease, preferably from a viral glycoprotein, and preferably one that is a receptor-binding protein. Preferably, such a protein also has a trimeric structure or is active in trimeric form.

In yet other preferred embodiments, the amino acid sequence of the heterologous protein is from a protein from a virus belonging to one of the following virus families:

Orthomyxoviridae, for example, haemagglutinin glycoprotein of influenza virus
Retroviridae, for example, surface glycoprotein SU of Rous Sarcoma virus (RSV), Feline Leukemia virus (FeLV), Human T-cell Leukemia viruses 1 and 2 (HTLV-1/2), Bovine Leukemia virus (BLV), Human Immunodeficiency viruses (HIV-1/2), Simian Immunodeficiency virus (SIV), Equine Infectious Anemia virus (EIAV), Feline Imunodeficiency virus (FIV), Caprine Arthritis Encephalitis virus (CAEV), Visna/Maedi
Filoviridae, for example, surface glycoprotein GP1 of Ebola, Marburg
Togaviridae, for example, E2 protein of alphaviruses such as Semliki forest virus, Sindbis virus, Ross River, Eastern/Western Equine Encephalitis, and rubiviruses such as Rubella
Coronaviridae, for example, S1 protein of Severe Acquired Respiratory Syndrome (SARS)
Reoviridae, for example, VP2 protein of orbiviruses such as Bluetongue virus, African horse sickness virus
Rhabdoviridae, for example, G proteins of vesiculoviruses such as Vesicular Stomatitis virus (VSV) and Lyssaviruses such as Rabies.
These proteins are all trimeric glycoproteins.

Flaviviridae, for example, E (E2) proteins of Tick Borne Encephalitis (TBE), Dengue, Hepatitis C virus (HCV), West Nile, Yellow fever. These proteins are dimeric in their native state but at low pH dissociation occurs and trimers form to allow fusion.

Alternatively, amino acid sequence from a protein from a virus of the family *Arenaviridae*, such as GP1 protein of Lassa, Venezuelan hemorrhagic fever, Lymphocytic

choriomeningitis may comprise the heterologous protein component. These proteins are tetrameric in their native state but may adopt a trimeric structure when in the chimeric protein format due to the influence of the HA protein components, retaining at least their antigenic properties.

In particularly preferred embodiments, the amino acid sequence of a heterologous protein is from an HIV Env glycoprotein. Preferably, the heterologous protein amino acid sequence is from an HIV-1 Env glycoprotein. Preferably, the HIV-1 Env glycoprotein is HIV-1 gp120. In preferred embodiments, the HIV-1 gp120 is from HIV-1 strain pNL43 or HIV-1 strain JRFL. However, the present invention provides the use of an Env glycoprotein amino acid sequence from any strain, isolate, clone or sub-clone of HIV. "HIV" is defined as a human immunodeficiency virus, such as HIV-1 or HIV-2. "HIV" also includes a virus equivalent to the human virus, but having a different host species, for example, SIV.

Preferably, the amino acid sequence of HIV-1 gp120 comprises cysteine residues at positions 119, 126, 131, 157, 194, 203, 216, 226, 237, 245, 294, 329, 376, 383, 416 and 443 as numbered from the N-terminal amino acid of gp120 from HIV-1 strain pNL43, or corresponding positions in other strains of HIV [15], where disulphide bonds are present between pairs of cysteine residues as found in native HIV-1 gp120. Alternatively, only some of these cysteine residues and their corresponding disulphide bonds may be present or further cysteine residues and their corresponding disulphide bonds may be present. Most preferably, the amino acid sequence of HIV gp120 comprises the amino acid from and including position 89

to the amino acid at and including position 495 as numbered from the N-terminal amino acid of gp120 from HIV-1 strain pNL43, or corresponding positions in other strains of HIV. Such a sequence is chosen to optimise joining of the HA1 subunit sequence and heterologous protein sequence at comparable positions in each of the proteins (in the "stalk" regions). The skilled person is able to determine the corresponding amino acid positions in gp120 from other HIV strains relative to those of gp120 from HIV-1 strain pNL43, for example by sequence homology comparisons. The term "corresponding position" of an amino acid is defined as an amino acid position in gp120 from HIV other than strain pNL43 which is equivalent to a given amino acid position in HIV-1 pNL43. Thus, an amino acid at an equivalent position has the same structural and/or functional role as the amino acid at the given position in HIV-1 pNL43. Preferably, the HIV gp120 amino acid sequence comprises one or more antigenic epitopes.

In a second aspect, the present invention provides a nucleic acid encoding the chimeric protein of the invention. The nucleic acid may be DNA or RNA. A nucleic acid, or fragments thereof, of the present invention may be obtained by standard methods known in the art. For example, a nucleic acid may be generated by chemical synthesis or preferably, by polymerase chain reaction (PCR) using specific primers. The generation of a nucleic acid of the present invention is described under Materials and Methods in the Examples.

In a further aspect, the present invention provides an expression vector comprising a nucleic acid of the invention. The type of vector used is not limited to any

particular type. The term "expression vector" is defined as a vector having the elements necessary for transcription of nucleic acid which are operably linked to that nucleic acid, for example, a nucleic acid of the invention. Nucleic acid is "operably linked" when it is placed in a functional relationship with another nucleic acid sequence. A promoter is operably linked to a nucleic acid if it effects transcription of that nucleic acid in a host cell. Such elements include a promoter, terminator and polyadenylation signal.

The expression vector may be a mammalian expression vector for expression of the chimeric protein in a mammalian cell. Suitable mammalian expression vectors include the pEE14tpa and pcDNA3.1 vectors. Alternatively, the expression vector may be a viral expression vector for expression of the chimeric protein by a virus infecting a susceptible host cell. Suitable viral expression vectors include, but are not limited to, the vaccinia virus shuttle vector pRB21 [16] and recombinant influenza viruses generated using the vector pHH21 [11].

In another aspect, the present invention provides a host cell comprising a nucleic acid of the invention or an expression vector of the invention. The invention also includes a method of producing a host cell of the present invention, which may be produced by introducing a nucleic acid or expression vector of the present invention into a suitable cell using techniques known in the art. A suitable host cell may be any cell which can express the introduced nucleic acid or expression vector. Suitable host cells including bacteria, in particular *E. coli*, yeast, fungi and higher eukaryotic cells are well known

in the art. For example, a suitable host cell for a mammalian expression vector includes mammalian cells such as Chinese Hamster Ovary (CHO) cells. Suitable host cells for viral expression vectors include mammalian cells such as CV-1, BHK, NS20, 293T and MDCK. Preferably, CV-1 cells are used with vaccinia expression vectors and 293T and MDCK cells are used with influenza expression vectors. In particular, suitable cells for use with influenza virus expression vectors of the invention which express a chimeric protein comprising an HIV Env protein include Jurkat, CEM C8166, HVS T and PMBC cells.

The invention also provides a method of producing a chimeric protein of the invention, comprising culturing a host cell of the invention under conditions allowing expression of the encoded chimeric protein and optionally recovering the chimeric protein from the host cell culture. Preferably, the host cell is a CHO cell. Suitable conditions for the expression of proteins in host cells are known in the art.

A further aspect of the present invention provides a method of producing a recombinant virus capable of expressing a chimeric protein of the invention, comprising contacting a susceptible cell with an expression vector of the invention and optionally isolating the virus from the cell. Preferably, the virus is a vaccinia virus, such as vaccinia virus strain vRB12. More preferably, the virus is an influenza virus, such as the human H1N1 influenza virus WSN. A susceptible cell is a suitable host cell infected with wt virus.

In embodiments relating to a chimeric protein of the invention which comprises an HIV Env glycoprotein, preferably specific mutations are made in the HA1 and/or HA2 domains, using techniques familiar to those skilled in the art, based on studies of influenza HA fusion mutants [17], in order to adjust the pH at which fusion occurs to ensure optimum infectivity of the viruses expressing the chimeric protein. The CD4 receptor, the presence of which is required for a cell to be infected by a virus expressing a chimeric protein comprising an HIV Env protein, is commonly found internalized in early endosomes in the absence of specific internalization stimulators. Thus, in the process of generating Env-expressing recombinant influenza viruses it may be preferable to adjust the pH at which fusion occurs to ensure good infectivity.

In another aspect, the present invention provides a virus obtained by the method of the invention for producing a recombinant virus. In one embodiment, a virus of the invention is further capable of expressing a conditionally lethal toxic protein. The conditionally lethal toxic protein may be herpes simplex virus thymidine kinase (HSVTK). This protein becomes toxic to a cell infected with recombinant virus expressing HSVTK when the drug ganciclovir is administered. Thus, use of such a virus allows ganciclovir-dependent killing of target cells. When the recombinant virus expresses HIV gp120, target cells will be those expressing CD4. Preferably, expression of HSVTK in a cell is also dependent on the target cell being infected with HIV. Thus, expression of the HSVTK gene under control of a modified HIV-2 LTR makes expression of HSVTK conditional on the presence of HIV, in particular the presence of

HIV-1, HIV-2 or SIV Tat proteins. Thus, recombinant virus expressing HIV gp120 and HSVTK under the control of HIV-2 LTR will selectively kill only CD4-expressing cells which are infected with HIV.

Alternatively, the conditionally lethal toxic protein may be an apoptosis inducer. Apoptosis is a normal physiological response that can occur in mammalian cells in response to different stimuli such as T-lymphocyte signalling or DNA damage. Upon stimulation, the cell enters a regulated suicide program; an efficient method of cell death where the cell contents are not spilled, such that inflammatory responses do not occur. A family of proteases, called cysteine-aspartic acid proteases (caspases), play a major role in apoptosis. There are at least eleven human caspases of which there are two main classes: initiators and effectors/executors. Initiators such as caspases-2, -8, -9 and -10 can activate themselves whereas effector/executor caspases-3, -6, and -7 require proteolytic processing by initiator caspases. They are all synthesized as two inactive heterologous subunits that require processing at a specific Asp site in the linker between the subunits in order to become active. Activation of caspases creates hierarchical caspase cascades that lead to proteolytic cleavage of cellular factors, DNA degradation and ultimately cell death. Thus, the use of a recombinant virus expressing a caspase allows targeted killing of a cell infected by the virus. By switching the two subunits of caspase-3, a reverse caspase-3 (revCasp3) capable of autocatalytic processing has been created [18]. Thus, expression of revCasp3 under control of a modified HIV-2 LTR makes expression of revCasp3 conditional on the presence of HIV, in particular the presence of HIV-1, HIV-2 or SIV

Tat proteins. Thus, recombinant virus expressing HIV gp120 and revCasp3 under the control of HIV-2 LTR will selectively kill only CD4-expressing cells which are infected with HIV.

Alternatively, the conditionally lethal toxic protein may be any of those developed or being developed for gene therapy purposes.

An alternative method of conferring conditional toxicity may result from including sequences corresponding to the HIV Rev Response Element (RRE) in the toxic gene segment. The RRE would serve to retain the toxic gene mRNA in the cell nucleus, thereby preventing production of the toxic protein [19]. This block would be overcome in cells expressing HIV Rev protein. Thus, recombinant viruses expressing HIV gp120 and toxic genes (HSVTK or revCasp3) containing the RRE will selectively kill only CD4-expressing cells which are infected with HIV.

In yet a further aspect, the present invention provides a target cell infected with a virus of the invention. Preferred target cells for infection by vaccinia virus include CV-1 cells. 293T and MDCK cells are preferably used for infection with influenza virus. In particular, preferred cells for use with recombinant influenza virus of the invention expressing a chimeric protein comprising an HIV Env protein include Jurkat, CEM C8166, HVS T and PMBC cells. These cells express CD4 which is necessary for their recognition and infection by recombinant viruses expressing HIV Env protein. Additionally, target cells such as 293T and MDCK, which support efficient influenza replication, are genetically modified to

express human CD4 and/or chemokine receptors using techniques known to those skilled in the art.

A virus and/or a chimeric protein of the invention may be used as a treatment or therapy for a mammal, preferably a human. A virus and/or a chimeric protein of the invention may be used as a vaccine for a mammal, preferably as a vaccine for a human. Further, a virus and/or a chimeric protein of the invention may be used for the preparation of a vaccine. A vaccine comprising a virus and/or a chimeric protein of the invention is also provided by the present invention. Further, a virus and/or a chimeric protein of the invention may be used for the preparation of a medicament for the treatment of a mammal. In embodiments where the heterologous protein component is from a protein associated with a disease, the virus and/or chimeric protein may be used to treat individuals suffering from the disease or at risk from suffering from the disease, and in therapeutic strategies against that disease. Thus, examples of diseases or conditions that may be targeted using a virus and/or chimeric protein of the invention include infectious diseases and cancer. In particular, the disease may be induced by a virus such as HIV, or may be a virus-induced disease, for example, a cancer induced by a virus.

Typically, a virus and/or a chimeric protein of the invention is used in combination with a suitable carrier as a vaccine. Virus and/or a chimeric protein at the desired degree of purity and in a sufficient amount to induce antibody formation is mixed with a physiologically acceptable carrier. A physiologically acceptable carrier is non-toxic to a recipient at the dosage and concentration employed in the vaccine. Generally, the

vaccine is formulated for injection, usually intramuscular or subcutaneous injection. Suitable carriers for injection include sterile water. Preferred suitable carriers include physiological saline solutions, such as normal saline or buffered salt solutions such as phosphate-buffered saline or Ringer's solution. The vaccine generally contains an adjuvant, for example an adjuvant which can enhance cellular or local immunity. Additional exipients that can be present in a vaccine include low molecular weight polypeptides, proteins, amino acids, carbohydrates including glucose or dextrans, chelating agents such as EDTA, stabilizing agents and anti-microbial agents.

A recombinant virus and/or a chimeric protein of the invention used as a vaccine, or a vaccine prepared using recombinant virus and/or a chimeric protein of the invention may be administered to uninfected individuals or infected individuals to enhance immune response to the heterologous protein comprised in the chimeric protein. Administration may be combined with administration of other vaccines, medicaments or compositions, or in combination with other treatments.

A preferred recombinant virus for use as a vaccine expresses a chimeric protein of the invention comprising an HIV Env glycoprotein, preferably a gp120 protein. Similarly, a preferred chimeric protein for use as a vaccine comprises an HIV Env glycoprotein, preferably a gp120 protein. Preferably, the gp120 has more than one antigenic epitope. Such a recombinant virus and/or chimeric protein, or vaccine prepared using such a virus and/or chimeric protein is administered to an HIV-infected individual or an uninfected individual at risk

of becoming infected. Preferably, multiple recombinant viruses, each expressing a chimeric protein comprising gp120 from a different HIV strain, clone or isolate and/or multiple chimeric proteins comprising gp120 from different HIV strains are used as a vaccine or to prepare a vaccine. Multiple recombinant viruses and/or chimeric proteins may be administered simultaneously or used in the preparation of a single vaccine. Alternatively, multiple recombinant viruses and/or chimeric proteins may be administered sequentially or used individually in the preparation of separate vaccines.

Furthermore, a virus of the invention may be used in gene therapy for a mammal. A virus of the invention which expresses a chimeric protein of the invention comprising an amino acid sequence of an HIV Env glycoprotein and/or a chimeric protein comprising an amino acid sequence of an HIV Env glycoprotein may be used in the treatment of an HIV-infected individual. Such a virus may be used as part of a therapeutic strategy to clear HIV from an infected individual. Such a virus and/or a chimeric protein may also be used for the preparation of a medicament for the treatment of an HIV-infected individual.

A virus of the invention may be used as a treatment either alone or in combination with one or more medicaments and/or treatments. For example, a number of medicaments and therapeutic strategies for the treatment of HIV-infected individuals are known to those skilled in the art.

A recombinant virus and/or a chimeric protein of the invention may also be used to provide a composition for

the treatment or therapy of a mammal, preferably a human. One or more recombinant viruses and/or one or more chimeric proteins can be formulated in pharmaceutical compositions. These compositions may comprise, in addition to one or more of the above substances, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant or an inert diluent. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included. Such compositions and preparations generally contain at least 0.1% by weight of the active ingredient(s). For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection.

Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Whether it is a polypeptide, antibody, peptide, nucleic acid molecule, small molecule or other pharmaceutically useful compound according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of the condition being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Oslo, A. (ed), 1980. The medicaments and pharmaceutical compositions of the invention may be administered by any of several routes, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes. The dose of the medicament or pharmaceutical composition is based on well known pharmaceutically acceptable principles. General dosages are based on mg of the active ingredient per kg body weight, for example, 0.01 mg/kg to 100 mg/kg, more preferably 0.5 mg/kg to 10 mg/kg. The dose will depend on the route of administration in addition to the factors described above.

The medicaments and pharmaceutical compositions are administered alone or in combination with other medicaments or compositions, or treatments or methods of managing a condition or disease.

Examples

Materials and Methods

Design of an EnvHA chimeric protein

Based on the known structure of the HA of influenza virus X31 [7] and the disulphide-bonding pattern and structure of HIV-1 gp120 [1, 3] a chimeric protein (EnvHA) that contained the entire stalk region of HA (the N- and C-termini of HA1 and the whole of HA2) and the globular head of gp120 was designed (Figure 1). The designed construct maintains disulphide-bonds between positions 68 and 293 of HA1, the eight in the globular head of gp120, that between residues 30 of HA1 and 153 of HA2, and that between residues 160 and 164 in HA2. Such a structure is expected to possess the stability and therefore the membrane fusion characteristics of native HA and the receptor-binding properties of HIV-1 Env.

Construction of *envHA* chimeric genes and generation of recombinant vaccinia virus expressing EnvHA chimeric proteins

To construct the *envHA* chimeric genes a clone of wt X31-*HA* was obtained [20] and the *env* genes of HIV-1 strains pNL43 [14] and JRFL [21] were rescued by polymerase chain reaction (PCR) using proviral DNA, cloned and sequenced as described [22]. pNL43 and JRFL were chosen as they are well characterised strains that use CXCR4 and CCR5 chemokine co-receptors respectively [23]. The required fragments of each gene were generated using PCR with

native *Pfu*-polymerase (Stratagene) and a panel of oligonucleotide primers (Table 1). The fragments were purified from 1.5% (<1 Kb) and 0.5% (>1 Kb) agarose gels using 45 μm filter units (Millipore #UFC30HVNB) according to manufacturer's instructions and the three required fragments joined together using PCR-splice overlap extension (PCR-SOE) with *Pfu*-polymerase and primers X31HAFU and X31HARU. All PCRs were performed on a PTC-100 cycler (MJ Research Inc) using 25 cycles of 96°C/2 min, 50°C/1.5 min, 72°C/10 min (for products up to 1300 bp) or 20 min (for final products of 2360-2600 bp). The *envHA* genes were ligated into the vaccinia shuttle vector pRB21 using Pst I and Hind III restriction sites, the resultant ligations used to transform DH5α *E. coli* and resultant clones sequenced prior to being used in the production of recombinant vaccinia (Copenhagen strain) viruses [16, 20]. A number of constructs were made with/without linker sequences (GGGS) at the HA1/gp120 junctions to allow a degree of flexibility such that the gp120 globular head might be more easily accommodated on the HA stalk to allow trimer formation (Table 2). As controls, recombinant vaccinia viruses allowing expression of X31 HA, rescued with primers X31HAFU and X31HARU, and HIV-1 NL43 and JRFL Envs, rescued with primers FgpV and RgpV (Table 2), were generated.

Expression of Env, HA and EnvHA chimeric proteins in mammalian cells using recombinant vaccinia virus

Recombinant vaccinia viruses were plaque purified twice in CV-1 cells before use in experiments [20]. To assess the expression of wt and chimeric proteins, CV-1 cells were infected with recombinant vaccinia virus at a moi of 1 and 16 h post-infection cell medium was removed and cells were resuspended in 1 ml of phosphate-buffered

saline (PBS) and centrifuged (6K/30 sec). Cell pellets were lysed with 120 μl SDS loading buffer (2% SDS, 62.5 mM Tris pH 6.8, 5% ß-mercaptoethanol, 0.1% bromophenol blue) and separated by SDS-PAGE. Western blotting was performed using a 1:5000-dilution of anti-Env sheep polyclonal serum (ARP401, UK Centralised Facility for AIDS Reagents; CFAR), and a 1:1000-dilution of anti-X31-HA rabbit polyclonal serum (supplied by D A Steinhauer [35]), with HRP-labelled donkey antisheep (Sigma, 1:5000-dilution) and HRP-labelled Protein A (Amersham Biosciences, 1:2000 dilution) secondary antibodies respectively. To allow specific detection of gp120 containing proteins a murine anti-V3 monoclonal antibody (EVA3012; CFAR) was used at 1:50-dilution with a 1:2000-dilution of HRP labelled goat anti-mouse (Promega). HA2 was detected with a rabbit polyclonal serum (R185 diluted 1:200; supplied by S A Wharton) raised against a HA2-derived peptide (115-125: MNKLFEKTRRQ) with HRP-labelled Protein A as secondary antibody. Blots were developed using Enhanced Chemiluminescence (ECL; Amersham Biosciences). Established procedures exist for the purification of HIV envelope and influenza HA proteins expressed using vaccinia [24, 25].

Surface expression of EnvHA proteins in mammalian cells using recombinant vaccinia virus

CV-1 cells were grown on 13 mm glass coverslips in 24-well tissue culture plates. Cells were infected with recombinant vaccinia virus at a moi of 1 for 16 h and fixed with 4% paraformaldehyde (w/v) in PBS. Surface expressed EnvHA proteins were detected using HIV-1 strain-specific anti-Env and anti-HA antibodies. A 1:20 dilution of the monoclonal antibody EVA3012 (CFAR) was used to probe NL43-based constructs, with a fluorescein

linked sheep anti-mouse secondary antibody (1:200 dilution, Amersham Biosciences). A polyclonal rabbit serum EVA435 (1:100-dilution, CFAR) and 1:200-diluted fluorescein linked donkey anti-rabbit secondary antibody (Amersham Biosciences) were used to probe JRFL-based constructs. In order to detect intracellular EnvHA, cells were permeabilised prior to immunofluorescent staining by treating with PBS/2% Triton X-100 (v/v) for 30min at room temperature. Cells were counterstained with 4',6' diamidino-2-phenylindole (DAPI) for 1 min at room temperature to visualize nuclei. Coverslips were mounted on glass slides and analysed on a Nikon Labophot-2 fluorescent microscope.

Trypsin-susceptibility of EnvHA chimeric proteins

To estimate the trypsin cleavability of expressed EnvHA proteins, CV-1 cells were infected with recombinant vaccinia virus at a moi of 1. At 16 h post-infection cells were washed with Dulbecco's modified Eagle medium (DMEM; Invitrogen) and incubated with a range of concentrations (0, 2.5, 5, 10, 20 μg/ml) of TPCK-treated trypsin (Sigma) for 10 min at 37°C. Cells were then incubated with equivalent concentrations of trypsin inhibitor (0, 2.5, 5, 10, 20 μg/ml; Sigma) for 10 min at 37°C, washed with PBS and cell lysates prepared. Lysates were analysed by SDS-PAGE and western blot as described above. Analysis of the oligomeric form of the EnvHA chimeric proteins was performed using recombinant vaccinia virus-infected CV-1 cell lysates prepared in the absence of ß-mercaptoethanol, separated by SDS-PAGE and probed by western blot.

Membrane fusion assays

Membrane fusion assays were performed following a method modified from that described previously [26]. Briefly, Ghost cells constitutively expressing either the CD4 receptor (g -parental), or CD4 and CCR5 co-receptor (g-R5), or CD4 and CXCR4 co-receptor (g-X4) [27] were infected at a moi of 1 with recombinant vaccinia virus. At 16 h post infection, cells were washed with DMEM and incubated with 5 μg/ml TPCK-treated trypsin for 5 min at 37°C. Cells were then washed and treated for 30 s with cell buffers (20 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$) that had been adjusted to particular pHs (6.0-5.0 in 0.2 steps) with citrate, as specified in the results section, after which pH was returned to neutral. Cells were incubated in DMEM with 5% fetal calf serum for 1 h at 37°C and the formation of heterokaryons/syncytia observed using light microscopy. Heterokaryons/syncytia were fixed with PBS/0.25% glutaraldehyde (v/v), stained with 1% Toluidine Blue (w/v; Sigma), and photographed using a Nikon Diaphot phase contrast microscope and F-301 camera.

Constitutive expression of EnvHA chimeric proteins in mammalian cells

The envHA genes were ligated into the vector pEE14tpa to generate pEE14tpa-envHA constructs. These constructs were used to transfect mammalian cells, in particular Chinese Hamster Ovary (CHO) cells. The cells are treated with the drug methyl-sulphoximine (MSX) to allow selection of clones which constitutively express the EnvHA protein. Thus, cell lines are generated which constitutively express EnvHA proteins. Established procedures exist for the purification of HIV envelope and influenza HA proteins from mammalian cells [24, 25].

Generation of recombinant influenza virus expressing EnvHA chimeric proteins

The envHA genes are modified using PCR with specific primers to allow incorporation of the genes into the pHH21 vector system [11] by methods familiar to those skilled in the art. The vectors are propagated in *E. coli* for subsequent generation of recombinant influenza virus. Recombinant influenza viruses bearing a chimeric protein of the invention are produced based on known methods [11, 12]. Essentially, up to 9 mRNA-sense plasmids (encoding influenza proteins) are used together with 8 vRNA-sense plasmids (carrying the eight influenza genes) to transfect host 293T cells. Suitable transfection reagents include, but are not limited to, Lipofectamine, Effectene, DOTAP and calcium phosphate. The mRNA-sense plasmids provide the viral proteins to allow virus replication and assembly and the vRNA segments provided by the vRNA-sense plasmids are packaged into progeny viruses. Replacement of the HA vRNA plasmid with that of a vector encoding a chimeric protein of the invention means that after one round of infection in the 293T cells, progeny viruses carry the nucleic acid encoding the chimeric protein and express the chimeric protein on their surfaces. Thus, replacement of the HA vRNA plasmid with that of a vector encoding EnvHA means that progeny viruses carry the *envHA* gene and express EnvHA on their surfaces. As a consequence, these recombinant viruses infect cells expressing CD4 and certain chemokine receptors instead of epithelial cells of the respiratory tract that express suitable sialic acid receptors.

Results

Generation of *envHA* chimeric genes

All primary PCRs and the subsequent SOE reactions with three templates and primers X31HAFU and X31HARU worked efficiently to yield products of the expected sizes (results not shown). Following insertion of the PCR-SOE products and the rescued genes for X31-*HA*, NL43-*env* and JRFL-*env* into the vector pRB21 and transformation of DH5α *E.coli*, individual clones were picked and the plasmids sequenced to ensure selection of cloned genes (Table 1) that matched the sequences of the parental genes (accession numbers J02090, M19921 and U63632 respectively).

Expression of EnvHA chimeric proteins in CV-1 cells

Chimeric EnvHA proteins were expressed in CV-1 cells and lysates were probed using anti-Env and anti-HA rabbit polyclonal sera. Immunoblotting demonstrated the presence of two protein bands for EnvHA chimeric proteins R3 to Y1 (Figure 2). The larger band, approximately 150 kDa, was detectable by both anti-Env and anti-HA antibodies, corresponded to the estimated sizes of the EnvHA proteins and was similar to that of wt Envs detected with anti-Env antibodies. The smaller band, representing a protein of approximately 28 kDa was detected only when lysates were probed with anti-HA serum. As expected, the latter serum did not detect wt Envs. To determine the oligomeric form of the EnvHA proteins expressed in infected cells, lysates were prepared in the absence of ß-mercaptoethanol to maintain the native protein structure, and separated by SDS-PAGE. Probing with anti-Env rabbit polyclonal serum demonstrated the presence of the major 150 kDa EnvHA monomer and also revealed the presence of two higher molecular weight bands (Figure 3). These bands

corresponded to the predicted sizes of dimeric and trimeric forms of the EnvHA protein (approximately 300 and 450 kDa respectively) indicating that a proportion of the EnvHA proteins were expressed in a trimeric form as for wt parental Env and HA.

Cell-surface expression of EnvHA chimeric proteins in CV-1 cells

Immunofluorescent staining with product-specific antibodies was used to assess expression on the surface of recombinant vaccinia virus-infected cells. Strain specific anti-Env antibodies, raised against either NL43 or JRFL Env proteins, showed good specificity for the parental wt Env proteins and detected EnvHA constructs based on the respective Env strains (Figure 4a, observed as peripheral band of staining (green fluorescein staining) around central stained area indicating nuclei (blue DAPI staining)). Whilst results are shown for EnvHA constructs R3 and Y1, it was possible to detect all EnvHA constructs. Similarly, the wt HA control was detected on the cell surface using the anti-HA antibody (Figure 4a, observed as described above). Cells were permeabilised to enable staining of intracellular EnvHA protein (Figure 4b). A comparison between non-permeabilised and permeabilised cells confirmed that the staining pattern observed in non-permeabilised cells was indicative of EnvHA surface expression (as described for Figure 4a).

Trypsin-susceptibility of EnvHA chimeric proteins

Influenza HA is susceptible to trypsin, cleaving the precursor HA0 into two subunits, HA1 and HA2. Such cleavage is essential for virus infectivity. Cells expressing EnvHA proteins were exposed to trypsin and cell lysates probed to assess the susceptibility of

EnvHAs. When probed with an anti-gp120 monoclonal antibody, neither EnvHA (R3) or wt NL43 gp160 appeared particularly sensitive to trypsin (Figure 5a), possibly reflecting the relatively low proportions of total EnvHA and gp160 present on the cell surface. However, on probing with anti-HA polyclonal serum detection of EnvHA0 decreased in line with increasing trypsin concentration and there was indication of an increase in intensity of the 28 kDa band. X31-HA0 was efficiently processed to HA1/HA2 at all concentrations used although significant amounts of HA0 remained in all samples, presumably representing intracellular protein that had not been accessible to trypsin (Figure 5b). These results suggested that the 28 kDa band seen in lysates containing EnvHA (Figures 2b and 5b) corresponded to the HA2 subunit of wt HA and indicated that, unlike for HA0, there is a certain level of processing in CV-1 cells of the trypsin-susceptible cleavage site situated between the gp120/HA1 and HA2 domains of the EnvHA constructs (Figure 1). By probing western blots with a rabbit serum raised against a HA2-specific peptide, the identity of the 28 kDa as HA2 was confirmed and levels shown to increase following exposure of EnvHA0 and HA to trypsin (Figure 6). Overall these results show that the EnvHA0 proteins can be processed to yield HA2 but they are less susceptible to trypsin processing than the wt HA.

EnvHA-mediated cell membrane fusion

To investigate whether the EnvHA proteins retained the fusogenic function of the influenza HA, a series of membrane fusion assays were performed. Recombinant vaccinia virus-infected cells expressing EnvHA, wt Env and wt HA proteins were either left untreated or treated with trypsin and then either maintained at neutral pH or

exposed to cell buffer adjusted to pH 5 to trigger the conformational changes required by wt HA to permit membrane fusion [20]. Examples of Ghost cell fusion seen after trypsin and low pH treatments are shown (Figure 7). The wt Env and HA proteins produced different patterns of fusion: large syncytia were formed by wt Envs, and heterokaryons were formed by wt HA. The pattern of fused cells produced by the EnvHA proteins resembled that of the wt HA and each EnvHA construct was able to initiate fusion in all of the Ghost cell lines indicating that the co-receptor specificity associated with wt Env had been lost (Figure 7, Table 3). Results confirmed that wt HA was low pH dependent and required trypsin treatment, whilst wt Env fused membranes at both low and neutral pH and was trypsin independent (Table 3). Further, whilst wt HA could fuse all cell types used including Baby Hamster Kidney (BHK) cells which do not express either CD4 or chemokine coreceptors, wt Envs maintained their co-receptor specificities with JRFL fusing Ghost cells expressing CCR5 only whilst NL43 preferentially fused cells expressing CXCR4 although there was some fusion observed on the other Ghost cell lines. The latter observation is probably related to the parental and CCR5 expressing Ghost cell lines both having low level endogenous expression of CXCR4. In contrast, EnvHA proteins displayed a mix of the properties of their parental proteins. All were able to mediate fusion after low pH treatment only, but the fusion was independent of trypsin treatment although the level of fusion was increased when trypsin treatment was applied prior to exposure to low pH (Table 3). Thus, it appears that the background levels of EnvHA processing seen in previous results (Figures 2, 5, 6) enable low levels of membrane fusion to occur in the absence of trypsin. To assess

more precisely the pH at which heterokaryon formation occurs with EnvHA, recombinant vaccinia viruses containing X31-HA and all eight EnvHA constructs were used to infect the three Ghost cell lines and 16 h later they were subjected to pH treatment in 0.2 unit steps covering the range 6.0-5.0. On leaving the cells for 1 h following each pH-treatment it was observed that whilst X31-HA yielded 80-100% heterokaryon formation at pH 5.0 5.2, all EnvHA constructs did so at pH 5.2-5.4 (results not shown). This 0.2 pH unit differential was conserved when cells were left for 4 h at which time X31-HA was active at pH 5.6 and EnvHA constructs at pH 5.8.

Discussion

The generation of viruses bearing chimeric proteins is not a novel concept, however, many of these proteins do not retain structural and functional properties of the donor proteins [9, 28]. Chimeric glycoproteins have now been generated that graft the globular head of the HIV-1 envelope protein (gp120) onto the HA1/HA2 stalk of the influenza haemagglutinin protein. Using a recombinant vaccinia expression system we were able to demonstrate the expression of these chimeric proteins in mammalian cells and their presence on cell surfaces. Due to the overall relative structural homology between the Env and HA proteins, i.e. both are type I membrane proteins and form trimers on their respective virus particles, we predicted that chimeric EnvHA proteins would be expressed and retain structural and functional capabilities of the parent proteins. The chimeras were designed to allow maintenance of important disulphide-bonds in the HA and gp120 components and short peptide linkers were incorporated at HA1/gp120 boundaries to give a degree of flexibility in these regions that might enhance the

likelihood of correct protein folding to produce functional proteins (Figure 1).

In order to use the EnvHA protein for structural studies of gp120, surface expression is preferable as it infers correct protein folding and can assist protein purification. Initial experiments involving the infection of mammalian cells with recombinant vaccinia viruses produced proteins of the size predicted for EnvHA chimeras. The antigenic characteristics of the Env and HA subunits have been retained as we were able to utilise anti-Env and anti-HA antibodies for the detection of EnvHA, a portion of which was trimeric, in cell lysates and at the cell surface (Figures 2-6).

Processing of influenza HA0 at an arginine residue (position 345 in X31 HA0) by trypsin-like proteases is essential for virus infectivity and the generation of the short hydrophobic N-terminus of HA2, the fusion peptide. This residue is then removed, leaving the adjacent glycine as the N-terminus of the fusion peptide. Proteases that cleave the HA1/HA2 processing site by removing the N-terminus glycine, e.g. bromelain and thermolysin, yield non-infectious viruses that cannot undergo fusion [20]. In agreement with others, in the absence of trypsin wt HA was present as the HA0 precursor, and it was processed into HA1 and HA2 by the protease (Figure 5b) [20]. The HA2 component was 28 kDa in size and corresponded to a protein seen in EnvHA expressing cell lysates in the absence of trypsin (Figure 2b) although the intensity of this band increased in the presence of trypsin (Figure 5b). The identity of this band was confirmed as HA2 by probing EnvHA-containing lysates with a rabbit polyclonal serum raised against a

peptide corresponding to residues 115-25 of X31-HA2 (Figure 6). This suggested that low level processing of the EnvHA protein was occurring during protein synthesis. It is likely that by substituting gp120 for the HA1 globular head, certain structural changes will have taken place in the remaining HA1/HA2 stalk for the two domains to be compatible. In wt HA the trypsin cleavage site forms a discrete loop that protrudes from the HA0 structure [25]. It is possible that this loop has adopted a novel conformation in EnvHA, extending further out from the EnvHA0 structure, thereby being more accessible to host cell proteolytic enzymes. Certain highly pathogenic avian influenza viruses have an insertion of a series of basic amino acids adjacent to the trypsin cleavage site thereby increasing the relative size of the "cleavage loop". The HAs of these viruses can be cleaved by enzymes other than those residing within the tissues of the respiratory and alimentary tracts allowing such viruses to infect other organs within the host resulting in serious systemic disease.

Following processing of HA0 into HA1/HA2, low pH treatment is required to induce conformational changes in HA that allow extrusion of the fusion peptide and membrane fusion to occur. Due to the HA1/HA2 stalk region having to accommodate a globular head approximately twice the size in EnvHA proteins (Figure 1), structural limitations might have been imposed upon it. However, all EnvHA constructs were able to induce membrane fusion of Ghost cells expressing human CD4 following low pH treatment whether or not they had been treated with trypsin (Table 3). This indicates that a portion of the EnvHA had been correctly processed in the host cells, whilst the increased levels of fusion seen after trypsin

treatment suggests that some of the surface expressed EnvHA is in an unprocessed state. These membrane fusion data support the EnvHA trypsin cleavage experiments where an increase in the 28 kDa band (HA2) was observed in the presence of trypsin (Figures 5b and 6). Overall these results show that EnvHA retains the susceptibility to processing by trypsin-like proteases and the requirement of a low pH environment to trigger membrane fusion, reminiscent of HA.

In membrane fusion assays the wt Env constructs of JRFL (R5-tropic) and NL43 (X4-tropic) induced fusion at neutral pH and retained their specificity for Ghost cells expressing the correct co-receptor (Figure 7, Table 3). However, co-receptor specificity was abolished in all EnvHA constructs, though the lack of membrane fusion in BHK cells confirmed that CD4 was still required. This suggests that the replacement of the gp120/gp41 stalk with the HA1/HA2 stalk had significant effects on the processes following the attachment of gp120 to CD4 receptors on target cells. Membrane fusion induced by HIV-1 Env has been shown to be a staged event with gp120 CD4 binding inducing conformational changes in gp120 that create/expose the chemokine co-receptor binding site, with the gp120/co-receptor interaction driving further conformational changes in the Env gp120/gp41 trimer to trigger the fusion event. Much effort has been put into mapping the sites on gp120 responsible for CD4-binding and co-receptor interaction, and whilst the V3-loop plays a major role, other variable domains and amino acid residues in constant regions are involved [1]. EnvHA constructs were designed (Figure 1) to contain all the gp120 residues identified as being important in these interactions. In the experiments reported here we have

not directly monitored co-receptor binding by EnvHAs but results suggest that it is not essential for induction of membrane fusion whereas CD4 binding is. However, it is possible that the stability conferred on the EnvHA constructs by the HA1/HA2 stalk region, notably with there being a disulphide bond between HA1 and HA2, impedes the conformational change induced by gp120-CD4 binding thereby preventing the creation/exposure of the co-receptor binding site. If this is so, clearly EnvHA-CD4 binding followed by a low pH trigger is sufficient to induce membrane fusion (Figure 7, Table 3).

It has been shown that the HAs of influenza mutants selected in the presence of high concentrations of amantadine contain amino acid substitutions at a number of positions in HA1 and/or HA2 that result in fusion occurring at elevated (more neutral) pHs, inferring less structural stability in HA1/HA2 interaction [17]. When membrane fusion was monitored against a pH range of 6-5 in 0.2 steps it was found that the pH at which fusion occurred was higher by approximately 0.2 pH units for EnvHAs when compared to wt HA. This shift is comparable to those observed for influenza mutants containing amino acid substitutions in the globular head of HA1 [17]. EnvHAs might be expected to be less stable than HA as the globular head of HA1 (224 amino acids and 2 N-linked glycosylation sequons) was replaced with gp120 proteins of approximately twice the size (NL43, 407 amino acids and 23 N-linked glycosylation sequons; JRFL, 401 amino acids and 22 N linked glycosylation sequons). However, the membrane fusion induced by influenza HA has been shown to be pH, temperature and time dependent [20, 26]. In the current work all fusion experiments were conducted at 37°C and there were no discernable time differences for

heterokaryon formation caused by X31-HA and EnvHAs. That all EnvHA constructs had the same membrane fusion characteristics is of interest as those with linkers included between respective Env and HA subunits, to provide some degree of flexibility to the protein structure, might have been expected to be more stable. Results indicate that the inclusion of these linkers had no effect on the functioning of the EnvHA chimeric proteins. This infers structural similarity between the eight EnvHA constructs and indicates tolerance of amino acid sequence changes flanking the HA1/Env and Env/HA1 junctions.

In the course of this work we have shown that recombinant vaccinia can drive the expression of EnvHA, a proportion of which is present on cell surfaces as a stable, trimeric protein that retains functions of receptor binding and membrane fusion. Preliminary studies indicate that the bromelain-sensitive cleavage site in the HA2 domain is conserved such that EnvHA glycoproteins can be purified for structural studies. Further, since EnvHA is expressed as a stable, functional protein, the opportunity to develop therapies for HIV-1 infection are presented.

In the context of the results presented here, recombinant influenza viruses expressing EnvHA chimeric proteins allow the development of strategies for the effective treatment of HIV-1. Such recombinant viruses target CD4-expressing cells. Recombinant viruses additionally carrying a conditionally lethal toxic gene, such as HSVTK [6] or reverse Caspase-3 that induces apoptosis [18], under the control of an HIV-2 LTR, allow the targeted killing of only HIV-infected CD4-expressing cells such

that uninfected CD4 cells remain viable to perform their immune functions. Since internalized CD4 is commonly found in early endosomes in the absence of specific internalization stimulators, in the process of generating EnvHA recombinant influenza viruses it may be preferable to adjust the pH at which fusion occurs to ensure good infectivity of the EnvHA containing viruses. This is done by making specific mutations in the HA1 and/or HA2 domains, using techniques familiar to those skilled in the art, based on studies of influenza HA fusion mutants [17].

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The references cited herein are all expressly incorporated by reference.

Table 1: Oligonucleotide primers used in the construction of *HA*-, *envHA*- and *env*-genes.

Primer	Sequence	Position[a]	Size[b]	Comment[c]
X31HAFU	AGTACCTGCAGAGCAAAAGCAGGGGATAATTC	11/1-21 HA		Pst I
X31Rint	CATGTTAAAATKTTCTGTYACGCATATTTTCCCCGTTGAGGA	HA 233-213/21 ENV	265	
X31RintL	CATGTTAAAATKTTCTGTYACAGATCCACCTCCGCATATTTTCCCCGTTGAGGA	HA 233-213/12/21 ENV	277	Linker = GGGS
ENVFOR	TCCTCAACGGGGAAAATATGCGTRACAGAAMATTTTAACATG	HA 21/262-282 ENV J		
		HA 21/265-285 ENV N		
ENVFORL	TCCTCAACGGGGAAAATATGCGGAGGTGGATCTGTRACAGAAMATTTTAACATG	HA 21/12/262-282 ENV J		Linker = GGGS
		HA 21/12/265-285 ENV N		
ENVREV	AGTGATGCATTCAGAAATACATGCTACTCCTAATGGTTCAAT	ENV J 1464-1444/21 HA	1245	
		ENV N 1485-1465/21 HA	1263	
ENVREVL	AGTGATGCATTCAGAAATACAAGATCCACCTCCTGCTACTCCTAATGGTTCAAT	ENV J 1464-1444/12/21 HA	1269	Linker = GGGS
		ENV N 1485-1465/12/21 HA	1287	
X31Fint	ATTGAACCATTAGGAGTAGCATGTATTTCTGAATGCATCACT	ENV 21/906-926 HA	892	
X31FintL	ATTGAACCATTAGGAGTAGCAGGAGGTGGATCTTGTATTTCTGAATGCATCACT	ENV 21/12/906-926 HA	904	Linker = GGGS
X31HARU	GAGTCAAGCTTAGTAGAAACAAGGGTGTTTT	11/1765-1746 HA		Hind III
FgpV[d]	TAAGAGGAATTCCAGAAGAYAGTGGCAATGARAGYGA	Start codon italicised		Eco RI
RgpV[d]	ACCACACCATGGTTTGACCAYTTGCCACCCATBTTA	Stop codon italicised	~2600	Nco I

Segments of the X31 *HA*-gene and the *env*-genes of HIV-1 isolates JRFL (J) and NL43 (N) were amplified and joined together by PCR splice-overlap extension (PCR-SOE). [a] The positions of hybridising sequence in the entire X31 *HA*-gene and the coding sequence of the *env*-genes are given. [b] The sizes of first round PCR products generated off the X31 *HA*- and HIV-1 *env*-genes are indicated. [c] The positions of restriction sites used for cloning into pRB21 are underlined in the primer sequences as are the nucleotides encoding the linker sequences at the *HA/env* junctions. [d] These two primers were used to rescue intact *env*-genes of JRFL and NL43. All primers were synthesised by Eurogentec Oswel.

Table 2: Composition of EnvHA chimeric proteins.

EnvHA Construct[a]	Parental HIV-1 Strain[b]	GGGS Linker[c]	
		HA1/Env	Env/HA1
R3	NL43	-	-
S4	JRFL	-	-
T2	NL43	-	+
U4	JRFL	-	+
V3	NL43	+	-
W2	JRFL	+	-
X2	NL43	+	+
Y1	JRFL	+	+

[a] Eight EnvHA constructs were generated (R-Y) and the clones indicated used in subsequent experiments. [b] Two HIV-1 strains, pNL43 [36] and JRFL [37] were used to source the gp120 components. [C] Short peptide linkers (GGGS) were incorporated at HA1/Env and Env/HA1 junctions as indicated (+: Figure 1).

Table 3: Membrane fusion medi ated by EnvHA proteins.

Construct	- Trypsin		+ Trypsin	
	pH7	pH5	pH7	pH5
R3	*	*X4, R5, P*	*	**X4, R5, P**
S4	*	*X4, R5, P*	*	**X4, R5, P**
T2	*	*X4, R5, P*	*	**X4, R5, P**
U4	*	*X4, R5, P*	*	**X4, R5, P**
V3	*	*X4, R5, P*	*	**X4, R5, P**
W2	*	*X4, R5, P*	*	**X4, R5, P**
X2	*	*X4, R5, P*	*	**X4, R5, P**
Y1	*	*X4, R5, P*	*	**X4, R5, P**
JRFL	**R5**	**R5**	**R5**	**R5**
NL43	**X4**, *R5, P*	**X4**, *R5, P*	**X4**, *R5, P*	**X4**, *R5, P*
X31	*	*	*	**X4, R5, P, B**
vRB12	*	*	*	*
NEG	*	*	*	*

Cells susceptible to fusion mediated by wt Env and/or wt HA {BHK (B), Parental Ghost cells (P) and those producing either CXCR4 (X4) or CCR5 (R5)}, were infected with recombinant vaccinia viruses to express the constructs indicated. After 16h incubation, cells were either left untreated (-) or treated (+) with 5ug/ml trypsin and then either maintained at neutral pH (pH7) or exposed briefly to low pH cell buffer (pH5). Membrane fusion was then monitored as described in materials and methods and scored as: no fusion detected (*) and fusion detected in specific cell lines at high (**X4, R5, P, B**) and low (*X4, R5, P*) levels.

Claims

1. A chimeric protein comprising:

(a) an amino acid sequence of the HA1 subunit of influenza virus haemagglutinin (HA) protein comprising cysteine residues at positions 30, 68 and 293 as numbered from the N-terminal amino acid of the HA1 subunit from influenza virus X31, or corresponding positions; and

(b) an amino acid sequence of the HA2 subunit of influenza virus HA protein comprising cysteine residues at positions 153, 160 and 164 as numbered from the N-terminal amino acid of the HA2 subunit from influenza virus X31, or corresponding positions; and

(c) an amino acid sequence of a heterologous protein positioned between the cysteine residues at positions 68 and 293 of (a); and having a disulphide bond between the cysteine residues at position 30 of HA1 and position 153 of HA2, at positions 68 and 293 of HA1, and at positions 160 and 164 of HA2, wherein the chimeric protein has the structural properties of the native HA protein such that the structure and function of the native heterologous protein are retained.

2. The chimeric protein of claim 1, wherein the amino acid sequence (b) further comprises the membrane anchor region from and including position 186 to and including position 207 as numbered from the N-terminal amino acid of the HA2 subunit from influenza virus X31, or corresponding positions.

3. The chimeric protein of claim 1 or claim 2 comprising in the direction from the N-terminus to the C-terminus:

(i) an amino acid sequence of the HA1 subunit from and including position 1 to and including position 68 as numbered from the N-terminal amino acid of the HA1 subunit from influenza virus X31, or corresponding positions;

(ii) an amino acid sequence of the heterologous protein;

(iii) an amino acid sequence of the HA1 subunit from and including position 293 to and including position 345 as numbered from the N-terminal amino acid of the HA1 subunit from influenza virus X31, or corresponding positions;

(iv) an amino acid sequence of the HA2 subunit from and including position 1 to and including position 237 as numbered from the N-terminal amino acid of the HA2 subunit from influenza virus X31, or corresponding positions.

4. The chimeric protein of any one of the preceding claims, further comprising an amino acid linker sequence at either or both of the junctions between the heterologous protein amino acid sequence and the HA1 subunit amino acid sequence.

5. The chimeric protein of claim 4, wherein the amino acid linker sequence is between sequences (i) and (ii) and/or (ii) and (iii) of claim 3.

6. The chimeric protein of claim 4 or claim 5, wherein the linker sequence is Gly-Gly-Gly-Ser.

7. The chimeric protein of any one of the preceding claims, wherein the influenza HA protein is from influenza virus X31.

8. The chimeric protein of any one of the preceding claims, wherein the heterologous protein is a protein that has a trimeric quaternary structure.

9. The chimeric protein of any one of the preceding claims, wherein the heterologous protein is a receptor-binding protein.

10. The chimeric protein of any one of the preceding claims, wherein the heterologous protein is a viral protein.

11. The chimeric protein of claim 10, wherein the heterologous protein is a viral protein selected from the group consisting of:
haemagglutinin glycoprotein of influenza virus; surface glycoprotein SU of Rous Sarcoma virus (RSV), Feline Leukemia virus (FeLV), Human T-cell Leukemia viruses 1 and 2 (HTLV-1/2), Bovine Leukemia virus (BLV), Human Immunodeficiency viruses (HIV-1/2), Simian Immunodeficiency virus (SIV), Equine Infectious Anemia virus (EIAV), Feline Imunodeficiency virus (FIV), Caprine Arthritis Encephalitis virus (CAEV) and Visna/Maedi; surface glycoprotein GP1 of Ebola and Marburg; E2 protein of Semliki forest virus, Sindbis virus, Ross River, Eastern/Western Equine Encephalitis and Rubella; S1 protein of Severe Acquired Respiratory Syndrome (SARS); VP2 protein Bluetongue virus and African horse sickness virus; G proteins of Vesicular Stomatitis virus (VSV) and Rabies; E (E2) proteins of Tick Borne Encephalitis (TBE), Dengue, Hepatitis C virus (HCV), West Nile and Yellow fever.

12. The chimeric protein of any one of claims 1 to 10, wherein the heterologous protein is an HIV Env glycoprotein.

13. The chimeric protein of claim 12, wherein the HIV Env glycoprotein is HIV gp120.

14. The chimeric protein of claim 12 or claim 13, wherein the HIV is HIV-1.

15. The chimeric protein of claim 14, wherein the HIV-1 is HIV-1 strain pNL43 or HIV-1 strain JRFL.

16. The chimeric protein of any one of claims 13 to 15, wherein the amino acid sequence of HIV gp120 comprises cysteine residues at positions 119, 126, 131, 157, 194, 203, 216, 226, 237, 245, 294, 329, 376, 383, 416 and 443 as numbered from the N-terminal amino acid of gp120 from HIV-1 strain pNL43, or corresponding positions, having disulphide bonds between pairs of cysteine residues as in native HIV gp120.

17. The chimeric protein of any one of claims 13 to 16, wherein the amino acid sequence of HIV gp120 comprises the amino acid from and including position 89 to the amino acid at and including position 495 as numbered from the N-terminal amino acid of gp120 from HIV-1 strain pNL43, or corresponding positions.

18. A nucleic acid encoding the chimeric protein of any one of the preceding claims.

19. An expression vector comprising the nucleic acid of claim 18.

20. The expression vector of claim 19 which is a mammalian expression vector for expression of the chimeric protein in a mammalian cell.

21. The expression vector of claim 19 which is a viral expression vector for expression of the chimeric protein by a virus.

22. A host cell comprising the nucleic acid of claim 18 or the expression vector of any one of claims 19 to 21.

23. A method of producing a host cell of claim 22, comprising contacting a susceptible cell with the nucleic acid of claim 18 or the expression vector of any one of claims 19 to 21 under conditions allowing uptake of the nucleic acid or expression vector into the cell.

24. A method of producing a chimeric protein of any one of claims 1 to 17 comprising culturing the host cell of claim 22 under conditions allowing expression of the encoded chimeric protein and optionally recovering the chimeric protein from the host cell culture.

25. A method of producing a recombinant virus capable of expressing the chimeric protein of any one of claims 1 to 17, comprising contacting a susceptible cell with the expression vector of claim 21 and optionally recovering the virus from the cell.

26. The method of claim 25, wherein the virus is a vaccinia virus or an influenza virus.

27. A virus obtained by the method of claim 25 or claim 26.

28. The virus of claim 27, further capable of expressing a conditionally lethal toxic protein.

29. The virus of claim 28 which expresses the chimeric protein of any one of claims 12 to 17, wherein the toxic protein is lethal conditional on the presence of HIV in the target cell which the virus infects.

30. The virus of claim 28 or claim 29, wherein the conditionally lethal toxic protein is herpes simplex virus thymidine kinase (HSVTK)and toxicity is conditional on the presence of the drug ganciclovir.

31. The virus of claim 28 or claim 29, wherein the conditionally lethal toxic protein is an apoptosis inducer, such as revCasp3.

32. The virus of any one of claims 27 to 31 for use as a vaccine for a mammal.

33. The virus of claim 32, wherein the vaccine is for a human.

34. The virus of any one of claims 27 to 31 which expresses the chimeric protein of any one of claims 9 to 17 for use in the treatment of an individual by gene therapy.

35. The virus of any one of claims 27 to 31 which expresses the chimeric protein of claim 10 or claim 11 for use in the treatment of an individual infected with the corresponding virus.

36. The virus of any one of claims 27 to 31 which expresses the chimeric protein of any one of claims 12 to 17 for use in the treatment of an HIV-infected individual by gene therapy.

37. The virus of any one of claims 28 to 31 which expresses the chimeric protein of any one of claims 12 to 17 for use in the treatment of an HIV-infected individual, wherein expression of the conditionally lethal toxic protein is conditional on the virus infecting a cell that is infected with HIV.

38. The virus of any one of claims 32 to 37, wherein the virus is for use in combination with one or more different medicaments or treatments.

39. A target cell infected with the virus of any one of claims 27 to 38.

40. Use of the virus of any one of claims 27 to 31 and/or the chimeric protein of any one of claims 1 to 17 for the preparation of a vaccine.

41. Use of the virus of any one of claims 27 to 31 which expresses the chimeric protein of any one of claims 12 to 17 and/or the chimeric protein of any one of claims 12 to 17 for the preparation of a medicament for the treatment of an HIV-infected individual.

42. A vaccine comprising the virus of any one of claims 27 to 38 and/or the chimeric protein of any one of claims 1 to 17.

43. A composition comprising the virus of any one of claims 27 to 38 and/or the chimeric protein of any one of claims 1 to 17.

44. The chimeric protein of any one of claims 1 to 17 for use as a vaccine for a mammal.

45. The chimeric protein of claim 44, wherein the vaccine is for a human.

46. The chimeric protein of any one of claims 1 to 17 for use in the treatment of a mammal.

47. The chimeric protein of any one of claims 12 to 17 for use in the treatment of an HIV-infected individual.

48. The chimeric protein of any one of claims 44 to 47, wherein the chimeric protein is for use in combination with one or more different medicaments or treatments.

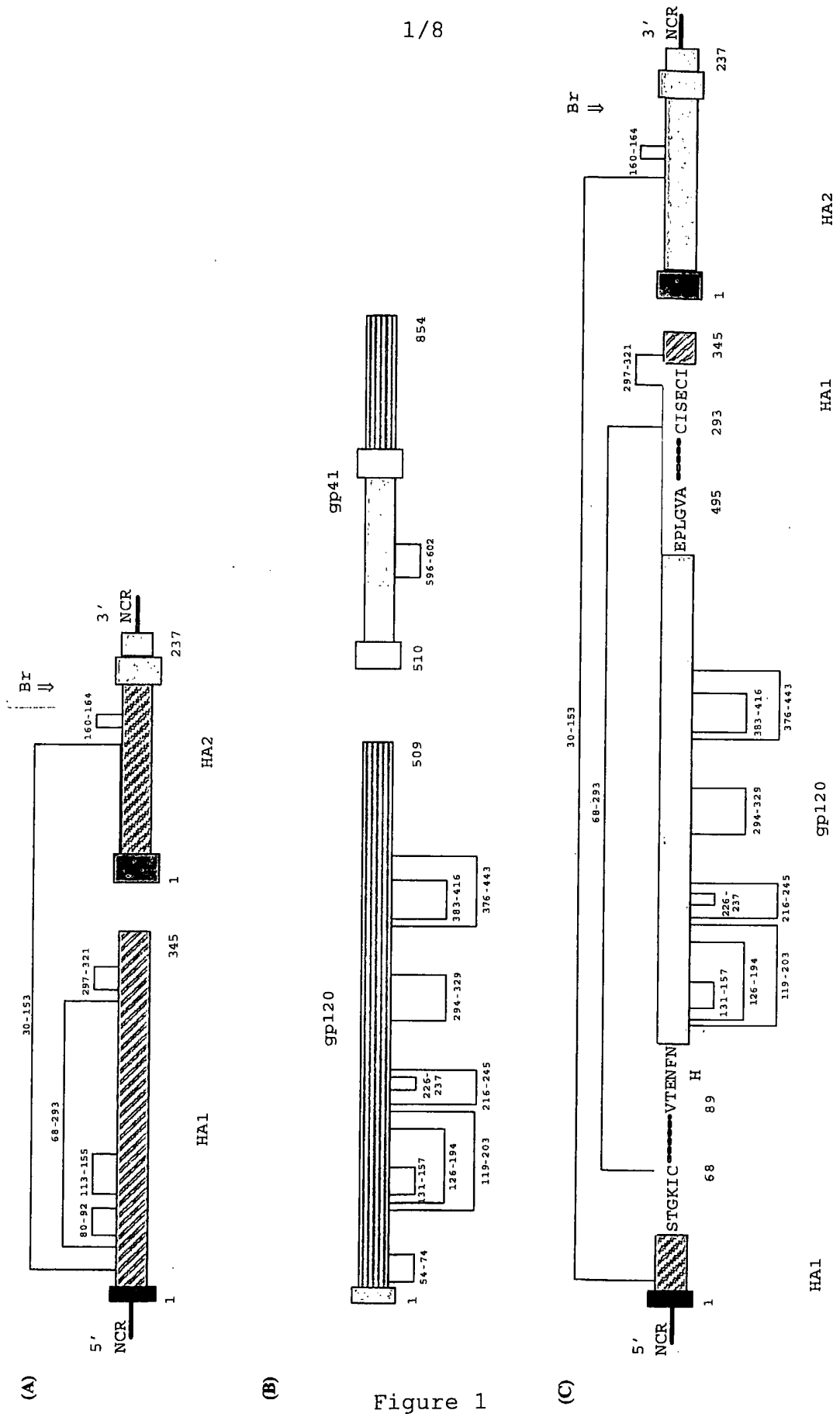

(A)
(B)
(C)
Br
3'
NCR
237
160-164
HA2
345
297-321
30-153
68-293
80-82
113-155
HA1
1
5'
NCR
gp41
854
596-602
510
509
gp120
383-416
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294-329
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Figure 1

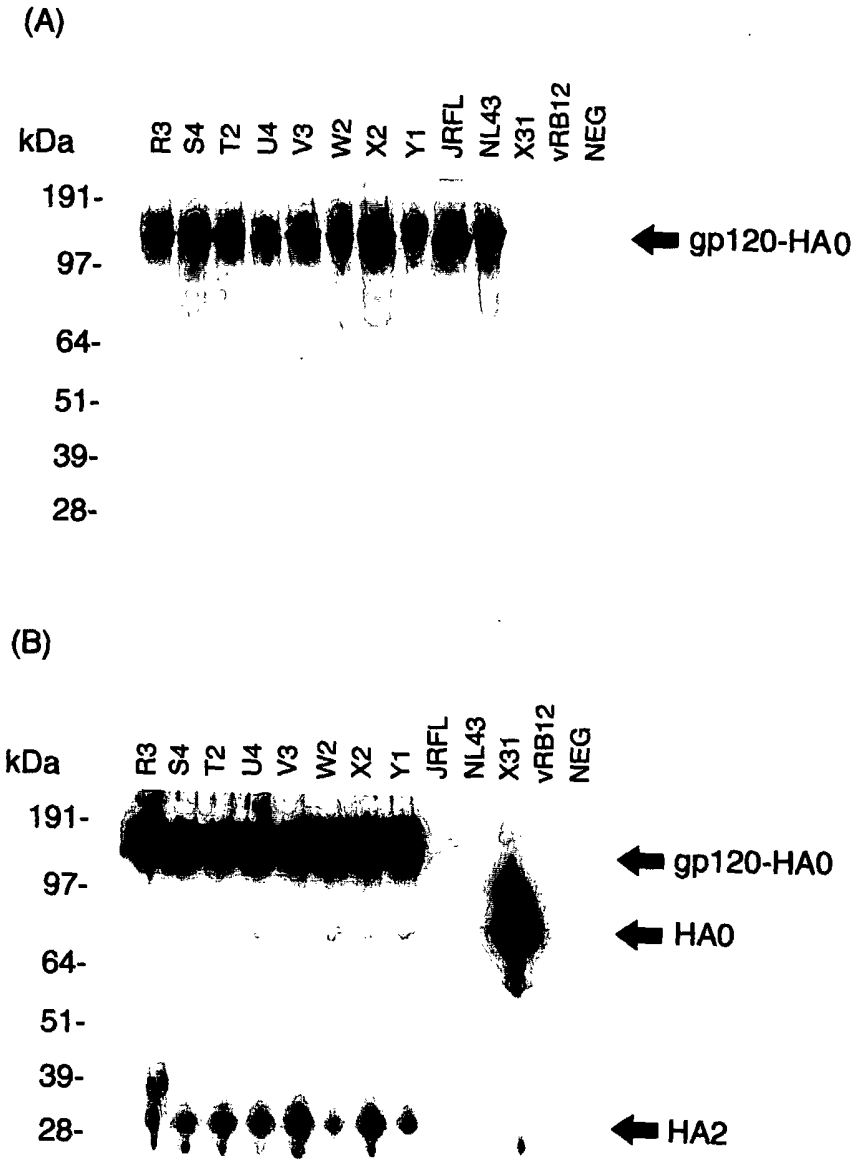

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S4
T2
U4
V3
W2
X2
Y1
JRFL
NL43
X31
vRB12
NEG
191-
97-
64-
51-
39-
28-
gp120-HA0
(B)
kDa
R3
S4
T2
U4
V3
W2
X2
Y1
JRFL
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HA0
HA2

Figure 2

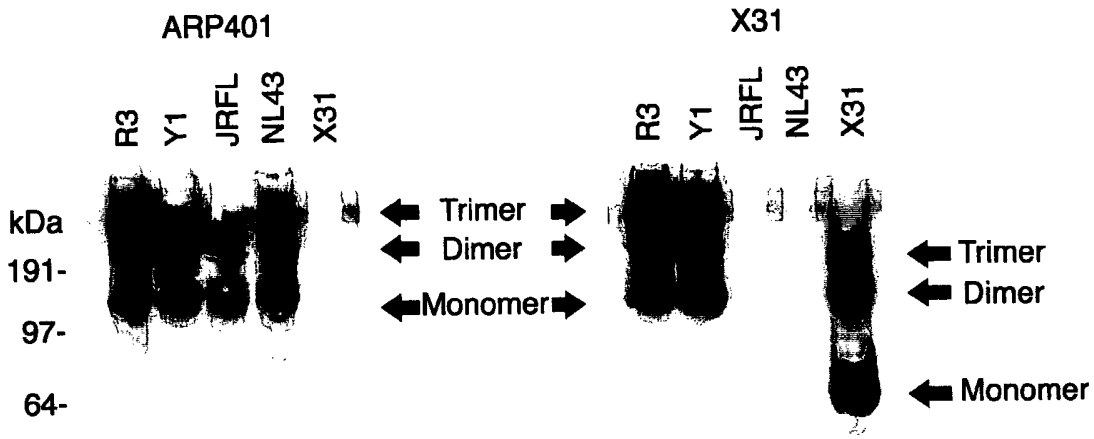

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JRFL
NL43
X31
R3
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kDa
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97-
64-
Trimer
Dimer
Monomer
Trimer
Dimer
Monomer

Figure 3

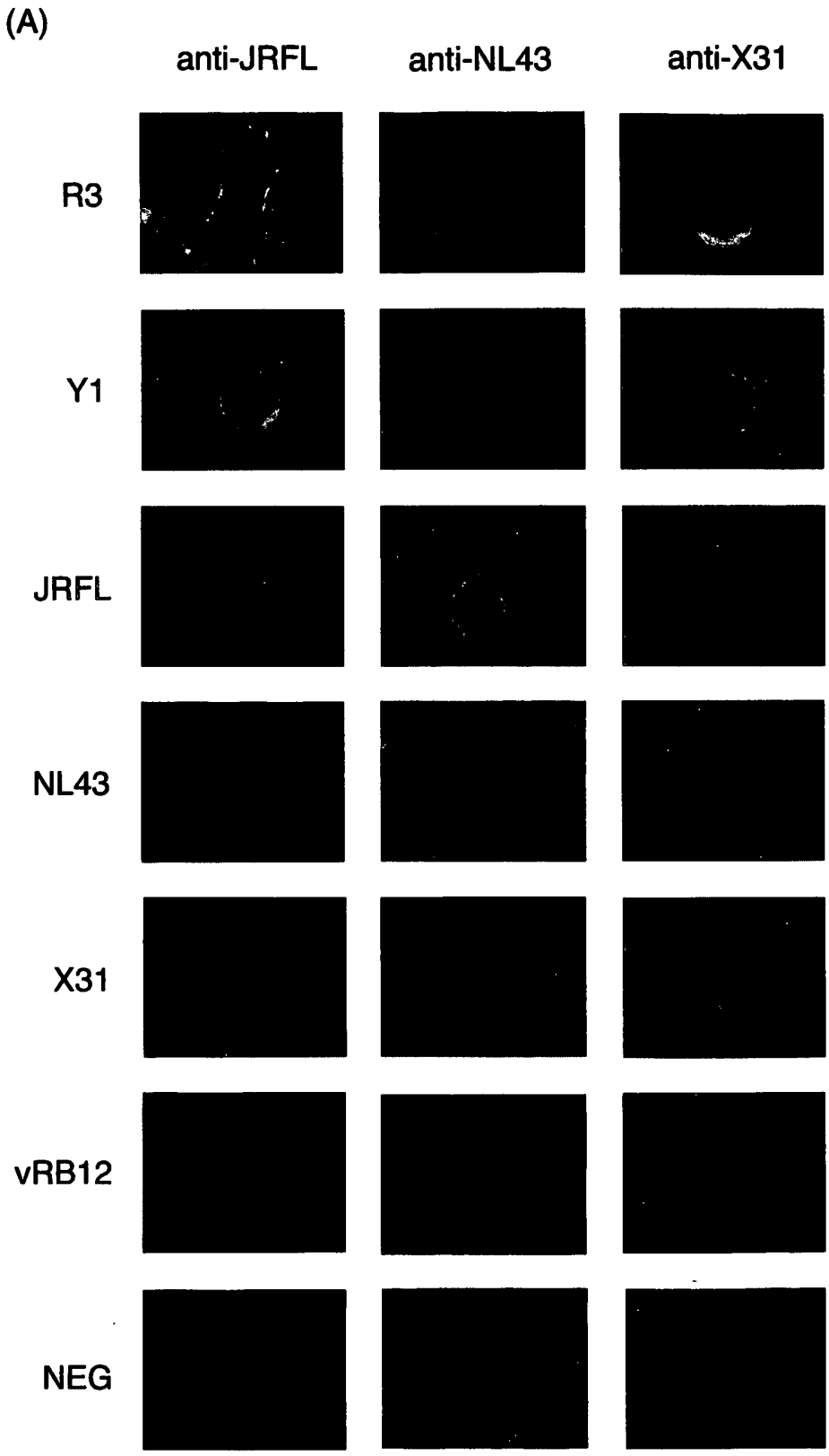


Figure 4

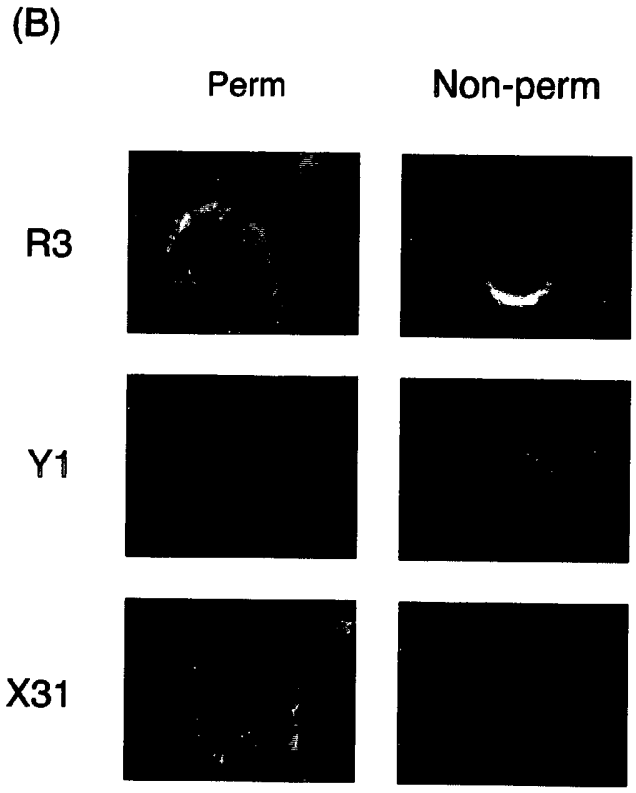


Figure 4

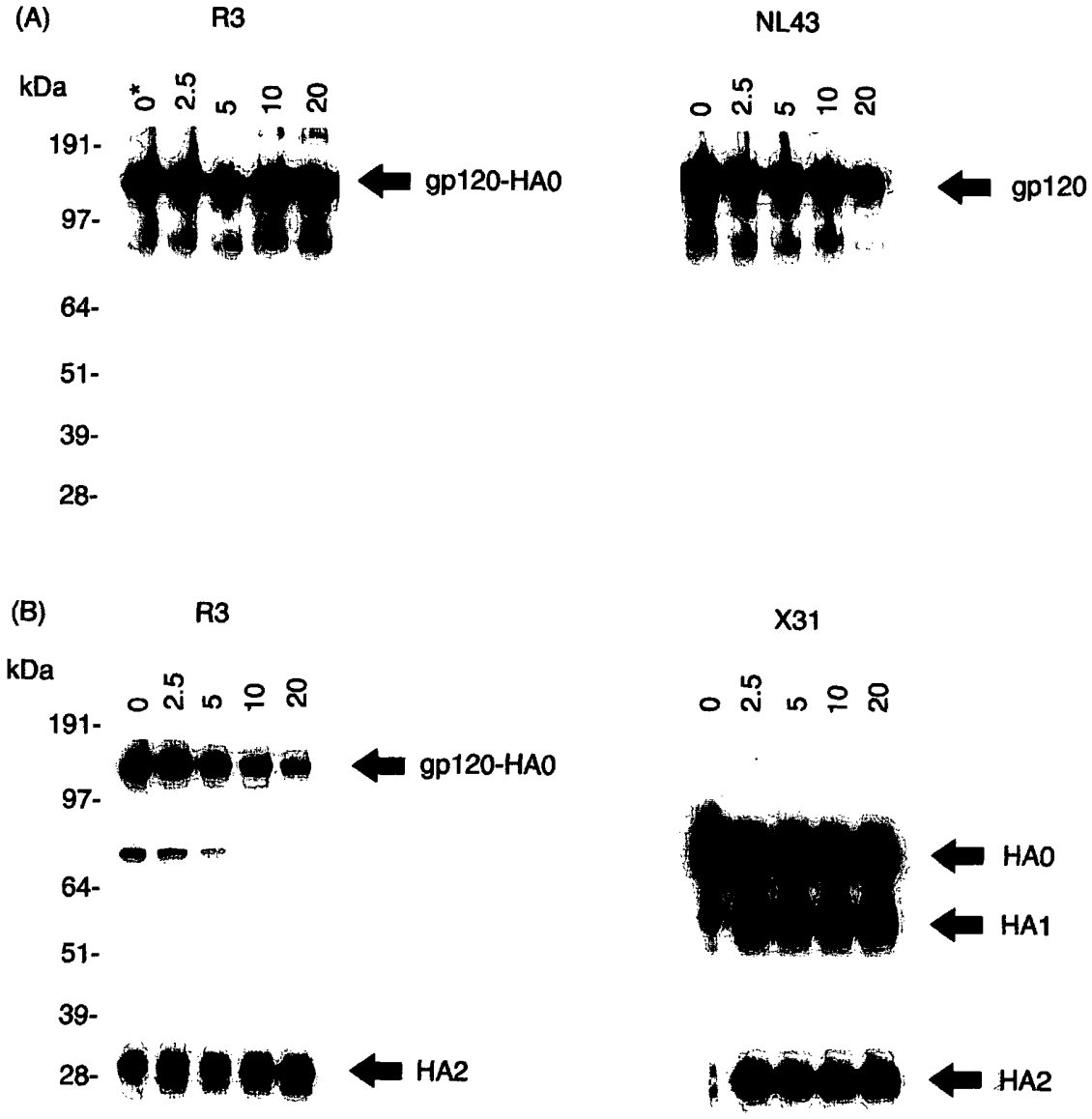

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2.5
5
10
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191-
97-
64-
51-
39-
28-
gp120-HA0
gp120
(B)
R3
X31
kDa
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HA1
HA2
HA2

Figure 5

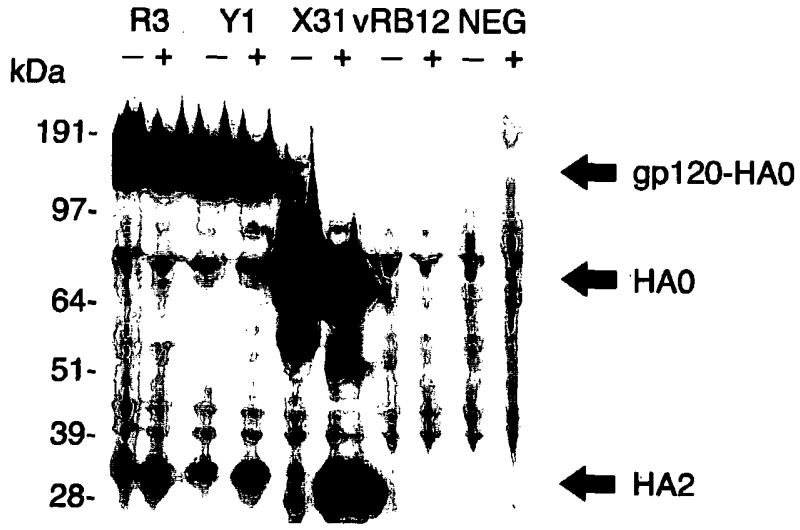


Figure 6

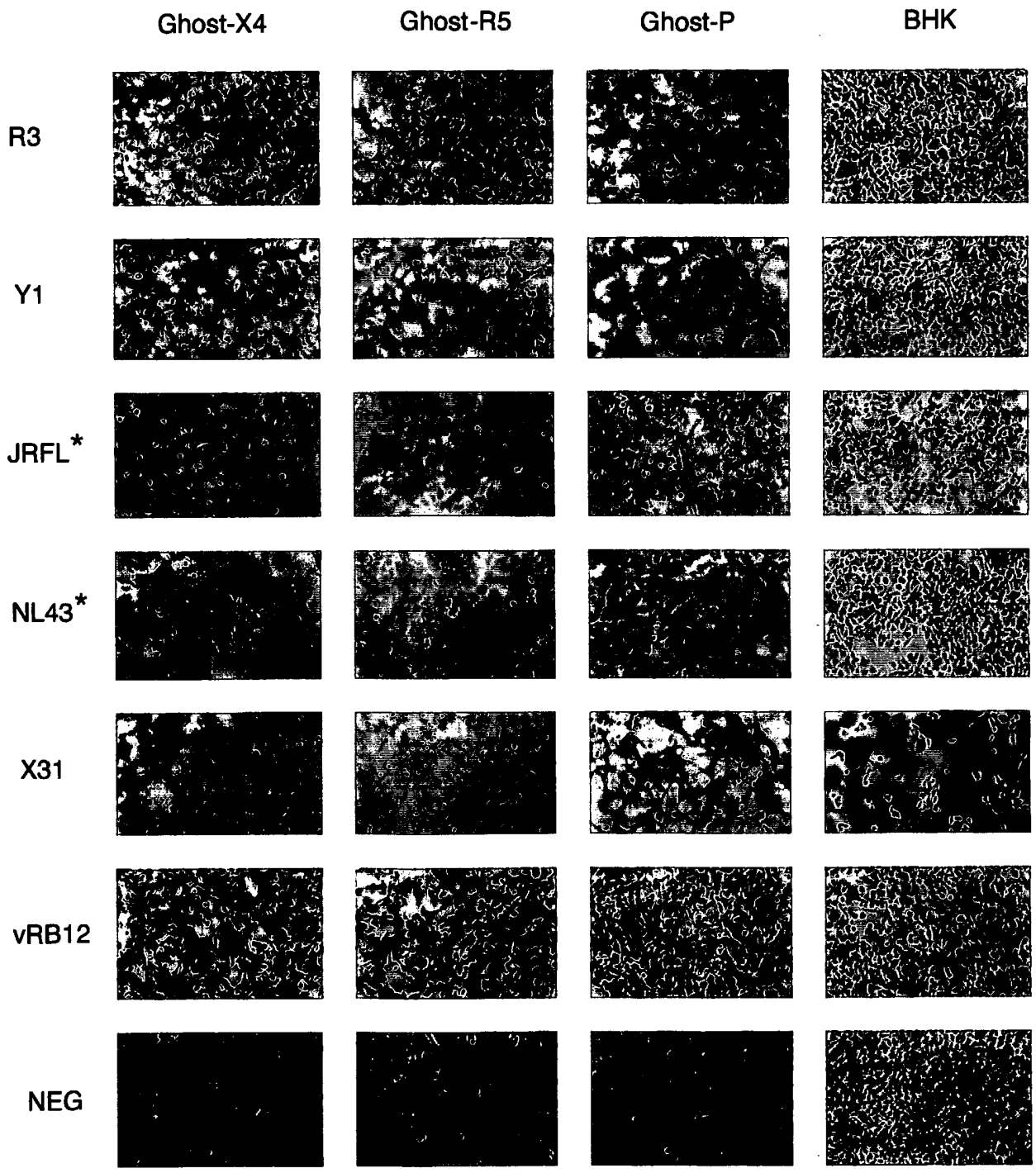


Figure 7