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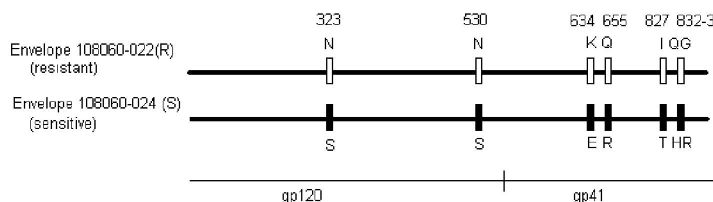
(54) Title: SELECTION OF HIV VACCINE ANTIGENS BY USE OF INTRAPATIENT SEQUENCE VARIATION TO IDENTIFY MUTATIONS IN THE HIV ENVELOPE GLYCOPROTEIN THAT AFFECT THE BINDING OF BROADLY NEUTRALIZING ANTIBODIES

FIGURE 1A: Amino Acid Differences between Neutralization Sensitive and Resistant Clones



(57) Abstract: Selection of HIV vaccine antigens by use of intrapatient sequence variation to identify mutations in the HIV envelope glycoprotein that affect the binding of broadly neutralizing antibodies and polypeptides identified by these methods.

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INTERNATIONAL APPLICATION FILED UNDER THE PATENT COOPERATION TREATY

Title: Selection of HIV vaccine antigens by use of intrapatient sequence variation to identify mutations in the HIV envelope glycoprotein that affect the binding of broadly neutralizing antibodies

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Sequence listing

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Field of the invention

The Invention relates to HIV vaccines and to methods using intrapatient sequence variation to identify mutations in the HIV envelope glycoprotein that affect the binding of broadly neutralizing antibodies.

Background

The identification of epitopes recognized by broadly neutralizing antibodies (bNAbs) in HIV+ sera is a major priority for HIV vaccine research. Previous methods used to identify these epitopes relied on monoclonal antibodies or selective immunoadsorbtion. The present disclosure describes a new method to identify epitopes recognized by bNAbs based on intra-patient sequence variation. Many lines of evidence suggest that for an HIV vaccine to be effective it needs to elicit broadly neutralizing antibodies. While it has been known that such antibodies exist in approximately 10-25% of HIV+ patient sera, none of the candidate vaccines described to date elicit these antibodies. Moreover, because of the complexity of the polyclonal antibody response it has not been possible to identify the epitopes recognized by these anti-

bodies. Clearly there is a need for a method to identify mutations in the HIV envelope glycoprotein that affect the binding of broadly neutralizing antibodies.

Brief description of the invention

Provided herein are methods comprising analyzing intra-patient HIV-1 virus variation to identify specific amino acid residues of the HIV-1 envelope glycoproteins, gp160, gp120, and gp41 that affect sensitivity or resistance to broadly neutralizing HIV-1 antibodies. Also provided are proteins identified by these methods, the nucleic acids encoding the proteins, and vaccines comprising the proteins and nucleic acids.

Brief description of the figures

FIGURE 1A: amino acid differences between neutralization sensitive and resistant clones from 108060.
FIGURE 1B: amino acid sequences of clones created by *in vitro* mutagenesis to map determinants of sensitivity and resistance to neutralization from subject 108060.
FIGURE 2. sensitivity of w/t and mutant clones from 108060 to neutralization by HIV-1+ serum Z23.
FIGURE 3. gp41 functional domains and comparison of sequences of functionally significant regions of the N36 and C34 helices.
FIGURE 4. shows a method to identify epitopes reactive with broadly neutralizing Abs in HIV+ sera
FIGURE 5. shows the sequences from subjects 108060, 108051, 108048 corresponding to neutralization sensitive and neutralization resistant variants.

General Representations Concerning the Disclosure

In this specification, reference is made to particular features of the invention. It is to be understood that the disclosure of the invention in this specification includes all appropriate combinations of such particular features. For example, where a particular feature is disclosed in the context of a particular embodiment or a particular claim, that feature can also be used, to the extent appropriate, in the context of other particular embodiments and claims, and in the invention generally. The embodiments disclosed in this specification are exemplary and do not limit the invention. Other embodiments can be utilized and changes can be made. As used in this specification, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a part" includes a plurality of such parts, and so forth. The term "comprises" and grammatical equivalents thereof are used in this specification to mean that, in addition to the features specifically identified, other features are optionally present. The term "consisting essentially of" and grammatical equivalents thereof is used herein to mean that, in addition to the features specifically identified, other features may be present which do not materially alter the claimed invention. The term "at least" followed by a number is used herein to denote the start of a range beginning with that number (which may be a range having an upper limit or no upper limit, depending on the variable being defined). For example "at least 1" means 1 or more than 1, and "at least 80%" means 80% or more than 80%. The term "at most" followed by a number is used herein to denote the end of a range ending with that number (which may be a range having 1

or 0 as its lower limit, or a range having no lower limit, depending upon the variable being defined). For example, "at most 4" means 4 or less than 4, and "at most 40%" means 40% or less than 40 %. When, in this specification, a range is given as " (a first number) to (a second number)" or "(a first number) - (a second number)", this means a range whose lower limit is the first number and whose upper limit is the second number. Where reference is made in this specification to a method comprising two or more defined steps, the defined steps can be carried out in any order or simultaneously (except where the context excludes that possibility), and the method can optionally include one or more other steps which are carried out before any of the defined steps, between two of the defined steps, or after all the defined steps (except where the context excludes that possibility). Where reference is made herein to "first" and "second" features, this is generally done for identification purposes; unless the context requires otherwise, the first and second features can be the same or different, and reference to a first feature does not mean that a second feature is necessarily present (though it may be present). Where reference is made herein to "a" or "an" feature, this includes the possibility that there are two or more such features.

This specification incorporates by reference all documents referred to herein and all documents filed concurrently with this specification or filed previously in connection with this application, including but not limited to such documents which are open to public inspection with this specification.

DEFINITIONS

The terms “amino acid” and “amino acid sequence” refer to an oligopeptide, peptide, polypeptide, or protein sequence, or a fragment of any of these.

“Amplification” relates to the production of additional copies of a nucleic acid sequence e.g., using polymerase chain reaction (PCR).

The term “antibody” refers to intact immunoglobulin molecules as well as to fragments thereof, such as Fab, $F(ab')_2$, and Fv fragments, which are capable of binding an epitopic determinant.

The term “similarity” refers to a degree of complementarily. There may be partial similarity or complete similarity. The word “identity” may substitute for the word “similarity.” A partially complementary sequence that at least partially inhibits an identical sequence from hybridizing to a target nucleic acid is referred to as “substantially similar.”

The phrase “percent identity” as applied to polynucleotide or polypeptide sequences refers to the percentage of residue matches between at least two sequences aligned using a standardized algorithm such as any of the BLAST suite of programs (e.g., blast, blastp, blastx, nucleotide blast and protein blast) using, for example, default parameters. BLAST tools are very commonly used and are available on the NCBI web site.

A “variant” of a particular polypeptide sequence is defined as a polypeptide sequence having at least 40% sequence identity to the particular polypeptide sequence over a certain length of one of the polypeptide sequences using blastp with the “BLAST 2 Sequences” tool set at default parameters. Such a pair of polypeptides may show, for example, at least 50%, at least 60%, at least 70%, at least 80%, at least 86%, at least 90%, at least 95%, or at least 98% or greater sequence identity over a certain defined length of one of the polypeptides.

Detailed description of the embodiments

Disclosed is a new method for identifying mutations in envelope proteins, which methods comprise analyzing intra-patient HIV-1 virus variation to identify specific amino acid residues of the HIV-1 envelope glycoproteins, gp160, gp120, and gp41 that affect sensitivity or resistance to broadly neutralizing HIV-1 antibodies. The mutations identified by the methods of the invention provide enhanced sensitivity (or resistance) to neutralization of a virus by anti-viral antisera; in particular neutralization of an HIV virus by anti-HIV antibodies, such as in antisera. The methods described identify epitopes recognized by broadly neutralizing antibodies. Such epitopes and the proteins of which they are a part may provide a powerfully immunogenic, protective vaccine against HIV. To identify polymorphisms and sequences that effect sensitivity or resistance to broadly neutralizing antibodies, viral envelope sequences (such as gp160, gp120, and gp41) from sensitive and resistant viruses were identified and compared and the differences were noted. Mutagenesis was carried out to identify specific residues that correlated with sensitivity or resistance to virus neutralization.

Essentially, the method consists of carrying out the following steps: (i) Providing a plurality of individual subjects who are seropositive for HIV antibodies and taking a biological sample such as blood or plasma from each subject, wherein the sample contains a multiplicity of HIV viruses with closely related genomes, wherein all subjects had been infected with HIV no more than one year before, and no less than one month before sample collection. (ii) Amplifying the *env* genes by the polymerase chain reaction (PCR) of the multiplicity of viruses to produce a library of different *env* genes. (iii) Cloning the amplified *env* genes into a plasmid shuttle vector allowing the plasmid to replicate in both bacteria (such as E. coli) and mammalian cells. Such vectors contain: a bacterial origin of replication, an origin of replication from a mammalian cell virus such as SV-40 or adenovirus, and a functional transcription unit that enables expression of a suitable drug resistance gene such as ampicillin, tetracycline, or kanamycin in order to allow selective growth of bacteria transformed with the shuttle vector. The shuttle vector must also contain the elements of a functional mammalian cell transcription unit. Beginning at the 5' end of the sense DNA strand, the transcription unit should contain a promoter sequence from a mammalian gene or virus, a splice donor/acceptor site, a segment of synthetic DNA containing either multiple restriction enzyme recognition sites or other sequences to allow directional cloning of PCR amplified envelope genes, a transcription termination codon, and a polyadenylation site. The transcription unit should also contain transcription enhancer sequences at either locater either 5' to the promoter or 3' of the polyadenylation site. Once PCR amplified HIV genes are ligated into the shuttle vector, the collection of plasmids containing the cloned envelope genes are transformed into E. coli by standard techniques, grown in a small volume of bacterial culture media and then plated onto agar plates containing the appropriate antibiotic so that only bacterial containing the shuttle vector plasmid containing the cloned envelope genes are able to form colonies. Individual colonies are then selected at random and plasmid DNA from each colony is prepared and analyzed by restriction digestion, and only those containing an insert of the proper size of the full length HIV envelope gene are retained and used for the preparation of pseudoviruses as described below. (iv) Co-transfecting mammalian cells (e.g. 293HEK) with the env-containing vector

and simultaneously with a plasmid containing a defective HIV provirus virus where the coding sequence of the env gene was replaced with the coding sequence of a marker gene such as one capable of emitting light, e.g. Luciferase) to produce pseudovirions containing the amplified env genes. (v) The pseudovirions are placed in contact with cells capable of being infected by HIV so as to produce colonies of infected cells. Such cells express the genes for CD4 and at least one chemokine receptor gene (either CCR5 or CXCR4). The cells can also express CD4 and both the CCR5 and CXCR4 chemokine receptor genes. Cell culture supernatants containing pseudoviruses are harvested from the transfected cells and individual stocks of pseudoviruses resulting from single purified expression plasmids represent virus stocks. (vi) The pseudotype virus colonies thus created are tested to determine infectivity; 20-50 pseudo virus stock are prepared from each individual and only those exhibiting good infectivity as measured by a significant higher level of relative light units relative to control pseudoviruses containing only defective envelope genes are advanced to neutralization assays. (vii) Then each infective pseudotype virus is tested for sensitivity or resistance to neutralization by one or more broad neutralizing antibodies. In neutralization assays two or more pseudovirions from the same individual are tested. Each pseudovirus stock is incubated with serially diluted plasma or sera from HIV infected individuals or purified polyclonal or monoclonal antibodies. A significant decrease in the emission of light relative to pseudoviruses incubated with a negative control specimen that does not contain antibodies to HIV envelope proteins. (viii) Then selection is done of pairs of plasmids containing specific env proteins which were used to prepare the pseudoviruses described above, wherein each pair contains one env gene that yielded a neutralization resistant pseudovirus and one env gene that yielded neutralization sensitive pseudovirion. (ix) The envelope genes from sensitive and resistant pseudoviruses are then sequenced and comparison was done to thus to identify amino acid sequence differences between the neutralization sensitive and neutralization resistant envelope genes. Only pairs of sequences with a minimal number of sequence differences (no more than for example 10%, 8%, 6%, 5% or 4% sequence difference over the entire coding region of the env sequence in question) are then selected for further analysis. (x) In vitro mutagenesis may then be performed to create envelope genes where the effect of each amino acid difference between the neutralization sensitive and neutralization resistant pairs can be determined when such mutant genes are incorporated into pseudovirions and tested for sensitivity and resistance to neutralization. In this step, amino acids at corresponding positions of neutralization sensitive member of the pair is introduced into the neutralization resistant member of the pair to see if it confers the neutralization sensitive phenotype. Conversely, specific amino acids from the neutralization resistant sequence can be introduced into the neutralization sensitive envelope gene by in vitro mutagenesis to identification of the specific amino acid responsible for the neutralization resistant phenotype.

It should be noted that it is an important feature of the invention that the samples be taken from individuals within a certain window. For various reasons more thoroughly explained elsewhere in this disclosure, the HIV virus population changes dramatically during the course of infection, and the inventors have reasoned that in order to successfully identify the polymorphisms of the invention, samples need to be taken within a certain window of time. In the present invention samples need to be taken from

subjects who had been infected with HIV no more than one year before, and no less than one month before sample collection. In various embodiments a wider window may be used and samples may be taken no more than 18 months before, and no less than two weeks before sample collection. In other embodiments a narrower window may be used and the earliest and latest times that bracket the sample window may be, for example, 14 months and 1 month, 12 months and 1 month, 10 months and 6 weeks, 8 months and 6 weeks, 6 months and 6 weeks, or any combination of these times from the date of infection. Obviously the date of infection is not always precisely known, and the dates that comprise the earliest and latest times since infection may vary, for example +/- 14 days or +/- 24 days. In one specific embodiment used to produce the current experimental results, all subjects had been infected with HIV 109 days +/- 58 days before specimen collection.

Although most of the viruses from an individual exhibited a predominant "neutralization sensitive" or "neutralization resistant" phenotype, variants were identified that differed in sensitivity from predominant forms. Because all of the samples compared were from recent infections the amount of intra-patient sequence variation in the envelope glycoprotein was minimal. Site directed mutagenesis enabled us to identify amino acids residues responsible for neutralization sensitivity or resistance. Mutations affecting virus neutralization were found in both gp120 and gp41.

The methods disclosed provide a novel strategy to enable quick and efficient identification of the epitopes recognized by bNAbs in HIV+ patient sera. Characterization of polymorphism at these sites will provide information to guide the formulation of multivalent vaccines. In one aspect, the invention discloses methods for identification of certain immunogenic epitopes, and further discloses the epitopes themselves. Broadly neutralizing antibodies recognize the specific epitopes of the HIV-1 envelope glycoproteins, including gp120, and gp41 and any gp160-derived protein, whether monomeric or oligomeric. Thus, aspects of the present invention include these HIV-1 envelope glycoproteins, nucleic acids encoding the polypeptides and vaccines comprising the polypeptides or nucleic acids.

Also described are methods for the identification of specific polymorphisms within, or having an effect upon, neutralizing epitopes that are suitable for inclusion in a protein or polypeptide that may be included in the formulation of a multivalent HIV vaccine cocktail. It should be noted that the polymorphisms of the invention need not be within or even close to the epitopes affected. The polymorphisms of the invention alter the conformation of the epitopes so as to reveal (or hide) a portion of the epitope in such a way that it becomes available to bind with (or hidden from) a corresponding antibody, such as a broadly neutralizing antibody. Further described is a method for identifying and purifying broadly neutralizing antibodies from HIV patient serum or plasma. HIV envelope genes were amplified from HIV+ plasma obtained in the VAX004 Phase 3 trial. See Flynn, N. M., D. N. Forthal, C. D. Harro, F. N. Judson, K. H. Mayer, and M. F. Para. 2005. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. J Infect Dis 191:654-65.

Also disclosed are vectors, pseudoviruses and other constructs that comprise specific polynucleotide sequences and mutations that encode antigens and epitopes described.

Also disclosed are generic and specific sequences, polymorphisms, mutations, antigens and epitopes that may be used for the treatment and/or prevention of viral infection such as HIV infection.

Also disclosed are medicaments and therapeutic formulations such as vaccines that comprise antigens and epitopes of the invention or that comprise polynucleotide sequences or vectors encoding antigens and epitopes of the invention. Vaccines of the invention may be used both to treat an infection once the infection has occurred, so as to prevent or cure a disease, and more commonly, to prevent an infection. Also disclosed are therapeutic methods that comprise delivering a vaccine to a subject wherein the vaccine may comprise one or more antigens or epitopes of the invention, or polynucleotide sequences or vectors encoding antigens and epitopes of the invention. Also described are specific glycoproteins, polypeptides, proteins and epitopes which may be formulated as part of an effective vaccine. Also described are polyclonal and/or monoclonal antibodies that may be used as therapeutic agents for passive immunization. The vaccines of the invention may be protein/polypeptide antigen vaccines, or may be polynucleotide vaccines wherein the polynucleotides express antigenic proteins that provoke a protective immune response.

Also disclosed are therapeutic methods that employ compositions such as drugs and small molecules or antibodies that interact with specific antigens or epitopes or regions of the glycoproteins or polypeptides described, thereby (i) exposing a previously unexposed epitope which epitope can bind specifically with a neutralizing antibody and/or (ii) limiting, inhibiting or preventing fusion of a viral membrane with a cell membrane, thereby inhibiting infection of a call by a virus. Also disclosed are the therapeutic compositions, drugs, small molecules or antibodies used in the above method.

Also described are compositions containing specific sequences and amino acid substitutions, deletions and additions that affect the confirmation of a protein or a polypeptide so as to hide or expose one or more particular epitope. Also described are methods of contacting a virus with such a composition to affect the confirmation of a protein or a polypeptide so as to hide or expose one or more particular epitope so as to expose a previously unexposed epitope which epitope can bind specifically with a neutralizing antibody and/or to limit, inhibit or prevent fusion of a viral membrane with a cell membrane.

Also described are polypeptides containing the epitopes of the invention, nucleic acids encoding the polypeptides, vaccines comprising the polypeptides or nucleic acids, and methods of attenuating or preventing HIV infection via administration of the vaccines.

Also described are nucleic acids encoding the polypeptides of the invention and vectors that comprise nucleic acids encoding the polypeptides of the invention, which vectors may be used for therapeutic and/or vaccination purposes.

Further, the invention isolated polynucleotides encoding the polypeptides of the invention, a polypeptide comprising a) an amino acid sequence selected from any sequence described herein, b) an amino acid sequence having at least 90% sequence identity to an amino acid sequence described herein, c) a biologically active or immunogenic fragment of an amino acid sequence described herein. The invention further provides an isolated polynucleotide comprising a polynucleotide sequence having at least 90% sequence identity to a polynucleotide described, or a polynucleotide sequence complementary to the

foregoing. In one alternative, the polynucleotide comprises at least 60 contiguous nucleotides. The invention also includes any of the polypeptides encoded by such polynucleotides. Additionally, the invention provides an isolated antibody which specifically binds to an amino acid sequence described herein.

The investigators have identified various specific polynucleotide and polypeptide envelope sequences that contain specific polymorphisms such as a substitution of arginine for glutamine at position 655 in gp41 ("Q655R"). The invention includes these sequences and also encompasses other similar and related sequences that display the same specific polymorphism at a location identifiable as being homologous to Q655R in the HIV env gene as disclosed in SEQ ID No. 1.

To say that a first particular sequence of amino acids, or a particular single amino acid residue or polymorphism "corresponds to" a particular (second) sequence, site or position on a known sequence means that the first sequence, residue or polymorphism is located at a position that is readily identifiable by virtue of sequence homology as being equivalent to a known sequence, site or position on a known sequence on the second, known sequence. The same reasoning may be applied to polynucleotides.

To say that a first particular sequence or specific polymorphism is "identifiable as being homologous to" a second particular sequence or polymorphism means that the sequences shows homology or sequence identity with each other so as to be identifiable as being homologues (and quite possibly, paralogs) of the same gene. Such homology is usually evident to one of skill in the art and can be determined by eye. Additionally various algorithms such as BLAST may be used.

In the present case, the region in which the polymorphism is found is highly conserved between variants, and the recognition of sequences or polymorphisms as being located at a site "identifiable as being homologous to" amino acid 655 in SEQ ID No.1 is clear and easily understood. In the present case the invention includes a substitution of Q to another residue such as R at a site identifiable as being homologous to amino acid 655 in SEQ ID No.1

The env polypeptide may be selected from any of the known env sequences, or may be a previously unpublished sequence having a certain degree of sequence similarity to one of the known env sequences.

For example, the env polypeptide of the invention may comprise a sequence with a substitution of arginine for glutamine at position identifiable as homologous to position 655 within in a gp41, wherein the env polypeptide has at least 60% identity (or, in other embodiments, at least 70%, at least 80%, or at least 87% or at least 90% or at least 95% or at least 98% or at least 99% identity) using BLASTP 2.2.21 with default settings (see Altschul et al., (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402) to one of the following sequences: SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5.

Alternatively, the for example, the env polypeptide of the invention may comprise a sequence with a substitution of arginine for glutamine at position identifiable as homologous to position 655 within in a gp41, wherein the env polypeptide has at least 65% identity (or at least 70%, 80%, 87%, 90%, 95%, 98% or at least 99% identity) using BLASTP 2.2.21 with default settings to one of the following se-

quences described in this application as: p1.10848_c2 Resistant, p1.10848_c11 Sensitive, 108051_c6 Sensitive, p1.108051_c5 Resistant, p1.108060_c22 Resistant, or p1.108060_c24 Sensitive.

Any of the above sequences may additionally include signal sequences of variable length or sequences that assist trimer at either the 5' or 3' ends.

Any of the above sequences may be truncated by deletion of sequences encoding the transmembrane domain and cytoplasmic tail of the gp41 region of the gp160 gene.

Any of the above sequences may also be expressed as a fusion protein where nucleotides encoding the signal sequence and 0-12 N-terminal residues of the mature HIV envelope protein are deleted from the HIV envelope gene and replaced by nucleotide sequences encoding the signal sequence from another highly expressed protein to facilitate expression in mammalian cells. Examples of suitable signal sequence include those of herpes simplex virus 1 glycoprotein D or the prepro signal sequence of human tissue plasminogen activator. It is also sometimes desirable to include nucleotide sequences encoding a flag epitope immediately adjacent to the signal peptidase cleavage site at the N-terminus of the mature gp140 protein, or a flag epitope adjacent to the C-terminal sequence of the gp140 protein to facilitate purification. The flag epitope can be any 4-30 amino acid sequence recognized by a monoclonal antibody suitable for immunoaffinity chromatography, or can be a cluster of amino acids such as a poly-histidine (his-tag) sequence that can mediate adherence to a insoluble matrix for affinity purification. In this regard it is important that a simple, non-denaturing process is available to elute the poly-histidine fusion containing fusion protein form the insoluble matrix. In some cases (e.g. herpes simplex virus glycoprotein D) the flag epitope can can be derived from the same protein as the heterologous signal sequence. The flag epitope can be attached to any amino acid within the first 20 amino acids of the gp120 portion of the molecule. An example of this is fusion adjacent to the conserved V at position 41 within the full length gp160 sequence and located at the sequence beginning VPVWKEA. Amino acid residues corresponding to a heterologous flag epitopes can be located either at the amino terminus of the mature protein.

Glycoprotein gp140 may be expressed as a fusion protein lacking the furin cleavage site. In another embodiment, it may be necessary to mutagenize the highly conserved furin cleavage site that occurs at the junction between gp120 and gp41 in order to insure that the gp41 domain is covalently attached to the gp120 domain during purification and possibly during immunization.

Glycoprotein gp140 may include sequences attached at the C-terminus of gp140 to facilitate oligomerization into gp140 trimers. In order to create an antigen that replicates the structure of the HIV envelope protein on the surface of virions, it is often desirable to produce gp140 trimers. To accomplish this goal, one can use one of the several strategies such as the addition of a GCN4 coiled coil domain or the T4 fibrin tag that have been described and successfully used by other investigators to produce stable gp140 trimers. Location where sequences could be attached are within 7 amino acids of the C terminus of gp140 as indicated.

Thus, for example, the invention includes a composition comprising a purified HIV env polypeptide, the polypeptide having a Q655R substitution, and having at least 90% amino acid sequence identity

to one of the following sequences: SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, and SEQ ID No. 5. Such compositions include vaccines.

Additionally, the invention encompasses an isolated antibody which specifically binds to a purified HIV env polypeptide, the polypeptide having a Q655R substitution, and having at least 90% amino acid sequence identity to one of the following sequences: SEQ ID 1, SEQ ID 2, SEQ ID 3, SEQ ID 4, and SEQ ID 5.

Vaccines of the present invention can be used in a prophylactic manner to prevent HIV infection or in a passive therapeutic manner to attenuate existing HIV infection. Vaccines of the present invention may be multivalent, i.e., contain multiple HIV antigens, for example, containing two more HIV-1 envelope glycoproteins, gp160, gp120, and gp41 which present one of more epitopes that bind specifically to broadly neutralizing antibodies. Vaccines of this invention may be administered alone or in combination with other HIV antigens and/or adjuvants, cofactors or carriers. The HIV-1 envelope protein or nucleic acid may be administered in combination with other antigens in a single inoculation "cocktail". Adequacy of the vaccination is determined by assaying antibody titers or the presence of T cells and/or the viral load may be monitored. The polypeptides of this invention may optionally be administered along with other pharmacologic agents used to treat AIDS or ARC or other HIV-related diseases and infections, such as AZT, CD4, antibiotics, immunomodulators such as interferon, anti-inflammatory agents, and anti-tumor agents.

The invention also encompasses constructs containing the sequence of gp160, gp140 or gp41 from neutralization resistant clone 22 from subject 108060 in which a mutation is present, the mutation (Q655R) created by replacement of glutamine with arginine at position 655. The mutation may be introduced by standard in vitro mutagenesis techniques. Note that the basic gp160 sequence (prior to the Q655R mutation) is that from a neutralization resistant, and not the neutralization sensitive clone. The Q665R neutralization resistant sequence appears to be more immunogenic than the Q665R neutralization sensitive sequence and confers a stronger neutralizing and protective antibody response. This is not what would have been predicted.

Possible preferred embodiments include constructs containing the sequences of SEQ ID Nos. 1, 2, 3, 4, and 5 described herein.

SEQ ID No. 1 is the full length gp160 854 residue sequence (from p1.108060_c22) with the Q655R mutation.

SEQ ID No. 2 is a truncated form of the envelope protein lacking the gp41 transmembrane domain and cytoplasmic tail, termed gp140. In this embodiment the gp160 gene is truncated by deletion of sequences encoding the transmembrane domain and cytoplasmic tail of the gp41 region of the gp160 gene. This is accomplished by introduction of a stop codon (e.g. TAA) and adjacent to introduction of a stop codon after any of the amino acids in the following sequence located adjacent to the start of the gp41 transmembrane domain: SWLWYIK.

SEQ ID No. 3 is a fusion protein where the signal sequence of HIV has been deleted and replaced with the signal sequence of another highly expressed protein. The fusion protein is designed to

facilitate expression in mammalian cells, and is termed gp140-FP. This embodiment includes at least 95% of gp120 and the extracellular domain of gp41. It specifically lacks the transmembrane domain and cytoplasmic tail of gp41. The molecule is best expressed as a fusion protein where nucleotides encoding the signal sequence and 0-12 N-terminal residues of the mature HIV envelope protein are deleted from the HIV envelope gene and replaced by nucleotide sequences encoding the signal sequence from another highly expressed protein to facilitate expression in mammalian cells. Examples of suitable signal sequence include those of herpes simplex virus 1 glycoprotein D or the prepro signal sequence of human tissue plasminogen activator. It is also desirable to include nucleotide sequences encoding a flag epitope immediately adjacent to the signal peptidase cleavage site at the N-terminus of the mature gp140 protein, or a flag epitope adjacent to the C-terminal sequence of the the gp140 protein to facilitate purification. The flag epitope can be any 4-30 amino acid sequence recognized by a monoclonal antibody suitable for immunoaffinity chromatography, or can be a cluster of amino acids such as a poly-histidine (his-tag) sequence that can mediate adherence to a insoluble matrix for affinity purification. In this regard it is important that a simple, non-denaturing process is available to elute the poly-histidine fusion containing fusion protein form the insoluble matrix. In some cases (e.g. herpes simplex virus glycoprotein D) the flag epitope can be derived from the same protein as the heterologous signal sequence. The flag epitope can be attached to any amino acid within the first 20 amino acids of the gp120 portion of the molecule. An example of this is fusion adjacent to the conserved V at position 41 within the full length gp160 sequence and located at the sequence beginning VPVWKEA. Amino acid residues corresponding to a heterologous flag epitopes can be located either at the amino terminus of the mature protein.

SEQ ID No. 4 is a gp140 from 108060_c22 Q655R containing gp120 and the extracellular domain of gp41 with Q655R mutation expressed as a fusion protein and lacking the furin cleavage site.

SEQ ID No. 5 is a gp140 from 108060_c22 Q655R containing gp120 and the extracellular domain of gp41 with Q655R mutation expressed as a fusion protein and containing sequences to facilitate or stabilize trimer formation.

Experimental procedures, materials, methods and results

Described is a new method to identify the epitopes recognized by broadly neutralizing antibodies by taking advantage of the naturally occurring amino acid sequence variation (intra-patient variation) that evolves within every HIV-infected individual. This method also allows one to define molecular determinants of sensitivity and resistance to antibody mediated neutralization, and allows for the design of a new class of antiviral drugs. We have used this method to identify a mutation in the HIV fusion protein, gp41, that markedly affects sensitivity and resistance of primary HIV-1 isolates to neutralization by HIV+ sera. The new approach that we describe provides a powerful and convenient method to identify epitopes recognized by bNAbs in HIV+ sera and will enable the development of new immunogens that target these sites.

Studies of the early events in infection have shown that transmission of HIV-1 involves a genetic bottleneck where, out of the myriad of genetic variants in each HIV infected donor, only a single homo-

geneous variant of HIV-1 successfully replicates in the recipient. This variant replicates to very high titers for the first days and weeks after HIV-1 infection and eventually starts to mutate in response to error-prone reverse transcription to generate a swarm of closely related variants. The swarm further diversifies in response to selective pressures imposed by both cellular and humoral antiviral immune responses. Virus variation, driven by the relentless error-prone reverse transcription and selection by immune responses, occurs throughout the course of HIV infection and is perhaps the greatest challenge in the development of vaccine and therapeutic products. In the present studies we have taken advantage of mutations occurring early in the course of HIV-1 infections to identify specific amino acid substitutions in the HIV-1 envelope glycoproteins gp120 and gp41 to address the problem of susceptibility and resistance to neutralization by bNAbs. For this purpose we have made use of a large collection of clinical specimens from new HIV infections collected in the course of a clinical trial (VAX004) of a candidate HIV-1 vaccine, AIDSVAX. See: Flynn NM, Forthal DN, Harro CD, Judson FN, Mayer KH, Para MF; "Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection." The Journal of infectious diseases 2005;191:654-65.

This collection of specimens is unique in that they were obtained within 6 months of infection (mean 109 +/- 58 days) from multiple sites throughout North America. We reasoned that by studying viruses from early infections, sequence variation would be limited compared to sequences collected at later times after infection, and that subsequent mutational analysis would be simpler than that which would be required if we used specimens collected from later time points.

Results and analysis

In initial experiments, we PCR amplified full length envelope genes from cryopreserved plasma using nested primers of the type described by Li et al. and cloned the swarm of PCR products into a plasmid expression vector. The vector was specifically designed to permit the construction of pseudoviruses for use in a well established and validated virus neutralization assay (Monogram Biosciences, Inc – see Schweighardt et al., 2007, J Acquir Immune Defic Syndr 46:1-11 and Whitcomb et al.,2007, Antimicrob Agents Chemother 51:566-75). However, instead of pooling all of the clones together and carrying out neutralization assays with a library of cloned genes from each infected individual for neutralization studies as had been done previously, we selected 24-48 clones from each individual and screened each for infectivity and chemokine receptor usage. We then selected approximately 10 CCR5-dependent pseudotype viruses with high infectivity for virus neutralization assays. Overall, viruses were prepared from each of 28 individuals and screened for sensitivity and resistance to neutralization (Table 1). In some cases (e.g. subject 108045) all 10 viruses were resistant to neutralization by a panel of four HIV+ sera known to contain broadly neutralizing antibodies (Table 2A). In other cases (e.g. subject 108073) most of the clones were sensitive to neutralization (Table 2B). However in approximately 85% of the specimens (e.g. subjects 108048 and 108051) we found a mixture of neutralization sensitive and resistant clones that showed differences in sensitivity or resistance to neutralization (Tables 3A and 3B).

After examining the results, 7 clones showing the greatest disparity in sensitivity and resistance to neutralization within the same individual were selected for oligonucleotide sequencing and further analysis. As we hypothesized, sequence variation in several of the sets of neutralization sensitive and resistant clones was limited and allowed for the possibility of *in vitro* mutagenesis to localize the amino acids responsible for conferring sensitivity and resistance to neutralization by HIV+ sera. To explore this possibility, we selected the viruses from subject 108060 for further analysis. It can be seen (Table 4A) that 3 of the 10 clones analyzed (clones 2, 18, and 24) were relatively sensitive to neutralization by all 4 HIV+ sera; and of the remaining 7 clones, most were resistant to neutralization by HIV+ sera Z1679, Z1684, and N16) and somewhat sensitive to HIV+ sera from Z23. When the gp160 sequences of the neutralization resistant variant (clone 22) and a neutralization sensitive variant (clone 24) were compared (Figure 1), it was found that they differed at only seven positions. There were 2 amino acid differences in gp120, two amino acid differences in the gp41 ecto-domain, and 3 differences in the cytoplasmic tail of gp41.

To determine which amino acids were responsible for the difference in sensitivity to neutralization between clone 22 and clone 24, a series of mutants were introduced onto the backbone of the neutralization resistant clone 22 (Figure 1B). We found (Table 5) that the replacement of asparagine for serine at position 323 (N323S) in the V3 domain of gp120 had no effect on sensitivity to neutralization. Similarly, the substitution of asparagine for glycine at position 530 in the C5 domain (N530G) of gp120 had no effect. Replacement of lysine at position 634 of the second heptad repeat domain (C34 helix) of gp41 with glutamic acid (K634E) also failed to show a significant difference in neutralization sensitivity. However the replacement of glutamine for arginine at position 655 (Q655R) resulted in a remarkable increase in neutralization sensitivity by all 4 of the HIV+ sera. The difference in neutralization sensitivity was seen with all four HIV+ sera tested, and titration data from the experiments carried out with HIV+ sera Z23 are presented in Figure 2. This result demonstrated that amino acid substitutions at some locations in the 34 helix, but not others, can cause a significant change in sensitivity and/or resistance to neutralization by antibodies in HIV+ sera.

To understand the impact of this mutation on the structure and function of the 108060 envelope glycoprotein, we examined the linear and 3 dimensional structures of gp41. Examination of the linear structure (Figure 3) revealed that position 655 was located in the conserved second heptad repeat of gp41 in a region also known as the C34 helix. This part of the molecule is known to play an integral role in virus fusion and indeed forms an essential component of the 6 coil bundle structure that is thought to mediate fusion of the viral membrane with T cell membrane in the course of HIV infection. Position 655 is also located in the T-20 peptide (Figure 2) that provides the basis for the antiviral drug, Fuzeon, that inhibits HIV infectivity by inhibiting virus fusion and entry. Finally the location of this mutation is only eight amino acids from the Membrane Proximal External Region (MPER) of gp41 that is known to contain two distinct epitopes recognized by the broadly neutralizing monoclonal antibodies 2F5 and 4E10. Taken together these results suggest that this mutation occurs in a region that is essential for virus fusion

and is in close proximity to a region known to contain other epitopes recognized by other broadly neutralizing antibodies.

The availability of a 3-D structure of the activated 6 coil structure of the gp41 fusion domain allowed us to evaluate the impact of the substitution of arginine for glutamine at position 655 on the structure and function of gp41. Using the structure of Chan and Kim we were able to determine that glutamine at position 655 is located at an internal position facing the interface with the adjacent between two adjacent gp41 monomers, two turns from the terminus of the C34 helix (Figures 4A and B). The glutamine 655 side chain contributes two hydrogen bonds that support both intra-molecular and intermolecular interactions. One hydrogen bond is formed by association with glutamine at position 553 of the N-terminal heptad repeat 1 (N36 helix) and the second hydrogen bond involves an inter-molecular interaction with the backbone of valine at position 551 of an adjacent C34 monomer in the 6 coil bundle (Figures 5A and B). When viewed in the context of the 6 coiled bundle, the hydrogen bonds contributed by glutamine 655, glutamine 551, and valine 551 form an inter-molecular ring structure (Figures 6A and B) that appears to stabilize the 6 coil oligomeric structure. Molecular modeling suggested that replacement of glutamine with arginine impacts the structure of the 6 coil bundle in two ways. First the longer arginine side chain disrupts the close packing of the C34 helix with the N36 helix on the adjacent monomers and precludes the possibility of a hydrogen bond between the arginine side chain with valine 551 (Figure 7). Although replacement of arginine for glutamine at 655 does permit a intra-molecular hydrogen bond between arginine with glutamine 553, this mutation precludes the possibility of the intermolecular ring of hydrogen bonds that appears to stabilize the quaternary interactions involved in the 6 coil assembly.

Monoclonal Antibody Sensitivity and Envelope Transfer - Sensitivity to neutralization by MAbs and fusion inhibitors. While the structural analysis provided insight into the functional consequences of mutations at position 655, two alternate hypotheses can account for a mechanism by which this mutation increases sensitivity to antibody-mediated neutralization. One possibility is that this mutation is located at or near an antibody binding site and that the Q655R mutation restores an epitope recognized by a population of neutralizing antibodies present in all four HIV-positive sera. Alternatively, it is possible that this mutation results in a significant conformational change that is transmitted to other parts of gp41 such as the adjacent MPER or the gp120/gp41 trimer complex in such a way as to increase exposure or access to antibodies at other locations on the molecule.

To explore these possibilities, antibody neutralization studies were carried out with a panel of neutralizing MAbs to epitopes in gp120 and gp41 as well as fusion inhibitors targeting either the gp120 or the gp41 portion of the HIV envelope glycoprotein. In these studies, we examined two broadly gp41-neutralizing MAbs, 2F5 and 4E10 (Muster, Steindl et al. 1993; Stiegler, Kunert et al. 2001; Zwick, Jensen et al. 2005); the broadly neutralizing b12 antibody able to block CD4 binding to gp120 (Burton and Barbas 1994; Burton, Pyati et al. 1994); and 2G12, an antibody that binds to a carbohydrate epitope in gp120 (Trkola, Purtscher et al. 1996). In addition, we tested the antiviral entry inhibitor CD4-IgG (Capon, Chamow et al. 1989), which binds to sequences in gp120 and is able to neutralize lab-adapted CXCR4-

dependent clinical isolates at low concentrations (0.01 to 0.1 μg/ml), and primary clinical isolates of HIV (Daar, Li et al. 1990) at high concentrations (10 to 100 μg/ml). We also examined the sensitivity of envelope mutants to enfuvirtide, a peptide virus entry inhibitor that consists of a gp41-derived peptide that includes sequences from the C34 helix containing Q655. The results of these studies are shown in Table 6, in which the sensitivities of clone 022 and clone 024 from subject 108060 to neutralizing MAbs were compared. It can be seen that the neutralization-resistant clone 022 is moderately sensitive to the 2F5 and 4E10 MAbs specific for the MPER of gp41 but resistant to neutralization by the b12 and 2G12 MAbs reactive with gp120. This virus was also sensitive to enfuvirtide and resistant to CD4-IgG. The high CD4-IgG concentration required for the neutralization of this virus is consistent with the concentration required to neutralize other primary, CCR5-dependent viruses (Daar, Li et al. 1990). We next examined the neutralization-sensitive clone 024 that differs from the neutralization-resistant clone 022 at only seven amino acid positions. We found that this clone was 15- to 20-fold more sensitive to the MPER-specific MAbs (2F5 and 4E10) than the 022 clone. Similarly, the neutralization-sensitive clone 024 was more than 20-fold more sensitive to CD4-IgG and 3.5-fold more sensitive to neutralization by enfuvirtide (Table 6). Thus, clone 024 exhibited significantly increased sensitivity to neutralization by MAbs and antiviral entry inhibitors as well as antibodies in HIV-positive sera. We then mutated the neutralization-sensitive clone 024 so as to replace R with Q at position 655. We found that the resulting mutant (108060_024 R655Q) became resistant to neutralization and showed a pattern of neutralization sensitivity closely resembling that of the neutralization-resistant clone 022. Conversely, when we mutated the neutralization-resistant clone 022 to replace Q at position 655 with R, the resulting mutant (108060_022 Q655R), which differed from the parental neutralization-resistant clone by a single amino acid, exhibited an extraordinary increase in neutralization sensitivity (Table 5). We observed a >125-fold increase in sensitivity to CD4-IgG compared to that of the wild-type clone 022 and a 30- to 35-fold increase in sensitivity to the MPER-reactive antibodies 2F5 and 4E10. We also noted a 17-fold increase in sensitivity to the antiviral drug enfuvirtide. These results highlight the importance of glutamine at position 655 and suggest that epistatic mutations at other sites in clone 024 moderate sensitivity to neutralization. The results of these studies are remarkable in that they show that a single amino acid substitution in gp41 not only confers sensitivity to neutralization by MAbs and entry inhibitors directed to gp41 but also increases sensitivity to CD4-IgG, a molecule that binds to gp120, an entirely different protein. Thus, the Q655R mutation appears to cause a conformational change in gp41 that affects not only the binding of antibodies and entry inhibitors (2F5, 4E10, and enfuvirtide) that bind close to the site of the mutation but also the binding of another inhibitor (CD4-IgG) that binds to a site on gp120 located a considerable distance from the mutation.

Transfer of the Q655R mutation to related and unrelated viruses. In order to determine whether the Q655R mutation could confer neutralization sensitivity and resistance to other viruses, this mutation was introduced into two unrelated viruses highly resistant to neutralization (from subjects 108069 and 108051) that normally possessed a Q at a position corresponding to 655 of the virus from subject 108060 (the 108060 virus). The results of these experiments are shown in Table 6. Interestingly,

we found that the replacement of Q655 with R had little or no effect on neutralization by any of the HIV-positive sera. However, these mutations significantly increased the sensitivity to neutralization by the 2F5 and 4E10 MAbs (25- to 35-fold). These mutations also increased the sensitivities to neutralization by the entry inhibitors enfuvirtide and CD4-IgG. Thus, the mutation of Q to R at a position corresponding to 655 in the 108069 virus increased the sensitivity to enfuvirtide by more than 17-fold and increased the sensitivity to CD4-IgG by more than 20-fold. The 108069 mutant with the Q655R mutation seemed to be somewhat more sensitive to enfuvirtide and possibly CD4-IgG than the corresponding mutant of the 108051 virus. Together, these results demonstrate that the mutation of Q to R at positions corresponding to 655 of the 108060 virus confers sensitivity to neutralizing MAbs to the MPER and antiviral compounds targeted to the C34 helix and the MPER of gp41. However, it was interesting that these mutations failed to increase the sensitivity to bNAbs in HIV-positive sera. We do not know whether neutralizing activity in HIV-positive sera is attributable to antibodies binding to the C34 region, the MPER, or other parts of the molecule. It has been recently reported (Sather, Armann et al. 2009; Shen, Parks et al. 2009) that antibodies with specificities similar to 2F5 and 4E10 are rare in HIV-positive sera, which might account for the lack of effect. Alternatively, the failure of the Q655R mutation to increase neutralization sensitivity by HIV-positive sera might be attributable to polymorphisms outside of the MPER and the C34 region that preclude the binding of otherwise bNAbs. This may well be the case since the 108069 and 108051 viruses were selected because of their resistance to neutralization by the HIV-positive sera selected for use in these studies.

Expression of envelope proteins derived from the 108060 clone 22 with the Q655R mutation. In certain embodiments it is desirable to express the protein as a fusion protein that includes a non-HIV signal sequence and a flag epitope for purification. In certain embodiments it is considered desirable to delete the furin cleavage site that is responsible for maturational cleavage of the gp160 precursor into the mature gp120 and gp41 proteins.

Figure 5 shows three pairs of sequences from neutralization sensitive and neutralization resistant viruses. Swam analysis was used to map the mutations conferring sensitivity and resistance to broadly neutralizing antibodies in HIV+ sera. Included are sequences from subject 108060 as well as sequences from subject 108051 and 108048. The preferred sequences for vaccines can be (1) the neutralization sensitive variant envelope proteins, or (2) the envelope proteins of the resistant viruses where a single amino acid substitution (e.g., Q655R) conferring neutralization sensitivity has been created by in vitro mutagenesis, or (3) any sequence derived from of such sequences. This second type of envelope protein construct appears to provide very strong immunogenicity. Insertion of the single amino acid substitution in a neutralization resistant variant envelope protein often results in a virus that is much more sensitive to neutralization than the original neutralization sensitive variant where there are multiple amino acid differences between the neutralization sensitive and resistant variants. An example of this can be seen in Table 5 where clone 22 with the Q655R mutation is much more sensitive to neutralization than the neutralization sensitive clone 24 variant.

Figure 5 shows the sequences from subjects 108060, 108051, 108048 corresponding to neutralization sensitive and neutralization resistant variants.

It is interesting to note that the resistant sequence from 108069, when altered to include the Q655R substitution, and analyzed using protein-blast, identified the following top three most similar sequence alignments:

gb|ABG67916.1| optimized HIV-1 subtype B consensus env gp [synthetic construct] Length=850
Score = 1482 bits (3836), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 736/863 (85%), Positives = 788/863 (91%), Gaps = 22/863 (2%)

gb|AAB64170.1| env polyprotein [Human immunodeficiency virus 1]Length=854
Score = 1461 bits (3783), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 723/864 (83%), Positives = 770/864 (89%), Gaps = 20/864 (2%)

gb|ACD41904.1| envelope glycoprotein [HIV 1] Length=855
Score = 1459 bits (3777), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 715/862 (82%), Positives = 775/862 (89%), Gaps = 15/862 (1%)

Clearly none of these have greater than 85% amino acid identity.

Discussion

In the present studies we describe a novel method useful for mapping epitopes recognized by bNAbs in HIV+ sera as well as mapping mutations that confer sensitivity and resistance to virus neutralizing antibodies. The method (Figure 8) relies on naturally occurring mutations in the swarm of closely related viruses that evolve during the course of HIV infection. Some of these mutations occur at epitopes or contact residues recognized by broadly neutralizing antibodies, and some of these appear to effect a conformational change that alters the binding of bNAbs at sites that are distinct from the site of mutation. In previous studies we noted a difference in the binding of a monoclonal antibody between two clones of the HIV-1 gp120 envelope protein obtained from a high risk volunteer that participated in a phase I trial of a candidate HIV vaccine. However, at the time the study was carried out it was not possible to study the effect of this mutation in a virus neutralization assay because technology was not yet available to re-introduce the mutant envelope protein back into the virus with assurance that the sequences were sTable 5nd wouldn't change as a consequence of errors in reverse transcription or selection induced by in vitro culture. However, the advent of pseudotype virus neutralization assays utilizing HIV envelope genes incorporated into a stable DNA plasmid vector as opposed to retroviruses with RNA genomes provided the opportunity to take advantage of naturally occurring mutations in HIV envelope genes without the fear of reversion or further mutations. Moreover high throughput sequencing strategies have since been

developed that have allowed us to quickly and conveniently sequence multiple variants from the same individual.

Previous attempts to characterized bNAbs in HIV patient sera have relied primarily on immunoadsorbtion studies or on the production of bNAbs from human or mouse B-cells. Immunoadsorbtion studies of HIV+ sera with recombinant gp120 has shown that some bNAbs appear to recognize conformation dependent epitopes, some of which are able to block the binding of gp120 to its cellular receptor, CD4. Studies with monoclonal antibodies prepared from HIV+ individuals have shown that broadly neutralizing antibodies recognize carbohydrate residues in gp120 (e.g. 2G12) or epitopes in the membrane proximal domain of gp41 (e.g. 2F5 or 4E10). The best characterized bNAb, 1B12, was isolated from mice immunized with gp120 and optimized for neutralizing activity by genetic engineering. This antibody binds to a complex conformational epitope and is able to block CD4 binding. However it is not clear whether any of these monoclonal antibodies are representative of antibodies found in HIV+ sera, and attempts investigate this possibility remain inconclusive.

In this study we validate the method of using intra-patient variation in the HIV envelope protein in the context of a pseudotype virus neutralization assay to identify mutations that sensitivity and resistance of viruses to neutralization by broadly neutralizing antibodies. Using this method we expect to be able to identify specific epitopes recognized by bNAbs as well as amino acid mutations that alter the sensitivity and resistance of viruses to neutralization by antibodies. <u>In the present studies we have identified a single amino acid substitution (Q655R) in the C34 helix of gp41 that appears to play an important and previously unrecognized role in maintaining the integrity of the 6 coil bundle in the viral membrane fusion apparatus of HIV-1</u>. X-ray crystallography studies demonstrate that this residue contributes two hydrogen bonds: one mediating an intra-molecular interaction with the N36 helix on the same monomer and the other mediating an inter-molecular interaction with the N36 helix on an adjacent monomer. This mutation appears to affect sensitivity to neutralization by bNAbs by altering 4 distinct interactions. First the Q655R mutation breaks a hydrogen bond that mediates an intra-molecular interaction (Q at position 655 of the C34 helix with valine at position 551 of the N36 helix). Second, the Q655R mutation disrupts an inter-molecular interaction (Q at position 655 with valine at position 553 in the N36 helix) with an adjacent monomer. Third, the longer arginine side chain in the Q655R mutation appears to alter the inter-helix packing interface between adjacent monomers by sterically hindering the close association between the C34 helix and the N36 helix on adjacent monomers. Finally, the Q655R mutation appears to prevent the formation of a ring structure involving 12 hydrogen bonds in the 6 coil bundle that occurs upon formation of the gp41 fusion complex. Although it is possible that R655 is able to form an intra-molecular hydrogen bond with position 551, it does not appear likely that this mutation allows for replacement of the inter-molecular hydrogen bond with a residue on the adjacent N36 helix essential for the formation of an inter-molecular hydrogen bonded ring structure.

The location and structural impact of the 655 mutation described in this paper appears to be fundamentally different from another recently described gp41 variant that that affects sensitivity and resistance to neutralization by bNAbs. First, the neutralization sensitive phenotype in this study requires two

mutations: an isoleucine to valine substitution at position 675 (I675V) in the MPER and a threonine for alanine subsititution at position 569 (T569A) in the first heptad repeat domain (N36 helix) of gp41. The MPER is a well known target of virus neutralizing monoclonal antibodies and is structurally distinct from the C34 helix. The T569A mutation does appear to occur at the interface of the intra-molecular interaction between the N36 and C34 helices. In this case, the substitution of the longer threonine for alanine at position 569 appears to preclude a classical "knob in hole" interaction between adjacent helices and does not appear to affect inter-molecular interactions.

Since the 6 helix coil structure appears to be a conserved structural element fundamental to many biologic processes involving membrane fusion, it may well be the case that hydrogen bond ring structures of the type we have identified for HIV-1 are present and essential for maintaining the functional integrity of coiled-coil bundles required for membrane fusion in other viruses such as influenza, Moloney leukemia virus, Ebola virus, and Visna virus.

If hydrogen bonded ring structures of the type we have identified for HIV are found to be present in other coiled-coil bundles involved in membrane fusion, they may provide a novel rationale for the development of vaccines for the prevention and treatment of other virus infections. Many viruses are thought to use homologous 6 coil bundles to mediate membrane fusion and virus entry, see: Flint SJ, Enquist LW, Krug RM, Racaniello VR, Skalka AM. Principles of Virology. 2nd ed.: ASM Press; 2004. We would expect that viruses with similar mutations that affect hydrogen bonded ring structures that stabilize 6 coil bundles may alter the structure of the virus in such a way as to expose important neutralizing sites and facilitate recognition by the immune system. We suggest that HIV envelope glycoproteins with mutations in gp41 that destabilize the 6 coil bundle structure such as that seen in clone 24 from subject 108060 may prove to be superior vaccine immunogens by providing better exposure of epitopes to B-cell receptors or T-cells required for the formation of broadly neutralizing antibody responses.

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TABLES

TABLE 1. Summary of Neutralization Sensitivity/Resistance Results from Clonal Analysis of Pseudoviruses using HIV Envelope Glycoproteins from 28 Individuals

Neutralization Sensitivity/Resistance Profiles of VAX004 Viruses (n=28)		
Profile	No.	Percentage
Sensitive	1	3.6
Resistant	3	10.7
Mixed	24	85.7

TABLE 2. Individuals with HIV-1 Clones Mostly Sensitive or Resistant to Neutralization by HIV+ Sera

2A. Patient 108045 Clone	Sera / Neutralization Titers				Chemokine Receptor Usage	
	Z1679	Z1684	N16	Z23	R5	X4
001	<40	<40	<40	53	2	0
008	<40	<40	<40	57	2	0
009	<40	<40	<40	71	2	0
010	46	50	58	86	1	0
012	<40	<40	<40	53	2	0
013	<40	<40	<40	61	2	0
015	<40	<40	<40	48	2	0
018	<40	<40	<40	49	2	0
020	58	<40	<40	78	2	0
021	<40	<40	<40	43	2	0

2B. Patient 108073 Clone	Sera / Neutralization Titers				Chemokine Receptor Usage	
	Z1679	Z1684	N16	Z23	R5	X4
001	**336**	**152**	**112**	**358**	2	0
005	**390**	**233**	**114**	**330**	1	0
006	**106**	64	<40	107	2	0
010	**308**	**183**	**131**	**253**	1	0
012	**392**	**245**	**160**	**555**	1	0
014	**373**	**185**	**148**	**265**	1	0
015	**378**	**183**	**104**	**390**	1	0
018	**386**	**200**	**151**	**325**	1	0
022	**259**	**167**	91	**229**	2	0
024	**989**	**595**	**402**	**1219**	1	0

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TABLE 3. Individuals with Both Neutralization Sensitive and Resistant Clones

3A. Patient 108048					Chemokine Receptor Usage	
Clone	Sera / Neutralization Titers					
	Z1679	Z1684	N16	Z23	R5	X4
002	53	<40	83	**143**	2	0
011	**804**	**524**	**564**	**1911**	3	0
012	**376**	**280**	**310**	**719**	3	0
013	**744**	**315**	**308**	**678**	2	0
016	120	78	**170**	**326**	2	0
018	**305**	**186**	**268**	**692**	3	0
024	**475**	**290**	**559**	**1684**	3	0

3B. Patient 108051					Chemokine Receptor Usage	
Clone	Sera / Neutralization Titers					
	Z1679	Z1684	N16	Z23	R5	X4
005	<40	<40	<40	<40	2	0
006	**824**	**490**	**354**	**1114**	1	0
009	<40	<40	<40	<40	2	0
011	<40	<40	<40	<40	2	0
013	<40	<40	<40	<40	2	0
015	**172**	**87**	**164**	**96**	1	0
016	<40	<40	<40	<40	2	0
018	56	<40	73	42	1	0
021	<40	<40	<40	<40	2	0
022	72	43	50	45	1	0

TABLE 4. Neutralization Sensitivity/Resistance of Wild-Type and Mutated Clone from Subject 108060

4A. 108060 Clonal Analysis					Chemokine
Clones	Sera / Neutralization Titers				Receptor Usage
	Z1679	Z1684	N16	Z23	R5/X4
R:022	53	58	51	**117**	R5
S:024	**804**	**609**	**612**	**1667**	R5
002	**303**	**160**	**195**	**379**	R5
003	69	57	67	**151**	R5
011	136	130	177	**222**	R5
012	62	57	70	**241**	R5
013	53	50	58	**158**	R5
018	**428**	**243**	**388**	**1378**	R5
019	44	<40	40	**145**	R5
021	47	47	70	**157**	R5

TABLE 5

TABLE A. Mutagenesis to Investigate the role of intramolecular and intermolecular hydrogen bonds at position 655 [a]

Clone	Mutation	Sera / neutralization titer for indicated HIV-positive sera			
		Z1679	Z1684	N16	Z23
022	wtR	**40**	<20	**36**	**281**
024	wtS	**1099**	**1193**	**545**	**4167**
022	Q655R	**14276**	**2876**	**2610**	**8422**
022	Q655K	**5486**	**8590**	**4276**	**19476**
022	Q655E	**564**	**132**	**366**	**2424**
022	Q655S	**1565**	**472**	**674**	**2650**
022	Q655N	**148**	**24**	**57**	**820**
024	R655Q	**50**	<20	**39**	**372**

[a] The neutralizing antibody titer (IC50) is defined as the reciprocal of the plasma dilution that produces a 50% inhibition in target cell infection. Values in bold represent neutralization titers that are significantly higher than the background value (see Materials and Methods). All clones tested were CCR5 tropic. Clones indicates gp160 envelope genes. wtR and wtS indicate wild type neutralization-resistant and -sensitive clones, respectively.

TABLE 6

TABLE B. Sensitivity to neutralizing monoclonal antibodies and entry inhibitors in 108060 clones and unrelated viruses (a)

Clone	Mutation	IC50 (μg/ml) of indicated MAb or fusion inhibitor					
		2F5	4E10	b12	2G12	Enfuvirtide	CD4-IgG
108060_022	wtR	**3.250**	**5.201**	>20	>20	**0.068**	>20
108060_022	Q655R	**0.093**	**0.156**	>20	>20	**0.004**	**0.161**
108060_024	wtS	**0.151**	**0.333**	>20	>20	**0.019**	**0.798**
108060_024	R655Q	**3.434**	**6.546**	>20	>20	**0.130**	>20
108069_005	wtR	**1.129**	**3.556**	>20	>20	**0.071**	>20
108069_011	wtS	**0.043**	**0.040**	>20	>20	**0.145**	>20
108069_005	Q655R[b]	**0.052**	**0.044**	>20	>20	**0.011**	**1.080**
108051_005	wtR	>20	>20	>20	>20	**0.088**	>20
108051_006	wtS	**1.176**	**1.369**	>20	>20	**0.008**	**0.231**
108051_005	Q655R[b]	**0.343**	**1.314**	>20	>20	**0.036**	**5.209**

[a] The neutralizing antibody titer (IC50) is defined as the concentration (μg/ml) of an MAb or entry inhibitor that produces a 50% inhibition in target cell infection.

Values in bold represent neutralization titers that are significantly above the background (see Materials and Methods). All clones tested were CCR5 tropic. Clones indicate gp160 envelope proteins. wtR and wtS indicate wild-type neutralization-resistant and -sensitive clones, respectively.

[b] Numbering with reference to subject 108060 protein.

Claims

1. A method of analyzing intra-patient HIV virus variation to identify specific amino acid residues of the HIV envelope glycoproteins that affect sensitivity or resistance to broadly neutralizing antibodies, the method comprising the steps of:

i) providing a plurality of individual subjects who are seropositive for HIV antibodies and taking a biological sample from each subject, wherein the sample contains a multiplicity of HIV viruses with closely related genomes, wherein all subjects had been infected with HIV no more than one year before, and no less than one month before sample collection,

ii) amplifying the env genes of the multiplicity of viruses to produce a library of different env genes,

iii) cloning the amplified env genes into a plasmid shuttle vector that allows the plasmid to replicate in both bacteria and mammalian cells,

iv) transforming bacterial cells with the shuttle vector and plating out the transformed bacterial cells onto a selective medium so that bacteria containing the shuttle vector plasmid containing the cloned envelope gene are selectable,

v) selecting individual colonies at random and preparing plasmid DNA from each colony selected and analyzing the plasmid DNA by restriction digestion so as to identify plasmids containing the full length HIV envelope gene, which plasmids are used to produce pseudoviruses,

vi) co-transfecting mammalian cells with the env-containing vector and simultaneously with a plasmid containing a defective HIV provirus plasmid where the coding sequence of the env gene has been replaced with the coding sequence of a marker gene, and culturing the co-transfected mammalian cells in a culture medium, to produce pseudovirions containing the amplified env genes, which pseudovirions are released into the cell culture medium,

vii) harvesting the supernatant from the cell culture medium, wherein the supernatant contains pseudoviruses from the transfected cells, and wherein each supernatant contains a stock of pseudovirus resulting from a single purified expression plasmid,

viii) testing the pseudovirion from the selected colonies to determine infectivity by culturing the pseudovirions with cells capable of being infected by HIV, wherein infectivity is measured by the degree of expression of the marker gene,

ix) selecting pseudovirions that exhibit high infectivity, and testing the selected pseudovirions for sensitivity or resistance to neutralization by one or more broadly neutralizing antibodies,

x) selecting pairs of plasmids from the same individual wherein each pair contains at least one neutralization resistant and at least one neutralization sensitive pseudovirus,

xi) sequencing the envelope genes identified from sensitive and resistant pseudovirus pairs,

xii) comparing the nucleotide sequences of the envelope genes of the neutralization sensitive and resistant pairs thereby identifying specific amino acid differences between the pairs and identifying polymorphisms that may affect sensitivity or resistance to neutralization by broadly neutralizing antibodies,

xiii) at each amino acid residue that differs between the neutralization sensitive and neutralization resistant envelope genes, site-by-site replacement of amino acids from the is performed, substituting one amino acid at a time from neutralization sensitive sequence into the neutralization resistant sequence,

xiv) each new construct is used to create a pseudotype virus which is tested for neutralization sensitivity so as to identify specific amino acid residues of the HIV envelope glycoproteins that affect sensitivity or resistance to broadly neutralizing antibodies.

2. The method of claim 1 wherein all subjects had been infected with HIV 109 days +/- 58 days before specimen collection.

3. A vaccine composition comprising an HIV envelope glycoprotein wherein a glutamine residue at a site identifiable as being homologous to position 655 of SEQ ID No.1 is replaced by a substitute amino acid such that the amino acid substitution disrupts an inter-molecular hydrogen-bonded ring structure between the N36 and C34 helices of the gp41 trimer.

4. The vaccine composition of claim 3 wherein possession of the HIV envelope glycoprotein confers greater neutralization sensitivity upon an HIV virus when it is exposed to 2F5 or 4E10 monoclonal antibodies, Enfuvirtide or CD4-IgG, than would be provided by another HIV envelope glycoprotein identical in all respects except for the substitution of the glutamine residue.

5. The vaccine composition of claims 3 or 4 wherein the substitute amino acid is arginine.

6. The vaccine composition of claims 3 or 4 wherein the substitute amino acid is Lysine, Serine or Glutamic acid.

7. The vaccine composition of claims 3, 4, 5 or 6 wherein the HIV envelope glycoprotein has at least 60% sequence identity to SEQ ID No.1.

8. The composition of claims 3, 4, 5 or 6 wherein the HIV envelope glycoprotein comprises a fusion protein that includes a non-HIV signal sequence and a flag epitope.

9. The vaccine composition of claims 3, 4, 5 or 6 wherein the HIV envelope glycoprotein has had a furin cleavage site deleted.

10. The vaccine composition of claims 3 or 4 wherein the HIV envelope glycoprotein comprises a full length gp160 wherein a glutamine residue at a site identifiable as being homologous to position 655 of SEQ ID No.1 is replaced by arginine.

11. The vaccine composition of claims 3, 4, 5 or 6 wherein the polypeptide comprises a truncated form of the envelope protein lacking the gp41 transmembrane domain and cytoplasmic tail.

12. A polynucleotide encoding an HIV envelope glycoprotein wherein a glutamine residue at a site identifiable as being homologous to position 655 of SEQ ID No.1 is replaced by a substitute amino acid such that the amino acid substitution disrupts an inter-molecular ring structure between the N36 and C34 helices of the gp41 trimer.

13. The polynucleotide of claim 14 formulated in an vector as a DNA vaccine.

14. A method for inhibiting the fusion of an HIV virus to a host cell, the method comprising exposing the HIV virus to a compound that disrupts the hydrogen-bonded ring structure between the N36 and C34 helices of gp41.

15. A method for increasing the immunogenicity of HIV envelope proteins the method comprising exposing the HIV virus to a compound that disrupts the hydrogen bonded ring structure between the N36 and C34 helices of gp41.

16. The method of claims 14 or 15 wherein the compound is a small molecule.

17. The method of claims 14 or 15 wherein the compound is an antibody.

FIGURES 1A and 1B. Location of Mutations in Neutralization Sensitive and Resistant HIV-1 Clones from Subject 108060.

FIGURE 1A: Amino Acid Differences between Neutralization Sensitive and Resistant Clones

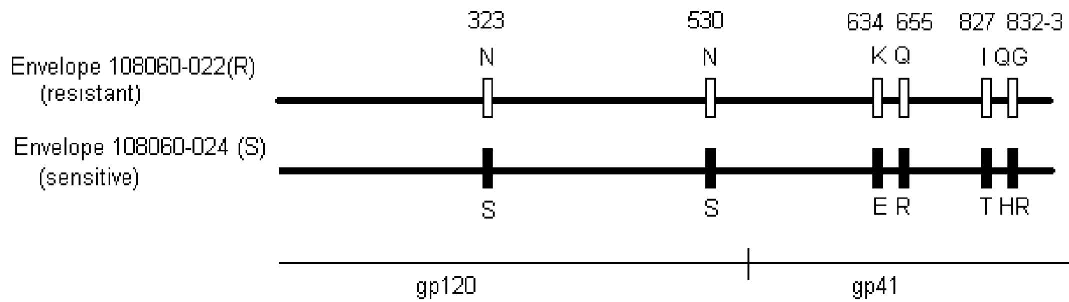


FIGURE 1B: Amino Acid Sequences of Clones Created by *in vitro* Mutagenesis to Map Determinants of Sensitivity and Resistance to Neutralization

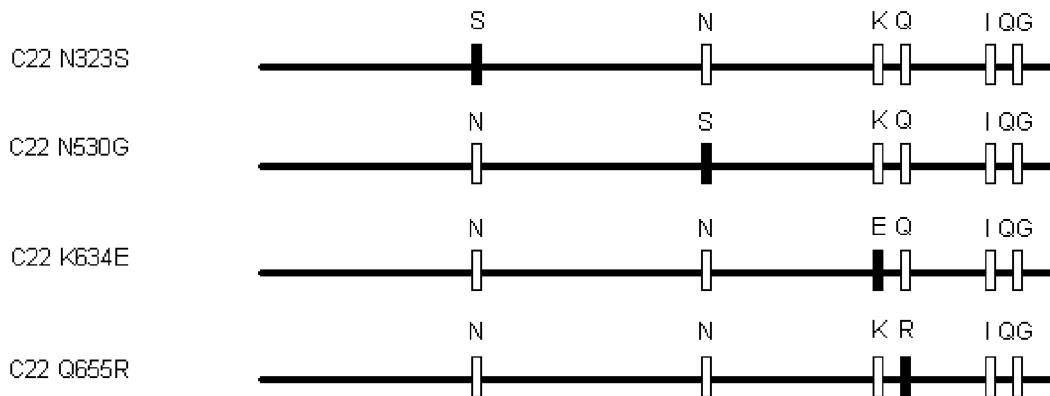
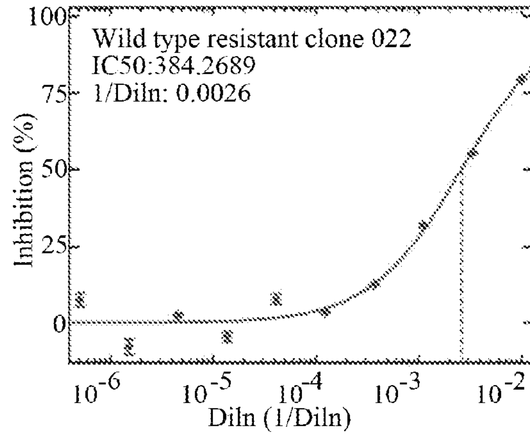
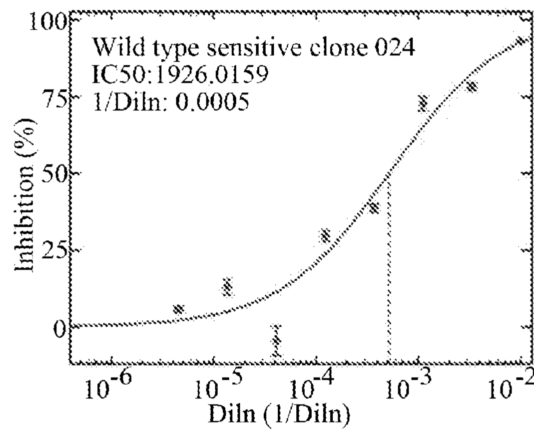


FIGURE 2 Sensitivity of Wild-Type and Mutant Clones from 108060 to Neutralization by HIV-1+ Serum Z23

2A



2B



2C

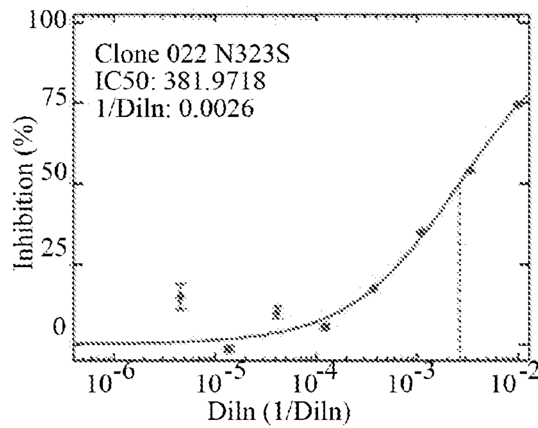


FIGURE 2 *(continued).* Sensitivity of Wild-Type and Mutant Clones from 108060 to Neutralization by HIV-1+ Serum Z23

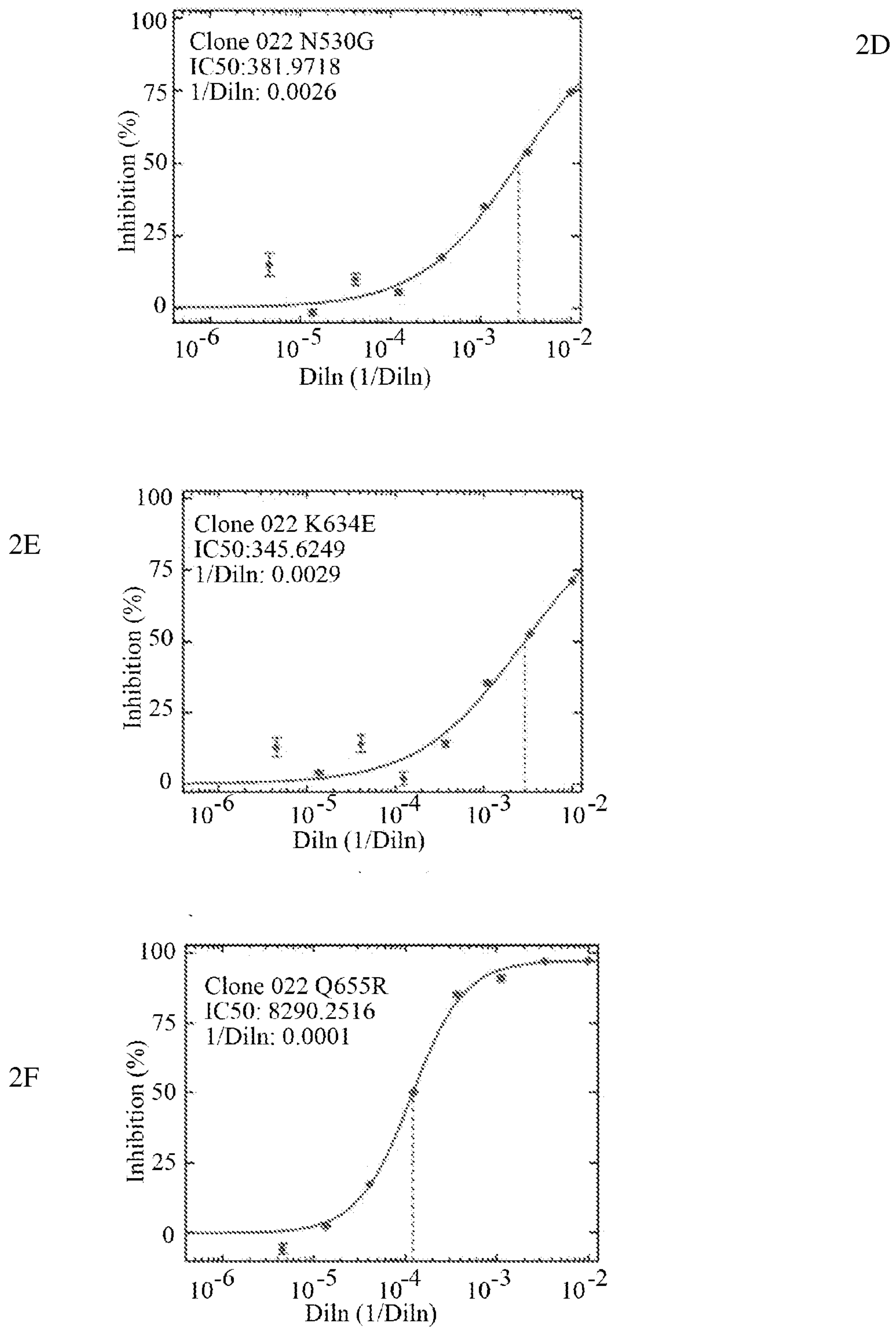


FIGURE 3

Gp41 Functional Domains and Comparison of Sequences of Functionally Significant Regions of the N36 and C34 Helices

FIGURE 4

Method to Identify Epitopes Reactive with Broadly Neutralizing Antibodies in HIV+ Sera

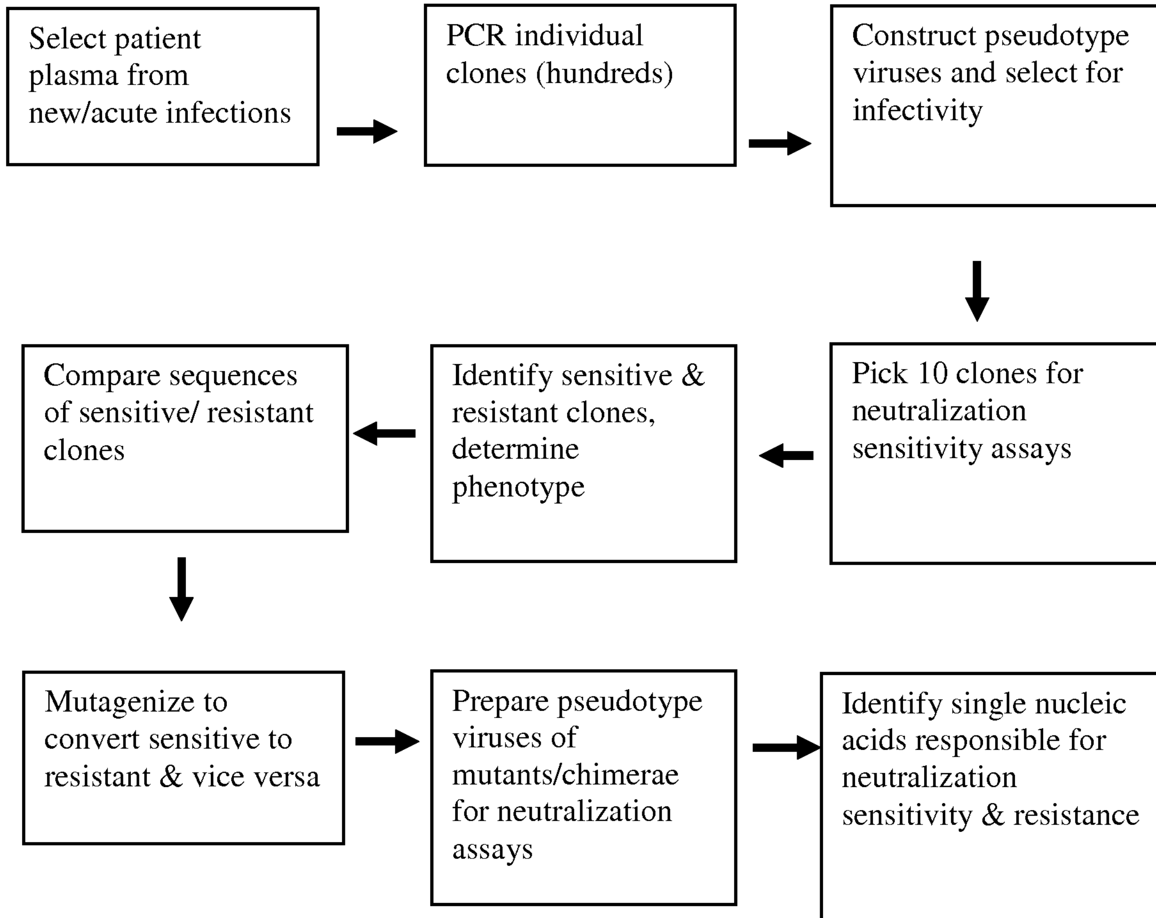


FIGURE 5

The sequences shown are:
Envelope sequence (subject 108060) - neutralization sensitive and neutralization resistant
Envelope sequence (subject 108051) - neutralization sensitive and neutralization resistant
Envelope sequence (subject 108048) - neutralization sensitive and neutralization resistant

```
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VPVWREATTTLFCASDAKSYETEVHNVWATHACVPTDPNP
QEILLENVTEDFNIWTNNMVEQMHEDIISLWDQSLKPCVK
LTPLCVTLNCTDLKNATNITNSEGGMREGGEIKNCSFNIT
TSLRDRVQKEYALFYKLDVEPIDDDKNSTDNNSTNYTNYR
LISCNTSVITQACPKVSFEPIPIHYCVPAGYALLQCNNKT
FSGKGQCKNVSTVQCTHGIRPVVSTQLLLNGSLAEEEVVI
RSENFTDNAKTIIVQLNETVEINCTRPNNNTRRSISIGPG
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KNKTIVFNHSSGGDPEIVMFSFNCGGEFFYCDSTKLFNST
WNGTEDDSGKNRTITLPCRIKQFINMWQEVGKAMYAPPIK
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QEILLENVTEDFNIWTNNMVEQMHEDIISLWDQSLKPCVK
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TSLRDRVQKEYALFYKLDVEPIDDDKNSTDNNSTNYTNYR
LISCNTSVITQACPKVSFEPIPIHYCVPAGYALLRCNNKT
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NLWNWFDITNWLWYIRIFIMIVGGLIGLRIVFAVLSIVRR
VRQGYSPLSFQTLLPVPRGPDRPEGTEKEGGEQDRGRSVR
LVDGFLALFWDDLRSLCLFLYHRLRDLLLIVTRIVGVLGH
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LTPLCVTLNCTDKLRNDAFGVNNTMEGEMKNCSFNTTTSL
RDKIQKEYALFYKLDVVQIKNNNNSNYTSYRLINCNTSVI
TQACPKVTFEPIPIHYCTPAGFAILKCNNKTFSGKGTCTN
VSTVQCTHGIRPVVSTQLLLNGSLAEEDVVIRSDNFSQNA
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IGNIRQAHCNVSSTKWNNTLQKIVEKLREQFGNKTIKFTS
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VSTVQCTHGIRPVVSTQLLLNGSLAEEDVVIRSDNFSQNA
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IGSIRQAHCNVSSTKWNNTLQKIVEKLREQFGNKTIKFTS
PSPGGDPEIVMHSFNCGGEFFYCDTTQLFNSTWDNTSTWN
NSNTQNKNDRNITLQCRIKQIINMWQEVGKAMYAPPIMGQ
IRCVSNITGLLLTRDGGNGSEAKNDTEIFRPGGGDMRDNW
RSELYKYKVVKIEPLGVAPTKAKRRVVQREKRAVGTIGAM
FLGFLGAAGSTMGAASLTLTVQARQLLSGIVQQQNNLLRA
IQAQQHLLQLTVWGIKQLQARVLAVERYLRDQQLLGIWGC
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TSLIYTLIEDSQNQREKNEQELLELDKWASLWNWFDITSW
LWYIKIFIMIVGGLIGLRIVFAVLSIVNRVRQGYSPLSLQ
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