Envelope Glycoprotein Determinants of HIV-1 Induced Membrane Fusion

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Envelop glycoproteïne determinanten van
HIV-1 geïnduceerde membraan fusie

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Promotor:	Prof. Dr. A.D.M.E. Osterhaus
Co-promotor:	Dr. M.L. Bosch
Overige leden:	Prof. Dr. E. Claassen
	Prof. Dr. J. Goudsmit
	Prof. Dr. M.C. Horzinek

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aan mijn moeder

voor Lizette

Abbreviations

AIDS	acquired immunodeficiency syndrome
bp	base pair
CAT	chloramphenicol acyltransferase
CDR-x	complementarity determining region-# x
CHO	chinese hamster ovary
Cx	conserved region # x
Dx	CD4 ectodomain # x
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
HA	hemagglutinin
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HuMoAb	human monoclonal antibody
LTR	long terminal repeat
MNC	peripheral blood mononuclear cells
NSI	non-syncytium inducing
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PHA	phytohemagglutinin
RIPA	radioimmunoprecipitation assay
sCD4	soluble CD4
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SI	syncytium inducing
SIR	syncytium inducing region
SIV	simian immunodeficiency virus
VN	virus neutralizing
Vx	variable region # x
wt	wild type

Contents

CHAPTER 1

General Introduction

General Introduction

In 1983 the isolation of a previously unknown human retrovirus was first associated with a newly recognized acquired immune deficiency syndrome (AIDS), characterized by unusual opportunistic infections and malignancies (11). Subsequently repeated retrovirus isolations from individuals with AIDS or from individuals known to be at risk of acquiring this disease were reported (53,99,133). These retroviruses were characterized as members of a separate group of primate lentiviruses, the human immunodeficiency viruses (HIV). They were indeed identified as the etiological agents of AIDS (29,143,153). Within this group two major subtypes are presently distinguished: HIV-1 and HIV-2 (26,27,64). The lentiviruses identified to date which may cause AIDS like syndromes in infected animals include simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV). Both SIV infection of macaques and FIV infections of cats are presently used as animal models for HIV infections in humans (97). Here a concise overview of the biology of HIV-1 is presented, with special attention for the process of HIV-1 induced membrane fusion which is at the basis of viral entry and syncytium formation.

1. HIV-I AND IT'S REPLICATION.

1.1. Virion morphology and structure. Mature HIV-1 particles, as typical lentivirus structures, are approximately 110 nm in diameter and consist of a cone-shaped capsid surrounded by a lipid envelope membrane (Fig. 1)(55). Electron microscopical studies have revealed 72 glycoprotein spikes projecting from the envelope membrane that are 9-10 nm in size (Fig. 1)(56,127,165). These glycoprotein spikes are tri- or tetrameric structures which each consist of heterodimers of a gp120 surface glycoprotein, associated with the gp41 transmembrane protein anchoring the heterodimer into the host cell derived virion membrane (38,43,180). In addition to the virus encoded envelope glycoproteins cellular membrane proteins are also present in the virus membrane (8). Biochemical and immunochemical studies have revealed a number of other viral proteins (Fig. 2). The nucleocapsid (core) is composed of the major Gag (group specific antigen) protein p24, which contains two positive sense, single-stranded genomic RNA molecules closely associated with the viral Gag proteins p7 (p9) and p6 in addition to the reverse transcriptase. Also the virus encoded integrase enzyme is present within the capsid (55,56). The myristylated matrix protein p17, forms an icosahedrycal layer between the core and the virion membrane and is required for incorporation of the envelope protein into mature virions (127,165,188).

1.2. Organization of the genome and encoded proteins. The linear proviral DNA integrated in host cell DNA is about 9.8 kb in size and is flanked by long terminal repeats (LTR) at each end. The LTR contains signals for integration, transcription initiation and regulation, and poly-adenylation of messenger RNA's. Like in other retroviruses the viral

genome contains Gag, Pol, and Env genes in the order 5' to 3'. However, in contrast to other less complicated retroviruses, the genome of lentiviruses including HIV-1 contains several additional genes that code for regulatory and accessory proteins (Fig. 2) (105). Gag and Gag-pol precursor polyproteins of HIV-1 are translated from full-length viral RNA, whereas the other (precursor) proteins are produced from single or multiple spliced smaller messenger RNA's (Fig. 3). The Gag-pol precursor protein, that is synthesized through a translational frameshift, is cleaved into a RNA and DNA-directed polymerase (reverse transcriptase), the integrase involved in viral DNA integration, and the viral protease (105).

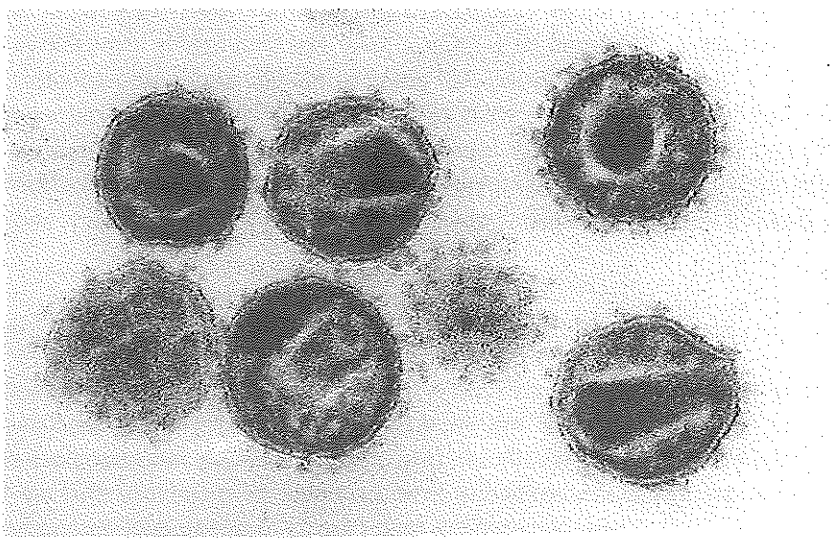

FIG. 1. Electron micrograph of mature HIV-1 particles, showing different core orientations. Virus surface projections represent the envelope glycoproteins. Photomicrograph courtesy of Dr. H.R. Gelderblom, Berlin.

The Gag polyprotein (p55 Gag) is cleaved into the matrix protein p17, the capsid protein p24, the nucleocapsid protein p7, and three smaller proteins p6, p2 and p1 (73,74). The translation, processing, and maturation of the envelope glycoproteins are discussed below in section 3.1. In addition to the structural genes, the HIV-1 genome contains at least six additional genes coding for the regulatory proteins Tat and Rev (34) and the four accessory proteins: Vif, Vpr, Vpu, and Nef (163). The Tat, Rev, and Nef proteins are synthesized from early Rev-independent (see below) multiply spliced mRNA's, while vif, vpr, and vpu are expressed late from Rev-dependent singly spliced mRNA's (163).

1.3. Replication cycle. The replication cycle of HIV-1 infection starts with the fusion of the target cell outer membrane and the viral membrane (108,161). For HIV-1 this membrane fusion is typically initiated by the binding of the envelope glycoproteins to the cellular CD4 receptor

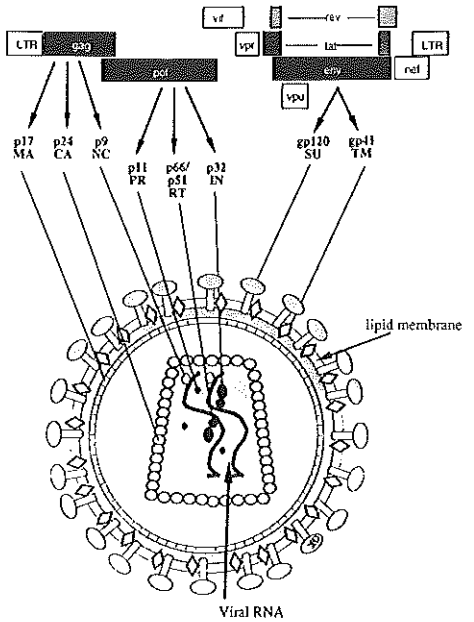

FIG. 2. HIV-1 genome organization and virion structure. The viral genome flanked by both LTR's, and the location of the genes is shown at the top. The viral proteins indicated in the virion are: matrix protein (MA), capsid protein (CA), nucleocapsid protein (NC), viral protease (PR), reverse transcriptase (RT), integrase (IN), surface glycoprotein gp120 (SU), and transmembrane protein gp41 (TM). Reproduced by kind permission from Annu. Rev. Biochem. 1991, 60:577-630.

and results in release of the nucleocapsid into the cytoplasm (36,133). This envelope glycoprotein controlled fusion process is discussed in detail in section 3. Upon entry in the cytoplasm viral replication is initiated with reverse transcription of the viral single stranded RNA genome, into double stranded DNA by a process requiring two strand-transfers (124). Negative-strand DNA synthesis by the viral reverse transcriptase is primed by cellular $tRNA_{lys}$ and is associated with RNase H activity of the reverse transcriptase, digesting the viral RNA once duplicated into DNA. Subsequent positive strand DNA synthesis by the same polymerase is presumably initiated at two sites on the first DNA strand but now primed by viral genome RNA sequences. This process of reverse transcription is particularly error prone since no proofreading is involved , and thus results in high genetic variation in progeny virus (135,138). Template switching during reverse transcription resulting in retroviral recombination also contributes to the high level of HIV-1 genetic variability (135). Linear double-stranded DNA with two identical LTR's is subsequently transported into the nucleus and inserted into host cell DNA by the viral encoded integrase (13,61). Both steps in viral replication, reverse transcription and integration, are only fully completed in dividing host cells (162,189) Once integrated, viral DNA (the provirus) represents a transcription unit ready to initiate the production of a next

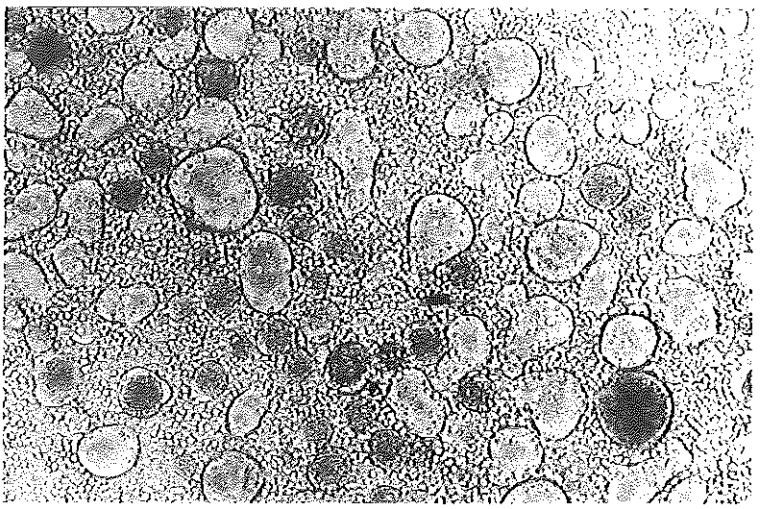

FIG. 3. Syncytia induced in transfected SupT1 cells upon expression of an SI virus derived envelope gene (env. gene 16.1, chapter 4).

generation of viruses. This transcription unit is also passed to progeny cells like resident cellular genes. HIV gene expression from the 5' LTR is controlled by both cellular and viral factors (105,141). The viral Tat protein plays a central role in HIV transcription regulation by binding to the Tat-responsive RNA-element (TAR) located at the 5' end of all viral mRNAs. Tat-TAR interaction upregulates viral gene expression including Tat gene expression itself, which results in a positive feedback loop, responsible for a strong increase of virus expression (33). Such a burst of expression may be initiated by a wide variety of external stimuli, including the lectin PHA, certain cytokines, phorbol esters, and cellular factors that may also be expressed upon co-infection with other viruses and thus induce HIV expression (141). The LTR directed transcription results in full length RNA molecules of which most are spliced into smaller messenger RNA species. The full length RNA transcripts serve either as genomic RNA to be encapsidated or as mRNA for the Gag and Gag-pol polyproteins. However, like the single spliced mRNA coding for the envelope glycoproteins, these long RNA molecules are only exported to the cytoplasm in the presence of the Rev protein. Rev acts as a post-transcriptional trans-activator, regulating the expression of viral structural genes in an indirect fashion (81). Newly enveloped viruses are generated through a budding process at the cell surface. This process is believed to start with the formation of immature cores of Gag and Gag-pol polyproteins at the interior surface of the cell plasma membrane (54,56). In the subsequent budding process the nucleocapsid Gag domain interacts with the psi packaging sequence on the viral genomic RNA (59) and the cytoplasmic tail of gp41 of envelope oligomers interacts with the myristylated matrix Gag domain (60). During the last stages of the budding process and in newly-released virions the Gag and Gag-pol precursors are cleaved in trans by the viral

protease. This cleavage process is required for viral infectivity (86) and is held responsible for the morphological maturation of virions in which the core condenses to form the typical electron-dense conical structure (56). The envelope glycoproteins expressed at the cell surface of infected cells may interact with the CD4 receptor expressed at the surface membrane of adjacent cells. Such an interaction may lead to membrane fusion similar to the situation in viral entry, and results in the formation of multinucleated cells (syncytia, Fig. 3) (100,101). CD4-gp120 based interactions between neighbouring cells may also be at the basis of direct cell to cell spread of the infection (144).

2. HIV-1 INFECTION IN HUMANS.

2.1. Course of infection. During the early stage of HIV-1 infection, about half of the individuals develop influenza like disease symptoms (30,170). In this early stage, high virus titers in plasma and high numbers of infected peripheral mononuclear blood cells (PBMC) are observed. The initial virus load is markedly reduced when the immune response develops (25,35). At this stage a transient low CD4:CD8 ratio is observed presumably due to an increased number of circulating CD8+ immune effector T-cells together with a reduced number of CD4+ cells (93). The immune response however, does not prevent early wide spread dissemination of HIV-1. In addition to the cells of the haematopoietic system, the virus may be detected in cells of several organs including the skin, the gastrointestinal tract and the brain (98). After returning to a near normal level after acute infection, the CD4+ cell numbers gradually decline during the subsequent asymptomatic phase of the infection (93). This asymptomatic phase may last for more than 10 years, but impaired immune functions are observed, as evidenced by hypo-responses to antigenic and mitogenic stimuli (114,156,166). Several mechanisms are postulated for the observed depletion of CD4+ cells contributing to the eventual immune collapse. These include direct cytopathic effects (100,158), immune mediated cyto-lysis (192), and programmed cell death (apoptosis) (63,112). Recently determined viral loads in the asymptomatic period are several orders of magnitude higher than previously estimated and the frequency of infected lymphocytes in lymphoid tissues proved to be significantly higher than in the peripheral circulation (44,129). Other more recent studies with experimental drugs that are potent inhibitors of viral replication revealed a highly dynamic process of HIV-1 replication (77,179). It was shown that replication of HIV-1 *in vivo* is continuous and highly productive, driving a rapid turnover of CD4+ cells. These data favour the view that AIDS is primarily the consequence of a direct virus- and immune-mediated killing of infected cells caused by a continuous high level of virus replication. AIDS is ultimately marked by the occurrence of normally rare malignancies like Kaposi's sarcoma, opportunistic infections, neurologic abnormalities and gastrointestinal disorders (98).

2.2.Immune response. Both the humoral and the cellular effector arms of the immune system are active in response to HIV-1 infection. These responses are detectable shortly after infection and persist throughout the lifetime of the host. Humoral responses have been detected

to both structural and non-structural viral proteins. However, most of the antibodies active in functional assays measuring virus neutralization or antibody dependent cellular cytotoxicity (ADCC) are directed to the viral envelope glycoproteins (46,102,181). Neutralizing antibodies are predominantly directed to the V3 loop and the CD4 binding domain of gp120 (82,117,136,193), but many other regions of gp120 and also of gp41 may elicit virus neutralizing antibodies (17,66,78). The role of the humoral immune response in the pathogenesis of HIV-1 infection is still controversial. Longitudinal studies have suggested a relationship between declining neutralizing antibody titers and disease progression (78,171,173) and similar studies using autologous virus - serum pairs demonstrated the rapid emergence of viral variants escaping from neutralizing antibody responses (1,177). Whether ADCC is clinically relevant in HIV-1 infection is not known either, and conflicting data have been presented on its correlation with an asymptomatic stage in patients (46,102,103,148). Finally the demonstration of enhancing antibodies has complicated the interpretation of experiments aiming at the elucidation of the role of antibody responses in the pathogenesis of HIV-1 infection (62,140).

The strong suppression of antigenemia in primary infection, often before the detection of neutralizing antibodies, suggests an important role for cell mediated immunity in HIV-1 infection (6). Indeed, the early appearance of T-helper and cytotoxic T-lymphocyte (CTL) activity was reported to coincide initial control of viraemia in HIV and SIV infections (88, personal communication A.M. Geretti). Also the prolonged asymptomatic period in certain individuals has been attributed to the presence of strong cellular responses (98).

2.3. HIV-1 genetic and biological variation related to pathogenesis. Comparison of the first nucleotide sequences available, revealed the variable nature of HIV-1 (65,137,187). Soon it was recognized that this diversity is not only reflected in antigenic variability of the virus, but also in significant variation of other biological properties shared by different HIV-1 isolates. These variable properties included cell tropism, syncytium inducing capacity, cytopathicity, and kinetics of replication(10,45). Correlating these biological characteristics of isolates with the stage of disease has been the subject of many studies. It was first recognized that viruses isolated from asymptomatic HIV-1 infected individuals frequently differed in their biological properties from viruses isolated from individuals with AIDS (10,22,50,167-169). Viruses isolated at a late time point in infection tended to have: an increased capacity to induce syncytia, an extended host range (replication in T-cell lines), and a higher replication rate relative to viruses isolated early in infection. Based on these biological differences two main viral phenotypes have been distinguished; the syncytium inducing (SI) and the non-syncytium inducing (NSI) variants (167). These two categories largely overlap with the so called rapid-high and slow-low phenotype designation used by others (50). Upon transmission, infection is likely to be established by NSI variants (174,186,190,191). The selective outgrowth of these variants may be based on their tropism for macrophages (113). The emergence of SI variants in the course of infection, as observed for about half of the infected individuals has been shown to correlate with an increased decline of CD4 cell numbers and was shown to be predictive for

progression to AIDS (87). In the studies presented in this thesis we used SI and NSI variants isolated from single individuals as representants of genetically highly homologous viruses differing in their capacity to mediate membrane fusion.

3. HIV-1 INDUCED MEMBRANE FUSION IN VIRAL ENTRY AND SYNCYTIUM FORMATION.

The process of membrane fusion underlying viral entry and syncytium formation in HIV-1 infection, is initiated by a complex interaction of the viral envelope proteins with the cellular CD4 receptor. Here the cellular expression and molecular structure of both membrane proteins is described, and related to the mutual interactions of these molecules in membrane fusion with a special focus on structure function relationship.

3.1. HIV-1 envelope proteins The HIV-1 envelope precursor protein is synthesized as an 88 kD polypeptide on membrane-bound ribosomes from a single spliced messenger RNA (3,139). The amino terminal hydrophobic signal sequence directs insertion of the growing polypeptide chain into the endoplasmic reticulum (ER). Upon translocation this signal peptide is removed by a host encoded endoprotease and the nascent protein is modified by the addition of oligosaccharide side chains through N-linked glycosylation of asparagine residues with predominantly mannose residues esterified into long chains (39,96). There are 30 to 38 potential asparagine-linked glycosylation sites present among different HIV-1 strains, most if not all of which are utilized for oligosaccharide attachment (96,125). A stop-transfer sequence near the carboxy terminus of the precursor protein holds the molecule anchored in the lipid membrane (51,52). Intramolecular disulfide bonds produce a folded monomer, which subsequently forms oligomeric complexes (Fig. 4). Both the formation of trimers (56,180) and tetramers (43,130,149) have been reported. Following transport to the Golgi complex, many mannose residues are trimmed from the side chains and other carbohydrates are added to yield envelope proteins containing complex carbohydrate side chains (58). Recently it has been demonstrated that the envelope proteins are also modified by O-linked oligosaccharides (12). The fully modified monomeric precursor envelope protein has a molecular mass of 160 kD, implying that the added carbohydrates contribute to approximately 45% of the total mass. Proteolytic cleavage of the envelope precursor into the surface protein gp120 and transmembrane protein gp41 in the oligomeric structure takes place in the Golgi compartment by a cell encoded trypsin-like protease (176,183). The subunits are not disulfide linked but associated through non-covalent interactions (90,111,176). On gp120 the most crucial residues for gp41 attachment cluster in the C1 and C5 regions (see below) respectively close to the amino and carboxy terminus (72). The complementary regions on gp41 have not been mapped conclusively, but the disulfide loop has been shown to be involved (104,152). Correctly folded and assembled envelope glycoproteins are transported out of the Golgi compartment and inserted into the cellular outer membrane. At the cell surface the envelope oligomers may be incorporated in the virions during the budding process or may mediate cell-cell fusion with CD4 expressing neighbouring cells resulting in syncytium formation. A considerable amount of the oligomeric envelope proteins

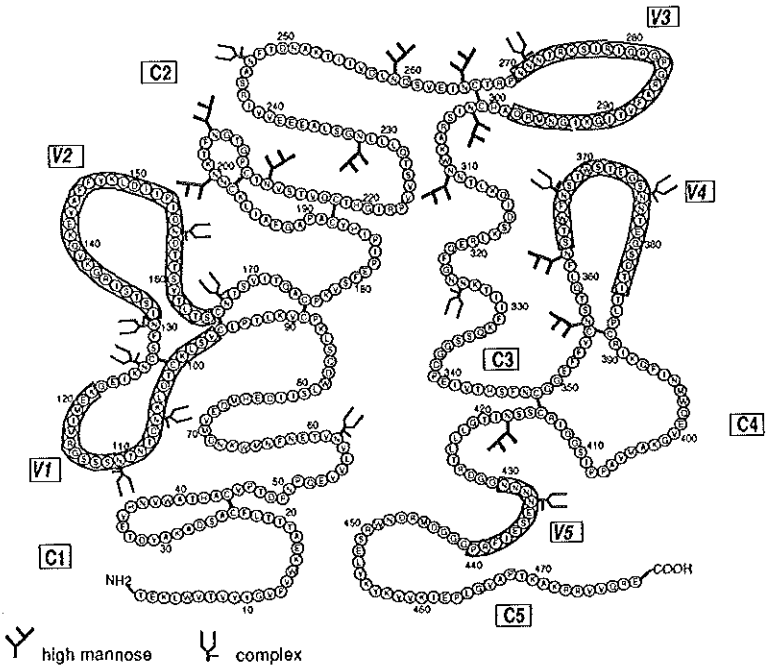

FIG. 4. Disulfide bond and carbohydrate (primary) structure of the HIV-1 surface glycoprotein gp120. Variable regions (V1-V5), conserved regions (C1-C5), and oligosaccharide structures are indicated. Amino acid numbering is based on the HIV-1 IIIB sequence. Reproduced by kind permission of Annu. Rev. Immunol. 1991, 9:649-678.

however dissociates to release the surface protein due to the weak binding of gp120 to gp41 (57,150). Different isolates of the virus displayed substantial heterogeneity in their DNA sequence, and much of it is clustered within the envelope gene, particularly the coding region for the gp120 glycoprotein (2,184). Genetic analysis of different HIV-1 isolates identified five distinct hypervariable regions (V1 - V5) which are interspersed with more conserved regions as indicated in Fig. 4 (116).

3.2. The CD4 receptor for HIV-1. The CD4 surface glycoprotein is predominantly expressed on cells of the T-cell lineage. The CD4 molecule acts in concert with the T-cell receptor in binding to class-II MHC molecules loaded with antigen on the surface of antigen-presenting cells. This binding governs thymic selection, the development of CD4+ T cells (T-helper cells) and their subsequent antigen dependent activation in the immune response (164). The progressive specific depletion of the CD4+ subset of T-cells led to the identification of CD4 itself as the major receptor for infection of T-helper lymphocytes and monocytes by HIV-1 (36,84,85). The CD4 glycoprotein has a molecular mass of 55 kd and is a member of the immunoglobulin superfamily which is reflected by its structural organization into four ectodomains designated D1 to D4 (three of which contain disulfide linked loops) (107). These

domains are tandemly arranged and consist of double anti-parallel beta-plated sheets (185). The first N-terminal domain (D1) is most similar to Ig variable domains, and although less strikingly D2 and D3 display sequence similarities with the constant domains of Ig (164). The third and the fourth domain each contain a N-linked glycosylation site that when expressed in CHO cells are both occupied by carbohydrates (19). The D1 domain is involved in both MHC-II association and gp120 binding (79). In the D1 domain complementarity determining regions (CDR's) are identified, especially CDR2 plays an important role in gp120 binding (see section 3.4). The short cytoplasmic domain (only 38 amino acid residues) is separated from the ectodomains by a typical transmembrane anchor of hydrophobic amino acids. The positively charged cytoplasmic domain interacts with tyrosine kinase $p56^{lck}$ and is thought to be involved in signal transduction (172,175).

3.3. A secondary receptor for HIV-1? Although the CD4 molecule functions as the receptor for HIV-1, and it is the specificity of this virus-receptor interaction that dictates HIV-1 tropism for T cells and monocytes/macrophages, CD4 expression alone is not sufficient for HIV-1 entry. The inability of HIV-1 to infect human CD4 expressing murine cells, may suggests that another molecule or receptor is required for entry (106). Most studies addressing this issue favour this option (15,40,67). The cellular restriction to virus entry however, varies with the virus-cell combinations tested (23,80). To explain this phenomenon, it has been postulated that the factor required may comprise different molecules or alternatively a single molecule belonging to a polymorphic family (157). No secondary receptor for HIV-1 has been identified unequivocally to date. The putative secondary receptor may interact directly with the envelope proteins or may interact exclusively with the CD4 receptor, inducing conformational changes in this molecule, essential to mediate fusion (see below).

3.4. CD4-gp120 binding regions. The observation that antibodies to CD4 could block HIV-1 induced membrane fusion processes, as demonstrated by inhibition of viral infection and syncytium formation, contributed to the identification of CD4 as the major receptor for HIV-1 (36,85). Subsequently on gp120 and CD4 regions involved in mutual binding have been identified.

Initial studies demonstrated that the C4 region of the surface glycoprotein gp120 is involved in CD4 binding (31,95). Other studies however showed the implication of additional gp120 regions in CD4 binding (32,90,126). It is now apparent that the CD4 binding site on gp120 contains multiple regions, including C2, C3, and C4, separated in the primary sequence but brought together in the folded molecule (126). This notion is consistent with the observation that most of the anti-gp120 monoclonal antibodies that interfere with CD4 binding, recognize conformational epitopes (76,134,154). These mutational studies do not discriminate between residues directly involved in CD4 binding or residues that interfere with CD4 binding by indirect conformational effects. Proteolysis protection studies suggested that especially regions in the C-terminal half of gp120 comprising C2 and C4 directly contact CD4 (131). The construction of a minimal CD4 binding surface glycoprotein in which the 62 N- and 20 C-terminal residues along with the entire V1, V2, and V3 regions were deleted is in line with

these data (132), which demonstrated that these deleted regions are dispensable for CD4 binding. While glycosylation of the envelope precursor protein is required for proper folding and processing, leading to a protein capable of binding CD4, deglycosylation after biosynthesis under non-denaturing conditions revealed that glycans only marginally contribute to CD4 binding (47,48). Similarly, studies in which potential glycosylation sites were removed, or in which carbohydrate addition or processing was inhibited, indicated that in general the glycans of the HIV-1 envelope proteins are dispensable for syncytium formation or viral entry (49).

The gp120 binding site on the CD4 molecule was first mapped with monoclonal antibodies that cluster with Leu3A and OKT4a (111,145). Extensive mutational analysis further characterized the gp120 high affinity binding site and localized it within in the first N-terminal domain D1. Finally a small region (residues 40-55) within the CDR-2 like sequence of D1 was identified as most critical for gp120 binding (7,9,14,28,92,115,128,151) but also residues outside this region have been identified as potential contact sites (9,16). Interpretation of these data is facilitated now that the structure of the D1 and D2 regions of CD4 is defined by crystallography (142,178). In these analyses the residues involved in the high affinity binding site form a protruding ridge (the C'-C'' ridge). Possibly the gp120 molecule has a complementary groove in the CD4-binding region. Several residues outside the CDR-2 region, which also appear to be involved in the interaction with gp120 were shown to reside on adjacent β-strands. Of these residues some have side chains projecting towards the C'-C'' ridge, suggesting direct interactions. Other residues are located further within the body of the protein and may only indirectly affect gp120 binding (18,142). Finally, it is postulated that binding to gp120 may involve dimerization of the CD4 molecule (42,94).

3.5. Receptor mediated activation of membrane fusion. The process of viral membrane fusion has been studied most thoroughly for influenza virus infection and this is the current paradigm for virus mediated fusion mechanisms (160). Upon viral attachment and receptor mediated endocytosis influenza virus mediated membrane fusion is triggered by a pH reduction within endosomes. This pH shift induces conformational changes in the viral glycoprotein (HA) resulting in the exposure of the hydrophobic fusion domain of the transmembrane protein. The fusion domain then perturbs the cellular lipid bilayer and initiates fusion with the viral membrane. Similarly HIV-1 induced membrane fusion is thought to be invoked by the gp41 N-terminal fusion domain exposed on the envelope glycoprotein multimere through conformational changes (Fig. 5). HIV-1 induced membrane fusion has been shown to be pH independent, and conformational changes are triggered through receptor binding, enabling viral entry at the cell surface at neutral pH (108,110,146,147,161). Such a receptor induced fusion mechanism was indicated by experiments in which HIV-2 infection of CD4 negative cells was demonstrated upon soluble CD4 (sCD4) incubation (24). Incubation with (subinhibitory) doses of sCD4 also enhances HIV-2 and SIV infection (4,24,155,182) of CD4 positive cells.

3.6. Receptor induced conformational changes. Indirect evidence for a receptor activated fusion mechanism was provided by several studies demonstrating that (soluble) CD4 binding induces conformational changes in the envelope protein complex. Recently, it was shown that

sCD4 binding results in increased exposure of gp120 regions recognized by antibodies (146,147), the dissociation of gp120 from gp41 (68,83,119,121,122) and the unmasking of gp41 epitopes (146,147). The complete array of conformational changes observed favours a model in which CD4 binding to gp120 leads to a fusogenic dissociation intermediate of the envelope protein complex (118). Binding studies revealed that the sCD4-virion binding affinity is temperature dependent (119,121). This may imply that initial contact of CD4 induces a conformational change in the envelope complex that converts a low affinity binding site for CD4 into a high affinity site (119,121). The CDR-2 region on CD4 serves as the initial contact site whereas other CD4 regions contribute to the subsequent increase of affinity. Since also CD4 induced shedding of gp120 appeared to be temperature dependent, it is likely that the conformational change that increases the affinity with CD4 directly destabilizes the gp120-gp41 interaction (121). This view is supported by the identification of monoclonal antibodies directed to CD4 that neutralize HIV-1 through inhibition of the high affinity binding of gp120 and that simultaneously reduce CD4 induced gp120 shedding from virions (123). Also the observation that primary isolate virions with reduced affinity for sCD4 have a more stable gp120-gp41 interaction compared to T cell line adapted HIV-1 strains, suggested that these processes are intimately linked (120). It remains to be determined whether complete gp120 dissociation is necessary for fusion to take place. The enhancement of infection by HIV-2 and SIV which both show reduced gp120 shedding upon incubation with sCD4 suggests that relatively stable dissociation envelope intermediates can cause fusion (5). However, associated gp120 might allow the virus to remain attached to the cell while e.g. gp41 dissociated from gp120 could trigger the actual fusion process. Furthermore other conformational changes, independent of gp120 shedding, have been described to occur after sCD4 binding including increased exposure of the V1, V2, and V3 loops on gp120, and two regions on gp41 (70,147). For all these regions a role in the fusion process post CD4 binding has been reported.

Retroviral transmembrane proteins have common structural features, such as two regions predicted to form alpha helices, separated by a cysteine loop, of which one displays a leucine zipper like motif and the N-terminal fusion domain (37,52) (Fig. 5). Since leucine zipper regions are frequently implicated in protein-protein interactions forming coiled-coil structures, the leucine zipper was supposed to constitute an oligomerization domain of gp41 (37). However, alpha-helix disrupting mutations in the leucine zipper domain inhibit membrane fusion but not oligomerization pointing to another function for the zipper motif (41). On the basis of these data and peptide inhibition experiments Matthews et.al. proposed a model in which a sequence of conformational rearrangements leads to a "fusion-attack complex" of gp41 in which the fusion domain interacts with the target membrane (109). In this fusion model leucine zippers on different gp41 molecules self-associate to form coiled-coils in the activated fusion-attack complex. Furthermore the alpha helical structure adjacent to the transmembrane region of gp41 either functions in preventing the formation of the coiled-coil in the native envelope complex or promotes coiled-coil formation in the fusion-attack complex (109). A recent report showing that amino acid residues in the C-terminal segment of the leucine zipper,

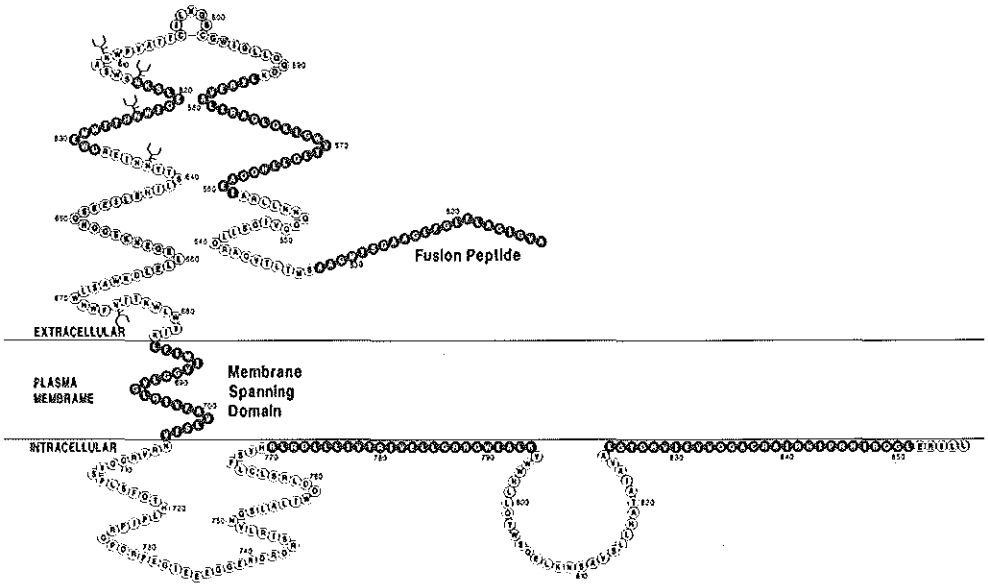

Fig. 5. Primary structure of the HIV-1 transmembrane protein gp41. Oligosaccharide structures are indicated by (Y). The disulfide loop (at the tip), the membrane spanning domain and the fusion peptide (fusion domain) are also shown. Amino acid numbering is based on the HIV-1 IIIB sequence. Reproduced by kind permission of AIDS Res. Human Retrov. 1992, 8:165-173.

that is located close to the cysteine loop, may form a gp120 contact site (21) suggests that this site transduces the trigger for gp41 conformational changes as started with CD4 binding to gp120. Conformational changes in CD4 are probably also essential for the fusion process (20). First, the virus binds to D1 of CD4, a region at the most distal end of the receptor molecule. To present the gp120/gp41 complex into close proximity of the target-cell membrane a considerable distance must be bridged. Secondly, it is likely that the conformational changes in the envelope protein complex that occur during the strengthening of binding CD4 parallel conformational changes in CD4 itself. It is postulated that CD4 bends at a putative hinge region between D2 and D3 (91) in the fusion process. This is supported by the fact that monoclonal antibodies directed to D3 close to this region block HIV-1 cell fusion without interfering with CD4 binding (69).

HIV-1 infection and syncytium formation generally both rely on the interaction of gp120 with CD4 and the subsequent conformational changes in both molecules. Additional information is however required to clarify the differences observed between syncytium formation and viral entry (71,75,89,159). Similarly, the differences in the membrane fusion processes responsible for the entry of different cell types are yet not resolved. Determinants responsible for the differences in theses processes are mapped on both the envelope proteins and cellular components other than CD4.

4. OUTLINE OF THIS THESIS.

The studies described in this thesis focuses on the role of the HIV-1 envelope proteins in virus induced membrane fusion processes. First the contribution of envelope glycoprotein sequence variation to HIV-1 syncytium formation and cell tropism is determined by genetic and functional analyses of two sets of envelope genes obtained from two different donors (chapter 2 and 3). Chimeric envelope genes generated from one of these two sets are constructed and transiently expressed, in order to identify envelope regions and the related sequence variation in these regions which determine syncytium formation and cell tropism (chapter 4 and 7). Subsequently, the impact of naturally occurring sequence variation in one of the envelope regions identified on the process of syncytium formation is studied in more detail (chapter 5). A large number of envelope gene nucleotide sequences is aligned, in order to allow the detailed analysis of variable regions with sequence length variation (chapter 6). Finally, the efficiency of HIV-1 entry in the presence of specific monoclonal and polyclonal antibodies as well as sCD4 is studied (chapter 8). The results of the studies presented in this thesis are discussed and summarized in chapter 9.

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CHAPTER 2

Genetic and Functional Analysis of a Set of HIV-I Envelope Genes Obtained from Biological Clones with Varying Syncytium-Inducing Capacities

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Genetic and Functional Analysis of a Set of HIV-1 Envelope Genes Obtained from Biological Clones with Varying Syncytium-Inducing Capacities

ARNO C. ANDEWEG,[1] MARTIJN GROENINK,[2] PAULA LEEFLANG,[1] RUUD E.Y. de GOEDE,[2] ALBERT D.M.E. OSTERHAUS,[1] MATTHIJS TERSMETTE,[2] and MARNIX L. BOSCH[1]

ABSTRACT

To study HIV-1 envelope-mediated syncytium formation we have amplified, cloned, expressed, and sequenced individual envelope genes from a set of eight biological HIV-1 clones. These clones were obtained from two patients and display either a syncytium-inducing (SI) or nonsyncytium-inducing (NSI) phenotype. Upon expression through recombinant vaccinia virus, individual envelope gene products display heterogeneous syncytium-inducing capacities which reflect the phenotype of the parental biological HIV-1 clones in all cases. For the eight biological HIV-1 clones presented here, variation of the envelope gene alone is sufficient to explain the observed variable syncytium-inducing capacity of the respective parental viruses. In addition we determined the complete nucleotide sequence of these envelope genes. The predicted amino acid sequence revealed a considerable amount of variation located mainly in the previously denominated variable regions. In various regions of envelope genes obtained from the same patient, phenotype associated amino acid variation was found. This phenotype associated amino acid variation however, is not conserved between the two sets of envelope genes derived from different patients. Four envelope sequences derived from clones obtained from one patient showed phenotype-associated amino acid variation in the fusion domain. Sequencing of 12 additional fusion domains revealed that this same variation is found in four additional clones. However, a functional test performed on recombinant vaccinia expressing mutant envelope genes showed that this observed fusion domain variation does not contribute to the variation in syncytium-inducing capacity of the envelope gene product.

INTRODUCTION

ISOLATES OF HUMAN IMMUNODEFICIENCY VIRUS type 1 (HIV-1) differ in their in vitro biological properties such as replication rate, cytotropism, and syncytium-inducing capacity.[1–3] Based on these in vitro properties one can distinguish syncytium-inducing (SI) isolates which have a high replication rate in primary cells and are generally able to grow in continuous cell lines on the one hand, and nonsyncytium-inducing (NSI) isolates on the other hand, which have a moderate to low replication rate in primary cells and do not grow in continuous cell lines.[3]

In an earlier longitudinal study, we demonstrated that detection of SI variants in asymptomatic individuals is strongly associated with subsequent rapid decline of $CD4^+$ T-cell numbers and progression to acquired immunodeficiency syndrome (AIDS), whereas from stable asymptomatic individuals only slow replicating NSI isolates can be recovered. This suggests different roles for SI and NSI variants in in vivo pathogenesis.[4,5] It has been demonstrated that syncytium formation is mediated by the envelope glycoproteins of HIV-1 although the exact mechanism has not been clarified.[6,7] Furthermore, several studies with pairs of recombinant proviruses have directly implicated the envelope gene as containing the primary determi-

[1]Laboratory of Immunobiology, National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven, The Netherlands.

[2]Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology of the University of Amsterdam, P.O. Box 9190, 1006 AD Amsterdam, The Netherlands.

nants in controlling biological variation of HIV-1,[8–10] whereas only minimal functional variation has been found so far in the regulatory genes or long terminal repeat (LTR) of HIV-1 isolates with varying biological properties.[11–15] In the present comparative study we analyze a set of envelope genes to elucidate the genetic basis of the observed variation in syncytium-inducing capacity.

The envelope glycoproteins are synthesized as a glycosylated precursor gp160, which undergoes several processing steps including oligosaccharide modification and proteolytic cleavage into two subunits; gp41 and gp120.[16] The transmembrane glycoprotein gp41 noncovalently anchors the external glycoprotein gp120. The mature glycoprotein complex probably forms tetramers or a mixture of tetramers and dimers on the surface of the virion and infected cells.[17–19] Several determinants on the envelope glycoproteins appear to be involved in the process of membrane fusion which is necessary for the infection of target cells by HIV-1 and for HIV-1-induced syncytium formation.[6,20] In the first step HIV-1 gp120 binds directly to the CD4 receptor molecule on target cells.[21,22] Variation in CD4 binding affinities of the gp120 molecules of different HIV-1 isolates suggests direct or indirect involvement of the CD4 binding site on gp120 in determining such biological properties of the virus as the ability to induce syncytia.[23,24] As a result of this binding event the hydrophobic amino terminus of gp41 is exposed probably through a series of conformational changes.[25] Mutational analyses have shown direct involvement of this region to the ensuing membrane fusion and it is therefore denominated the fusion domain.[26–29] Mutations in the fusion domain of simian immunodeficiency virus (SIV) resulted in either reduction or enhancement of SIV envelope-induced syncytium formation depending on the hydrophobicity of the introduced amino acid which indicates that natural variation in this region of HIV-1 may contribute to determining the biological phenotype of the virus.[26] Other regions of the envelope glycoproteins (e.g., the hypervariable V3 region) appear also to be involved in the process of membrane fusion following the gp120-CD4 binding.[30] In addition, variation in the V3 region has been shown to affect the biological properties of recombinant HIV-1 proviruses especially with regard to cell tropism.[31]

Here we describe the amplification, cloning, expression and sequencing of eight complete envelope genes derived from two sets of biological clones of HIV-1 with different in vitro biological properties. This approach allows us to correlate the syncytium-forming capacities of the individual envelope genes to their respective amino acid sequences and to the syncytium inducing capacity of the parental HIV-1 clones.

MATERIALS AND METHODS

Subjects

Virus was recovered from seropositive individuals visiting the outpatient clinic of the Academic Medical Centre, Amsterdam, or participating in a large cohort study.[32]

Clonal virus isolation

To obtain clonal populations of HIV-1 directly from patient peripheral blood mononuclear cells (MNC), virus isolation was performed in 96-well microtiter plates as described previously.[33] Briefly, patient MNC (10,000/well) were cocultivated with 100,000 phytohemaglutinin-stimulated MNC from healthy seronegative blood donors. Under these conditions productive HIV-1 infection was observed in less than a third of the wells, indicating clonality according to the Poisson distribution. The thus obtained biological HIV-1 clones were then characterized as syncytium-inducing (SI) or nonsyncytium-inducing (NSI) according to previously published criteria.[3] Following this procedure, multiple clones were obtained from MNC of 30 seropositive individuals. For this study four individuals (HIVach320, HIVams16, HIVams93, and HIVach373 further referred to as, respectively, #320, #16, #93, and #373) were selected whose uncloned HIV-1 isolates, obtained as described previously,[3] had a SI phenotype. By clonal isolation both NSI and SI clones could be obtained from MNC of these individuals. From one individual (#373) in whom over time a transition from NSI to SI phenotype had been observed,[34] HIV-1 clones obtained from two time points (as indicated) were included in this study.

Polymerase chain reaction (PCR) amplification of envelope sequences.

High-molecular weight DNA from MNC infected with clonal isolates were subjected to 35 cycles of PCR, using a set of primers either spanning the complete envelope gene or a 423 bp subregion of the envelope gene (position 7092-7514, HIV-HXB2[35]). The 423 bp region encodes the carboxy terminal part of gp120, the envelope cleavage site, and the fusion peptide located at the amino terminal end of gp41. The primers "FUS1" and "FUS2" (5′-GGAAAAGCAATGTATGCCCCTCC-3′ and 5′-CCAGACTGTGAGTTGCAACAGATGC-3′, respectively) were used to amplify this 423 bp envelope region. To amplify the complete envelope gene the primers "5000" and "7500" (5′-GACGTCCCCGGGCAGAAGACAGTGGCAATGA-GAG-3′and 5′-GCATGCCCCGGGCTTTTTGACCACTTGC-CACCC-3′) were used. The primer "5000" includes the first seven nucleotides of the envelope open reading frame, fixing these nucleotides for all amplified envelope genes. XmaI restriction sites (underlined) were incorporated into the primers "5000" and "7500" to facilitate cloning. PCR conditions were: 35 cycles of 1′95°C, 1′55°C, and 2.5′ 72°C with 0.7 μM primers, 2 mM Mg^{2+}, and 200 μM dNTP for the complete envelope gene and 35 cycles of 1′ 95°C, 1′ 55°C, and 2′ 72°C with 5 μM primers, 4 mM Mg^{2+} and 200 μM dNTP when amplifying the 423 bp region.

Construction of recombinant vaccinia virus

Envelope genes derived from biological clones obtained from patient #320 and #16 were cloned into the XmaI site of plasmid vector pSC11.[36] Hela cells infected with vaccinia virus (strain WR) were transfected with calcium phosphate-precipitated pSC11-*env* constructs as previously described.[36] Upon 5-bromodeoxyuridine (BudR) selection in 143 cells, recombinant virus plaques were visualized by their blue color upon overlay with 5-bromo-4-chloro-3-indolyl-βD-galactopyranoside (XGal) due to the coexpression of the lacZ gene derived from the pSC11 vector. Independent plaques were plaque-purified three times

and the recombinant vaccinia viruses were grown to large stocks in RK13 cells. All recombinant vaccinia viruses were assayed for HIV-1 envelope expression by radioimmunoprecipitation (see below). The recombinant vaccinia virus containing the envelope genes from clones 320.2a.7 and 16.2 failed to express the expected gp160/gp120 products and these *env* genes were subsequently reamplified from the same genomic DNA. Recombinant vaccinia virus containing the *env* genes from this second PCR now expressed the expected bands and these were used for all further experiments.

Radioimmunoprecipitation

For radioimmunoprecipitation (RIPA) HeLa cells were metabolically labeled for 8 hours with [^{35}S]methionine and [^{35}S]cysteine (ICN Biomedicals) 12 hours after infection with 10 plaque-forming units (PFU) of recombinant vaccinia virus per cell. Supernatant was collected and cells were lysed 5 hours after reconstitution of the medium with unlabeled methionine and cysteine. Subsequent immunoprecipitation was performed with polyclonal sheep anti-gp120 serum (kindly provided to us by Dr. M. Page through the MRC AIDS reagent program) as previously described.[37] The precipitated proteins were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% acrylamide), according to Laemmli.[38]

CD4 binding

Secreted envelope glycoproteins were metabolically labeled as described for RIPA. Supernatant was incubated with 150 ng soluble CD4 for 3 h at 4°C and subsequently immunoprecipitated with excess monoclonal antibody to CD4 (OKT4, Ortho Diagnostic Systems Inc.). The precipitated proteins were analyzed as described for RIPA.

Syncytium-forming assay

The MNC (3×10^5) from different donors were infected with recombinant vaccinia virus or wild-type vaccinia virus (wt) at a multiplicity of infection (MOI) of 30. Infections were allowed to proceed for 48 h, at which time the cultures were assayed for syncytia. Relative sizes and numbers of syncytia found per well were scored independently on coded samples by two independent researchers.

Sequence analysis

Sequence analysis of eight complete envelope genes was performed: four were sequenced by the dideoxynucleotide chain termination method[39] using T7 polymerase (Amersham) and four others were sequenced on an Applied Biosystems Inc. automated DNA sequencer by the Central European Facility for HIV genome analysis (Georg-Speyer-Haus, Frankfurt, Germany). The amplified 423 bp fragments were analyzed by direct DNA sequencing. For direct sequencing the PCR products were purified by Centricon 30 (Amicon) filtration, denatured with NaOH and ethanol precipitated after primer annealing (FUS1 or FUS2). The precipitated DNA was then sequenced using a modified T7 polymerase according to the protocol provided by the manufacturer (U.S. Biochemical). From each isolate unambiguous DNA sequences were obtained, even in the hypervariable regions,[40] confirming the clonality of these isolates. The sequence data were analyzed using the Lasergene (DNAstar) computer programs. All sequence comparisons were performed with the gap penalty set to zero.

Site-directed mutagenesis

Oligonucleotide-directed mutagenesis was performed on two envelope genes cloned in the filamentous phage M13 by the method described by Kunkel et al.[41] using supplies from Bio-Rad. Oligonucleotides 5'-CATAGCTCCTATCGTTC-CCACTGCTC-3' and 5'CATAGCTCCTATCATTCCCACT-GCTC-3' were used for mutating the fourth codon of gp41 coding region of the envelope gene of biological clones #16.1 and #16.3 from ATG to ACG and from ACG to ATG, respectively. The mutagenesis reaction changed the fourth amino acid residue of the fusion domain from methionine to threonine and reverse. The mutant envelope genes were partially resequenced for examination of the desired mutation.

RESULTS

Construction of recombinant vaccinia viruses that express env *genes derived from biological HIV-1 clones*

To examine the role and function of the envelope glycoproteins in determining the varying syncytium-inducing capacity of HIV-1, we constructed a collection of eight recombinant vaccinia viruses expressing the envelope genes of a set of biological HIV-1 clones. These biological HIV-1 clones were obtained from two patients; selected clones show similar replication rates in MNC (data not shown) but differ in their capacity to induce syncytia in MNC cultures (Table 1). The complete envelope genes of these eight clones were amplified by PCR using a set of primers introducing XmaI sites to facilitate cloning of the envelope genes downstream the P7.5 promoter of the vaccinia expression vector pSC11.[42]

Expression of HIV-1 envelope glycoproteins

Recombinant vaccinia virus-mediated expression of the HIV-1 envelope glycoproteins was demonstrated by RIPA. HeLa cells infected with recombinant vaccinia virus or wild-type vaccinia virus, were radiolabeled and the proteins were analyzed by immunoprecipitation followed by SDS–PAGE and autoradiography. Figure 1, panel A shows the recombinant vaccinia virus induced expression of gp160. Only in the lysates or recombinant vaccinia virus infected cells the precursor glycoprotein gp160 was precipitated by polyclonal sheep serum directed to the HIV-1 envelope protein. Under these labeling conditions the cleaved gp120 was also demonstrated in the lysates of most of the infected cell cultures (Fig. 1, panel A), and could easily be detected in the supernatants of all the recombinant vaccinia virus infected cell cultures (Fig. 1, panel B). In order to demonstrate the receptor-binding capacity of the expressed envelope proteins, gp120 present in supernatant was also coprecipitated with soluble CD4 (sCD4). Coprecipitation of gp120 by the monoclonal antibody OKT4 (directed to CD4) after incubation with sCD4, demonstrated that the eight expressed envelope proteins were all able to bind CD4 (results not

TABLE 1. SYNCYTIUM-INDUCING CAPACITY OF RECOMBINANT VACCINIA VIRUS EXPRESSED ENVELOPE GENE PRODUCTS IN RELATION TO THE PHENOTYPE OF THE PARENTAL BIOLOGICAL CLONES

Parental biological HIV-1 clones		*Syncytium-inducing capacity[c] of corresponding* env *genes expressed by rVV*
Clone #[a]	*Phenotype[b]*	
320.2a.5	SI	+++++[d]
.7	SI	+++++
.3	NSI	−
.6	NSI	−
16.1	SI	+++++
.2	SI	+++++
.3	NSI	−
.4	NSI	+

[a]The origin of each clone (and/or the corresponding recombinant vaccinia virus expressed envelope gene) is indicated as #xx.yy.z (or xx.z) where xx indicates patient code, yy the number of the particular isolate (if applicable), and z represents the clone number.

[b]Syncytium-inducing capacity of the parental biological HIV-1 clones is determined in MNC according to Tersmette et al.[3]

[c]Syncytium-inducing capacity of the recombinant vaccinia virus expressed gene products are determined in MNC.

[d]Symbols: −, no syncytia; +, <20 small syncytia per well; +++++, >200 large syncytia per well.

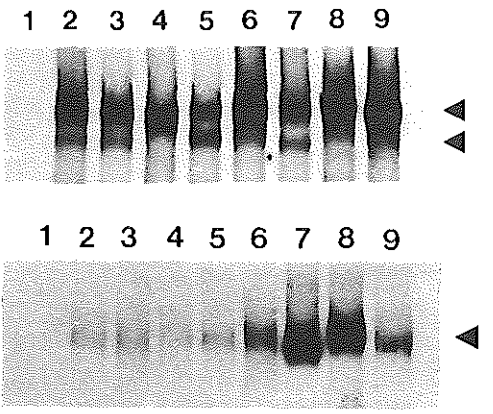

FIG. 1. Expression of HIV-1 envelope gene products by HeLa cells infected with recombinant vaccinia virus (or wt vaccinia virus). Metabolically labeled proteins from infected cell lysates (panel A) or from supernatant (culture medium) of infected cells (panel B) were immunoprecipitated with sheep polyclonal antiserum to gp120 and analyzed by SDS-PAGE. Lane 1, wt; lane 2, 320.2a.5; lane 3, 320.2a.7; lane 4, 320.2a.3; lane 5, 320.2a.6; lane 6, 16.1; lane 7, 16.2; lane 8, 16.3; lane 9, 16.4. See legend to Table 1 for description of the expressed envelope genes. Arrows indicate the precursor glycoprotein and the surface glycoprotein gp120 (panel A) or gp120 shed in the medium (panel B), respectively.

shown). This coprecipitation of gp120 was dependent on addition of soluble CD4, showing the specificity of the reaction.

Biological activity of the expressed envelope proteins

To determine the syncytium-forming capacity of the envelope proteins, human MNCs were infected with recombinant vaccinia virus or wild-type vaccinia virus. Formation of syncytia was assayed 48 h after infection. Syncytium induction by envelope genes expressed in MNCs through recombinant vaccinia viruses is shown in Figure 2. The results are summarized in Table 1. The syncytium-forming capacity of individual HIV-1 envelope genes expressed by recombinant vaccinia virus corresponds to that of the HIV-1 clones from which the envelope genes were derived. The syncytium-forming capacity of each envelope glycoprotein expressed in MNCs obtained from different donors yielded no significant differences.

Envelope gene sequence analysis

In order to define the genetic determinants of the syncytium-inducing property of HIV-1, the complete nucleotide sequence of the eight expressed envelope genes was determined. Figure 3 shows the predicted amino acid sequences of the amplified envelope genes with their origin (patient and biological clone) and the in vitro determined phenotype (SI or NSI). Comparison of these sequences revealed a substantial amount of amino acid variation located mostly in the hypervariable regions V1 through V5.[40] In these regions mutations as well as sequence length variations were observed. The overall conservation is 74.8%: 653 of the 873 predicted amino acid residues of the envelope glycoprotein are identical for all eight clones, mean amino acid homology is 88.9%. Envelope genes derived from biological clones obtained from the same patient display a higher degree of conservation: 90.2% en 95.0% for patients #320 and #16, respectively. Table 2 shows the percentage amino acid homology for all single pairs of envelope sequences. Envelope genes obtained from the same patient and displaying the same phenotype upon expression, show the highest degree of sequence homology; 95.6–99.0%.

Fusion domain variation

We have demonstrated previously that mutations in the SIV envelope fusion domain that affect the hydrophobicity of this region influence the syncytium forming capacity of the SIV envelope glycoproteins.[26] The predicted amino acid sequences of the four envelope glycoproteins obtained from patient #16 display a phenotype associated sequence variation at position number four of the fusion domain (Fig. 3). The SI envelope proteins share a methionine at this position (the fourth N-terminal amino acid residue of gp41, the fusion domain) whereas the NSI biological clone-derived envelope genes have a threonine at the same position. To examine if this mutation, which affects the hydrophobic character of the fusion domain, prevails in more HIV-1 isolates, we amplified and sequenced specifically an envelope gene fragment around the fusion domain of twelve additional biological clones. Figure 4 shows the predicted amino acid sequences with their origin (patient and isolate) and in vitro-determined phenotype of the parental biological clone (SI or NSI). This extended sequence analysis of the fusion domain

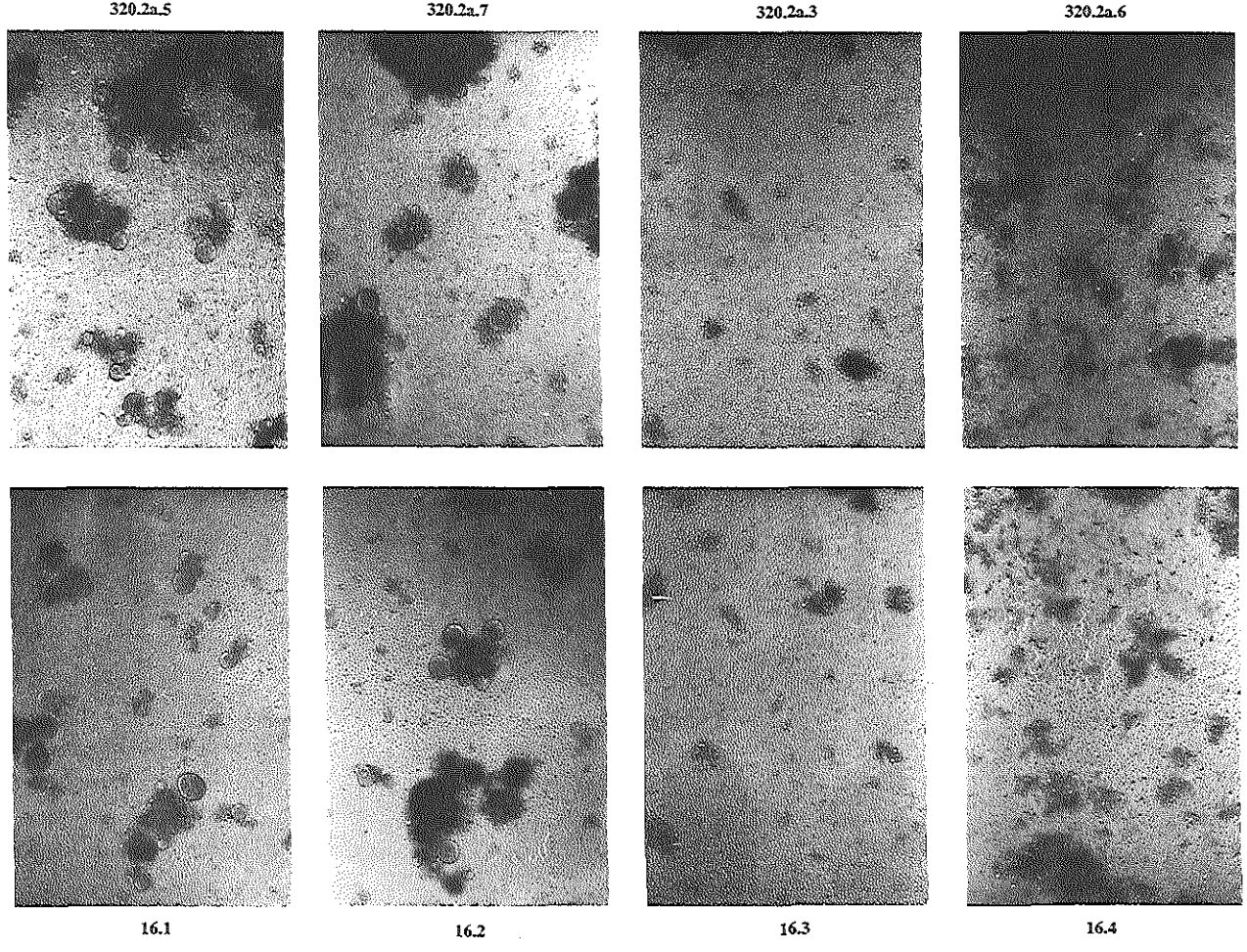

FIG. 2. Syncytia-induced in MNC upon infection with recombinant vaccinia viruses. Indicated are the biological HIV-1 clones which envelope genes are expressed by the recombinant vaccinia viruses (see legend of Table 1).

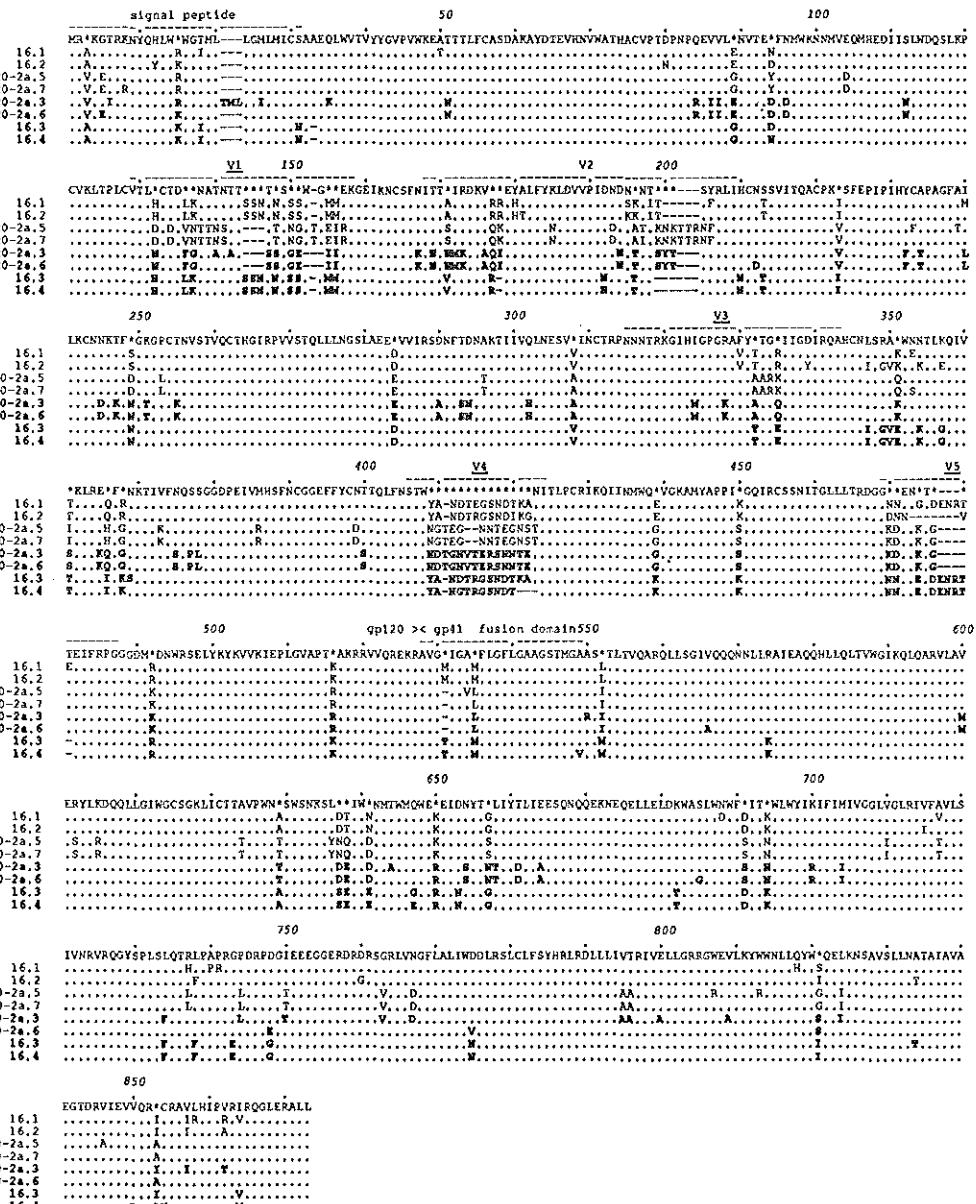

FIG. 3. Predicted amino acid sequence of eight envelope proteins. Sequences were obtained from PCR-amplified envelope genes derived from biological HIV-1 clones (codes as indicated in Table 1). The amino acid sequence is presented in the 1-letter amino acid code and alignment was performed using Clustal. In the top line a consensus sequence derived from these clones is given. Identity with the consensus sequence is indicated by (.), (−) indicates the absence of an amino acid residue at the particular position. The symbol * in the consensus sequence indicates that no consensus amino acid residue could be assigned for that position. Amino acid numbers are indicated as well as the signal peptide, the hypervariable regions V1 through V5, the gp120-gp41 cleavage site, and the fusion domain. Sequences obtained from NSI clone derived envelope genes are bold printed.

TABLE 2. SIMILARITY MATRIX OF EIGHT PREDICTED ENVELOPE AMINO ACID SEQUENCES

320.2a.5[a]	100[b c d]							
320.2a.7	**99.0**	100						
320.2a.3	86.1	**86.7**	100					
320.2a.6	86.1	86.6	**96.9**	100				
16.1	**86.6**	**87.1**	85.1	86.1	100			
16.2	**86.2**	**86.6**	85.0	86.2	**95.6**	100		
16.3	86.1	86.5	**84.8**	**86.3**	93.5	94.5	100	
16.4	86.5	86.9	**84.6**	**86.4**	93.2	93.9	**99.0**	100
	320. 2a.5	320. 2a.7	320. 2a.3	320. 2a.6	16.1	16.2	16.3	16.4

[a]Origin of envelope sequences as indicated in the legend of Table 1.

[b]Numbers represent percentage amino acid homology between each pair of sequences according to alignment of Figure 3 with gap penalty set to zero.

[c]Bold print is the observed amino acid variation occurring between HIV-1 biological clone envelope sequences displaying the same syncytium-inducing capacity.

[d]Shaded area represents observed amino acid variation between amino acid sequences obtained from the same patient.

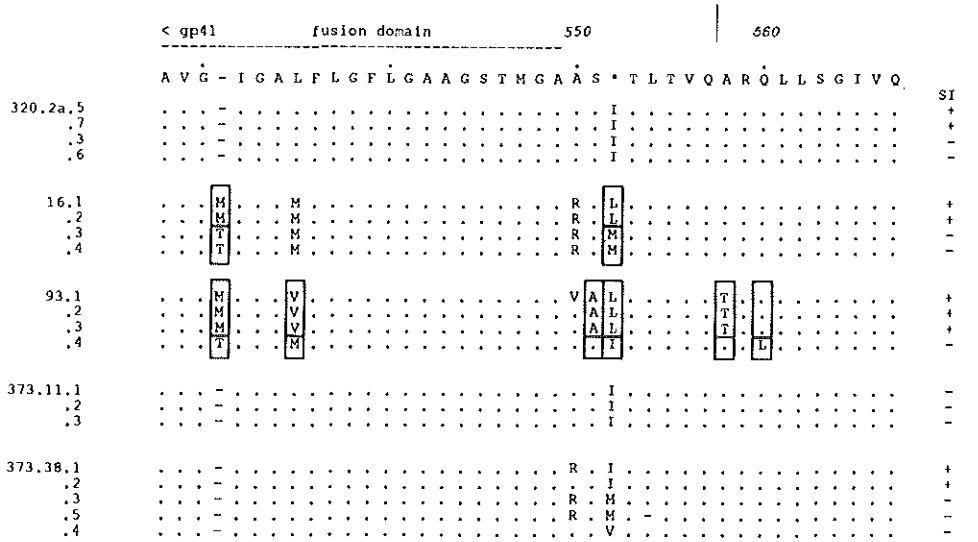

FIG. 4. Predicted amino acid sequences of the fusion domain containing N-terminal region of the transmembrane glycoprotein gp41 of 20 biological HIV-1 clones. In the top line a consensus sequence derived from these 20 clones is given. Codes indicate the biological HIV-1 clones from which the envelope sequences are obtained (see legend of Table 1). Identity with the consensus sequence is indicated by (.), (−) indicates the absence of an amino acid residue at that particular position. The symbol * in the consensus sequence indicates that no consensus amino acid residue could be assigned for that position. The phenotype of the clones is indicated: +:SI and −:NSI. Phenotype associated (SI vs. NSI) amino acid variations are boxed.

A

rVV #	parental phenotype	fusion domain amino acid sequence (first 11 residues)	syncytium inducing capacity
16.1	SI	N-Ala-Val-Gly-**Met**-Ile-Gly-Ala-Met-Phe-Leu-Gly-	+++++
16.3	NSI	N-Ala-Val-Gly-**Thr**-Ile-Gly-Ala-Met-Phe-Leu-Gly-	-
mutant 16.1	"SI"	N-Ala-Val-Gly-**Thr**-Ile-Gly-Ala-Met-Phe-Leu-Gly-	+++++
mutant 16.3	"NSI"	N-Ala-Val-Gly-**Met**-Ile-Gly-Ala-Met-Phe-Leu-Gly-	-

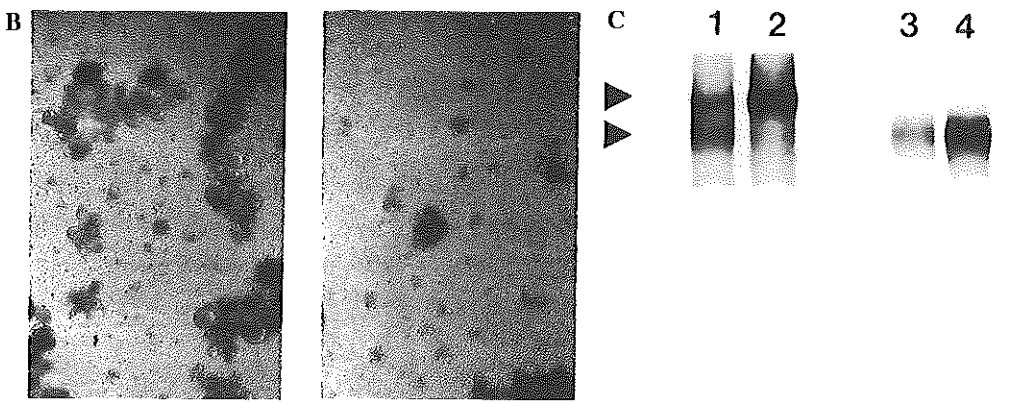

FIG. 5. *Panel A:* Syncytium-inducing capacity of parental and mutated envelope gene products. The first eleven amino acid residues at the amino terminus of gp41 are shown. Codes and symbols as indicated in table 1. *Panel B:* Syncytia induced in MNC upon infection with recombinant vaccinia virus expressing an envelope gene with a mutated fusion domain as indicated in panel A. *Panel C:* Expression of HIV-1 mutated envelope gene products by HeLa cells infected with recombinant vaccinia virus. Metabolically labeled proteins from infected cell lysates (lanes 1 and 2) or from supernatant (culture medium) of infected cells (lanes 3 and 4) were immunoprecipitated with sheep polyclonal antiserum to gp120 and analyzed by SDS-PAGE. Lane 1, mutant 16.1; lane 2, mutant 16.3; lane 3, mutant 16.1; lane 4, mutant 16.3. Arrows indicate the precursor glycoprotein and the surface glycoprotein gp120, respectively.

revealed that the in patient #16 observed phenotype associated sequence variation also occurred in patient #93 but not in patients #320 and #373 (both isolates).

Functional study of the observed phenotype-associated fusion domain variation

To assess the functional contribution of the observed phenotype associated fusion domain variation to the phenotype of HIV-1 isolates, the methionine/threonine mutation was inversely introduced in the envelope glycoproteins originally derived from biological clones #16.1 and #16.3 as shown in Figure 5 panel A. When expressed through recombinant vaccinia virus, these mutated envelope genes did not display an altered syncytium-forming capacity (Fig. 5 panels A and B). Figure 5, panel C shows that these mutated envelope glycoproteins were expressed properly. A coprecipitation with sCD4 demonstrated that these mutated gp120 molecules were still able to bind the CD4 receptor (results not shown).

DISCUSSION

In this study we determined the syncytium-inducing capacity and the nucleotide sequence of envelope genes derived from a set of phenotypically well-characterized biological HIV-1 clones obtained from two patients.

The envelope gene product of lab-adapted syncytium-inducing HIV-1 isolates like HIV-1 IIIB has been shown to induce syncytia when expressed in heterologous expression systems which demonstrates that the envelope glycoproteins of HIV-1 are by themselves sufficient to induce syncytia.[6,7] It is not known whether the lack of syncytium-inducing capacity of NSI field isolates must be attributed to functional differences between the envelope proteins of SI and NSI isolates or whether determinants located outside the envelope gene (e.g., regulatory genes, LTR, or otherwise) modulate this syncytium-inducing capacity. A limited number of studies using recombinant HIV-1 proviruses have implicated the envelope gene products as the major culprit in determining biological variation of HIV-1,[8–10] although evidence pointing to other viral genes has also been presented.[11,43]

We have expressed the complete envelope genes of eight biological clones of HIV-1 (4 SI and 4 NSI clones) to determine their SI capacity in activated human MNC. The results show that only the envelope gene products obtained from SI clones readily induce large syncytia when expressed by recombinant vaccinia virus whereas those obtained from NSI clones did not, despite the fact that they are functionally intact as demonstrated by their ability to bind sCD4. Only the NSI clone 16.4-derived gene product induced some small syncytia in MNC culture but always much less than was observed for the SI clone derived envelope genes (Table 1). This observation formally proves that envelope gene variation alone is sufficient to explain variable syncytium inducing capacities of HIV-1 isolates. For the eight HIV-1 clones presented here genetic variation outside the envelope gene need not to be invoked to explain the viral phenotypic variation.

In addition to the phenotype we also determined the nucleotide sequence and thus the predicted amino acid sequence of the eight expressed envelope genes. The predicted protein sequences exhibited considerable amino acid variation which is mainly located in the previously denominated variable regions. Mean amino acid homology is 88.9% (range 84.6–99.0%; Table 2). Mean amino acid variation of envelope sequences obtained from the same individual was less, 90.2% and 95.0% homology for patient #320 and #16, respectively. Envelope glycoprotein sequences derived from biological clones obtained from the same individual with the same phenotype with regard to syncytium induction, exhibited the least variation (95.6%–99.0% homology; Table 2). This is a reflection of the presence of many amino acid positions displaying phenotype-associated variation between sequences with different phenotypes but obtained from the same patient. This phenotype-associated amino acid variation can be found throughout the whole sequence (e.g., in the V1 and V2 regions, in the V3 region which plays a role in the fusion process and which is the major determinant in HIV-1 neutralization, in the CD4 binding region, in the fusion domain, and in the external region of gp41). None of these variations appear to be conserved between the biological clones from the two patients (#16 and #320), at least not with regard to the exact sequences. At one position in the V3 region (amino acid 21), however, we observe consistent variation concerning charged amino acids. This change in particular could have functional consequences based on observations by others that charge density of the V3 region be important for fusion.[44,45] These and other mutations that could induce structural changes in gp120 or gp41 are of particular interest for further studies, since the lack of exact sequence conservation could mean that structure of individual regions of the envelope protein is more important than sequence, at least in determining biological properties. Alternatively, it might indicate that in different sets of envelope genes different determinants control biological variation. It is presently unclear whether in different viral populations (e.g., obtained from different patients) the biological variation of HIV-1 is controlled by the same determinants. In addition, much of the phenotype-associated amino acid variation may be the result of the presence of different viral populations distinguished by phenotypic properties other than their syncytium inducing capacity alone (e.g., cytotropism). Such shared or linked phenotypic properties will complicate the elucidation of the genetic basis of the separate phenotypic properties (e.g., the syncytium-inducing capacity in the present comparative study).

The 23 cysteine residues appeared to be highly conserved throughout these eight expressed envelope glycoproteins. Only the envelope gene obtained from clone 320.2a.5 codes for a phenylalanine at position #233 and the envelope gene of clone 16.4 codes for a tyrosine at position #854, whereas all other genes code for a cysteine at these positions (Fig. 3). The cysteine at position #854 of gp41, however, is not conserved over other known HIV-1 envelope sequences, a tyrosine at this position is frequently found.[46] In contrast to the cysteine at position #854, the cysteine at position #233 appeared to be highly conserved among HIV-1 sequences.[46] Interestingly the envelope gene product of clone 320.2a.5 which does not contain this cysteine residue does induce syncytia. Reexamination of this region by direct sequencing of a PCR-derived envelope gene fragment could not confirm the presence of this phenylalanine in the genomic DNA of MNC infected with HIV-1 clone 320.2a.5 (data not shown) which implies that it may have arisen during the envelope cloning procedure (e.g., during PCR amplification). This amino acid switch however, does not abolish CD4 binding or the syncytium-inducing capacity of the envelope glycoprotein, illustrating that the cysteine at position #233 is not crucial for these biological properties of the envelope protein.

Eight out of 20 analyzed envelope genes show amino acid variation at two positions in the fusion domain. The variation at position #531 affects the hydrophobic character of the fusion domain whereas the variation at position #535 does not (Fig. 4). Mutational studies with SIV_{mac} indicated that changing the hydrophobicity of the fusion domain could significantly modulate the fusogenic potential of the envelope protein.[26] Although the sequence analysis of the extended set of envelope genes revealed only phenotype-associated amino acid variation in some of the envelope genes, we assessed the functional contribution of this variation by inversely introducing the methionine/threonine mutation. When expressed by recombinant vaccinia virus, these mutated envelope genes do not display an altered syncytium-forming capacity, showing that this fusion domain variation does not contribute to the variation in the syncytium-inducing capacities of this subset of studied envelope genes.

The expression of chimeras of the here studied genetically highly related genes, together with site-directed mutagenesis studies, should allow the identification of the specific domains within the envelope protein that control the variable syncytium-inducing capacity of HIV-1.

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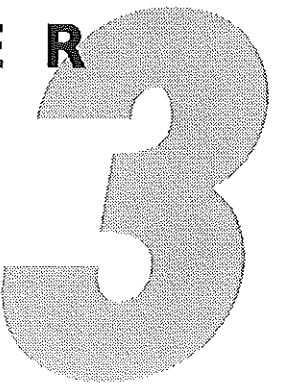
Address reprint requests to:
Marnix L. Bosch
Laboratory of Immunobiology
National Institute of Public Health and
Environmental Protection
P.O. Box 1
3720 BA Bilthoven, the Netherlands

CHAPTER



Phenotype Associated env Gene Variation among Eight Related Human Immunodeficiency Virus Type 1 Clones: Evidence for In Vivo Recombination and Determinants of Cytotropism outside the V3 Domain

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Phenotype-Associated *env* Gene Variation among Eight Related Human Immunodeficiency Virus Type 1 Clones: Evidence for In Vivo Recombination and Determinants of Cytotropism outside the V3 Domain

MARTIJN GROENINK,[1]* ARNO C. ANDEWEG,[2] RON A. M. FOUCHIER,[1] SILVIA BROERSEN,[1] ROBERT C. M. VAN DER JAGT,[1] HANNEKE SCHUITEMAKER,[1] RUUD E. Y. DE GOEDE,[1] MARNIX L. BOSCH,[2] HAN G. HUISMAN,[1] AND MATTHIJS TERSMETTE[1]

Central Laboratory of The Netherlands Red Cross Blood Transfusion Service and Laboratory For Experimental and Clinical Immunology of the University of Amsterdam, Amsterdam,[1] and Laboratory of Immunobiology, National Institute of Public Health and Environmental Protection, Bilthoven,[2] The Netherlands

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The nucleotide sequences of the *env* genes of eight phenotypically heterogeneous human immunodeficiency virus type 1 (HIV-1) clones recovered from a single individual within a 3-week period were compared. In addition, the accessory gene sequences for four of these clones were obtained. Variation among most accessory genes was limited. In contrast, pronounced phenotype-associated sequence variation was observed in the *env* gene. At least three of these clones most likely resulted from genetic recombination events in vivo, indicating that this phenomenon may account for the emergence of proviruses with novel phenotypic properties. Within the *env* genes of the eight clones, four domains could be defined, the sequence of each of which clustered in two groups with high internal homology but 11 to 30% cluster variation. The extensive *env* gene variation among these eight clones could largely be explained by the unique manner in which the alleles of these four domains were combined in each clone. Experiments with chimeric proviruses demonstrated that the HIV-1 *env* gene determined the capacity to induce syncytia and tropism for T-cell lines. Amino acids previously shown to be involved in gp120-CD4 and gp120-gp41 interaction were completely conserved among these eight clones. The finding of identical V3 sequences in clones differing in tropism for primary monocytes and T-cell lines demonstrated the existence of determinants of tropism outside the *env* V3 region.

Human immunodeficiency virus type 1 (HIV-1) isolates possess distinct biological properties with respect to cytotropism, replication rate, and capacity to induce syncytia (4, 7, 30, 31). Previously, we have demonstrated that from stable asymptomatic individuals, only non-syncytium-inducing (NSI) HIV-1 variants which cannot be transmitted to the H9 or MT-2 T-cell lines can be recovered. In contrast, the detection of syncytium-inducing (SI) T-cell-line-tropic HIV-1 variants is strongly associated with rapid CD4 T-cell depletion and progression to AIDS (20, 31).

Recently, several studies have identified mechanisms and regions of the HIV-1 genome which control biological variation of HIV-1. We and others have shown that differences in cytotropism are determined at an early level of the virus replication cycle prior to provirus formation, which suggests involvement of the *env* gene (3, 13, 27). Studies with chimeric proviruses have formally identified the *env* gene as the major determinant responsible for differences in biological properties such as cellular tropism and cytopathogenicity (24, 28).

We previously described the generation of a panel of phenotypically distinct yet genetically highly homologous infectious molecular HIV-1 clones (13). These molecular clones were derived from HIV-1 isolates, mostly recovered by direct clonal isolation, from a single individual who developed AIDS within half a year after seroconversion. The clones were obtained from patient peripheral blood mononuclear cells collected at about the time of transition from an NSI to an SI HIV-1 phenotype. All eight clones were able to replicate in peripheral blood lymphocytes. The molecular and biological HIV-1 clones exhibited differences in SI capacity and in tropism for T-cell lines and monocyte-derived macrophages (13, 27). To genetically compare phenotypically heterogeneous HIV-1 variants and to analyze regions of the HIV-1 *env* gene previously identified as determinants of biological properties, we analyzed eight envelope sequences obtained from four infectious molecular HIV-1 clones (13) and from four biological HIV-1 clones through polymerase chain reaction amplification (1). In addition, we determined the sequences of the accessory genes of the four molecular clones. The biological phenotypes of the four molecular HIV-1 clones and the four biological clonal HIV-1 variants are shown in Table 1. Clones 320.2A 6, 320.2A 3, 320.2A 2.1, 320.2A 1.1, 320.2A 1.2, 320.2A 5, 320.2A 7, and 320.3 1 will be referred to as clones 6, 3, 2.1, 1.1, 1.2, 5, 7, and 1, respectively.

Although the SI HIV-1 clones differed in their abilities to replicate in the H9 and Sup T1 T-cell lines, all SI clones productively infected the MT-2 T-cell line, whereas NSI HIV-1 clones did not infect the MT-2 cell line (Table 1). This finding is in accordance with the strong correlation between the abilities of field isolates to induce syncytia and tropism for the MT-2 cell line (20). SI capacity also appeared to be required, although not sufficient, for H9 tropism (Table 1). In contrast, Sup T1 tropism was also observed for NSI clones, indicating that entry in Sup T1 cells is at least partially

* Corresponding author.

TABLE 1. Biological phenotypes of biological clonal HIV-1 variants and molecular HIV-1 clones derived from a single individual

Patient PBMC[a] sample	Clone no.[b]		PBL SI[c]	Transmission to:						MDM for replication[d]
	Biological	Molecular		MT-2		H9		Sup T1		
				Replication[e]	SI	Replication	SI	Replication	SI	
320.2A	6		−	−	−	−	−	+	−	NT
	3		−	−	−	−	−	−	−	NT
	2	1	−	−	−	−	−	++	−	+
	1	1	+	++	+	−	−	−	−	−
		2	(+)	++	+	−	−	−	−	−
	5		+	++	+	NT		+	+	NT
	7		+	++	+	+	+	+	+	NT
320.3		1	+	++	+	++	+	++	+	+

[a] PBMC, peripheral blood mononuclear cell.
[b] Biological clonal HIV-1 isolates were obtained from patient PBMC sample 320.2A, and molecular clones were derived from biological clonal HIV-1 isolates, except for clone 1.
[c] PBL, peripheral blood lymphocyte; −, no syncytia observed; (+), reduced SI capacity; +, SI.
[d] MDM, monocyte-derived macrophages; −, not transmissible; +, transmissible; NT, not tested.
[e] −, not transmissible; +, delayed growth kinetics; ++, readily transmissible; NT, not tested.

independent of the mechanisms involved in syncytium formation. Compared with clones 2.1 and 1, clones 5 and 7 exhibited delayed growth kinetics in the H9 and Sup T1 T-cell lines. In contrast to NSI Sup T1-tropic clone 2.1, NSI clone 6 replicated less efficiently in the Sup T1 cell line.

The alignments of the deduced amino acid sequences of the accessory genes are shown in Fig. 1. Except for the *vpu* gene of clone 1, which did not contain a methionine start codon, the reading frames of all sequenced genes encoded full-length proteins. In contrast to the other three molecular clones, which were obtained from biologically clonal infected peripheral blood lymphocyte cultures, clone 1 was obtained from infected H9 cells (13). A nonfunctional *vpu* gene is often found in molecular HIV-1 clones derived from cell lines (23). The maximum amino acid sequence variability for the separate HIV-1 genes is shown in Table 2. The *vif*, *vpr*, and *nef* genes showed only minor amino acid variation (1.0, 1.0, and 1.9%, respectively), confirming the genetic relationship of these clones (Table 2 and Fig. 1A, B, and F). In contrast, the *tat*, *rev*, and *vpu* genes showed a maximum amino acid variability of 7.9, 10.2, and 8.6%, respectively (Table 2). The amino acid variability in the *tat* gene was concentrated in the second exon (exon 1, 4.2%; exon 2, 17.2%) of this regulatory gene, which is located within the *env* gene. This amino acid variation, however, was not phenotype associated. In spite of the prominent amino acid variation in the *tat* and *rev* genes, the *tat* and *rev* proteins were completely homologous in regions thought to be critical for their functions (Fig. 1C and D) (9, 12, 14, 17, 22, 26). This is compatible with the finding that the clones did not exhibit significant differences in replication rate in peripheral blood lymphocytes (13). Although the sequence variability in the *vpu* gene was completely restricted to NSI clone 2.1, the absence of the initiator methionine codon of the *vpu* gene in SI clone 1 indicates that the *vpu* gene is not required for syncytium induction or for replication in T-cell lines and primary monocytes (Fig. 1E and Table 1).

Alignments of the deduced amino acid sequences of the *env* genes are shown in Fig. 2. Given the fact that all clones except clone 1 were derived from the same time point, the level of variation (up to 14.9% [Table 2]) was surprisingly high. Closer inspection, however, revealed the existence of four distinct domains within the *env* gene (termed A through D in Fig. 3). For each separate domain, the sequences of the eight clones fell apart into two distinct clusters (intercluster variation, 11 to 30%) with high internal similarities (96 to 100%). In each of the eight clones, the alleles of these four domains were combined in a unique manner, implying that exchange of genetic material had occurred. In particular, clones 1.1, 1.2, and 2.1 appeared to result from recombination events between a clone similar to clone 6 or 3 and a clone similar to clone 5 or 7 (Fig. 2 and 3). Clone 2.1 possessed striking sequence similarity to clones 6 and 3 between amino acids (aa) 87 and 426 (domain A in Fig. 3) and to clones 5 and 7 between aa 653 and 705 and aa 852 and 863 (domains C and D in Fig. 3). Clones 1.1 and 1.2 are highly similar to clones 5 and 7 between aa 87 and 426 (domain A in Fig. 3), to clones 6 and 3 between aa 599 and 738, and to clone 3 between aa 852 and 863 (Fig. 2 and 3). The transitions of sequence similarity between the HIV-1 variants were also observed at the nucleotide level (data not shown). Previously, it has been demonstrated that genetic recombination events between HIV-1 proviruses can occur in vitro (5). Since the *env* genes studied belonged to single-clone HIV-1 isolates recovered directly from patient peripheral blood mononuclear cells under limiting dilution conditions, these findings imply that recombination events occur in vivo as a mechanism to generate clonal diversity.

The *env* gene has been identified as the major determinant responsible for differences in biological properties (24, 28). To investigate whether in this set of molecular HIV-1 clones the *env* gene is involved in determining differences in the capacity to induce syncytia and cytotropism, chimeric proviruses were constructed. In the chimeric provirus 320.2A 1.2 *env* 2.1, an *Nsi*I-*Xho*I fragment of clone 2.1 coding for amino acid residues 108 to 873 of the *env* gene and amino acid residues 1 to 43 of the *nef* gene was exchanged for the corresponding fragment of the Sup T1-tropic NSI clone 2.1. In the chimeric provirus 320.2A 1.2 *env* 1, the same fragment was replaced by the corresponding fragment of the H9- and Sup T1-tropic SI clone 1. Construction of the chimeric proviruses and determination of the biological properties of these constructs were performed as described elsewhere (13, 28). The finding that these two chimeric proviruses, with variations only within the *env* coding sequence, were different in their SI capacities and tropisms for the MT-2 and H9 T-cell lines provided proof that these phenotypic traits are conferred by determinants in the *env* gene (Table 3).

Comparison of the biological phenotypes of the eight clones and their differences in *env* gene sequence composi-

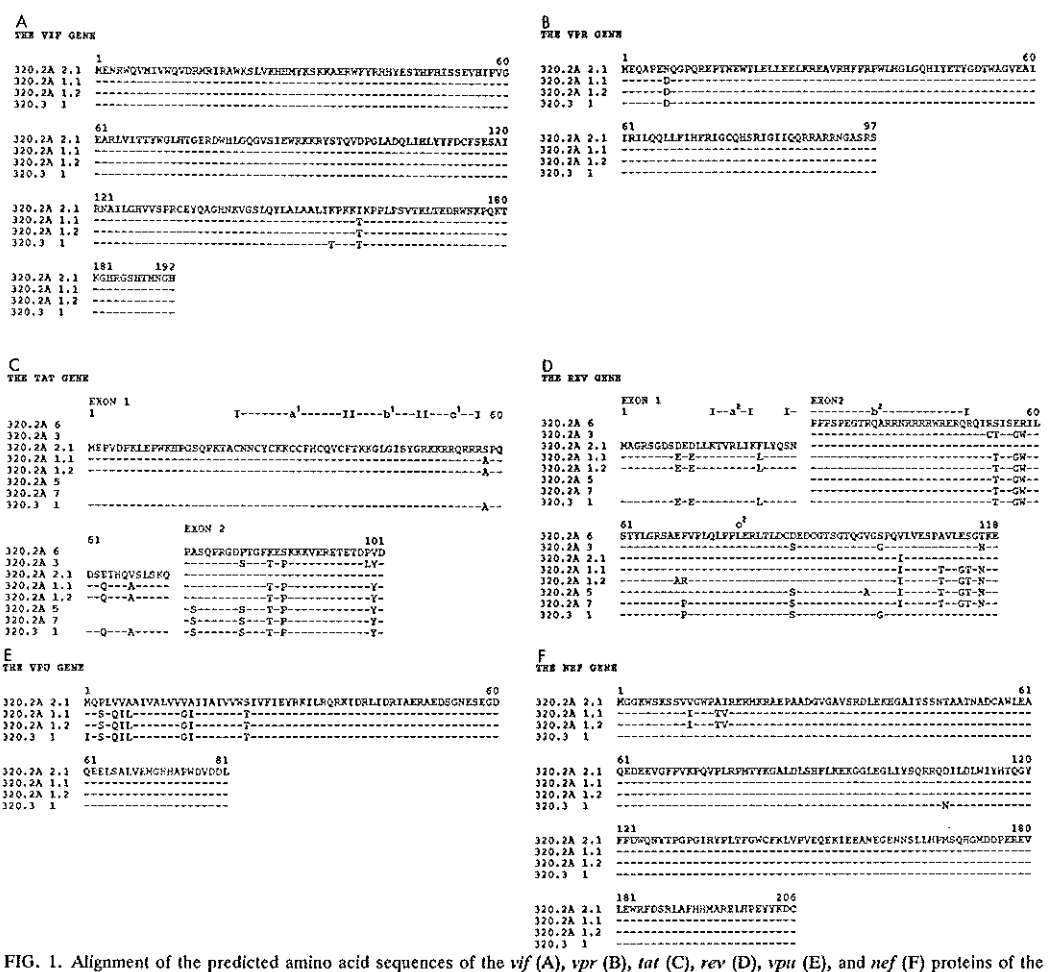

FIG. 1. Alignment of the predicted amino acid sequences of the *vif* (A), *vpr* (B), *tat* (C), *rev* (D), *vpu* (E), and *nef* (F) proteins of the molecular HIV-1 clones 2.1, 1.1, 1.2, and 1. For the biological clonal HIV-1 variants 6, 3, 5, and 7, the amino acid sequences of the second exons of the *tat* (C) and *rev* (D) genes are shown. Sequence analysis was performed by the dideoxy chain termination method using Sequenase (USB). Amino acid alignments were performed by using CLUSTAL (16). The predicted amino acid sequences are presented in the one-letter amino acid code. Amino acid numbers are indicated. Dashes indicate amino acid identities, whereas points indicate gaps. Functional regions of the *tat* and *rev* proteins are indicated. a^1, cysteine-rich region and proposed metal-binding region, which may mediate protein-protein interaction; b^1, possible activation domain; c^1, nucleolar localization signal and putative nucleic acid-binding region; a^2, region involved in nuclear localization and transactivation; b^2, nuclear localization signal possibly involved in binding to the *rev* responsive element; c^2, region near amino acid residues 78 and 79 involved in transactivation.

tion revealed a remarkable association between particular phenotypic traits and each of the four domains described above. On the basis of their sequences in the gp120 C1-V4 region (domain A in Fig. 3), the clones clustered into two groups, each with >98.5% internal homology, with one containing all SI MT-2-tropic clones and the other comprising all NSI non-MT-2-tropic clones. On the other hand, all H9-tropic clones clustered in the same group for domains A, C, and D, in contrast to all non-H9-tropic clones. In addition, clone 1, replicating most efficiently in H9 cells, had a unique sequence between 426 and 535 (domain B). The Sup T1-tropic clones 2.1, 5, 7, and 1 clustered in domains C and D. Clone 6, which replicated inefficiently in Sup T1 cells, clustered only in domain D with clones 2.1, 5, 7, and 1. Finally, clones that could not be transmitted to H9 or Sup T1 cells (3, 1.1, and 1.2) clustered in domains B, C, and D (100% similarity in all three regions), regardless of their abilities to induce syncytia or their MT-2 tropisms (Fig. 3). This indicates that the C1-V4 region of the gp120 molecule is responsible for differences in the capacity to induce syncytia and tropism for the MT-2 T-cell line, whereas determinants of tropism for the H9 and Sup T1 T-cell lines are located

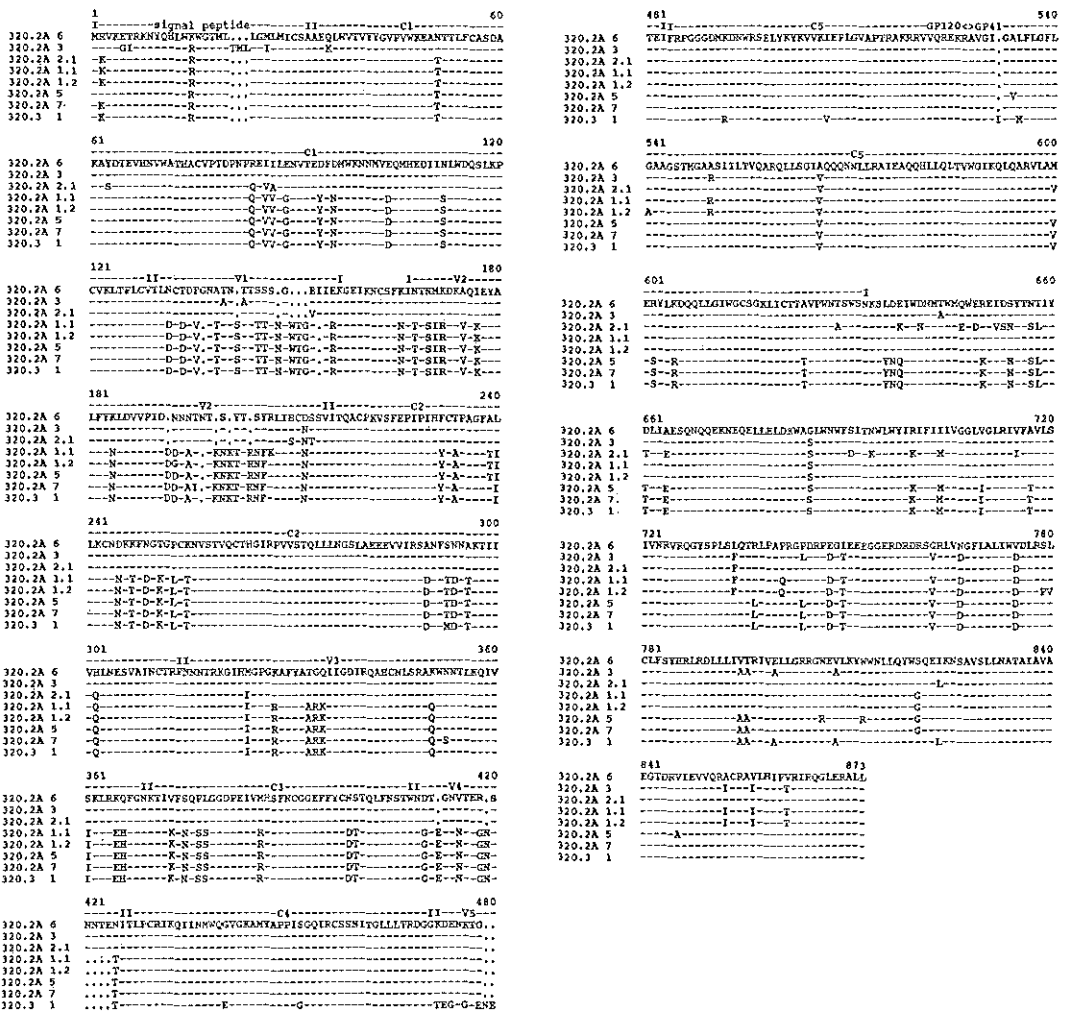

FIG. 2. Alignment of the *env* proteins of molecular and biological clonal HIV-1 variants 6, 3, 2.1, 1.1, 1.2, 5, 7, and 1. Sequence analysis was performed by the dideoxy chain termination method using Sequenase (USB). Amino acid alignments were performed by using CLUSTAL (16). Final alignments were adjusted by the eye. The predicted amino acid sequences are presented in the one-letter amino acid code. Amino acid numbers are indicated. Dashes indicate amino acid identities, whereas points indicate gaps. The signal peptide, hypervariable regions (V), constant regions (C), and gp120-gp41 proteolytic cleavage site are indicated.

downstream of aa 426 in the C-terminal portions of the gp120 and gp41 molecules. Although there is an association between particular phenotypic traits and the overall patterns of amino acid sequence arrangements in the four domains described, it remains possible that single amino acid alterations are responsible for the observed phenotypes. However, this possibility is heard to reconcile with the observed stability of recessive phenotypic traits of these clones in vitro.

Previous studies have identified regions of the gp120 glycoprotein involved in CD4 binding and association with the gp41 transmembrane glycoprotein. Amino acid residues Thr-272 and Trp-439 in hydrophobic domains of the C2 and C4 regions and amino acid residues Asp-381, Glu-383, and Asp-469 in hydrophilic domains of the C3 and C4 regions appear to be important for CD4 binding (25). Amino acid residues Val-38, Tyr-42, Trp-47, Ile-507, and Lys/Ala-516/517 located at the amino and carboxyl termini of the gp120 molecule contribute to the noncovalent association with the gp41 molecule (15, 19). The corresponding residues as well as the residues directly adjacent were completely conserved among the eight clones, except in clone 1, which possessed

TABLE 2. Maximum amino acid variability present in accessory and *env* genes of phenotypically divergent HIV-1 variants

Gene	Variability[a] (%)
vif	1.0
vpr	1.0
tat	7.9
rev	10.2
vpu	8.6
env	14.9
nef	1.9

[a] Variability was determined for the accessory genes of the four molecular HIV-1 clones and the *env* genes of the four molecular HIV-1 clones and the four biological HIV-1 clones.

TABLE 3. Biological properties of wild-type and chimeric HIV-1 proviruses

Molecular clone	SI in PBL[a]	Transmission to[b]:		
		MT-2	Sup T1	H9
2.1	–	–	+	–
1.2	(+)	+	–	–
1	+	+	+	+
1.2 *env* 2.1[c]	–	–	+	–
1.2 *env* 1[c]	+	+	+	+

[a] PBL, peripheral blood lymphocyte; –, no syncytia observed; (+), reduced SI capacity; +, SI.
[b] –, not transmissible; +, transmissible.
[c] aa 106 to 873 of the HIV-1 *env* gene and the first 34 aa residues of the HIV-1 *nef* gene were exchanged.

a Val instead of an Ile at position 507. Moreover, all clones exhibited an Arg instead of a Lys at position 516 (Fig. 2).

The amino terminus of gp41, which resembles the fusion peptides of ortho- and paramyxoviruses (11), has been proposed as a determinant of SI capacity (2, 21). However, the sequence identity between NSI clones 6 and 2.1 and SI clones 1.1 and 7 excludes the possibility that this region is the sole determinant of syncytium formation.

Recent studies indicate that the V3 loop not only is involved in type-specific neutralization but also might be a determinant of biological properties such as cytopathic properties and cellular tropism. Amino acid changes in the tip of the V3 loop affect the capacity to induce syncytia and alter cellular tropism (6, 10, 29). A high degree of sequence variation within the V3 loop was observed between the groups of NSI and SI clones. Recently, we have shown that SI HIV-1 variants contain V3 sequences with significantly

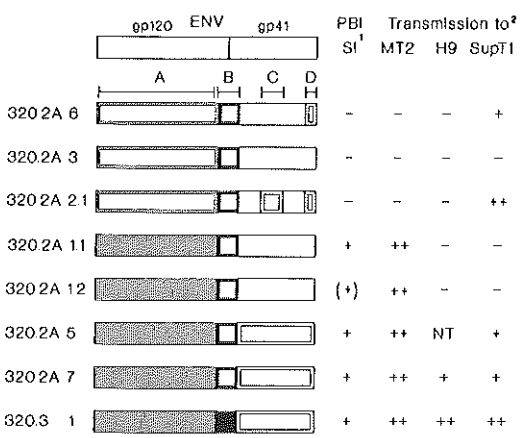

FIG. 3. Amino acid sequence similarity in *env* gene glycoproteins of the eight phenotypically heterogeneous HIV-1 variants 6, 3, 2.1, 1.1, 1.2, 5, 7, and 1. Sequence similarities are indicated by identical patterns. The minimum sequence similarities in regions with identical patterns are 98.5% for domain A (aa 87 to 426), 100% for domain B (aa 426 to 535), 96% for domain C (aa 653 to 705), and 100% for domain D (aa 852 to 863). Biological phenotypes of the corresponding molecular and biological clonal HIV-1 variants are indicated. [1]–, no syncytia observed; (+), reduced SI capacity; +, SI. [2]–, not transmissible; +, delayed growth kinetics; ++, readily transmissible; NT, not tested.

higher positive charges than those of NSI HIV-1 variants (8). In the highly variable V3 domain, two amino acid residues located on either side of the V3 loop are responsible for these differences in charge. In NSI HIV-1 variants, these two residues are negatively charged or uncharged, whereas in SI HIV-1 variants, one or both are positively charged. This correlation is also observed in the presently studied NSI and SI HIV-1 variants (Fig. 2, aa 321 and 335). Experiments with chimeric proviruses suggested that the V3 loop is also a determinant for tropism for T-cell lines and primary monocytes (18, 24, 28, 32). However, the SI HIV-1 variants analyzed in the present study, which differ from each other in their tropisms for the H9 and Sup T1 T-cell lines and primary monocytes, possessed completely identical V3 regions (Fig. 2).

Analysis of these eight HIV-1 clones demonstrated extensive phenotype-associated sequence variation dispersed nonrandomly over the *env* gene. The observed considerable genetic distances between clones with different phenotypes may explain the stability of viral phenotypes even after prolonged propagation in vitro. Four clones with intermediate phenotypes most likely were the products of genetic recombination events in vivo, indicating that this mechanism, next to mutations resulting from errors during reverse transcription, may account for the emergence of proviruses with novel phenotypic properties during the course of the HIV-1 infection (31).

In agreement with previous reports (24, 28), the *env* gene appeared to be the major determinant responsible for differences in biological properties. The amino acids previously shown to be involved in gp120-CD4 binding and gp120-gp41 association and their directly surrounding residues were completely conserved among these eight clones. This argues against variation in gp120-CD4 binding affinity or variation in gp120 shedding as a cause of differences in the capacity to induce syncytia or cytotropism.

Sequence variation associated with SI capacity and tropism for the MT-2 T-cell line was concentrated in the C1-V4 region of the gp120 glycoprotein. In contrast, sequence variation associated with tropism for the H9 and Sup T1 T-cell lines was completely limited to the gp120-V5 region and the gp41 glycoprotein. In contrast to observations with other HIV-1 clones, the V3 region of the gp120 molecule of these clones appeared not to be critical for their tropisms for primary monocytes and the H9 and Sup T1 T-cell lines. This finding demonstrates the existence of additional determinants for cytotropism next to the V3 loop, most probably located within the gp120-V5 region and the gp41 molecule.

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CHAPTER 4

Both the V2 and V3 Regions of the Human Immunodeficiency Virus Type 1 Surface Glycoprotein Functionally Interact with Other Envelope Regions in Syncytium Formation

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Both the V2 and V3 Regions of the Human Immunodeficiency Virus Type 1 Surface Glycoprotein Functionally Interact with Other Envelope Regions in Syncytium Formation

ARNO C. ANDEWEG, PAULA LEEFLANG, ALBERT D. M. E. OSTERHAUS, AND MARNIX L. BOSCH*

Laboratory of Immunobiology, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands

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To map the regions of the external envelope glycoproteins of human immunodeficiency virus type 1 (HIV-1) involved in the process of membrane fusion, we determined the syncytium-inducing capacity of a panel of transiently expressed chimeric envelope genes. This panel was generated by exchanging gene fragments between four previously studied envelope genes that exhibited a high degree of sequence homology yet displayed marked differences in syncytium-inducing capacity when expressed by recombinant vaccinia virus. The results demonstrate that multiple regions of the HIV-1 envelope glycoproteins are involved in syncytium formation. Some fragments, most notably those containing the V2 or V3 region, can transfer syncytium-inducing capacity to envelope proteins previously not capable of inducing syncytia. Moreover, it is shown that such regions functionally interact with other envelope regions, especially one encompassing the V4 and V5 regions of gp120 or a region encompassing part of gp41, to exert their function in membrane fusion.

Human immunodeficiency virus type 1 (HIV-1) enters the cell through a multistep process. Binding of the viral external glycoprotein gp120 to the cellular receptor CD4 ultimately results in fusion of the viral envelope and the cellular membrane (6, 22). A similar fusion process between HIV-1-infected cells expressing viral envelope glycoproteins and (uninfected) cells expressing CD4 may lead to the formation of syncytia (23, 29).

HIV-1 isolates differ in their capacity to induce syncytia in vitro. On the basis of both this capacity and other in vitro biological properties, such as replication rate and cytotropism, syncytium-inducing (SI) and non-SI (NSI) HIV-1 isolates are distinguished (35). These two categories largely overlap with the "rapid-high" and "slow-low" viruses, respectively, described by others (10). The isolation of HIV-1 variants with an SI phenotype correlates with the subsequent rapid decline of $CD4^+$ cell numbers and progression to AIDS (4, 21, 36), which suggests that the emergence of viruses with a cytopathic SI phenotype plays a role in the pathogenesis of AIDS. Dissection of the process of membrane fusion that leads to syncytium formation may contribute to our understanding of its role in HIV-1 biology and pathogenesis.

HIV-1 envelope glycoproteins contain all of the viral information required for syncytium formation (22, 34), and several of the determinants that mediate the underlying membrane fusion process have been identified. Genetic variation in such determinants could theoretically result in differences in SI capacity between different HIV-1 envelope genes. For efficient binding to the cellular receptor CD4, an intact CD4-binding region is needed. Amino acid residues involved in CD4 binding are scattered among at least four different regions of gp120 (26). The potential effects of variation in these regions on syncytium formation has not yet been studied in detail. Syncytium formation by both simian immunodeficiency virus and HIV-1 envelope genes could be experimentally manipulated by introducing mutations in the fusion domain at the amino terminus of the transmembrane glycoprotein gp41 (2, 12). Although natural sequence variation in the fusion domain of SI and NSI isolates was also found, such variation does not contribute significantly to the differences in syncytium formation (1). Naturally occurring sequence variation in the V3 loop, however, does seem to be relevant for the differences in the capacities of HIV-1 isolates to induce syncytia. Site-directed mutagenesis experiments have demonstrated a direct role for this region in HIV-1-induced membrane fusion (13, 27), and genetic exchange experiments between molecular clones of HIV-1 with different SI capacities have shown that such differences may map to the V3 region (5, 8). This same region also contains determinants that control HIV-1 cell tropism (20). Taken together, these data suggest a pivotal role for the V3 region of HIV-1 envelope.

Neutralizing antibodies directed against both the CD4-binding region and the V3 region have been described previously (16, 17, 28, 30, 32, 37). Such antibodies exert their function upon binding to their target sites either by inhibition of CD4 binding or through interference with an essential step in the process that leads from CD4 binding to fusion. Neutralizing antibodies that are directed against envelope regions for which no function in the membrane fusion process is known (e.g., the V2 region [14] or the central region of gp41 [7]) have also been described. These data and the results of studies employing site-directed mutagenesis (19, 38) suggest that other regions than those described above may be involved in the membrane fusion process. Nevertheless, in studies in which gene fragments are exchanged between pairs of molecular clones with different SI capacities, the determinants involved are repeatedly mapped to an envelope gene fragment that always includes the V3 region that has already been identified (5, 8). In an attempt to screen for additional membrane fusion-mediating determi-

* Corresponding author.

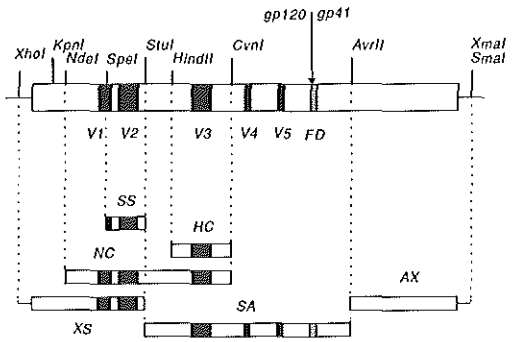

FIG. 1. Positions of the generated gene fragments relative to the complete envelope gene (top bar). The hypervariable regions V1 to V5, fusion domain (FD), cleavage site (arrow), and the restriction enzymes used are shown. The gene fragments are named after the flanking restriction sites.

nants, we performed the mapping experiments described in this report.

The starting material for this study consisted of four complete HIV-1 envelope genes described elsewhere (1). Since these envelope genes were obtained from one patient at a single time point, these genes are highly related genetically, yet they display heterogeneous SI capacities: two induce syncytia in peripheral blood mononuclear cells and in SUP-T1 cells, whereas the two others do not. We have generated 40 chimeric envelope genes, mainly between SI and NSI envelope genes, and tested these genes in a syncytium-forming assay. Our results demonstrate that multiple regions of the HIV-1 envelope glycoproteins control SI capacity; most notably, those regions that contain the V2 or V3 region can transfer SI capacity to envelope proteins previously not capable of inducing syncytia. Moreover, our data show that the capacity of the V2 and V3 regions to mediate syncytium formation also depends on additional envelope regions encompassing the V4 and V5 regions of gp120 or a region encompassing part of gp41.

MATERIALS AND METHODS

Construction of chimeric envelope genes. Four previously characterized HIV-1 envelope genes from biological clones obtained from patient 16 at a single time point and designated 16.1 to 16.4 (1) were used to generate a set of chimeric envelope genes. These parental genes were originally cloned in the pSC11 vaccinia virus expression vector (3) and in the plasmid pGEM7$^-$ (Promega). Chimeric envelope genes were constructed by exchanging gene fragments generated with restriction enzymes (*Nde*I, *Spe*I, *Stu*I, *Hin*dII, *Cvn*I, and *Avr*II) with conserved restriction sites in all four envelope genes and the restriction enzymes *Xho*I and *Xma*I, which provide convenient sites in the cloning vectors, as depicted in Fig. 1. The resulting envelope chimeras are designated according to the exchanged gene fragment and the two parental envelope genes (see Fig. 3). All chimeric envelope genes were tested by restriction mapping using restriction sites that were not conserved in the original four envelope genes. Occasionally, chimeric envelope genes were partially sequenced to verify the cloning procedure.

Cloning of chimeric envelope genes in transient expression vector. The chimeric envelope genes were cloned in the simian virus 40-based expression plasmid pSRHS (a generous gift of Eric Hunter, University of Alabama at Birmingham, Birmingham [9]). The HIV *env* gene sequences were cloned by exchanging the original *env* gene of HXB2 with *Kpn*I and *Sma*I restriction enzymes (Fig. 1). All the expressed chimeric envelope genes thus contain the signal peptide and the first 13 amino acids of the HXB2 envelope protein.

DNA transfection and syncytium formation assay. All generated constructs were transfected in CEMx174 cells (31) by electroporation. Five million cells were mixed with 20 μg of DNA in 250 μl of RPMI medium (GIBCO) supplemented with 10% fetal calf serum (FCS) in a 4-mm-wide electroporation cuvette (Eurogentec S.A.). After electroporation (200 V, 960 μF; Bio-Rad gene pulser), the cells were kept on ice for 10 min and then transferred to small culture flasks containing 10 ml of RPMI medium. Forty-eight hours after electroporation, viable cells were isolated on a Ficoll density gradient, and 2×10^5 of these cells were cocultured for 16 h with an equal amount of SUP-T1 cells (33). Syncytia were only found after cocultivation. The relative sizes and numbers of syncytia found in each culture were scored independently by three researchers. The size of syncytia was quantified as follows: small (+), up to five times the size of single cells; large (+++), giant syncytia estimated to contain over 100 fused cells (scored as being larger than 250 μm in diameter), or intermediate (++). For examples of syncytium size, see Fig. 2.

***env* expression.** At the time that the cultures were scored for syncytium formation, separately cultured portions of the Ficoll-purified cells were treated as follows: (i) spotted onto microscope slides and fixed with ice-cold ethanol (70%) for immunofluorescence and stored at −20°C and (ii) lysed with 1% empigen BB (25) and frozen at −70°C for enzyme-linked immunosorbent assay. Finally, a sample of the culture supernatants was frozen and also tested in an ELISA.

(i) Immunofluorescence. The percentage of envelope protein-expressing cells was determined by an immunofluorescence assay. The fixed cells on microscope slides were incubated with patient serum (1:70 dilution) and exposed to fluorescein isothiocyanate (FITC)-conjugated sheep anti-human immunoglobulin G1. The percentages of positive cells were scored with a fluorescence microscope.

(ii) gp120 ELISA. To determine the relative amounts of envelope protein produced in the different transfected cell cultures, a gp120 ELISA that is based on an assay developed by Moore et al. (24, 25) was used. Capture antibodies D7324 (Aalto BioReagents, Dublin, Ireland) were adsorbed on to ELISA plates (Costar) by incubation overnight in 40 mM $NaHCO_3$, pH 9.6, at 4°C. D7324 is an affinity-purified polyclonal sheep antiserum to the conserved carboxy-terminal 15 amino acids of gp120. The predicted amino acid sequences of the four envelope genes used in this study are completely homologous to this peptide (1). Unbound antibodies were removed by washing each well four times with 200 μl of the assay buffer (0.1 M NaCl, 0.1 M Tris, 0.1% Tween 20, pH 7.5). All incubations were performed for 1 h at 37°C with assay buffer supplemented with 1% FCS and 0.5% normal goat serum and terminated by washing each well four times with 200 μl of assay buffer unless stated otherwise. The wells were subsequently blocked with assay buffer supplemented with serum. Next the wells were incubated with 100-μl portions of a serial dilution of cell lysate or culture supernatant for 2 h. Detection of the attached gp120 was achieved by successive incubation of the wells with the

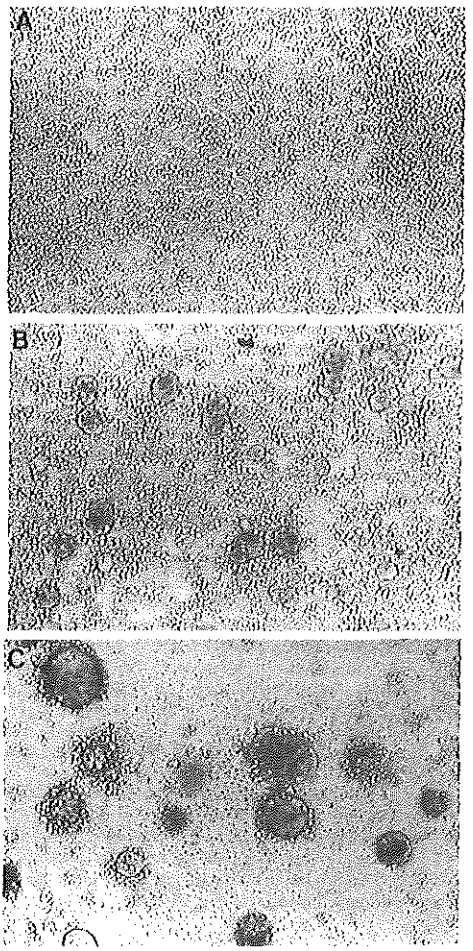

FIG. 2. Syncytia induced upon cocultivation of transfected CEMx174 cells with SUP-T1 cells. (A) No syncytia; intermediate-size syncytia (B); and large syncytia (C).

following: a 1:2,000 dilution of patient serum, a 1:2,000 dilution of biotinylated goat antihuman IgG (Amersham), and streptavidin-horseradish peroxidase conjugate (strept-ABComplex/HRP [Dako A/S]). Finally, the plates were incubated with tetramethylbenzidine-H_2O_2. The reaction was stopped by adding 50 μl of 2 M H_2SO_4, and the optical density at 450 nm was read with a Titertek Multiskan (Flow Laboratories). All gp120 ELISA values for the different constructs were related to each other and expressed as a percentage of the maximum value measured. The assay can routinely detect 20 pg of recombinant gp120 (Celltech, provided to us through the Medical Research Council AIDS reagent project).

(iii) CD4 ELISA. The CD4 ELISA used was very similar to the gp120 ELISA described above. The only difference was that the envelope proteins are now captured to the solid phase by binding to attached CD4. Recombinant soluble CD4 (100 ng/well; American Bio-Technologies; provided to us through the Medical Research Council AIDS reagent project) was adsorbed on to ELISA plates (Costar) by incubation overnight in phosphate-buffered saline (pH 7.2) at 4°C. All subsequent incubations were performed as described for the gp120 ELISA, except that the detecting patient serum was diluted 1:3,000.

Nucleotide sequence accession numbers. The GenBank accession numbers for the envelope gene sequences derived from biological clones 16.1 to 16.4 are L08655 to L08658, respectively.

RESULTS

Construction of chimeric envelope genes. Chimeric envelope genes were constructed from the four parental envelope genes 16.1, 16.2, 16.3, and 16.4 described elsewhere (1). We exchanged six different fragments between these four parental envelope genes to generate the 40 chimeric envelope genes (Fig. 1 and 3). The chimeric envelope genes were expressed in CEMx174 cells in transient expression experiments.

Expression of gp120. Expression of the envelope protein in the transfected cells was monitored by ELISA and immunofluorescence. In Table 1, the relative amount of gp120 produced (cell associated and shed in the culture medium) is listed for each construct tested. The percentage of HIV-1 envelope protein-expressing cells in the population of transfected CEMx174 cells varied from 0.6 to 4.6% of the total (Table 1).

CD4 binding of expressed gp120. By immunoprecipitation we have previously shown that all four parental envelope gene products were able to bind soluble CD4 (1). For the present study, we also tested the expressed chimeric envelope proteins for their ability to bind CD4. All chimeric envelope proteins produced upon transfection of the generated constructs were able to bind CD4, as demonstrated by the CD4-binding ELISA (results not shown). The exchange of envelope gene fragments apparently did not interfere with the CD4-binding abilities of these expressed gene products.

SI capacities of expressed envelope genes. In a previous study (1), we have shown that upon expression in a recombinant vaccinia virus, the two parental SI envelope gene products 16.1 and 16.2 induce syncytia in SUP-T1 cells, whereas the NSI envelope gene products do not. For the experiments described here, we therefore used SUP-T1 cells as CD4-positive fusion partners. Upon transfection of CEMx174 cells and subsequent cocultivation with SUP-T1 cells, almost all expressed envelope chimeras gave rise to the formation of syncytia. The sizes of the induced syncytia differed substantially for the different constructs and were highly reproducible. We have defined the SI capacity of the chimeric envelope gene products as the maximum size of the syncytia they induce in SUP-T1 cells. Within any range of the quantitative parameters shown in Table 1, small and large syncytia were observed. These results indicate that syncytium size is a qualitative property of the (chimeric) envelope and that the syncytium size is not dependent on experimental variables that influence the amount of envelope proteins produced at least not within the range observed in these experiments.

Figure 3 shows the SI capacities of all generated chimeric envelope constructs. The data presented are derived from single representative experiments. Several control experiments were performed, including multiple testing of the

TABLE 1. Quantitative expression of gp120 by (chimeric) envelope genes and sizes of the induced syncytia

env code[a]	Syncytium size[b]	Envelope protein-expressing cells by IFA (%)[c]	gp120 ELISA (%)[d]
16.1	+++	2.6	18
16.2	+++	1.9	42
16.3	–	3.8	36
16.4	–	2.7	27
12XS	++	NT	76
21XS	+++	1.9	23
12NC	+++	1.7	17
21NC	+++	3.4	23
12HC	+++	4.1	45
12AX	+++	2.2	23
21AX	+++	2.8	20
13XS	++	3.3	36
14XS	++	1.3	27
13SS	++	NT	52
14SS	++	2.0	22
31SA	+++	0.6	38
31AX	+++	1.0	67
31HC	+++	4.6	100
14NC	+++	2.9	25
13SA	+++	1.6	77
14SA	+++	2.3	25
31XS	+++	1.8	45
41XS	+++	1.8	19
13HC	+++	2.7	28
14HC	+++	4.2	39
13AX	+	0.9	54
31NC	++	3.7	58
41NC	+	3.2	69
24XS	++	1.8	29
23XS	+	1.2	44
42SA	++	1.5	38
23HC	++	NT	30
23NC	+	2.6	26
42NC	+	1.9	31
32NC	++	3.3	26
24AX	–	1.5	29
42AX	+++	1.8	45
32AX	+++	1.2	18
42HC	+++	1.8	42
42SSCX	+++	1.5	25
32HC	+++	3.3	50
24SA	+++	1.3	34
23SA	+++	4.6	31
32XS	+++	1.9	80

[a] Chimeric genes were named so that the name reflects construction of the chimeric gene. ABCD is a chimeric envelope gene in which gene fragment CD from parental *env* gene A is transferred to gene B. The envelope genes obtained from biological clones 16.1 to 16.4 are referred to as 1 to 4.

[b] SI capacity of expressed envelope genes. Symbols: –, no syncytia; +, small syncytia; ++, intermediate-size syncytia; +++, large syncytia.

[c] Percentage of envelope protein-expressing CEMx174 cells as monitored in an immunofluorescence assay (IFA). NT, not tested.

[d] Relative amount of gp120 produced by transfected CEMx174 cells (shed in culture medium and cell associated) expressed as a percentage of the maximum observed gp120 expression.

same construct and complete reconstruction of some envelope chimeras. In all of these cases, essentially similar results were obtained.

Mapping of envelope determinants controlling SI capacity. Figure 3A first shows the four parental envelope genes with the sizes of the induced syncytia in SUP-T1 cells. As in our experiments in which these same envelope genes were expressed by recombinant vaccinia virus, only SI clone-derived envelope gene products were capable of inducing (large) syncytia (1). The same panel also lists seven chimeric envelope constructs in which gene fragments between the two SI clone-derived envelope genes (16.1 and 16.2) have been exchanged. With the exception of construct 12XS that induces intermediate-size syncytia, all constructs consisting of solely SI clone-derived envelope gene sequences were able to induce large syncytia.

For further analysis, we divided our constructs in two subsets that each had sequences of one of the parental SI envelope genes as a common denominator. Figure 3B and C summarize the data for the subsets based on the SI genes 16.1 and 16.2, respectively, which were expected to yield information on regions that positively influence syncytium formation. Interestingly, dissimilar results were obtained for both subsets.

Analysis of the constructs based on 16.1 sequences revealed two small distinct regions containing determinants that are important in syncytium formation. These regions are the SS fragment located in the amino-terminal part of gp120 (Fig. 3B, top) and the central HC fragment (Fig. 3B, center). Both small fragments transfer considerable SI capacity from the 16.1 SI envelope to both NSI envelope backgrounds. The 16.1 HC fragment alone transforms the NSI envelopes into full SI envelopes upon expression. The 16.1 SS fragment (or the larger XS fragment of 16.1) by itself transfers only the capacity to form medium-size syncytia but in the presence of other 16.1-derived fragments can transfer full SI capacity (Fig. 3B, top).

Chimeric envelopes based on 16.2 sequences show also that the same two distinct envelope regions are important in syncytium formation. However, like the SS region of 16.1, now both regions transfer full SI capacity only in the presence of additional SI-derived sequences in the same chimeric envelope construct (Fig. 3C).

DISCUSSION

We are studying the process of syncytium formation as a model system to unravel the underlying mechanism of HIV-1-induced membrane fusion. In the present study, we have mapped the regions of the HIV-1 envelope proteins which determine the SI capacity.

It has been demonstrated that syncytium formation is mediated by the envelope glycoproteins of HIV-1, although the exact mechanism has not been clarified (22, 34). In a previous study (1), we provided evidence that differences in the SI capacities of field isolates are dependent on differences in the envelope gene itself. To avoid possible interference of long terminal repeat or regulatory gene functions with envelope-induced syncytium formation, we have chosen to analyze chimeric envelope genes that are transiently expressed out of the context of the viral genome. The highly homologous HIV-1 envelope genes that were obtained from a single donor at the same time point and that were previously analyzed genetically and functionally served as the basis for the experiments described here.

Since transient expression experiments lack internal con-

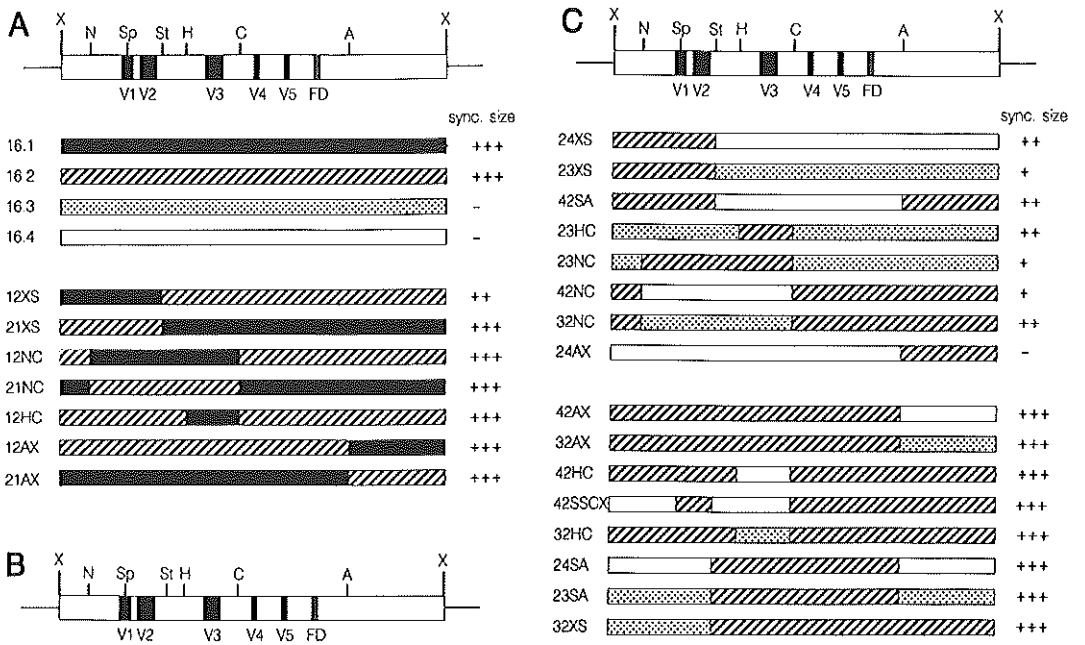

FIG. 3. Syncytium size induced by (chimeric) envelope gene products. (A) The syncytium sizes of the expressed parental envelope genes (16.1 to 16.4) which were used to generate the chimeric envelope genes and chimeric envelope gene products based on solely SI clone-derived sequences (16.1 and 16.2). (B and C) Syncytium sizes of chimeric envelope genes based on envelope gene SI 16.1 (B) or SI 16.2 (C). At the top of each panel, the complete envelope gene is represented by a bar and the restriction enzymes used to exchange gene fragments are indicated. Abbreviations: FD, fusion domain; X (left end), *Xho*I; N, *Nde*I; Sp, *Spe*I; St, *Stu*I; H, *Hin*dII; C, *Cvn*I; A, *Avr*II; X (right end), *Xma*I; sync., syncytium. Symbols: +, small syncytia; ++, intermediate-size syncytia; +++, giant syncytia; −, no syncytia (for more details, see Materials and Methods).

trols for protein expression and function, we first studied the levels of envelope protein production and the CD4-binding capacity. For each transfection, experimental variables, such as the percentage of gp120-producing cells, the relative amount of gp120 produced, the number of syncytia, and the CD4-binding capacity of the expressed recombinant protein, were monitored. Within any range of the quantitative parameters, small and large syncytia were observed, which excluded the possibility that envelope protein expression was a limiting factor in our experiments. This notion was supported by the observation that none of the quantitative parameters correlated with the readouts of the experiments; the size of the induced syncytia appears to be a genetic trait of each individual construct. Finally, all expressed (chimeric) envelope proteins were able to bind CD4.

In our approach, we have transferred fragments of SI envelope genes to NSI envelope genes searching for regions that positively influence the SI capacity. All of these constructs therefore contain genetic information from both SI and NSI envelope genes. We found no evidence for NSI clone-derived envelope regions that negatively influence the SI capacity, but we were able to identify regions from SI genes that transfer SI capacity to NSI backgrounds. The mapping experiments show that multiple determinants located on several envelope gene fragments contribute to syncytium formation. In our opinion, the effects described

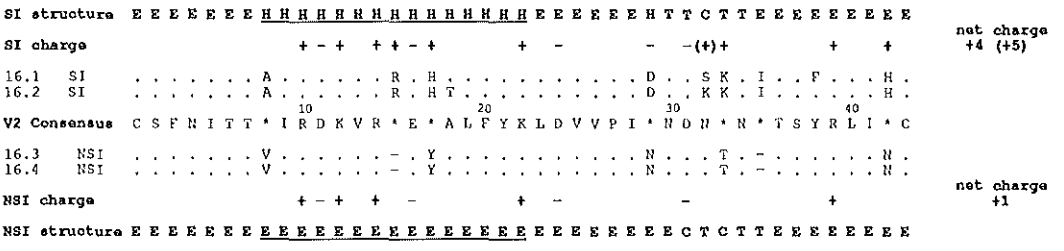

FIG. 4. Predicted amino acid sequences of the hypervariable region V2 together with the predicted secondary structures and charge differences. Amino acids identical to those of the consensus sequence (.) and deletions (−) are indicated. The predicted secondary structures of the SI and NSI gene products are also given, at the top and bottom of the figure. The corresponding amino acid is predicted to be in the following configurations; helical (H), extended stretch (E), turn (T) and coil (C). The underlined regions are predicted to have significantly different secondary structures for the SI and NSI clone-derived V2 amino acid sequences. The charges are given in boldface type.

here are the results of multiple functional interactions between different regions of the envelope glycoproteins, we distinguish SI regions (SIRs), regions which are by themselves capable of transferring considerable SI capacity, and enhancing regions that enhance the SI capacity of SIRs but by themselves are not or hardly capable to induce syncytia. Both the SS and HC gene fragments of the 16.1 and 16.2 SI envelopes can independently transfer considerable SI capacity to an NSI envelope which identifies these regions as SIRs. The 16.1 HC fragment alone is sufficient to transfer full SI capacity. This SIR apparently functions independently of the background sequence into which it is cloned. For the other identified SIRs (the 16.2 HC fragment and the SS fragment of 16.1 and 16.2) function is context dependent: their SI capacity can be increased by the simultaneous transfer of an enhancing region. An alternative explanation for the described enhancements of syncytium formation by enhancing regions could be a purely additive effect, since the enhancing regions by themselves can also transfer some SI capacity to NSI gene products. However, such additive effects are not seen when two SIRs, e.g., the V2 and V3 regions of 16.2, are combined in one construct in the absence of an enhancing region (construct 23NC [Fig. 3C]). It is therefore more likely that the effects observed upon transfer of enhancing regions from SI genes to NSI genes result from the "activation" of cryptic SIRs present in the NSI background through the functional interactions postulated here.

The SIR-containing HC fragment codes for 107 amino acids and encompasses the hypervariable region V3, which suggests that the V3 loop region is a major determinant for syncytium formation for the envelope genes studied here. The SI clone-derived V3 loops (16.1 and 16.2) share a positively charged amino acid (arginine) at position 25 of the V3 loop, whereas the NSI clone-derived V3 loops (16.3 and 16.4) both have a negatively charged glutamic acid at this position. This same phenotype-correlated amino acid variation has also been found in a large set of V3 loop sequences (11). The four parental genes, however, also showed considerable amino acid variation just C terminal of the V3 loop itself. This region, which is present in the small HC fragment, also contains phenotype-associated amino acid variations, e.g., the SI genes have a positively charged residue (lysine) at position 351, where the NSI genes have a negatively charged glutamic acid residue (1). Additional experiments to demonstrate exactly which residues on the HC fragment are responsible for the observed transfer of SI capacity are needed.

Interestingly, the extremely small SI gene-derived SS fragment alone is also capable of transferring SI capacity to an NSI gene product. This SS fragment, coding for only 71 amino acids, encompasses part of the variable region V1 and the complete variable region V2. The V1 region is identical for all four parental envelope proteins, and all amino acid variation of the SS fragment is confined to the V2 region. Constructs 24XS, 42HC, 32HC, and 42SSCX, all based on 16.2 SI envelope sequences, demonstrate that the V2 region is also an important determinant in syncytium formation for the 16.2 envelope. Examination of the amino acid variation of the V2 region revealed that the SI clone-derived V2 sequences differ from the NSI clone-derived sequences in charge and predicted secondary structure. The variable amino acids of the SI V2 sequences are often positively charged residues, whereas the NSI V2 sequences mostly carry polar but uncharged residues at these positions which results in a higher net positive charge for this region in SI genes (Fig. 4). Computer analysis (Garnier-Robson analysis [15]) shows that the variation of residues 8 and 17 greatly affects the predicted secondary structure of the V2 loop. In contrast to the NSI clone-derived V2 region, the SI clone-derived sequence of the N-terminal part of the V2 region is very likely to form a stable α-helix (Fig. 4). The importance of these V2 loop features in syncytium formation is currently being tested in site-directed mutagenesis studies. A previous report describing the mapping of a virus-neutralizing antibody to the V2 region also suggested a role for the V2 region in the infection process (14). Our data support this notion and point to a direct role for the V2 region in HIV-1 gp120-gp41-mediated membrane fusion.

Our data suggest that the V2 and V3 regions are mutually independent in controlling syncytium formation: when derived from 16.1, both regions transfer considerable SI capacity, with that of the V2 region further enhanced by an enhancing region. Similarly, the same two regions derived from 16.2 transfer full SI capacity when the proper enhancing region, which is the same for both, is present. Thus, the V2 and V3 regions are the primary SIRs that functionally interact with the enhancing regions identified here. Syncytium formation can apparently be achieved through at least two different determinants on gp120, and in the SI envelope genes studied here, both are functional. For the V3 region, basic residues at positions 11 or 28 are linked to the SI phenotype, and all SI isolates so far appear to have this configuration (11). However, it is not known whether the SI phenotype of these isolates is mediated solely through the

TABLE 2. SIRs with the corresponding enhancing regions

SIR	Syncytium size induced by SIR	Protein or fragment causing enhanced SIR function
16.1		
V2	++	16.1 gp41 (AX fragment)
V3	+++	?
16.2		
V2	++/+	16.2 CA fragment
V3	++/+	16.2 CA fragment

V3 region or whether the V2 region also plays a role. It would be of interest to determine whether a consensus SI configuration for V2 can be found in order to answer these questions.

For the context-dependent SIRs identified in this study, additional sequences are required for transfer of maximal SI capacity. The transfer of SI capacity by the 16.2 V3 region is enhanced by simultaneous exchange of at least the *Cyn*I-*Avr*II envelope region at the carboxy terminus of gp120 (constructs 24SA and 23SA [Fig. 3C]). To increase transfer of SI capacity by the V2 region of the same (16.2) SI envelope, this *Cyn*I-*Avr*II region is apparently necessary (constructs 42HC, 42SSCX, and 32HC). This fragment contains the V4-C4-V5 regions on gp120 and part of gp41. Interestingly, evidence for physical interaction between the C4 region and the V3 loop has been described by others (39) and may well explain the observed functional interaction between the V3 as an SIR and this enhancing region.

Transfer of SI capacity by the 16.1 V2 region is enhanced by the AX region of the same SI gene (construct 31SA). This same AX region from 16.1, but not 16.2, could also transfer some SI capacity to NSI envelope 16.3, probably through activation of a cryptic context-dependent SIR on 16.3. For an overview of the functional interactions between SIRs and enhancing regions, see Table 2.

Identification of any region as an SIR or an enhancing region heavily depends on the sequences of all other regions involved. Probably even the context-independent SIR (V3 region 16.1) functionally interacts with the enhancing regions identified here, but the exact sequence of the enhancing region is less restricted than for the other SIRs. Cryptic SIRs on NSI envelope genes (for instance, 16.3) can be activated by acquisition of an enhancing region with the proper sequence, e.g., through recombination. We have previously demonstrated that recombination does occur in vivo in an infected individual (18). This result means that regions involved in syncytium formation do not necessarily have to coevolve in the same virus to generate an SI variant. Even recombination between two NSI envelope genes, one containing a cryptic SIR and the other containing an enhancing region could therefore lead to an SI virus. We are currently testing this hypothesis. Such mechanisms greatly enhance the dynamics of the evolution of HIV-1.

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CHAPTER 5

Impact of Natural Sequence Variation in the V2 Region of the Envelope Protein of Human Immunodeficiency Virus Type 1 on Syncytium Induction: a Mutational Analysis

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Impact of Natural Sequence Variation in the V2 Region of the Envelope Protein of Human Immunodeficiency Virus Type 1 on Syncytium Induction: a Mutational Analysis

ARNO C. ANDEWEG[1,3], PATRICK H.M. BOERS[1], ALBERT D.M.E. OSTERHAUS[1,2] and MARNIX L. BOSCH[1,4]

[1]Laboratory of Immunobiology, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands. [2]Department of Virology, Erasmus University Rotterdam, The Netherlands. [3]Present address: Laboratory of Viral Pathogenesis, Biomedical Primate Research Centre, Rijswijk, The Netherlands. [4]Present address: Regional Primate Research Center and Department of Pathobiology, University of Washington, Seattle, USA.

Several studies have demonstrated a functional role for the V1-V2 region of the HIV-1 envelope surface glycoprotein gp120 in membrane fusion processes underlying viral entry and syncytium induction. In a study with chimeric primary envelope genes, we have demonstrated previously that the exchange of V2 regions was sufficient to transfer syncytium inducing capacity to a non-syncytium inducing envelope protein. The exchanged V2 regions, comprising a number of variable amino acids, conferred changes to both the predicted secondary structure and to the net positive charge of the V2 loops. In a syncytium forming assay based on transient envelope protein expression in CD4 positive SupT1 cells, we have extended this observation by mutating the variable positions of the V2 region to determine the relative contribution of individual amino acids to syncytium formation. It is shown that simultaneous mutation of multiple amino acids is needed to interfere with the V2 region determined syncytium inducing phenotype. Single amino acid changes either influencing charge or predicted secondary structure of the V2 loop proved to be insufficient to abolish V2 region controlled syncytium formation. This robust V2 organization may allow the virus to accumulate mutations, while retaining it's biological phenotype.

Human immunodeficiency virus type 1 (HIV-1) induced syncytium formation and viral entry are the result of membrane fusion processes that are both initiated by the binding of the viral surface glycoprotein gp120 to the CD4 receptor molecule expressed on target cells (5,22,23,27). This receptor binding induces conformational changes resulting in the increased exposure of regions of gp120, dissociation of gp120 from the transmembrane glycoprotein gp41, and

unmasking of gp41 epitopes (16,25,26,28,29). Both cellular and viral determinants involved in these events, thus involved in viral entry and syncytium formation have been identified. The principal viral determinants involved in these processes are located on the envelope glycoproteins (22,32) and their further identification will contribute to the understanding of the HIV-1 induced membrane fusion processes.

In addition to studies with mutant - mostly T cell line adapted - viruses, also studies with chimeric viruses have helped to identify determinants that control the biological properties of HIV-1. Studies with chimeric viruses have shown that the V3 region of gp120 is a major determinant for cell tropism and syncytium induction (18,31). Similarly, it has been demonstrated that the variable envelope regions V1 and V2 are also involved in these processes (2,4,10,15,19,35). In a study with chimeric envelope genes generated from a small set of genetically highly related envelope genes, obtained from a single patient at a single time point we recently identified a region on the transmembrane protein gp41 determining syncytium formation and tropism (Andeweg *et al.* unpublished results).

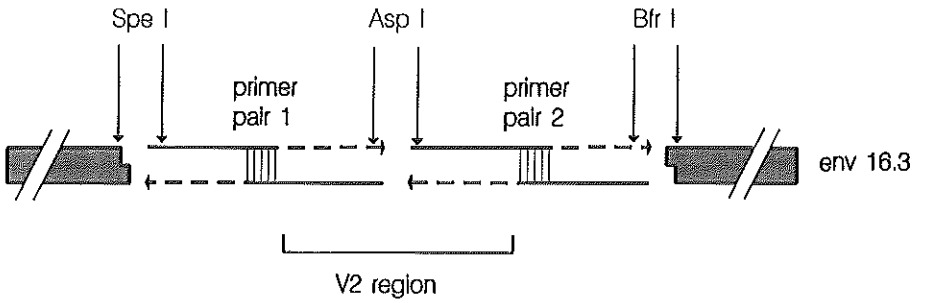
The V2 region together with the V1 region of the surface glycoprotein gp120 form a double loop structure through disulfide bonds at a common basis (21). In multimeric envelope proteins expressed at the surface of infected cells the V2 loop exposure is increased as a result of sCD4 binding (26,29), and mutation of conserved amino acid residues of the V2 region was shown to interfere with the capacity to induce syncytia while sCD4 binding capacity was retained (33). These data collectively suggested a role for the V2 loop in a post-receptor binding event in membrane fusion. A functional role for the V2 region in membrane fusion was also suggested by the detection of neutralizing antibodies directed to the V2 region (6,11,13,17,24). The screening of a large set of V2 regions from viruses with different syncytium inducing capacities suggested a correlation between V2 region length and syncytium forming capacity (15). In a similar study we demonstrated that the increased length of the V2 region is also associated with the insertion of N-linked glycosylation sites through AAT triplet reiteration, suggesting a mechanism by which epitopes on the V2 loop are masked from immune surveillance (3).

We have demonstrated that the variation in the V2 region alone is sufficient to considerably change the syncytium inducing capacity of transiently expressed chimeric envelope proteins (2). The exchanged V2 region harbouring a number of variable amino acids conferred changes to both the predicted secondary structure and to the net positive charge of the V2 loops (2). In the present study we have extended these observations by mutating the variable positions of the V2 region to determine the relative contribution of individual amino acids to syncytium formation.

MATERIAL AND METHODS

Construction of mutant chimeric envelope genes. Previously we genetically and phenotypically characterized a set of envelope genes from biological HIV-1 clones obtained from a single donor at a single time point that were designated 16.1 to 16.4 (1). For the present study we have introduced a series of synthetic V2 regions into the 16.3 NSI envelope gene. The V2

A



B

primer pair 1

5' primer:

```
          Spe I
5'-CTAATACCACTAGTAGTAATACCAATAGTAGTAGTTGGGGAATGATGGAGAAAGGAGAAA

   TAAAAAACTGCTCTTTCAATATCACCACAG-3'
```

3' primer:

```
          Asp I                          H      R
5'-CTATTGGGACTACGTCAAGTTTATAAAAAAGTGCATGTTCTCTCCGCACCTTATCTCTTA
     A
   TGGCTGTGGTGATATTGAAAGAGC-3'
```

primer pair 2

5' primer:

```
          Asp I          D         S  K      I         F
5'-TAAACTTGACGTAGTCCCAATAGATAATGATAGTAAAAATATTACCAGCTTTAGGTTGAT
     H
   ACATTGTAACACCTCAGTCATTACACAGGCC-3'
```

3' primer:

```
        Bfr I
5'-GTTACACTTAAGAATCGCAAAACCAGCCGGGGCACAATAGTGTATGGGAATTGGCTCAAA

   GGATATCTTTGGACAGGCCTGTGTAATGACTGAGGTGTTAC-3'
```

FIG. 1. In panel A the cloning strategy of synthetic 16.1 based V2 regions into the NSI 16.3 envelope gene is illustrated. T7 DNA polymerase (Sequenase, USB) extended products of two mutually priming long oligonucleotide pairs were ligated at a designed central Asp I site and subsequently inserted into the NSI 16.3 envelope gene using the V2 region flanking restriction sites Spe I and Bfr I (the latter generated by site directed mutagenesis) as indicated . In panel B the primer sequences used to construct the synthetic wt 16.1 V2 region are given. Indicated are: the incorporated restriction sites (double underlined), the complementary regions for mutually priming (single underlined), and the triplets that were individually or simultaneously changed (bold printed) to produce the set of different V2 regions as shown in figure 2. For each marked wt 16.1 triplet the encoded amino acid is indicated in the 1-letter amino acid code.

regions were produced by cloning T7 DNA polymerase (Sequenase, USB) extended products of two mutually priming long oligonucleotide pairs as depicted in figure 1 panel A. The primers used to construct the wild type 16.1 V2 region are shown in figure 1 panel B. Mutant V2 regions were produced by using similar primers containing the desired sequence changes and/or by site directed mutagenesis of yet constructed (mutant) V2 regions according to the method described by Deng and Nickoloff (7). The generated synthetic V2 regions were cloned into the 16.3 NSI envelope gene using the unique Spe I restriction site (already present at a position corresponding to amino acid residue #144) (1). and a Bfr I site created by site directed mutagenesis (located at a position corresponding to amino acid residue #242). The introduction of this Bfr I restriction and of the designed Asp I restriction site inside the V2 region did not change the predicted parental amino acid sequences. This cloning strategy resulted in chimeric envelope genes identical to the parental 16.3 NSI gene but coding for different V2 amino acid sequences as shown in figure 2. The entire cloned region of all generated chimeric-mutant envelope genes was sequenced to verify correct assembling.

```
                                                                net pos    Helix
                                                                charge     Predicted
                  +-+ ++-+     + -     - - +      +  +
16.1  CSFNITTAIRDKVRREHALFYKLDVVPIDNDSKNITSFRLIHC                4          +
      |           |           |           |          |
      1           10          20          30         40

AV    .......V.....................................              4          -
R-    ..............-..............................              3          +
HY    ..............Y..............................              3          -
HR    ..............R..............................              4          -
DN    ............................N..............              5          +
SN    .............................N.............              4          +
KT    ..............................T.............              3          +
I-    ...............................-............              4          +
FY    ..................................Y.....                  4          +
HN    .....................................N.                    3          +

mt1   .......V........Y.....................                  3          -
mt2   .......V........R.....................                  4          -
mt3   ..............-.............N...T........N.               2          +
mt4   .......V......-.Y..........N...T........N.               1          -


16.3  .......V......-.Y..........N..NT.-..Y...N.               1          -
```

FIG. 2. Predicted amino acid sequences of the two parental V2 regions (16.1 SI and 16.3 NSI) aligned with the generated single and multiple V2 region mutants. The amino acid sequences are presented in the 1 letter amino acid code. Identity with the 16.1 sequence is indicated by (.), (-) represents a deletion. Charged residues in the SI 16.1 sequence are indicated at the top (+ or -), the net positive charge of all listed V2 regions is indicated at the right. The last collum lists whether an α-helical structure is predicted (+) or not (-).

Transient envelope gene expression. All generated chimeric envelope genes were cloned in the simian virus 40 based expression vector pSRHS as previously described (2,8). Subsequently these constructs were transfected into Sup-T1 cells by electroporation. Five

million cells were mixed with 20 μg DNA in 250 μl RPMI (Gibco) supplemented with 10% foetal calf serum (FCS) in a 4 mm electroporation cuvette (Eurogentec S.A.) and after electroporation (Biorad genepulser, 200 volts, 960 μF) the cells were transferred to culture flasks with RPMI medium. One day post transfection, viable cells were isolated on a Ficoll density gradient and cultured for an additional four days. At day three post transfection the relative expression of envelope protein was determined in both supernatant and cell lysates in an ELISA as previously described (2). The catching antibody D7324 (Aalto BioReagents, Dublin, Ireland) used in this ELISA is directed to the conserved carboxy-terminal 15 amino acids of gp120, implicating that both gp120 and gp160 will contribute to the signals obtained. Finally envelope protein processing - and expression kinetics was examined by radioimmunoprecipitation assay (RIPA) and by testing multiple cell lysate and supernatant samples over time in ELISA. For RIPA: COS-7 cells transfected with the envelope constructs were metabolically labelled 48 hours after electroporation for 30 minutes with a mixture of [^{35}S] methionine and [^{35}S] cysteine (Amersham). Cells were lysed and supernatants were collected at 16 and 40 hours after labelling. Immunoprecipitation, using 2 μg of a pool of three different monoclonal antibodies; gp13 (30), 391/95-D (14) and K14 (34), was performed as previously described (1).

Syncytium forming assay. Previously we defined the syncytium inducing capacity of (chimeric) envelope genes as the size of the syncytia induced in SupT1 cells since syncytium size and not the number of syncytia emerged in our experiments as a qualitative property of the expressed genes (2). Therefor each day the transfected cell cultures were examined for the presence of syncytia and relative sizes of the observed syncytia were quantified as small: up to five times the size of single cells to large: giant syncytia estimated to contain over 100 fused cells (scored as being larger than 250 μm in diameter) and intermediate sizes. To this end the cultures were examined at the respective time points, independently by three different coworkers. Each envelope gene construct was tested for expression and syncytium inducing capacity at least three times in independent assays.

RESULTS

Construction of mutant chimeric envelope genes. Sixteen chimeric envelope genes were constructed on the basis of the 16.3 NSI envelope gene in which a series of synthetic V2 regions were inserted. The set of inserted V2 regions represents the wild type V2 sequence of the 16.1 SI envelope gene and mutants thereof which were mutated toward the 16.3 NSI sequence at single and multiple positions (Fig. 2). The transient expression of these chimeric mutant envelope genes allowed us to study the contribution of the observed variable amino acids in the V2 region to the membrane fusion process underlying syncytium formation.

Transient envelope gene expression. Expression of the chimeric envelope genes in the transfected cells was monitored by ELISA. In table 1, the relative amounts of gp120/gp160 present at day three after transfection are listed, both for cell associated and shed glycoproteins

TABLE 1. Relative envelope gene expression in the syncytium forming assay.[a]

env code	cell lysate	supernatant	ratio[b]
wt 16.1	48	37	68
V2 chimeras:			
16.1	97	78	71
AV	52	30	52
R-	69	40	51
HY	80	64	69
HR	55	33	53
DN	80	55	60
SN	85	48	49
KT	66	49	65
I-	60	34	51
FY	80	43	47
HN	100	86	75
mt1	93	75	71
mt2	80	37	41
mt3	88	100	100
mt4	79	65	72
wt 16.3	74	35	41

[a] Values given are relative OD_{450} levels obtained by ELISA in a single representative experiment. The level of envelope gene expression for each construct in cell lysate or supernatant was expressed relative to the construct for which the highest expression level was observed (HN and mt3 for respectively env expression in cell lysate and supernatant).

[b] Ratio between the amount of gp120 present in the culture medium and the amount of envelope proteins determined in cell lysate, expressed relative to the highest ratio as observed for construct mt3.

as shown for a single representative experiment. The ratio between the amount of gp120 present in the culture medium and the amount of envelope proteins determined in cell lysate for each construct is also presented. The relative amounts of envelope proteins present in cell lysate and supernatant varied two to three fold. On average 35 percent of the envelope proteins present at day three was present in the culture medium (data not shown). The observed ratio varied two fold when comparing all expressed chimeric genes (Table 1). SupT1 cells transfected in parallel by electroporation with a ß-galactosidase encoding control plasmid (Pharmacia) demonstrated that under the conditions described 3 to 5 percent of the cells are successfully transfected (data not shown). Since we observed a considerable delay in syncytium formation

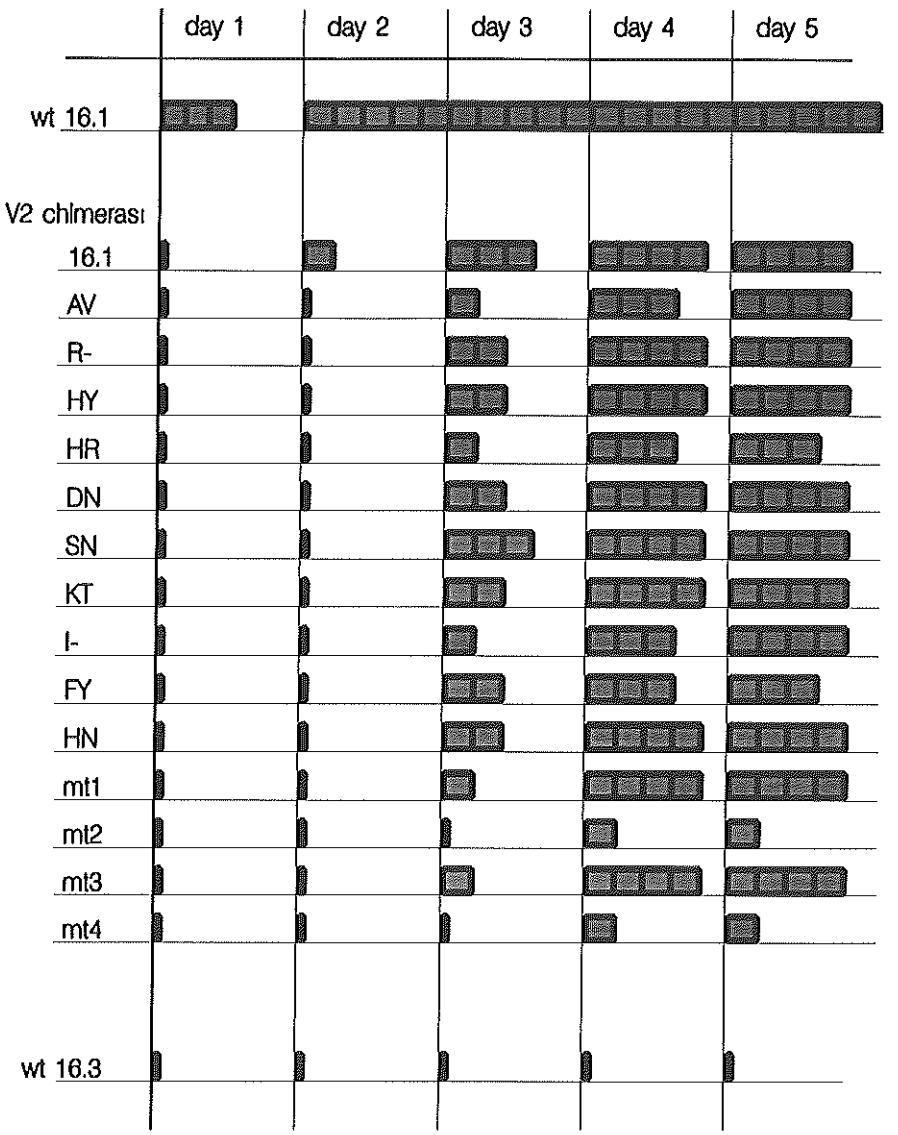


FIG. 3. Kinetics of syncytium size induced by envelope gene products. The size of the syncytia was quantified by classifying the observed syncytia in five arbitrarily chosen categories. The smallest syncytia were about five times the size of single cells and indicated by a single square. The largest syncytia were estimated to contain over 100 fused cells and exceeding 250 μm in diameter are indicated by a bar of five squares, intermediate size syncytia are indicated by bars with a number of squares corresponding to their relative sizes. Wt 16.1 and wt 16.3 represents the unmodified 16.1 SI and the unmodified 16.3 NSI envelope gene constructs. All other codes refer to the (mutated) V2 sequences of Fig. 2 expressed in the 16.3 NSI background. The data presented are derived from single representative experiments.

for some of the constructs, envelope processing kinetics were determined by RIPA using COS-7 cells. In this assay we used adherent COS-7 cells since these cells are efficiently electroporated (up to 25% of the cells become transfected, data not shown). As demonstrated in figure 4, gp160 expression, cleavage and gp120 shedding into the supernatant occurred within 16 hours for all envelope constructs analyzed.

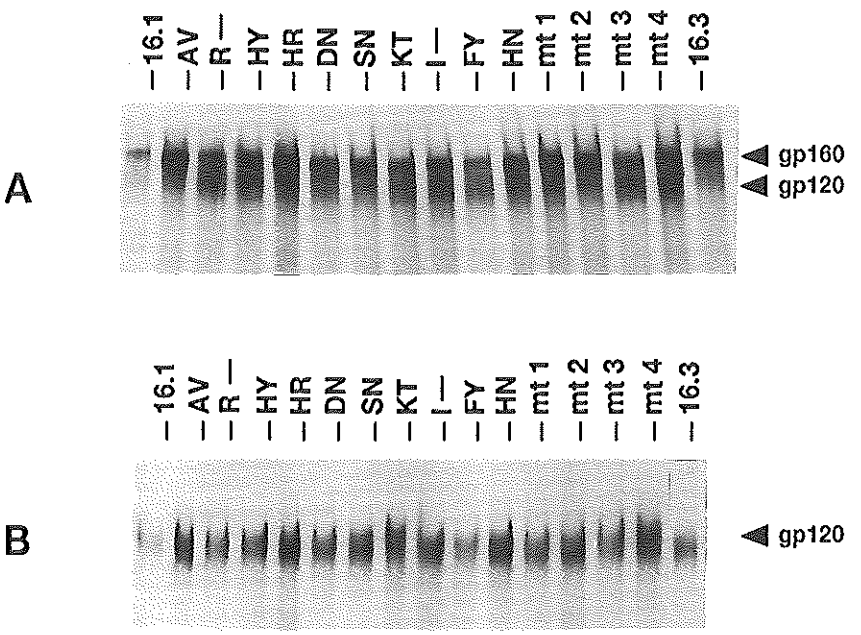


FIG. 4. Expression of envelope gene products by COS-7 cells transfected with the chimeric envelope gene constructs as demonstrated by RIPA (all V2 sequences indicated were expressed in the 16.3 NSI background). Pulse labelled proteins from transfected cell lysates (panel A) or from culture medium (panel B) were immunoprecipitated 16 hours after transfection with a mixture of monoclonal antibodies and analyzed by SDS-PAGE. Arrows indicate the precursor glycoprotein gp160 and the surface glycoprotein gp120.

Syncytium forming assay. Expression of the chimeric NSI envelope gene 16.3 with a V2 region sequence derived from the 16.1 SI gene resulted in syncytium formation in Sup-T1 cells as shown in figure 3, which confirmed data from previous studies (2). Monitoring the transfected cell cultures for 5 days revealed that all other chimeric constructs induced syncytia upon expression but significant differences in kinetics and size were observed (Fig. 3). Relative to the complete unmodified 16.1 SI envelope the chimeric envelope construct "16.1" containing the 16.1 SI derived V2 region in an NSI envelope background displayed delayed syncytium formation. Further delay in syncytium induction for some of the chimeric V2 envelope

constructs was observed upon the introduction of one or more mutations in this region. The most prominent delay was observed with mutant #2 and mutant #4. In contrast to the other chimeric envelope proteins, both induced only small syncytia and this after prolonged times in culture (Fig. 3). The mutations introduced influenced either the overall net charge of the V2 region, the predicted secondary structure, or both. These changes are indicated in figure 2. Garnier-Robson analysis (12) predicted the formation of an alpha-helix for the SI 16.1 V2 region, which was not the case for the NSI 16.3 V2 region or for mutants AV, HY, HR, or mutant #1. These mutants did not display a significantly altered syncytium forming capacity compared to the wild type "16.1" construct. Likewise, mutants R-, HY, DN, KT, HN, mt1, and mt3, which differ in net positive charge from the 16.1 V2 region, did not significantly reduce the size of the induced syncytia. Combination of multiple mutations which introduced differences in charge and two mutations which abolished the predicted secondary structure (mutant #4) however almost completely abolished syncytium formation. Mutant #2 contains the histidine to arginine change, that was also found in mutant HR. The introduced arginine is different from all other changes discussed in this paper in that an arginine was never found in this position in all the HIV-1 envelope genes sequenced to date, all the other changes were based on their "natural" occurrence in 16.3. The HR mutant was constructed since it affects only the predicted helix whereas the natural HY variation at this position also affects the net charge of the V2 region. As shown in figure 3 the histidine to arginine change at this position was not tolerated in combination with the alanine to valine mutation also present in mutant AV. The combination of these two mutations in mutant #2 abrogated syncytium formation.

DISCUSSION

In the present paper we have shown by mutational analysis based on the natural sequence diversity in the V2 region of the HIV-1 envelope glycoproteins obtained from one individual at a single time point that simultaneous mutation of multiple amino acids is needed to interfere with V2 region determined syncytium induction.

Monitoring transfected cell cultures for 5 days revealed that all chimeric envelopes constructed in these experiments induced syncytia upon expression, but that also significant differences in kinetics and size were observed (Fig. 3). The envelope expression as demonstrated by ELISA three days post transfection revealed no major differences in the levels of envelope expression in cell lysates and supernatants. Furthermore the ELISA data demonstrated that expression of the chimeric envelope genes resulted in the shedding of similar amounts of gp120 in culture supernatant for all the envelope constructs. These envelope protein expression levels were similar to levels routinely detected in experiments in which we showed that the envelope protein expression was not a limiting factor in syncytium formation. The expression levels corresponded to levels at least eight times those sufficient for immediate massive syncytium formation (data not shown). Finally all envelope expressing constructs were assayed independently for their syncytium inducing capacity at least three different times.

Essentially identical results were obtained for each of the constructs tested. These data showed that limited or variable envelope protein expression did not introduce a bias in the observed syncytium inducing capacity of the envelope genes tested.

Relative to the complete unmodified 16.1 SI envelope the chimeric envelope construct "16.1" containing the SI derived V2 region in an NSI envelope background, displayed delayed syncytium formation, indicating that the kinetics of syncytium formation is at least in part determined by regions outside the V2 domain. Further delay in syncytium induction of most of the chimeric V2 envelope constructs was observed upon the introduction of one or more mutations in this region. The most prominent delay was observed with mutant #2 and mutant #4 which, in contrast to the other chimeric envelope proteins, induced small syncytia after prolonged periods of time in culture (Fig. 3). To examine a possible correlation between the delay in envelope function (syncytium induction) on the one hand and envelope protein processing - and expression kinetics on the other hand we performed a radioimmunoprecipitation assay (RIPA) and tested multiple cell lysate and supernatant samples over time in ELISA. The results presented in figure 4 demonstrate that gp160 expression, cleavage and gp120 shedding into the supernatant occurred within 16 hours for all envelope constructs analyzed including mutant #2 and mutant #4. Moreover cell lysates and supernatant from cultures transfected with mutant #2 or mutant #4, taken 24 hours after electroporation, already showed similar levels of envelope expression as obtained with the other constructs when tested in ELISA (data not shown). These results indicate that the delayed appearance of syncytia as seen for mutants #2 and #4 is not due to reduced processing - or expression kinetics of these envelope proteins. Therefor we conclude that the phenotypic differences observed are the direct consequence of the amino acid sequences of the envelope glycoproteins expressed. The efficient processing, transport, cleavage and shedding of gp120 for all the different constructs suggested that the phenotypic differences observed were due to differences in envelope proteins that are present at the cell surface and differ qualitatively in the ability to mediate the membrane fusion process. Our data indicate that the naturally occurring sequence variation in the V2 region does not significantly influence the precursor processing and subunit association of the envelope proteins. This in contrast to mutations of conserved amino acids in this region of the T cell line adapted HXBc2 envelope glycoprotein as demonstrated by Sullivan et.al. (33).

Garnier-Robson computer analysis suggested that the V2 variation at residues 8 and 17 greatly affects the predicted secondary structure of the V2 loop (2). The N-terminal part of the SI derived V2 region is very likely to form a stable α-helix which is not predicted for the NSI V2 region. In addition, the amino acid variation observed results in a more positively charged V2 region for the SI envelope gene than for the NSI gene (2).

We have shown here that the envelope function in syncytium induction is not lost when variable residues affecting only the predicted α-helix or the charge of the V2 region are changed. Only multiple simultaneously introduced mutations interfered with the SI phenotype demonstrating that the SI configuration is a solid property of the V2 region. Mutants R-, HY, DN, KT, HN, mt1, and mt3, which differ in net positive charge from the 16.1 V2 region, do not display a

significantly decreased capacity to induce syncytia. This observation demonstrates that the observed differences in syncytium inducing capacity of primary envelope proteins determined by the V2 region, is not controlled by charged amino acid residues. These results may indicate a fundamentally different role for the V2 region in syncytium formation as compared to that of the previously defined role of the V3 region in this process, since the presence of positively charged residues in this V3 region strongly correlates with the syncytium inducing phenotype (9). Several studies indicated physical or functional interactions of the V2 region with other envelope regions like C4 (2,10,26). Interestingly, in the presently expressed chimeric envelope proteins, syncytium formation is not affected by numerous changes in the V2 region. Since we have shown that the 16.1 V2 region supports syncytium formation in different NSI backgrounds (2), functional interactions with other envelope regions in the here described chimeric envelopes are not seriously disturbed or lost. This is probably due to the high degree of sequence homology in the parental envelope genes used in this study (1). Apparently, the robust V2 organisation allows the virus to accumulate mutations as a means of escape from immune selection pressure (20,36), without necessarily having direct consequences for the biological properties. This flexibility would be additional to that achieved by anchoring important biological properties in different regions of the envelope glycoproteins, like the association of determinants for syncytium formation and cell tropism with multiple variable regions of gp120 (V1, V2 and V3) and gp41 either independently or in concert. This complex but flexible organization may largely hamper attempts to interfere with the biology of HIV-1 through immunological or other intervention strategies.

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CHAPTER 6

Insertion of N-Linked Glycosylation Sites in the Variable Regions of the Human Immunodeficiency Virus Type 1 Surface Glycoprotein through AAT Triplet Reiteration

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Insertion of N-Linked Glycosylation Sites in the Variable Regions of the Human Immunodeficiency Virus Type 1 Surface Glycoprotein through AAT Triplet Reiteration

MARNIX L. BOSCH,[1]* ARNO C. ANDEWEG,[1]† RONALD SCHIPPER,[2] AND MARCEL KENTER[1]‡

Laboratory of Immunobiology, National Institute for Public Health and Environmental Protection, 3720 BA Bilthoven,[1] and Laboratory of Immunohaematology and Blood Bank, University Hospital E3-Q, 2333 AA Leiden,[2] The Netherlands

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Variable regions with sequence length variation in the human immunodeficiency virus type 1 envelope exhibit an unusual pattern of codon usage with AAT, ACT, and AGT together composing >70% of all codons used. We postulate that this distribution is caused by insertion of AAT triplets followed by point mutations and selection. Accumulation of the encoded amino acids (asparagine, serine, and threonine) leads to the creation of new N-linked glycosylation sites, which helps the virus to escape from the immune pressure exerted by virus-neutralizing antibodies.

The surface glycoprotein gp120 of the human immunodeficiency virus type 1 (HIV-1) contains a number of regions with a relatively high degree of amino acid sequence variation, designated variable regions 1 through 5 (V1 to V5 [11]). Recently, evidence has been presented that, like variation in, e.g., the V3 region, variation in the V1 and V2 regions contributes to the determination of viral tropism and cytopathic potential (2, 3, 8, 17, 19), indicating that these regions are probably involved in the process of envelope-mediated membrane fusion resulting in virus entry or in syncytium formation. Sequence variation in these regions can then be reflected in these processes, resulting in variations in cell tropism and cytopathicity as described above. Virus-neutralizing antibodies to the V2 region that probably directly interfere with virus entry have also been described previously (7, 10a, 14), and sequence variation could enable the virus to escape from such antibodies. The addition of carbohydrates can alter the recognition of envelope glycoproteins by the immune system (1, 6). Here we describe a process of sequence length variation in the HIV-1 envelope gene that results in the selective insertion of new N-linked glycosylation sites in the variable regions of the envelope glycoproteins as a mechanism to escape immune pressure exerted by neutralizing antibodies.

The alignments of the variable-length sequences found in V1 and V2 are shown in Fig. 1. We have used the HIV-1 envelope sequence alignment presented by Myers et al. (12) as a starting point and readjusted the alignment by eye. The numbers at the top indicate the codons incorporated in the regions of variable sequence length. The underlined blocks of three codons indicate encoded potential N-linked glycosylation sites (see below). We have noticed an apparent skewed usage of codons in these regions. A relatively high number of codons begin with A and end with T, with an especially high frequency of AAT codons.

To confirm this observation, we have counted the frequencies of the four nucleotides adenosine (A), cytidine (C), guanosine (G), and thymidine (T), at the first, second, and third positions of each codon in the variable-length sequences of the V1 and V2 regions. We find sharp deviations from the distributions as found in the whole of gp120 (we have used the HXB2R gp120 sequence for comparison). Most notably, we find that approximately 84% of the codons in these regions start with A (<1% start with C); only 1 to 4% of codons have a T at position 2 (compared with 26% in gp120); and 67 to 76% of codons end with T, with sharp decreased frequencies of A and G at position 3. We have subsequently analyzed the frequency distributions of all four nucleotides at each codon position in the whole envelope gene alignment by a sliding window method (see legend to Fig. 2 for details). The results obtained for A at position 1 (A1), T at position 2 (T2), and T at position 3 (T3) are plotted in Fig. 2 for a window size of 20 codons shifted by 1 codon, as are the number of sequences counted in each window to map the regions with sequence length variation (bottom panel). It can be seen that sharp increases in frequencies of A1 and T3 with concomitant decreases in T2 occur in the V1 and V2 regions and also in two other regions that display a high degree of sequence length variation, the V4 and V5 regions. No other regions of the envelope gene display similar concomitant nucleotide frequency distributions. The A1 and T3 peaks are not as pronounced for V5 because of the relatively small size of the region of variable sequence length in V5 relative to the window size.

We postulate here that the skewed distribution of nucleotides at the first, second, and third positions of the codons that make up the regions of variable sequence length is the result of the insertion of AAT triplets at these sites followed by point mutations and selection for sequences that provide a selective advantage to the virus by the insertion of N-linked glycosylation sites, resulting in escape from the immune system, as well as selection against disadvantageous (in this case, hydrophobic) sequences. The implications of this mechanism will be discussed below.

The use of a severely limited set of nucleotides at position 1

* Corresponding author. Present address: Regional Primate Research Center and Department of Pathobiology SC-38, University of Washington, Seattle, WA 98195. Phone: (206) 543-7619. Fax: (206) 543-3873.

† Present address: Laboratory of Viral Pathogenesis, Biomedical Primate Research Center, Rijswijk, The Netherlands.

‡ Present address: Institute of Virology, Erasmus University, Rotterdam, The Netherlands.

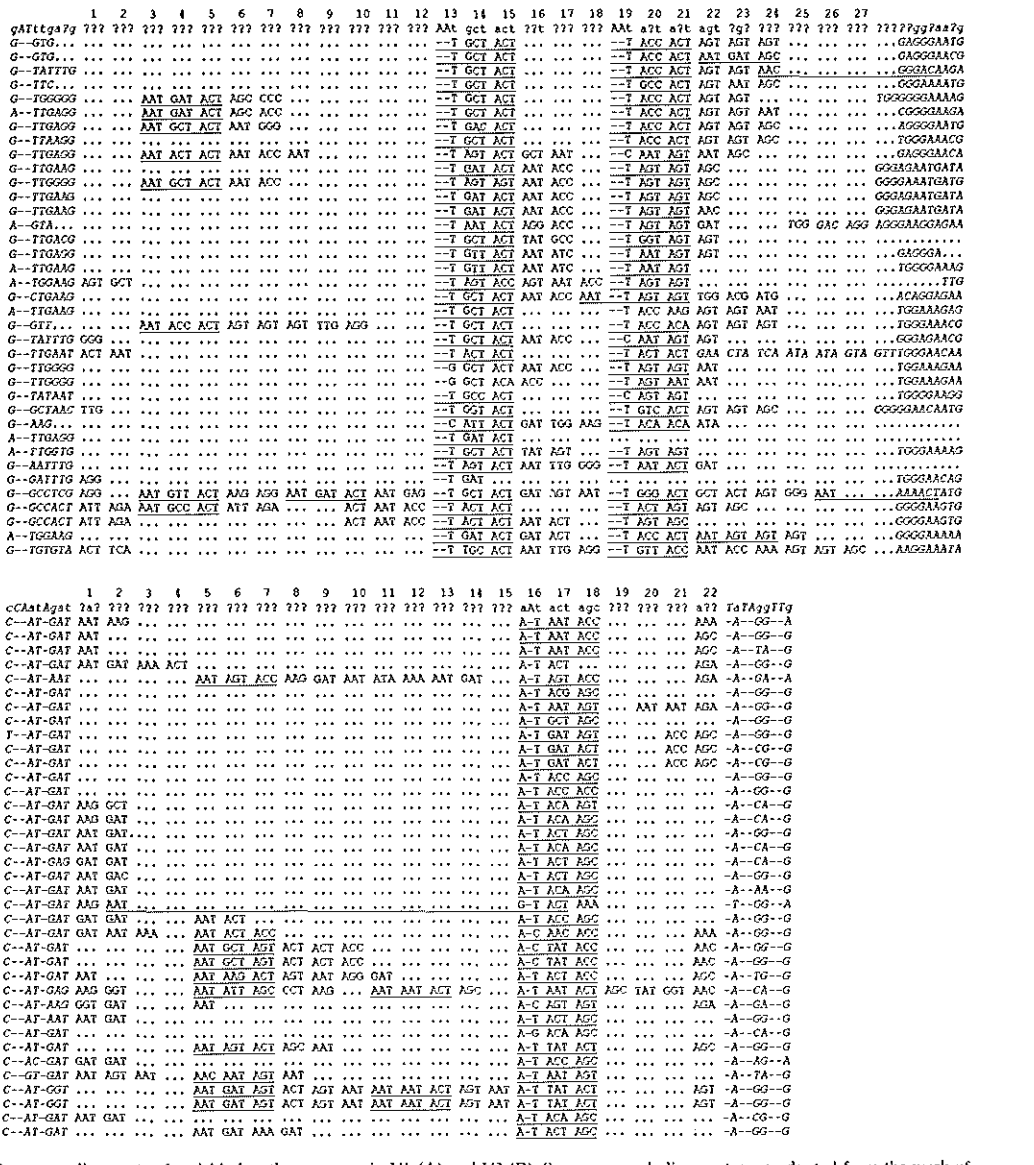

FIG. 1. Sequence alignments of variable-length sequences in V1 (A) and V2 (B). Sequences and alignment were adopted from the work of Myers et al. (12) and adjusted by hand. Numbers in the top row indicate codons of the variable-length sequence region. Sequences outside of the variable-length sequences discussed here are in italics. Dashes indicate nucleotides identical to the consensus sequence (top row), and dots indicate deletions relative to the consensus sequence. Uppercase letters in the consensus sequence indicate bases conserved throughout all aligned sequences; lowercase letters indicate bases found in at least 50% (but less than 100%) of the aligned sequences in reference 12. Blocks of three codons encoding a potential N-linked glycosylation site (asparagine-X-serine/threonine) are underlined.

(as, in this case, mostly A) is reflected in the encoded amino acid sequence. The preferential use of codons starting with an A (>80% in the V1 and V2 variable-length sequences) results in predominant insertion of isoleucine, methionine, asparagine, serine, threonine, lysine, and arginine. The virtual absence of T's at position 2 results in the exclusion of isoleucine (Ile) and methionine (Met), the only two hydrophobic amino acids whose codon starts with an A. Apparently, the insertion

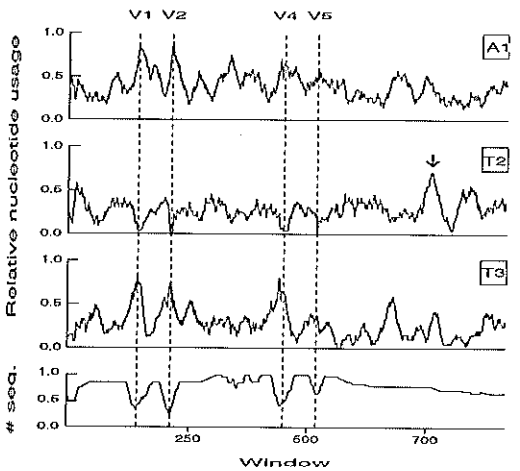

FIG. 2. Frequency distributions of A1 (top panel), T2 (second panel from top), and T3 (third panel from top) in the HIV-1 envelope gene, as well as the relative number of sequences counted in each window (bottom panel). Valleys in the bottom panel plot indicate regions of variable sequence length. Frequencies of each nucleotide at each position were counted in a window of 20 codons, shifted by 1 codon, with the aligned sequences taken from reference 12. Only A1, T2, and T3 are shown. Variable regions V1, V2, V4, and V5 are indicated (except V3). The 95% confidence interval upper and lower limits, calculated as mean ± 1.96× standard deviation following square root transformation of the data to achieve normality, are as follows: A1, 0.71 and 0.15; T2, 0.56 and 0.08; and T3, 0.63 and 0.05, respectively. The A1 and T3 peaks in V1, V2, and V4 exceed these limits, as do the T2 valleys in V1, V2, V4, and V5. A number of A1 peaks are found besides the V1, V2, V4, and V5 regions. The lack of concomitant T2 valleys and T3 peaks indicates that these have been generated through mechanisms different from the one postulated in this paper. A T2 peak is found at positions corresponding to windows 754 to 769 (arrow). All codons with a T at position 2 code for hydrophobic amino acids, and this peak corresponds to the location of the hydrophobic membrane anchor sequences in gp41. Both the alignments and the computer program used to calculate this plot are available from the authors upon request.

of hydrophobic amino acids in this region of env is disadvantageous to the virus. The strong bias towards T, a remnant of the postulated originally inserted AAT, and to a lesser extent towards C as a result of mutation and selection, at position 3 now selectively inserts threonine, asparagine, and serine at these positions. Both threonine and serine codons can be generated from the AAT and AAC asparagine codons through one mutation. Four of the six codons that can potentially code for serine start with a T rather than with an A, namely, T-C-x. These codons are not used in the insertion sequences described here, although they normally compose approximately 60% of all serine codons in mammalian genes (18) and are used throughout the Env open reading frame (not shown). Random insertions and mutations followed by selection would have resulted in a predominant use of T-C-x codons for serine. The exclusive use of AGT and AGC codons to code for serine is therefore a strong point in favor of the postulated mechanism. We have counted the frequency of asparagine, threonine, and serine in V1 and V2 and compared them with those in the gp120 amino acid sequence of HIV_{HXB2R}. In the V1 and V2 variable-length sequences, the combination of Asn, Thr, and Ser makes up 75% of all amino acids, compared with only 22% of all amino acids in gp120.

Insertion of AAT triplets followed by mutation and selection results in the insertion of predominantly asparagine (N), threonine (T), and serine (S) as described above. Random combination of these three amino acids will result in the frequent occurrence of N-x-T and N-x-S sequence blocks, where "x" stands for any amino acid. Both N-x-T and N-x-S are potential N-linked glycosylation sites, and indeed the variable-length sequences are characterized by the occurrence of multiple potential glycosylation sites. Approximately 55% of all amino acids in the variable-length sequences are actually involved in the formation of N-linked glycosylation sites (Fig. 1), demonstrating the efficiency of the postulated mechanism for the generation of such sites. If glycosylation of these regions confers selective advantage to the virus, then it might be expected that virus genotypes that contain such a novel glycosylation site would persist and appear in future virus generations. Examples of such glycosylation sites that have become stable features of the HIV-1 genome can be found in both the V1 and V2 variable-length sequences (V1 codons 13 to 15 and 19 to 21 and V2 codons 16 to 18 [Fig. 1]) and the V4 and V5 regions (not shown). The occurrence of sequences with variable length 5′ and 3′ of these conserved sites indicates that the mechanism of AAT triplet insertion is ongoing; we postulate that the accumulation of more glycosylation sites provides further selective advantages to the virus through better masking of the epitopes in this region from the immune system. We predict, therefore, that the number of glycosylation sites in these regions of the Env glycoproteins of viruses isolated from infected individuals is directly related to the immune pressure exerted on these regions, which may vary from person to person, but in all probability will increase over time, especially during the asymptomatic period of infection. Others have shown a statistically significant sequence length increase and addition of a novel N-linked glycosylation site at the V2 insertion sequence that correlates with increased viral virulence (8). More virulent virus variants generally arise over time in infected individuals and, as discussed above, so will the length of the insertion sequences because of selective advantages of the viruses that have acquired more glycosylation sites. The observed correlation between viral virulence and sequence length variation in V2, therefore, probably reflects the parallelism in time of these two processes. Accumulation of sequences rich in potential glycosylation sites (both N-linked and O-linked) has been reported for the V1 regions of simian immunodeficiency virus (SIV_{MNE}) isolates obtained from monkeys that progressed towards AIDS (13). This region in SIV shows evidence of ACA triplet reiteration (based on the SIVenv alignment in the work of Myers et al. [12] and on the work of Overbaugh and Rudensey [13], not shown), which results in the accumulation of threonine residues, thereby creating potential targets for O-linked glycosylation (13). It appears that the highly related lentiviruses SIV and HIV have adopted very similar but not identical strategies (AAT versus ACA reiteration) to achieve the same goal: glycosylation of sites on the envelope glycoproteins that are important for the virus life cycle, thereby shielding these sites from recognition by antibodies.

The sequences in the V1 and V2 region play a direct role in virus entry, as discussed above. No such functional properties have been described for either the V4 or the V5 region, and yet it appears from our analysis that they may be subject to the same selective pressures as V1 and V2. The high degree of variation in these regions hampers functional studies, e.g.,

because of the difficulties of raising cross-reactive antibodies. It is conceivable that functional roles for V4 and V5 will be described in the future. Alternatively, glycosylation at V4 and V5 could help mask other functional regions that are brought into the proximity of V4 and/or V5 in the folded molecular complex.

The mechanism by which the described triplet reiterations arise is unclear. Recently, a number of human genetic diseases characterized by trinucleotide repeats have been described (reviewed in reference 15), and strand slippage during DNA replication (5) has been proposed as a mechanism that could introduce such repeats (16). Tandemly repeated sequences are also observed in human hypervariable minisatellite sequences (10), and an alternative mechanism involving double-stranded breaks and gap repair has been proposed (9). Although it is tempting to speculate that the viral reverse transcriptase is primarily responsible for the observed phenomenon, in vitro studies of the HIV-1 reverse transcriptase have not revealed direct evidence for triplet insertion, although strand slippage during replication of DNA or RNA is observed (4). The examples quoted above are compatible with the idea that cellular polymerases may be involved.

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CHAPTER 7

A Region of the Transmembrane Glycoprotein of Human Immunodeficiency Virus Type 1 Involved in Syncytium Induction and Viral Tropism

Submitted for publication

A Region of the Transmembrane Glycoprotein of Human Immunodeficiency Virus Type 1 Involved in Syncytium Induction and Viral Tropism.

ARNO C. ANDEWEG[1,3], MARTIN SCHUTTEN[1,2], PATRICK H.M. BOERS[1], ALBERT D.M.E. OSTERHAUS[1,2] and MARNIX L. BOSCH[1,4]

[1]Laboratory of Immunobiology, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands. [2]Department of Virology, Erasmus University Rotterdam, The Netherlands. [3]Present address: Laboratory of Viral Pathogenesis, Biomedical Primate Research Centre, Rijswijk, The Netherlands. [4]Present address: Regional Primate Research Center and Department of Pathobiology, University of Washington, Seattle, USA.

Human immunodeficiency virus type 1 entry and virus induced syncytium formation are both membrane fusion processes that are initiated by the binding of the viral envelope glycoproteins to the CD4 receptor. To identify critical regions of the envelope glycoproteins that are involved in these complex processes, we have generated chimeric genes from genetically highly homologous envelope genes obtained from a single donor, which display differences in syncytium inducing capacity. Through transient expression in SupT1 cells we identified a region of the transmembrane glycoprotein gp41 involved in syncytium formation. Similarly we have previously identified the V2 and V3 regions of the surface glycoprotein gp120 as natural determinants of syncytium formation. In an envelope complementation assay the contribution of this region, and also of the V2 and V3 regions, to viral entry of different cells was determined. Exchange of only the gp41 region, and not the V2 or the V3 region, transferred the capacity of entry into SupT1 cells to an envelope protein previously not able to mediate infection of SupT1 cells. Likewise infectibility of MT2 cells could be transferred through exchange of only the V3 region. These results demonstrate that different regions of the envelope protein may play distinct roles in membrane fusion processes underlying syncytium formation and viral entry. Furthermore these results show that different envelope regions independently control viral entry of different cell types. They also indicate that functional interactions of different viral determinants with cellular determinants can result in unique combinations leading to the entry of specific target cells.

Human immunodeficiency virus type 1 (HIV-1) enters the cell via direct membrane fusion at the cell surface (39,55). The fusion process is initiated by the binding of the viral surface glycoprotein gp120 to the cellular CD4 molecule. This receptor binding induces conformational

changes that result in an increased exposure of regions of gp120, dissociation of gp120 from the transmembrane glycoprotein gp41, and unmasking of gp41 epitopes (28,41,42,47,48). A basically similar membrane fusion process of infected cells, expressing envelope glycoproteins at the cell surface, with adjacent CD4-positive cells may result in the formation of multinucleated syncytia (37,38,53). Cellular factors other than CD4 are also involved in viral entry and syncytium formation (5,8,14,19,21,27).

HIV-1 variants differ in their capacity to enter different cell types (15,22,26,46). Both macrophage tropic and T cell tropic HIV-1 variants have been described and such variants may play different roles in the pathogenesis of HIV-1 infection (13,56). A select group of HIV-1 isolates has the ability to replicate to high titers in immortalized human T-cell lines. This has allowed the study of characteristics of HIV-1 that could be relevant for in vivo pathogenesis like e.g. viral entry and sensitivity to virus neutralizing antibodies. However, T-cell tropic viruses represent only a small subset of HIV-1 variants present in vivo, with properties that may be absent from most viruses isolated directly from HIV-1 infected individuals.

Numerous studies have linked HIV-1 cell tropism to cytopathicity. T-cell tropic viruses appear to induce syncytia in vitro whereas this is not reported for macrophage tropic viruses (49). In particular these syncytium inducing (SI) viruses are able to replicate in T-cell lines like e.g. MT2, which allows the rapid detection of such variants in clinical specimens (35). Nevertheless insufficient data are available to positively state that cell tropism and syncytium formation are genetically linked features of HIV-1, especially in situations that closely mimic in vivo circumstances. Molecular clones of HIV-1 reveal a less than complete correlation between T-cell line tropism and syncytium formation. Non syncytium inducing (NSI) HIV-1 clones that replicate in T-cell lines like SupT1, as well as SI clones that replicate in macrophages have been described (16,26). A recent study reveals the widespread tissue distribution of HIV-1 variants in vivo with a genetic signature of macrophage tropic variants (see below) but with evidence of syncytium formation, again pointing to potential discrepancies between cytopathicity and cell tropism (18).

Genetic mapping of determinants involved in either cell tropism or syncytium formation has identified several regions of the envelope gene products of HIV-1 involved in these processes. Initial evidence pointed to the hypervariable V3 region as the major determinant in both tropism and syncytium formation (32,52) with a more positively charged V3 region associated both with T-cell tropism and enhanced syncytium formation (57). Based on these data T-cell tropic and macrophage tropic signatures were assigned to the V3 regions of HIV-1 isolates (23). More recently also genetic variation in other envelope regions like the V1 and V2 regions has been implicated in determining either cell tropism or cytopathic potential of HIV-1 (4,7,24,25,34,57). Mutational analysis demonstrated that no simple distinction can be made between SI and NSI variants of HIV-1 on the basis of differences in the V2 region (2), although sequence length variation in this region has been linked statistically to cytopathic potential (25). The lack of such length variation between V2 regions with widely differing influences on envelope mediated syncytium formation however, indicates that the observed correlation is not absolute.

Next to the influence of genetic variation of individual regions of the envelope gene products on cell tropism and syncytium formation the overall conformation of the envelope protein complex is also of prime importance since evidence of specific interactions between different envelope regions for syncytium formation has been presented recently (24). Identification of all of the viral determinants involved in determining cell tropism and cytopathicity will eventually lead to the understanding of these complex processes.

Since HIV-1 viral entry and syncytium formation depend on both the amino acid sequence and the conformational structure of the envelope glycoproteins, we used naturally occurring sequence variation to probe envelope glycoprotein function in these processes. We have relied on gain-of-function experiments where syncytium forming capacity was transferred from a syncytium inducing envelope gene into a genetically highly homologous but non syncytium forming envelope background. This approach minimizes the risk of corrupting the protein structure, although such undesired consequences cannot formally be ruled out. In this way we have previously demonstrated the involvement of both the V2 and V3 regions in syncytium formation (4). However in our previous experiments not all transfer of syncytium forming capacity could be explained by variation in V2 and / or V3, since in some experiments especially the carboxy-terminal half of the envelope gene products, including gp41 sequences, appeared to be involved. In this paper we demonstrate that genetic variation in a discrete region of gp41 directly influences syncytium forming capacity of the envelope genes involved, independent of V2 and V3. Furthermore we demonstrate that, like the V3 region, this region is also involved in determining cell tropism.

MATERIAL AND METHODS

Construction of chimeric envelope genes. Previously a set of chimeric HIV-1 envelope genes was generated starting from four phenotypically well characterized envelope genes with known nucleotide sequence (3). These parental envelope genes were derived from biologically cloned viruses obtained from a single individual (#16) at a single time point and were designated 16.1 to 16.4 (3). For the present study chimeric envelope genes were constructed by exchanging gene fragments between envelope gene 16.2 and 16.3. We selected this envelope gene pair since it represents the pair with highest amino acid sequence homology (94.5%) yet displaying different syncytium inducing capacity when expressed (3). The gene fragments were obtained by digestion with restriction enzymes (Spe I, Stu I, Cvn I, EcoN I, Avr II, Bgl II and Pst I) with conserved restriction sites in these envelope genes and with restriction enzymes (Xho I and Xma I) cutting the cloning vector pGEM7- (promega). Occasionally envelope gene fragments were subcloned in puc18 to enable the exchange of gene fragments generated by enzymes that are cutting at multiple sites in the envelope gene. The resulting envelope chimeras were named according to the exchanged gene fragment(s) and the two involved parental envelope genes (see Fig. 1 and 3). All chimeric envelope genes were tested by restriction mapping using restriction sites that were non-conserved in the parental envelope genes or by

partial sequencing to verify the cloning procedure. The similarly produced chimeric envelope genes 13SS, 23SS, 13HC, and 23HC were previously described (2,4).

Transient env expression and syncytium formation assay. All generated chimeric envelope genes were cloned in the simian virus 40 based expression plasmid pSRHS as previously described (4,20). Subsequently the constructs were transfected into Sup-T1 cells by electroporation. Five million cells were mixed with 20 μg DNA in 250 μl RPMI (Gibco) supplemented with 10% foetal calf serum (FCS) in a 4 mm electroporation cuvette (Eurogentec S.A.) and after electroporation (Biorad genepulser, 200 volts, 960 μF) the cells were transferred to culture flasks with RPMI medium. One day post transfection, viable cells were isolated on a Ficoll density gradient and cultured for an additional three days during which the transfected cell cultures were examined for the presence of syncytia. Relative sizes of the syncytia found at day three in each culture was quantified as: small (+): up to five times the size of single cells, large (+++): giant syncytia estimated to contain over 100 fused cells (scored as being larger than 250 μm in diameter) or intermediate (++). At day three post transfection the relative expression of envelope proteins was determined in both supernatant and cell lysates in an ELISA as previously described (4). Each construct was tested at least three times in independent assays.

Envelope complementation assay. The envelope complementation assay was performed basically as described by Helseth et.al. (29). COS cells were cotransfected with 15-20 μg of the pHXBΔenvCAT plasmid and the same amount of a pSRHS construct coding for one of the (chimeric) envelope genes. Electroporation of COS cells was performed as described in the previous section but pulsed at 240 volts. The next day culture medium was replaced and the adherent viable cells were cultured for three days. Subsequently cell-free supernatants with complemented virions were collected by centrifugation, aliquoted and frozen at -135°C. Prior to infection the amount of p24 antigen, as determined with the V5 Elisa kit from Organon Teknika, was standardized and the level of envelope protein expression was checked in an Elisa as previously described (4). Infection of two million cells (SupT1, MT2 or H9) or five million human peripheral blood mononuclear cells (PBMC) was performed overnight in 1 millilitre RPMI (Gibco) complemented with 10 % foetal calf serum. The next day fresh medium was added and cells were cultured for an additional two days. Finally the target cells were washed in PBS, pelleted, lysed, and assayed for chloramphenicol acyltransferase (CAT) activity using a phase-extraction procedure as described elsewhere (51).

RESULTS

Gp41 determinant in envelope mediated syncytium formation. Chimeric envelope genes were constructed from two genetically highly homologous parental envelope genes 16.2 and 16.3 described elsewhere (3). The transfer of a series of small 16.2 SI gene derived fragments to the 16.3 NSI background envelope gene resulted in a set of 13 new chimeric envelope genes.

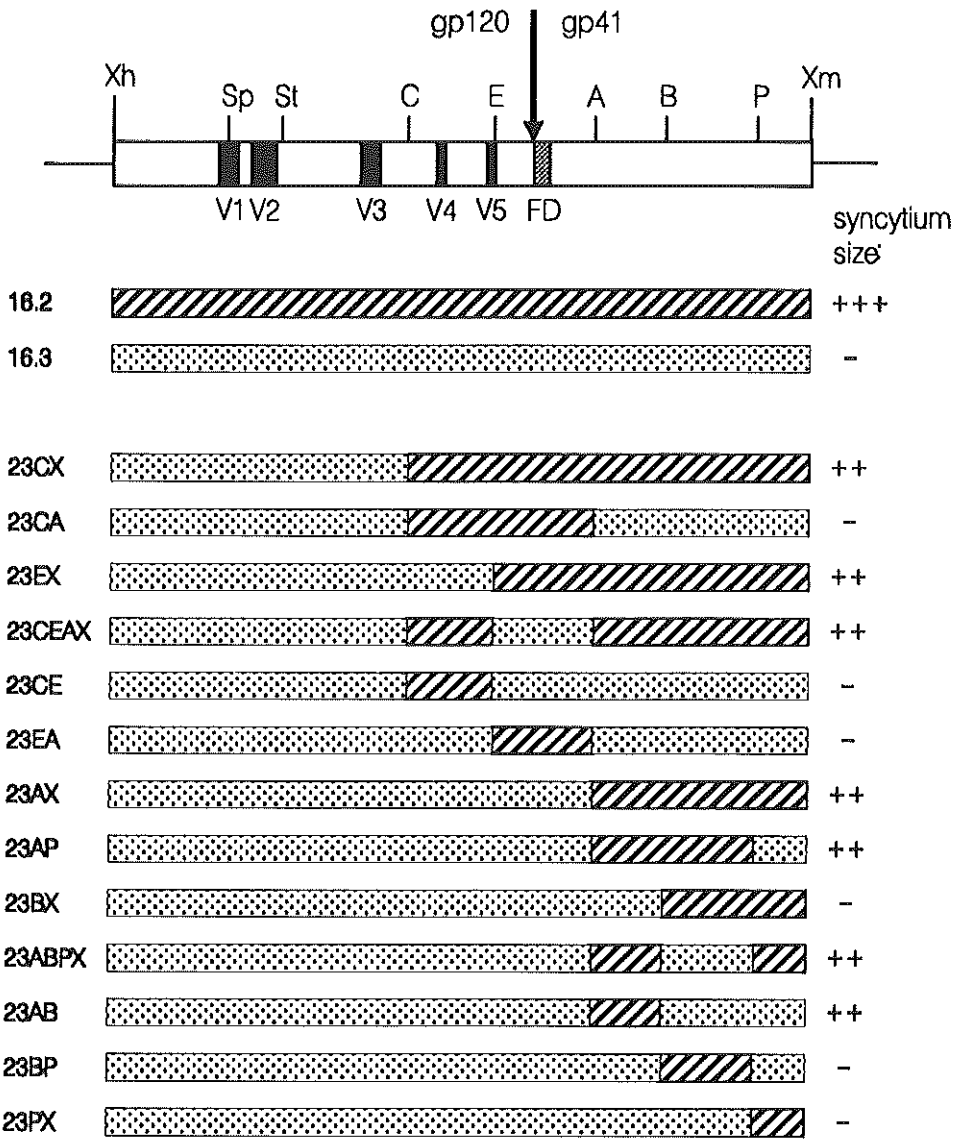

FIG. 1. Syncytium size induced by (chimeric) envelope gene products. The syncytium sizes of the expressed parental envelope genes (16.2 SI and 16.3 NSI) together with the chimeric envelope genes generated are shown. At the top the complete envelope gene is represented by a bar and the restriction enzymes used to generate the chimeras are indicated. Abbreviations: V, variable region; FD, fusion domain; Xh, Xho I; Sp, Spe I; St, Stu I; C, Cvn I; E, Eco NI; A, Avr II; B, Bgl II; P, Pst I; Xm, Xma I. Symbols: arrow, cleavage site; +, small syncytia; ++, intermediate size syncytia; +++, large syncytia; -, no syncytia (for more details, see material and methods section).

Mainly 16.2 SI sequences derived from the 3' half of the envelope gene coding for the transmembrane protein were transferred as shown in Fig 1. This set of chimeric envelope genes was transiently expressed in SupT1 cells to determine the contribution of the transferred SI envelope regions to syncytium formation. The expression of envelope protein in the individual cell cultures was determined in both supernatants and cell lysates using an ELISA. Relative envelope protein expression for all constructs was at the level routinely detected for all our pSRHS envelope gene constructs expressed in SupT1 or CEMx174 cells. These levels of envelope production corresponded to levels at least eight times those already sufficient for the immediate induction of large size syncytia (4) (results not shown).

Fig. 1 shows the syncytium inducing capacity of the parental envelope genes together with the novel constructed set of chimeric envelope genes upon transfection in SupT1 cells. As previously shown the 16.2 derived envelope induced very large syncytia, in contrast to the 16.3 envelope, despite their high degree of amino acid homology (3,4). The syncytium inducing capacity of chimeric envelope 23CX supports our previous observation that also the carboxy terminal part of the encoded envelope protein contributes to the overall syncytium inducing capacity of SI envelopes (4). Subsequent repeated transfer of progressively smaller regions of the 16.2 SI gene to the 16.3 NSI background demonstrated that the transfer of syncytium inducing capacity could be achieved by the exchange of only the small fragment generated by the Avr II-Bgl II restriction enzyme digestion (Fig. 1). All other separately transferred gene fragments in this region failed to contribute to syncytium formation, also when transferred simultaneously with the AB region.

Fig. 2 shows the amino acid sequence and location of the AB fragment on the transmembrane protein gp41 for all four envelope genes originally obtained from patient #16. As few as seven of the only 52 amino acid residues that differ between the two parental envelope protein sequences 16.2 and 16.3 are situated in the AB region. With the exception of the lysine to threonine change at position 682 all variable residues were tightly clustered around position 645. Most of the observed amino acid variation is non-conservative and frequently involves charged residues. The variation of charged residues in this region however did not result in a significant altered overall net (negative) charge when the amino acid sequence of the SI and NSI envelope proteins of patient #16 are compared (Fig. 2).

Contribution of discrete envelope regions to cell tropism. Exchange experiments in which discrete gene fragments were transferred between four envelope genes obtained from patient #16 revealed that at least three regions are functionally involved in membrane fusion. In addition to the presently identified determinant located on the AB gene fragment, also natural sequence variation present in the V2 and the V3 region was previously demonstrated to transfer syncytium inducing capacity to the 16.3 NSI envelope (4). In order to evaluate the contribution of these same three regions to determining cell tropism, the complementation assay described by Helseth et.al. was adopted (29). Cotransfection of cells with the generated (chimeric) envelope constructs together with the IIIB molecular clone in which the original envelope gene was replaced by the CAT reporter gene resulted in virions that may introduce CAT activity in

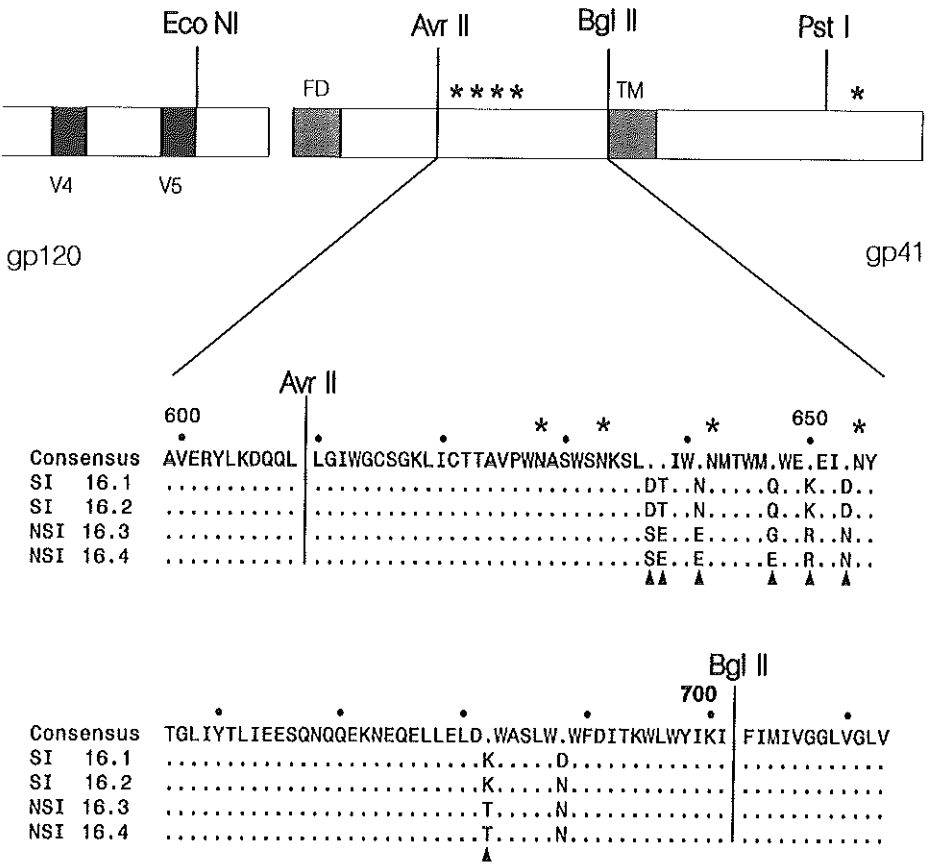

FIG. 2. Location and predicted amino acid sequences of the gp41 AB region of the four envelope genes obtained from patient 16. Amino acids identical to those of the consensus sequence are indicated by (.), the amino acid positions given correspond to the complete envelope protein amino acid sequence as previously published (3). Abbreviations: V, variable region; FD, fusion domain; TM, transmembrane region. Symbols: *, potential glycosylation site; ▲, amino acid variation between envelope genes 16.2 and 16.3.

target cells when the envelope protein used for complementation supports entry of these cells. Depending on the envelope gene - target cell combination, up to 100 fold increase in CAT activity was observed as compared to a control infection in which the plasmid controlling envelope expression was omitted or when a potent neutralizing monoclonal antibody directed to the envelope protein was present during infection (data not shown). These control experiments demonstrated that a true envelope mediated phenomenon was assayed. Each complemented virus stock was furthermore monitored for p24 and envelope protein contents. The relative amounts of p24 and envelope proteins produced after cotransfection was similar

to the amounts of these proteins expressed upon transfection of a single complete molecular clone. The relative quantity of envelope proteins present in the virus stocks after p24 standardization varied up to threefold. No correlation between capacity to infect and relative envelope protein expression was observed (not shown). Finally, repeated functional testing of the (chimeric) envelope genes in the envelope complementation assay gave similar results for every single construct.

Fig. 3 shows the results of infection experiments using the four original parental genes and a selected set of chimeric genes as envelope protein donors and SupT1, PBMC, MT2, and H9

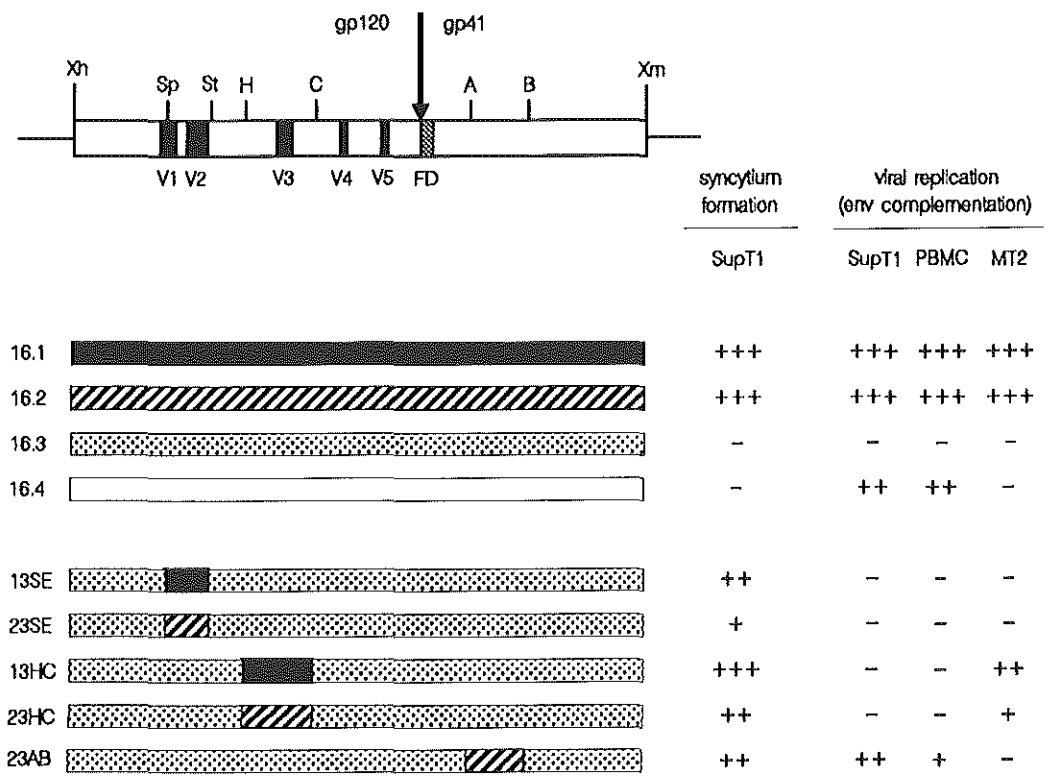

FIG. 3. Summary of parental and chimeric envelope genes products (see legend of figure 1) together with their syncytium inducing capacity in SupT1 cells and capacity to be involved in viral entry as shown in the envelope complementation assay. Syncytium inducing capacity of the 13SS, 23SS, 13HC and 23HC chimeric envelope genes have been determined previously (2,4). Arbitrary estimates of syncytium formation: +, small syncytia; ++, intermediate size syncytia; +++, large syncytia; -, no syncytia (for details, see material and methods section). Arbitrary estimates of CAT activities obtained in the envelope complementation assay are expressed relative to the CAT activity induced by virus complemented with the 16.2 envelope glycoproteins: -, background or low CAT activities less than 5 percent; +, 10 to 20 percent CAT activity; ++, 21-35 percent CAT activity; +++, more than 70 percent CAT activity.

as target cells. We chose SupT1 cells since these cells were used in our syncytium forming assays, MT2 cells since these are used by others to discriminate between SI and NSI isolates of HIV-1 (35), H9 as a generic T cell line and PBMC's as primary cells since our envelope genes are derived from viruses that have not been adapted to replicate in T-cell lines. None of the complemented virus stocks was able to significantly infect H9 cells and only very low CAT activities (less than three times background activity) were found. Both the SI envelope genes 16.1 and 16.2 however mediated infection of the two other target T-cell lines tested (SupT1 and MT2) which is compatible with the notion that syncytium inducing HIV-1 isolates can productively infect continuous T cell lines. In all cases the 16.2 SI envelope gene resulted in the highest CAT activities induced in the target cells (58000-69000 cpm). The CAT activities obtained in the envelope complementation assay for all envelope genes are expressed relative to the CAT activity induced by virus complemented with this 16.2 envelope gene. The NSI 16.4 envelope gene products also conferred tropism for SupT1 and PBMC (respectively 25 and 33 percent), but not for MT2 cells. No CAT activity over background was detected in any of the target cells tested when the 16.3 NSI envelope gene was used in the complementation assay.

The chimeric envelope genes tested next all contained one SI envelope gene fragment in the 16.3 NSI background. The SS fragment contained the V2 region, the HC fragment contained the V3 region and the AB fragment contained the AvrII-BglII fragment of the gp41 coding region of the envelope gene identified as described above (Fig. 1 and 2). No significant increases of CAT activity were found for the 13SS and 23SS constructs in any of the cells tested (up to 3 percent). Using the HC fragment, either from 16.1 or 16.2 SI envelope, significant increase in CAT activity was detected in MT2 cells (respectively 21 and 10 percent). The gp41 AB fragment from 16.2 in the 16.3 background conferred tropism for SupT1 cells and PBMC (respectively 24 and 14 percent).

DISCUSSION

Functional analysis of chimeric HIV-1 envelope genes constructed from genetically highly homologous envelope genes demonstrated that a region of the transmembrane glycoprotein of HIV-1 is involved in syncytium induction and viral tropism.

Exchange of gene fragments between phenotypically well characterized envelope genes obtained at a single time point from a single donor has enabled us previously to identify the variable regions V2 and V3 as natural determinants in syncytium formation (4). Here we have shown that also the small "AB region" of gp41 controls syncytium induction (Fig. 1). Thus the differences in syncytium inducing capacity of the simultaneously obtained parental viruses is apparently based on amino acid variation situated in at least three envelope regions located in either the transmembrane - or the surface glycoprotein.

The envelope complementation assay as described by Helseth et.al. (29) was used to test selected chimeric recombinant envelope proteins for their ability to support membrane fusion in envelope protein mediated entry of different target cells. The parental and the chimeric

envelope proteins displayed different capacities to mediate infection (Fig. 3). Both parental SI envelopes mediated entry in PBMC, SupT1, and MT2 cells in contrast to the 16.3 NSI envelope. The complementation experiments with the 16.3 based chimeric envelope genes demonstrated that the gp41 AB region determined tropism for PBMC and SupT1 whereas the V3 region from either 16.1 or 16.2 SI origin provided tropism to MT2 cells. This observation is in line with the use of MT2 cells as an indicator cell-line for convenient discrimination of SI versus NSI virus variants and this MT2 tropism appears to be determined by the V3 region at the level of viral entry. These results indicate that both membrane fusion processes are governed by overlapping envelope determinants. However, interesting differences were observed. First, the SI derived V2 and V3 regions which transfer syncytium inducing capacity to the 16.3 NSI envelope in SupT1 cells did not transfer the capacity to mediate entry into these cells. Furthermore, different envelope regions appeared to control the process of entry into different cells.

The 16.3 NSI envelope differs in only 11 amino acid positions throughout the transmembrane - and the surface glycoprotein, from the 16.4 NSI sequence (3). As for 16.4 no syncytia were detected in SupT1 cells upon expression of the 16.3 envelope gene (4). However the 16.3 complemented virus differs from 16.4 in its inability to infect SupT1 cells and PBMC. All the four parental envelope genes were obtained by PCR from viruses biologically cloned on peripheral blood mononuclear cells. Nevertheless the 16.3 gene products did not support infection of PBMC. Since chimeric envelope proteins containing the V2 region, the V3 region or the gp41 AB fragment of SI origin in the 16.3 background, were all capable of inducing syncytia, and two of these chimeric envelopes mediated viral entry, it is unlikely that the 16.3 envelope proteins were not functional. The inability of the parental 16.3 envelope gene products to support replication in PBMC may indicate that the gene products used in the complementation assay must interact with another HIV-1 gene product encoded by the HXB2 background and that this interaction is not operational in the case of 16.3. In this view it should be postulated that the gp41 AB fragment contains a determinant for this interaction which is essential for replication in PBMC and SupT1 but not for replication in MT2. Alternatively, the inability of the 16.3 envelope proteins to support replication in PBMC, may be due to the fact that the 16.3 envelope gene may have been amplified from virus replicating in macrophages, present during the biological cloning procedure but absent in the complementation assay since CAT activity was only determined in the non adherent cell fraction. We have attempted to address this question by using monocyte derived macrophages as target cells in the complementation assay. No CAT activities over background were found in these cells for any of the four parental envelope genes tested (results not shown). However, it remains to be determined whether the envelope complementation assay is sufficiently sensitive to detect low level entry or replication in these target cells.

Since the chimeric envelope proteins tested in the envelope complementation assay were only selected on the basis of their actual capacity to induce syncytia in SupT1 cells, we can not exclude that also other envelope regions, in addition to the AB region of SI envelope origin,

may transfer capacity to enter SupT1 cells. However even within the limited set of chimeric envelope proteins tested, amino acid variation in different envelope regions was shown to contribute to a different extent to membrane fusion processes involved in syncytium formation or viral entry. The observation that amino acid variation in three different envelope regions contributes to syncytium formation in SupT1 cells whereas only viral entry in supT1 cells is transferred by the AB region, suggests that membrane fusion involved in viral entry is more restricted, or that the threshold of syncytium formation is lower. This may be due to functional differences of envelope proteins on the virion or on the cell surface. Alternatively, it may be based on differences in additional membrane interactions like e.g. those exerted by ICAM and LFA-1, since it has been reported that these proteins may be involved in syncytium formation although not in the process of viral entry (10,30,45). The observation of a less restricted fusion process in syncytium formation that is controlled by amino acid variation in several regions of the envelope proteins is in line with the results of several other studies in which syncytium formation usually proved to be inhibited more easily than viral entry (11,29,36,54). As a consequence of the observed extension of the cellular host range of viruses isolated from the same individual over time, which may be based on amino acid variation in different envelope regions for different cell types (like e.g. V3 for MT2 and AB region for SupT1), the extension of the range of permissive cells is likely to coincide with the increased capacity of these viruses to induce syncytia. In this view the syncytium inducing phenotype of HIV-1 is property emerging as a direct consequence of the extended range of permissive cells. Different envelope regions which may independently be involved in viral entry, may all individually contribute to syncytium formation.

A functional role for the AB region in the membrane fusion underlying viral entry is supported by reports of neutralizing antibodies directed to this region (9,31,43). In this light it is interesting to note that the observed cluster of variable residues in the AB region is surrounded by four of the five potential glycosylation sites in gp41 (3) (Fig. 2) of which three are highly conserved in HIV-1 (17,44). Such an addition of carbohydrates may offer the virus the opportunity to escape from neutralizing antibody since addition of glycans may alter recognition by the antibody (1,6). Studies with T cell line adapted virus strains also support a role for the gp41 AB region in membrane fusion. Several mutations of conserved amino acids around position 675 were shown to result in increased syncytium forming capacities (12), and recently two studies reported an inhibitory effect of synthetic peptides, derived from sequences overlapping the here identified AB region, on syncytium formation (58) and virus replication (33). It has been postulated that this region, predicted to form an alpha-helix, is involved in the conformational rearrangements which lead to a "fusion-attack complex" of gp41 in which the fusion domain finally interacts with the target membrane (40). Since the AB region links the gp41 cysteine loop, that may form part of the gp120 interaction site (50), with this alpha-helix, it may be speculated that the AB region plays a role in transducing the trigger for gp41 conformational changes as initiated with CD4 binding to gp120. Natural sequence variation in the AB region may thus modulate the capacity of the envelope complex to mediate membrane

fusion.

In conclusion, the present studies demonstrated that natural sequence variation in multiple HIV-1 envelope regions, on both envelope proteins gp41 and gp120, control syncytium formation. These regions however seem to play distinct roles in the membrane fusion process underlying viral entry. While different envelope regions independently control viral entry of different cells, functional interactions of different viral determinants with (multiple) cellular determinants apparently result in unique combinations leading to entry into specific target cells.

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CHAPTER 8

Enhancement of a Infectivity of Non-Syncytium Inducing HIV-1 by sCD4 and by Human Antibodies that Neutralize Syncytium Inducing HIV-1

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Enhancement of Infectivity of a Non-Syncytium Inducing HIV-1 by sCD4 and by Human Antibodies that Neutralize Syncytium Inducing HIV-1

M. SCHUTTEN, A. C. ANDEWEG*·†, M. L. BOSCH*·‡, A. D. M. E. OSTERHAUS

Erasmus University Rotterdam, Institute of Virology, Rotterdam, The Netherlands
**National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands*

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Enhancement of virus infectivity after sCD4 treatment has been documented for SIVagm and HIV-2. It has been suggested that a similar phenomenon may play a role in HIV-1 infection. In the present study we have analysed biological activities of virus neutralizing polyclonal and monoclonal human antibodies and of sCD4, towards HIV-1 chimeras with envelope proteins derived from one donor, which display different biological phenotypes. The antibodies, which recognize the V3 and/or the CD4 binding domains of the glycoproteins of these viruses and also sCD4 showed different levels of virus neutralizing activity toward the syncytium inducing HIV-1 strains. In contrast, they all dramatically enhanced the infectivity of an HIV-1 chimera with an envelope glycoprotein displaying the non-syncytium-inducing phenotype. Given the relatively conserved nature of non-syncytium-inducing HIV-1 surface glycoproteins early after infection, these data suggest a major role for antibody mediated enhancement of virus infectivity in the early pathogenesis of HIV-1 infection.

Professor Dr A. D. M. E. Osterhaus, Erasmus University Rotterdam, Institute of Virology, Dr Molewaterplein 50, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

INTRODUCTION

The identification and characterization of virus neutralization inducing (VNI) antigenic sites on the glycoproteins of HIV-1 have been the focus of many studies (for review see [1]). For practical reasons, HIV-1 strains and primary HIV-1 isolates which efficiently replicate in T cells have predominantly been used to study virus neutralization [1]. In this context it is important to note that macrophage-tropic non-syncytium-inducing (NSI) variants with low efficiency of replication in T cells predominate in the asymptomatic stage and T cell line-tropic syncytium inducing (SI) variants are almost exclusively found in late stages of the infection [2, 3]. The macrophage-tropic NSI strains have been shown to exhibit a relatively high level of conservation in their VNI V3 loop [4]. It has been suggested that escape from virus neutralizing (VN) antibodies is based on the relatively high mutation rate generally observed in the virus envelope glycoprotein. Given the fact that also the CD4 binding domain (bd) is relatively conserved, another mechanism of escape from VN antibodies may be postulated for the relative success of NSI/macrophage-tropic viruses to persist in the host. To study the basis of this apparent paradox, we have used an *env* complementation assay in which the biological activities could be compared of human HIV-1-specific monoclonal and polyclonal antibody preparations towards identical HIV-1 strains only differing in their glycoproteins which displayed an SI or an NSI phenotype.

MATERIALS AND METHODS

Human antibodies, sCD4 and HIV-1 envelope glycoproteins. The human monoclonal antibodies (HuMoAbs) used are directed against the CD4 binding domain (GP13 and GP68 [5]), a highly conserved conformational epitope on gp41 (K14 [6]) and the HIV-1 gp120 V3 loop (257-D [7]). Furthermore sCD4 (kindly provided by Dr I. Jones through the MRC AIDS directed programme) and HIVIG [8]) (kindly provided by Dr H. Schuitemaker) were used as VN agents. The *env* genes used [16.1 (SI), 16.2 (SI), 16.4 (NSI), MN and HIB] for

†Current address: BPRC, Lange Kleiweg 151, 2288 GJ Rijswijk, The Netherlands.

‡Current address: Regional Primate Research Center, I-421 Health Sciences Center SJ-50, University of Washington, Seattle WA 98195, USA.

the production of envelope glycoproteins in the capture antibody ELISA and for the production of chimeric viruses have previously been characterized and described [9, 10–12].

Capture antibody ELISA for measurement of relative affinity. The relative affinity of the HuMoAbs used in the present study was determined in a previously described ELISA. In short, ELISA plates (Costar, Cambridge, MA 02140, USA, cat. no. 3590), coated with affinity purified sheep immunoglobulins directed against the carboxy terminus of gp120 (Aalto BioReagents, Dublin, Ireland, cat. no. D7324), were incubated for 2 h with cell lysate of recombinant vaccinia virus (rVV) infected HELA [13] cells (16 (NSI) and 16 (SI)) or HIV-1 infected SupT1 [14] cells (MN [11] and IIIB [12]). Plates were subsequently washed and incubated with serial dilutions of previously described HuMoAbs (GP13, GP68 [5] and 257-D [7]). The ELISA was completed and read as previously described [10]. The relative amount of antigen bound to the ELISA plates was calibrated by using the same ELISA protocol with serial dilutions of the antigen and a standard excess concentration of polyclonal serum from seropositive individuals. The data presented are the mean values of duplicates in one assay which was performed at least twice with similar results.

Envelope trans-complementation assay. This assay was based on a previously described *trans*-complementation assay in which an *env*-defective HIV-1 provirus, encoding the bacterial CAT reporter gene [15], was complemented for a single round of replication by a set of recently described HIV-1 envelope glycoproteins [9]. The *env* genes encoding these glycoproteins were derived from biologically cloned SI or NSI viruses, isolated from one single donor (number 16) at a single point in time in natural target cells (peripheral blood mononuclear cells [PBMC]). In the complementation assay the genes encoding these envelope glycoproteins were co-transfected into a $CD4^-$ cell line (COS [16]) together with the *env*-defective molecular clone of the HIV-1 IIIB strain. This resulted in the production of virions carrying heterologous envelope glycoproteins which could infect $CD4^+$ cells, like the SupT1 cells or PHA stimulated PBMC used in this study. After 3 days cell free supernatants were collected by centrifugation and frozen at −135°C. For infection the amount of p24 antigen, as determined with a p24 ELISA kit (V5, Organon Teknika, Boxtel, The Netherlands) was calibrated and the level of envelope expression was checked with the capture antibody ELISA (see above). Infection was performed overnight at 37°C in 1 ml medium. After 24 h the cells were washed and cultured for 72 h. CAT activity in these cells was measured in a previously described CAT assay using a two-phase extraction system [17]. CAT activities expressed in the cells were considered a direct measure of virus infectivity in these assays.

Table 1. Relative affinities of HuMoAbs for different HIV-1 envelope glycoproteins

		HIV-1 strain (phenotype)				
HuMoAb	Specificity	16.1 (SI)	16.2 (SI)	16.4 (NSI)	MN (SI)	IIIB (SI)
K14	gp41	83*	67	67	33	67
GP13	CD4 bd	83	83	33	250	250
GP68	CD4 bd	67	67	67	125	250
257-D	V3 domain	125	125	125	125	–†

* Reciprocal of dilutions of MoAbs, starting at 1 μg/ml, still giving 50% of maximal binding with the respective envelope glycoproteins in a capture antibody ELISA as a measure of relative affinity. The data presented are the mean values of duplicates in one assay which was performed at least twice with similar results.

† No binding: OD450 nm values remained at background levels at the concentrations tested.

RESULTS

Relative affinity of HuMoAbs for HIV-1 envelope glycoproteins

The HuMoAbs used in these experiments were selected on basis of their epitope specificities and relative affinities in the capture antibody ELISA for different HIV-1 envelope glycoproteins (Table 1). The same recombinant envelope glycoproteins were used in the *env* complementation assay (Table 2). Both CD4 bd specific HuMoAbs GP13 and GP68 showed an overall higher relative affinity for the glycoproteins of the T cell line adapted HIV-1 strains IIIB and MN than for the glycoproteins derived from the donor number 16 HIV-1 strains. The V3 loop specific HuMoAb 257-D showed a high relative affinity for the glycoproteins of the MN-like HIV-1 strains and no binding was observed in this ELISA with the glycoproteins of HIV-1 IIIB (Table 1).

Virus neutralization and enhancement of viral infectivity by sCD4 and human antibodies

The VN activities of human antibodies and sCD4 toward T cell line adapted strains HIV-1 MN and HIV-1 IIIB (Table 2) have been documented previously [10–13]. The CD4 bd specific HuMoAbs GP13 and GP68 as well as HIVIG and sCD4, inhibited infectivity of HIV-1 16.2 (SI) only to a limited extent (< 50%) when tested at a concentration (15 μg/ml) that significantly inhibited infectivity of HIV-1 IIIB in the *env* complementation assay (reduction to 15%, 12%, 9% and 8% respectively) (Table 2). The V3 domain specific HuMoAb tested at the same concentration, neutralized HIV-1 16.2 (SI), as was shown by a reduction of CAT activities to 0.7% (Table 2). No major biological activity of any of the antibodies tested or sCD4, was observed against HIV-1 16.1 (SI) as was shown by a reduction of CAT activity with 2–9% only, using 15 μg/ml antibody (Table 2).

In contrast to the VN activities observed with the SI viruses, the same concentration of all these VN antibodies and sCD4 caused a dramatic enhancement of infectivity of HIV-1 16.4 which has a NSI envelope (> six-fold increase in CAT activity) (Table 2). In order to demonstrate the dose dependency of these phenomena, dose response curves were made with the V3 domain specific HuMoAb 257-D, which exhibited the highest affinity for all the envelop proteins

Table 2. Virus neutralizing and enhancement activities of human antibody preparations and sCD4

		Virus strain (biological phenotype)					
Antibody preparation	Epitope	MN* (SI)	IIIB (SI)	IIIB (SI)	16.1 (SI)	16.2 (SI)	16.4 (NSI)
none		–‡	–	100§	100	100	100
				72.461*	55.065	54.428	8.239
K14	gp41	–	–	103	98	106	96
GP13	CD4 bd	+	+	15	97	65	732
GP68	CD4 bd	+	+	12	93	65	741
sCD4	CD4 bd	+	+	9	92	84	620
257-D	V3	+	–	97	91	0.7	655
HIVIG	poly	+	+	8	95	95	744
		VN assay		*env* complementation assay			

‡ Due to differences in the VN assays used, biological activities are indicated as high (+) or undetectable (–).

§ Percentage of CAT activity relative to the CAT activity observed in the absence of antibody. The mean value within one experiment obtained with SupT1 cells is given. The antibody preparations were tested at least twice with the same chimeric virus yielding similar results.

* CAT activity in counts per minute (cpm), background values range from 300 to 800 cpm.

tested (Table 1). Clear dose related VN and enhancement activities were found with this antibody in the *env* complementation assay with HIV-1 16.2 (SI) and HIV-1 16,4 (NSI) respectively (Fig. 1). When these experiments were repeated in PHA stimulated PBMC essentially the same phenomena were observed. The overall differences were however less pronounced in this system (average enhancement about 50%) probably due to a limited cellular host range of this virus (A. C. Andeweg *et al*, unpublished observations).

DISCUSSION

Using an assay that is highly sensitive for measuring viral entry we have shown in the present study that both sCD4 and human antibodies which neutralize SI HIV-1 strains may enhance infectivity of NSI HIV-1 strains.

HIVIG, sCD4 and the CD4 bd specific HuMoAbs GP13 and GP68 readily neutralized the HIV-1 IIIB strain whereas limited or no VN activity was observed against the primary HIV-1 strains from donor number 16. This supports previous observations that sCD4, CD4 bd specific HuMoAbs and HIVIG have little or no neutralizing activity against primary virus strains (M. Schutten, personal observation and [18]). It has been shown by several groups that VN capacity of V3 loop specific MoAbs toward T cell line adapted HIV-1 strains is dependent on the affinity of the antibody for the V3 loop of the HIV-1 strain used [7, 19]. However, in the present study we showed that VN activity of V3 loop specific HuMoAb 257-D toward the SI HIV-1 strains from donor number 16 is not directly related to its relative affinity for the glycoproteins involved: identical relative affinities for the 16.1 (SI) and 16.2 (SI) envelope glycoproteins were observed, but reductions in the *env* complementation assay proved to be 9 and 99.3% respectively. This was unexpected since both envelopes exhibit SI capacity and a 95% overall predicted amino acid sequence homology exists between these envelopes [9]. Since we have previously shown that other domains than the V3 domain are involved in membrane fusion [10], it may be speculated that HIV-1 16.1 is less dependent on the V3 domain to establish membrane fusion and infection.

The efficiency of entry into T cell lines of the NSI HIV-1 strain 16.4 was significantly lower than that of the SI HIV-1 strains 16.1 and 16.2 as shown by a >six-fold lower CAT activity. The infectivity of the NSI HIV-1 strain was however enhanced to levels comparable to those of the SI HIV-1 strains by the administration of sCD4 or antibodies that neutralized these SI HIV-1 strains. Different mechanisms for HIV-1 specific antibody mediated enhancement have been described, including Fc receptor- and complement-mediated enhancement [20, 21]. Two observations argue against the same mechanism in our experiments. Firstly, SupT1 cells do not express Fc receptors [14] and complement was not present in the assays. Secondly, enhancement of HIV-1 16.4 (NSI) was also observed with sCD4 (Table 2). An explanation for the observed enhancement can be found in a mechanism previously described for the enhancement of non-cytopathic SIV agm infectivity and induction of HIV-2 mediated cell fusion of $CD4^+$ cells, by sCD4. In these systems sCD4 does not induce dissociation of surface glycoprotein (SU) from infected cells [22, 23]. In contrast, sCD4 does

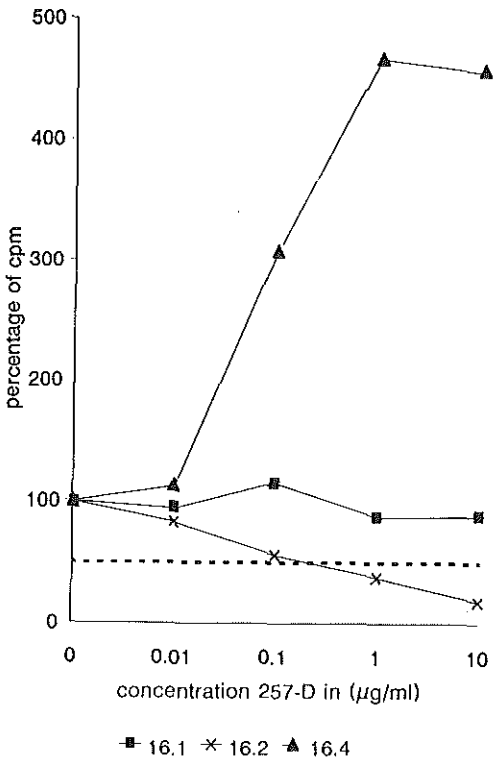

Fig. 1. Virus neutralizing and infectivity enhancing activities of serial dilutions of HuMoAb 257-D using SupT1 cells in the *env* complementation assay with HIV-1 16.1 (SI), HIV-1 16.2 (SI) and HIV-1 16.4 (NSI) chimeric viruses. Counts per min (cpm) obtained in the assays with antibodies, are expressed as percentages of cpm obtained in the assay carried out in the absence of antibodies. Dotted line represents 50% inhibition of virus infectivity.

induce dissociation of SU from cells infected with T cell line adapted HIV-1 strains which are readily neutralized by sCD4 [24]. We hypothesize that both sCD4 and the antibodies we studied, cause conformational changes in the viral envelope complex of certain NSI/macrophage-tropic HIV-1 strains exposing cryptic fusion sites as was also shown in the SIVagm and HIV-2 systems [22, 23]. These changes would allow a more efficient virus-cell fusion that is less dependent on the presence of CD4 on the target cell membrane.

Taken together our data suggest that naturally occurring antibodies to the so-called HIV-1 VNI antigenic sites, may cause either neutralization or enhancement dependent on the biological phenotype of the virus recognized. If indeed our findings are a reflection of a more general phenomenon of enhancement also found in infections with other lentiviruses like HIV-2 [23] and SIVagm [22], they may explain why early after HIV-1 infection predominantly viruses with an NSI/macrophage-tropic phenotype are found and why these viruses are able to persist in the infected host despite the conserved nature of their 'VNI' antigenic sites. The observed enhancement would actually favour replication of viruses displaying the NSI/macrophage-tropic phenotype, whereas viruses displaying the SI/T cell line-tropic phenotype are neutralized.

These observations may not only have implications for our understanding of the pathogenesis of HIV-1 infection but also for the development of immunization strategies. If this observation is the reflection of a more general phenomenon, the induction or administration of antibodies that neutralize viruses of the SI/T cell-tropic phenotype, which is a commonly followed strategy at present, may actually be counter productive.

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CHAPTER 9

Summary and General Discussion

Summary and General Discussion

The studies presented in this thesis aimed at identifying and characterizing envelope protein determinants involved in HIV-1 induced membrane fusion. Identification of such determinants adds to the understanding of the complex interactions of the HIV-1 envelope proteins with the target cell, that lead to membrane fusion as an integral part of viral entry and syncytium formation. It also provides information on virus evolution under selective conditions in the host. When these studies were initiated most attention was focused on the V3 loop as the principal determinant controlling cell tropism and syncytium inducing capacity of HIV-1. The results of the studies presented here demonstrate however, that in primary virus isolates at least three determinants located in different envelope regions control these membrane fusion processes. Furthermore, they demonstrate that antibodies may directly modulate the function of primary HIV-1 envelope proteins in membrane fusion.

Experimental approach. The studies presented focused on a limited number of genetically highly homologous primary envelope proteins which had been obtained from selected viruses differing in their syncytium inducing capacity. In most of these studies envelope protein functions were studied independently of the rest of the background of the viruses from which they had been obtained. The capacity of individual envelope proteins to induce membrane fusion was determined in syncytium forming assays upon expression in PBMC or T cell lines. To this end recombinant vaccinia viruses expressing envelope genes were generated or envelope genes were transfected into target cells upon cloning in an eukaryotic expression vector. To determine the capacity of envelope proteins to mediate viral entry (cell tropism) an envelope complementation assay was used. In this assay an envelope gene deleted genome of HIV-1 was cotransfected into cells with envelope genes of other viruses in order to study envelope gene determined phenotypes. The contribution of individual envelope regions to membrane fusion was determined by functionally testing chimeric envelope genes generated from parental envelope genes that display different syncytium inducing capacities. These chimeric envelope genes were tested in the syncytium forming assay and in the envelope complementation assay. This approach allowed the independent study of the contribution of different envelope regions in the processes underlying syncytium formation and viral entry. The inherent limitation of the studies presented in this thesis is that they focus on a set of glycoproteins from one or a limited number of individuals. This may however also be considered the strength of these studies, since the consequences of envelope protein variation can be studied against one and the same background. Due to the limitation indicated it should be realized that additional regions beyond the ones identified here may be involved in the membrane fusion process. However the approach chosen resulted in the identification of three regions involved in syncytium formation and viral entry, illustrating the complex nature of the phenomena studied. Finally it may be postulated that apart from the positive effects of certain envelope regions on membrane fusion activity, also negative effects may exist, although no evidence for this supposition was found in the studies described in this thesis.

HIV-1 phenotype analysis: the role of the envelope glycoproteins. During primary HIV-1 infection, isolated virus populations display a remarkable homogeneity, both in genetic sequence (31,60,61) and in biological properties (61). Intra-patient virus variability increases with time during which also the specific immune response develops. Extensive sequence analyses of virus populations later in the course of HIV-1 infection have revealed the presence of quasispecies; populations of highly homologous viruses yet all differing from each other (19,46,55). For the experiments described in chapters two and three, sets of envelope genes were studied from biologically cloned viruses simultaneously obtained from patients at a time that both SI and NSI variants were present. These genes may be considered samples taken out of a pool of quasispecies. The complete envelope sequences of eight biological clones obtained from two individuals (#16 and #320) were studied in chapter 2. Recombinant vaccinia expression of these envelope genes demonstrated that variation of the envelope gene alone may be sufficient to explain the observed variable syncytium inducing capacities of the respective parental viruses. The predicted protein sequences obtained from the two individuals exhibited amino acid variation which was mainly located in the previously denominated variable regions. Intra-patient phenotype associated sequence variation (SI versus NSI) was present in many envelope regions but appeared not to be conserved between the sets of envelope sequences obtained from the two different donors (chapter 2). This analysis did not provide information on the location of putative determinants for syncytium formation. Interestingly, analysis of an extended number of envelope genes obtained from individual #320, as described in chapter 3, showed that amino acid variation determining the syncytium inducing capacity was most likely located in the C1 to V4 region of gp120. The analysis also revealed, albeit by exclusion, that determinants for cell tropism resided in envelope regions outside the V3 region. Furthermore comparison of the sequences demonstrated that some of the envelope genes most likely resulted from genetic recombination events *in vivo*.

In chapter 4 and 7, genetic exchange experiments between envelope genes of SI and NSI origin were presented. For these studies the envelope genes obtained from patient #16 were used. It was shown that at least three envelope regions contain determinants for membrane fusion. In addition to variation in the V3 region, for which already a role had been demonstrated, also variation in the V2 region of gp120 and in a small region of gp41 (the "AB region") was shown to contribute to differences in syncytium inducing capacity. Furthermore within this set of envelopes, the V3 region and the AB region were identified as natural determinants involved in HIV-1 cell tropism.

The V3 region. Since it was shown that antibodies directed to the V3 loop neutralize HIV-1 infection (21,27,37) many studies have focused on this envelope region. It was demonstrated that infection and syncytium formation are blocked by anti-V3 antibodies after binding of gp120 to CD4 (35,51). Furthermore it had been demonstrated that changes in the V3 loop could abolish membrane fusion capacity (9,16,22,24) and could influence cell tropism (5,10,26,36,50,57). In addition, amino acid variation at two positions at both sides of the relatively conserved tip of the V3 loop, was shown to correlate with syncytium inducing

capacity and cell tropism (13). Indeed also the V3 loop of the envelope proteins studied here, displayed phenotype correlated amino acid variation at these positions. Exchange of a region containing the V3 loop, transferred syncytium inducing capacity after transfection of SupT1 cells from SI to NSI envelope backgrounds. Furthermore, the experiments presented in chapter 4 suggested a functional interaction between the V3 region and other envelope regions in syncytium formation. This finding is in favour of a multi-determinant process of membrane fusion.

As shown in chapter 7, the SI derived V3 region contributes to MT2 tropism but not to SupT1 tropism. Apparently the process of SupT1 entry is a more restricted event than the process of SupT1 syncytium formation, since this SI derived V3 region did confer the capacity to induce syncytia in SupT1 cells to an NSI envelope background. Upon sCD4 incubation the V3 region exposure is increased (47,48). It has been postulated that the strongly positively charged V3 loop interacts with the negatively charged target membrane or its components. Others postulated that V3 loop cleavage by a cell surface protease might be necessary to expose a cryptic fusion domain (7,29,53). The V3 loop physically interacts with other envelope regions (59) and if not directly involved in the process of fusion may transfer conformational changes from one region to another and may thus modulate the function of other envelope regions in membrane fusion. It is however unknown at present through what mechanism the V3 region is involved in the process of membrane fusion.

The V2 region. The experiments with chimeric envelope proteins presented in chapter 4 identified the V2 region as a determinant involved in syncytium formation in SupT1 cells. This region alone did not transfer cell tropism to an NSI envelope background for any of the cells tested, including SupT1 cells. This again pointed at differences between membrane fusion processes involved in syncytium formation and virus entry. A role for the V2 region in syncytium formation, was also supported by results obtained by others with chimeric primary viruses (23) and by mutagenesis studies of this region in HIV-1 IIIB (54). Neutralizing antibodies directed to the V2 region support a functional role for this region in membrane fusion processes underlying viral entry (8,17,20,40). Indeed, such a role in controlling tropism for macrophages in concert with the V3 loop was demonstrated for V2 (28). It is therefore conceivable that also for SupT1 tropism the V2 and the V3 regions must act in concert, whereas the transfer of each of these SI derived regions alone is sufficient to confer syncytium inducing capacity to an NSI envelope background. The V2 region together with the V1 region forms a double loop structure through disulfide bonds at a common basis (34). Like the V3 region, also exposure of the V1/V2 regions is increased after sCD4 binding (47,48). Functional interactions of V2 and V3 with other envelope regions leading to syncytium formation and viral entry parallel results of studies demonstrating that V1/V2, V3, and C4 interact physically (14,28,59)(chapter 4). These interactions provides a rationale for the roles of V2 and V3 in membrane fusion processes since the C4 region is involved in both CD4 binding and the association of gp120 to gp41 (25,43). The observation that alteration of conserved amino acid residues in V2 affect association between gp120 and gp41(54) and that amino acid variation in

V2 determines sensitivity to sCD4 neutralization (28), both support the importance of these interactions in envelope function.

In chapter 5 it was demonstrated that single mutations in the V2 region based on the observed natural sequence variation in this region, did not interfere with V2 controlled syncytium formation. Such a functionally robust organization of the V2 region may allow escape from immune control without immediate functional consequences for the virus. The analysis of genetic envelope variation as presented in chapter 6, suggested such a selective pressure on several envelope regions, including the V2 region. The postulated mechanism of AAT triplet insertions leading to an increase of N-linked glycosylation sites may contribute to the masking of exposed envelope regions (chapter 6). This mechanism may explain why the initially reported correlation of syncytium inducing capacity with sequence length of the V2 region could not be unequivocally confirmed (12). Possibly increase in V2 sequence length is the result of a viral mechanism of escape by means of insertional addition of carbohydrates to this functionally import region. In this view, sequence length variation is a reflection of immune pressure and not directly linked to functional aspects of membrane fusion.

Other variable regions of gp120. For the envelope genes studied here, obtained from donor #16, no evidence for a role of the V1 region in membrane fusion processes was obtained (chapter 4). However, a contribution of V1 amino acid variation to cell tropism (4) and syncytium formation (54) has been demonstrated by others. The analysis presented in chapter 6 do reveal sequence length variation of the V1 region associated with a preferential insertion of AAT triplets. The observation of an excess of non-synonymous over synonymous substitutions in this region (and the V2 region) in HIV-1 infected individuals, strongly pointed at a positive selection for genetic variation (32) and is in line with reports describing neutralizing antibodies directed to this region (40). In a study probing the structure of gp120 with a large panel of monoclonal antibodies it was demonstrated that on oligomeric gp120-gp41 complexes, only the V2, the V3 loop, part of the C4 region and perhaps the V1 loop are exposed (42). Most of the conserved regions might lie within the core of the molecule, which is consistent with the hydrophobic nature of these regions. Since upon CD4 binding the V1, V2, and the V3 region exposure is increased (47,48), this gp120 conformation may explain why viral phenotype determinants are mapped to these exposed regions. It is tempting to speculate that these regions directly interact with the target cell membrane or its components, resulting in membrane fusion. Alternatively, there exposure may also be associated with rearrangements in the oligomeric envelope complex in which these regions e.g. interact with other gp120 subunits. Few studies have focused on a role for the V4 and V5 regions in membrane fusion processes. The genetic exchange experiments described in chapter 4, pointed at a possible contribution of an envelope region, that included the V4 and V5 regions, to syncytium formation. It appeared that this region enhanced the functionality of the V2 and V3 regions in the process of syncytium formation. Others demonstrated that partial deletion of V4 did not abolished syncytium formation (30), but complete deletion of the V4 region disrupted processing of the envelope precursor and a small insertion in V5 inhibited CD4 binding (30,58).

Additional information is needed to address the functional role(s) of the V4 and V5 regions in envelope function. Interestingly the process of AAT triplet reiteration was also observed for these sequence length displaying regions V4 and V5 (chapter 6).

Regions of gp41. On the basis of an observed sequence homology with paramyxoviruses, a conserved hydrophobic fusion domain at the amino terminus of the transmembrane protein of immunodeficiency viruses was identified (18). Insertion of hydrophillic amino acid residues inhibited syncytium formation and mutations that increase hydrophobicity tend to enhance fusion capacity (3,15). These observations are consistent with the postulated role for this domain in direct target cell membrane interactions (18,47) as is the prerequisite of gp160 cleavage for membrane fusion (39). The sequence analysis presented in chapter 2, predicted phenotype-associated amino acid variation in the fusion domain of four envelope genes obtained from one of the two patients analyzed (patient #16). The fusion domain of the SI derived genes of this patient shared a methionine at a position where the NSI genes shared the hydrophillic amino acid threonine. Sequencing of 12 additional fusion domains revealed that the same variation was also present in four other genes derived from another individual. Mutational analysis on recombinant vaccinia expressed envelope genes demonstrated however that this observed phenotype associated fusion domain variation alone did not interfere with the syncytium inducing capacity of the expressed genes. It has however not been excluded that this sequence variation contributes to syncytium formation in concert with the V2 or the V3 regions (chapter 4). Indeed, the functional role for this domain in membrane fusion may be more complex since it was observed that insertion of hydrophillic residues does abolish syncytium formation of HIV-2 whereas the process viral entry was almost unaffected (52). It is also demonstrated that sequence variation in this region of HIV-1 can be involved in determining cell tropism (2).

In chapter 7, a discrete region on gp41 (the "AB" region) close to the transmembrane region was identified to be involved in determining both syncytium formation and viral entry of SupT1 and PBMC's. This AB region contained only seven variable amino acid residues. It is conceivable, as discussed in chapter 7, that amino acid variation in this region controls the membrane fusion processes by influencing the conformational changes in gp41 that may lead to the activation of a "fusion-attack complex" in which the fusion domain finally interacts with the target membrane (38). Interestingly, similar to the variable regions V1, V2 and V3 of gp120, exposure of this region is also increased upon sCD4 binding (47). The presence of a conserved cluster of potential glycosylation sites may help to protect viruses from antibodies directed to this region. No evidence for insertion of additional glycosylation sites through AAT triplet insertions in this region is detected (chapter 6). The analysis of eight envelope proteins obtained from donor #320 presented in chapter 3, pointed at a possible determinant for SupT1 tropism on gp41. Comparison of the amino acid sequence variation in the AB region of envelope sequences obtained from individual #320 and #16 however, did not reveal a consensus pattern even when conserved amino acid changes that share biochemical properties were considered. Functional and sequence analysis of additional envelope proteins will be needed to

clarify if this gp41 AB region is controlling membrane fusion more commonly, as is most likely to be the case for the V2 and V3 regions.

HIV-1 entry in the presence of monoclonal antibodies and sCD4. The highly variable nature of HIV-1 was also demonstrated in the experiments presented in chapter 8. The envelope genes of patient #16 were tested for their capacity to mediate viral entry of SupT1 cells in the envelope complementation assay in the presence of a selected set of neutralizing antibodies. With the 257-D monoclonal antibody directed to the V3 loop, SupT1 infection mediated by envelope proteins 16.1, 16.2 (both SI), and 16.4 (NSI) was respectively unaffected, strongly inhibited, or considerably enhanced (seven fold). Similar results, although less pronounced, were obtained with PBMC's, . Enhancement of the infection of 16.4 complemented virus was also observed in the presence of monoclonal antibodies directed to the CD4 binding site and with sCD4. These results show that primary HIV-1 infections may similarly be enhanced by sCD4 as previously reported for HIV-2 and SIVagm (1,6,49,56). This similarity may be a reflection of the relative strong association of gp120 with gp41 for HIV-2, SIVagm, and primary HIV-1 in contrast to T cell line adapted HIV-1 strains which are neutralized by sCD4 (41). If preferential antibody dependent enhancement of infection of NSI viruses is a more general phenomenon, it is likely that such a mechanism may contribute to the apparent success of NSI viruses in infection.

Interestingly, infectivity of the 16.4 NSI complemented virus could be enhanced by antibodies directed to both, the CD4 binding site and the V3 loop. Since the here observed mechanism of enhancement differed from other previously described mechanisms in which enhancement was mediated by Fc receptor or complement (see chapter 8), the enhancement found is likely to be the result of conformational changes of gp120 introduced by direct antibody binding. Such conformational changes might be induced by cross linking of two gp120 molecules in the multimeric envelope complex by the bivalent antibodies. In this view, cross linking through either the V3 loops or the CD4 binding domains is apparently functionally similar. A very similar cross linking of two gp120 molecules may be the result of sCD4 binding to the envelope protein complex since it is likely that CD4 binds to the envelope protein complex as a dimer (11,33). Recent results revealed that Fab fragments of a CD4 binding site specific antibody did not induce enhancement, in contrast to bivalent antibodies (personal communication M. Schutten), which supports a role for cross linking in the observed mechanism of enhancement.

Conclusions. The studies presented in this thesis demonstrate that the HIV-1 envelope protein induced membrane fusion processes may be controlled by at least three determinants located in variable regions on both the surface glycoprotein gp120 or the transmembrane protein gp41. These determinants were identified on the basis of their capacity to contribute to syncytium formation in SupT1 cells. The gp41 AB region was furthermore shown to be involved in determining differences in tropism for these same cells. The V3 regions contributed to tropism for other cells (MT2) and on the basis of recent data obtained by others it is most likely that also the V2 region will be involved in membrane fusion processes underlying viral

entry (28). Thus within the limitations of the experimental approach, it appeared that for both membrane fusion processes studied, amino acid variation in multiple regions may be involved. Furthermore the results obtained suggest, that all these regions may also contribute individually to the cell specificity in the membrane fusion process underlying viral entry and possibly also membrane fusion in syncytium formation. Whether each of these regions physically interacts with the target cells in membrane fusion remains to be resolved. Further progress in the detailed understanding of these interactions and their specificities is seriously hampered due to a possible control by yet unidentified cellular accessory protein(s).

It is evident that the regions involved in membrane fusion are all subject to selective pressures. Evidence was however provided that the V2 region determined syncytium inducing capacity for the envelope genes studied here, was not easily abolished by single mutations. This may also hold true for the putative function for this region in the process of viral entry. This may be speculated to allow the virus to escape from e.g. humoral responses by mutations in this region, without affecting the process of entry. It is estimated that the highly error prone viral RT polymerase may produce up to seven mutations in each progeny viral genome (44,45). Furthermore it is likely that envelope regions develop "immunity" from disadvantageous immune responses by the addition of carbohydrates e.g. by the presented mechanism of AAT triplet insertion. Additional to these mechanisms that may lead to escape, the multi-determinant nature of HIV-1 induced membrane fusion process (in viral entry) provides another option for the virus to persist in hostile environments. The exchange of segments of genetic information between different viruses, may enhance the kinetics of viral adaptation considerably. Such an exchange by recombination may frequently occur *in vivo* as shown in chapter 3. Adaptation of HIV-1 to a broader cellular host range possibly related to escape from immune selection in the course of infection, may directly lead to a change in the capacity to induce syncytia. Finally, the extremely flexible organization of HIV-1 is perhaps best illustrated by the observation that binding of antibodies to the envelope proteins can even promote viral entry. Enhancement of infectivity was notably observed with antibodies that could neutralize another highly homologous primary virus.

Taken together, the results of this thesis have demonstrated that several envelope regions, either alone or in concert, contribute to the extreme capacity of HIV-1 to adapt its phenotype to the continuous and permanently changing immune response of the host, enabling the virus to persist in a hostile environment.

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Samenvatting

Het in dit proefschrift beschreven onderzoek had als centraal doel het identificeren en karakteriseren van determinanten van de envelop-eiwitten die betrokken zijn bij membraanfusie-processen die geïnduceerd worden door het humaan immuundeficiëntievirus type 1 (HIV-1). Deze processen vormen de basis van virus-"entry" en van de vorming van reuzencellen of syncytia. De bestudering van deze determinanten beoogde het inzicht te vergroten in de complexe interacties van de HIV-1-envelop-eiwitten met de gastheercel. Een tweede doelstelling was informatie te verkrijgen over virusevolutie onder selectieve condities zoals aanwezig in het geïnfecteerde individu. Bij de aanvang van dit onderzoek was de meeste aandacht gericht op de functie van het derde variabele domein (V3) van het envelop-eiwit gp120 van HIV-1 als determinant van membraanfusie-processen. Het hier beschreven onderzoek leidt echter tot de conclusie dat tenminste drie envelop-eiwit-determinanten de membraanfusie-capaciteit van primaire envelop-eiwit-complexen bepalen; deze determinanten zijn gelocaliseerd in drie verschillende envelop-eiwit-domeinen (waaronder V3). Bovendien wordt aangetoond dat interactie tussen door de gastheer aangemaakte antistoffen en de envelop-eiwitten, niet alleen kan leiden tot virus-neutralisatie, maar ook tot een toename van HIV-1-"entry" in de gastheercel.

De algemene introductie - hoofdstuk 1 - geeft een kort overzicht van de moleculaire biologie van HIV-1 en de pathogenese van de HIV-1-infectie bij de mens. In het tweede gedeelte van dit hoofdstuk wordt de huidige kennis over de HIV-1-envelop-eiwitten en hun functie in membraanfusie-processen samengevat.

In het tweede hoofdstuk wordt de genetische en functionele analyse beschreven van acht HIV-1-envelop-genen die centraal staan in het verdere onderzoek. Deze envelop-genen zijn afkomstig van biologisch gekloneerde virussen. Zij werden verkregen uit perifere bloedlymfocyten van twee deelnemers (#16 en #320) aan een Amsterdamse cohortstudie van HIV-1-geïnfecteerde individuen. Deze virussen zijn onderling genetisch sterk verwant maar verschillen toch in hun syncytium-inducerend vermogen. Door middel van recombinant vacciniavirus gemedieerde expressie van de geïsoleerde HIV-1-genen wordt aangetoond dat de variatie in de envelop-genen voldoende is om het verschil in syncytium-inducerende vermogen van de oorspronkelijke virussen te verklaren. Mutatie-experimenten sluiten echter uit dat de waargenomen genetische variatie in het gp41-fusiedomein alleen verantwoordelijk kan zijn voor het verschil in syncytium-vormende capaciteit van deze envelop-gen producten.

Sequentieanalyse van een groter aantal envelop-genen afkomstig van één van de twee

donoren (hoofdstuk 3), laat zien dat hoogst waarschijnlijk aminozuurvariatie in gp120 verantwoordelijk is voor de waargenomen verschillen in het syncytium-inducerend vermogen van de bestudeerde virussen. Deze sequentieanalyse toont ook aan dat sommige van de envelop-genen het product zijn van *in vivo* recombinatie en dat determinanten voor SupT1-celtropisme gelegen moeten zijn buiten het V3-domein van gp120.

In hoofdstuk 4 en 7 worden genetische uitwisselingsexperimenten beschreven. De localisatie van drie envelop-determinanten die betrokken zijn bij membraanfusie-processen die de basis vormen voor syncytiumvorming, wordt mogelijk gemaakt door transiënte expressie van chimere envelop-genen, geconstrueerd van syncytium-inducerende (SI) en niet-syncytium-inducerende (NSI) envelop-genen. Naast aminozuurvariatie in het V3- en het V2-domein van gp120 blijkt ook een klein, variabel gebied van gp41 (het "AB-domein") bij te dragen aan het syncytium-inducerend vermogen van primaire envelop-eiwitten. Met behulp van zogenaamde envelop-complementatie-experimenten wordt bovendien aangetoond dat variatie in zowel het V3-domein als in het AB-domein de capaciteit beïnvloedt van de virale envelop-eiwitten om bepaalde cellen te infecteren. Deze beide domeinen worden dan ook aangemerkt als determinanten van celtropisme (hoofdstuk 7).

In hoofdstuk 5 wordt de bijdrage bepaald die afzonderlijke variabele aminozuurresiduen in het V2-domein van de bestudeerde envelop-genen leveren aan syncytium-vorming. Hierbij bleek dat enkelvoudige mutaties in het V2-domein, gebaseerd op de waargenomen natuurlijke aminozuurvariatie, niet interfereren met syncytium-vorming die bepaald wordt door het V2-domein. De kennelijk robuuste organisatie van het V2-domein lijkt aan het virus een mogelijkheid te bieden om te ontsnappen aan bijvoorbeeld immuundruk zonder directe functionele consequenties.

Het V2-domein vertoont evenals enkele andere envelop-eiwit-domeinen ook variatie in lengte. In hoofdstuk 6 wordt door analyse van een grote verzameling "B-clade"-envelop-gen-sequenties aangetoond dat gebieden met een variabele lengte een afwijkend codongebruik hebben. Dit afwijkende codongebruik leidt tot accumulatie van asparagine-, serine- en threonineresiduen en daarmee tot accumulatie van potentiële asparagine-geassocieerde glycosyleringsplaatsen. Gepostuleerd wordt dat het afwijkende codongebruik veroorzaakt wordt door repeterende insertie van AAT-tripletten die gevolgd worden door puntmutaties en selectie. Het is waarschijnlijk dat de additie van suikergroepen zoals waargenomen in het V2-domein, het virus een selectief voordeel oplevert door maskering van dit gebied voor neutraliserende antistoffen.

In hoofdstuk 8 wordt aangetoond dat de HIV-1-"entry" kan worden bevorderd door de aanwezigheid van oplosbare CD4 receptor moleculen. Eenzelfde fenomeen is in

eerdere studies door anderen al waargenomen voor SIV_{agm}- en HIV-2-infecties. De toename in HIV-1-"entry" wordt eveneens gevonden in envelop-complementatie-experimenten waarbij monoclonale of polyclonale antistoffen aanwezig zijn. Het blijkt dat dezelfde HIV-1-specifieke antistoffen een verschillend effect kunnen hebben op virussen met een verschillend fenotype. Antistoffen die een SI-virus krachtig neutraliseren blijken daarentegen de "entry" van een genetisch zeer verwant virus met een NSI-fenotype te kunnen bevorderen.

De algemene diskussie - die in hoofdstuk 9 is opgenomen - bespreekt tenslotte de resultaten van het in dit proefschrift beschreven onderzoek tegen de achtergrond van de virusvariatie die in de gastheer wordt waargenomen. Gepostuleerd wordt dat de complexe organisatie van de HIV-1-gemedieerde membraanfusie-processen, bijdraagt aan het vermogen van HIV-1 om te persisteren in de gastheer.

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Curriculum Vitae

Arno Andeweg werd op 4 juni 1961 geboren in Den Haag. Aan het 2e Vrijzinnig Christelijk Lyceum, ook in Den Haag, werd in 1979 het VWO diploma behaald. Na een intermezzo van een jaar werd in 1980 de studie Biologie aangevangen aan de Rijksuniversiteit Utrecht. Na de kandidaatsfase werd deze studie anderhalf jaar onderbroken voor een reis door Zuid-Amerika. Begin 1989 werd het doctoraal examen biologie behaald. De doctoraalfase was samengesteld uit een hoofdvak Virologie en een bijvak Experimentele Parasitologie (beide aan de Faculteit Diergeneeskunde, Utrecht) alsmede een bijvak Bio-informatica en een nevenrichting Didaktiek (beide aan de Faculteit Biologie, Utrecht). Na een korte periode werkzaam te zijn geweest op de afdeling Moleculaire Virologie van de Animal Health Division van de firma Solvay-Duphar in Weesp, werd in december 1989 het promotieonderzoek gestart aan het toenmalige Laboratorium voor Immunobiologie van het Rijks Instituut voor de Volksgezondheid en Milieuhygiëne in Bilthoven. Sinds maart 1994 is hij werkzaam aan het Laboratorium voor de Virale Pathogenese van het Biological Primate Research Centre in Rijswijk.