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]† Patrick H. M. Boers,[1] Albert D. M. E. Osterhaus[1,2]* and Marnix L. Bosch[1]‡

[1] *Laboratory of Immunobiology, National Institute of Public Health and Environmental Protection, A. van Leeuwenhoeklaan 9, 3720 BA Bilthoven and* [2] *Institute of Virology, Erasmus University, Dr Molewaterplein 50, PO Box 1738, 3000 DR Rotterdam, The Netherlands*

Several studies have demonstrated a functional role for the V1–V2 region of the human immunodeficiency virus type 1 (HIV-1) envelope surface glycoprotein gp120 in the membrane fusion processes underlying viral entry and syncytium induction. In a study with chimeric primary envelope genes, we have previously demonstrated 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 $CD4^+$ 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 can be 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 its biological phenotype.

Introduction

Human immunodeficiency virus type 1 (HIV-1)-induced syncytium formation and viral entry are the result of membrane fusion processes and are both initiated by the binding of the viral surface glycoprotein gp120 to the CD4 receptor molecule expressed on target cells (Dalgleish *et al.*, 1984; Lifson *et al.*, 1986*a*, *b*; Popovic *et al.*, 1984). 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 (Hart *et al.*, 1991; Moore *et al.*, 1990, 1993; Sattentau *et al.*, 1993; Sattentau & Moore, 1991). Both cellular and viral determinants involved in these events, and thus involved in viral entry and syncytium formation, have been identified. The principal viral determinants are located on the envelope glycoproteins (Lifson *et al.*, 1986*a*; Sodroski *et al.*, 1986) and their further identification will contribute to the understanding of HIV-1-induced membrane fusion processes.

In addition to studies with mutant (mostly T cell line-adapted) viruses, studies with chimeric viruses have also 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 (Hwang *et al.*, 1991; Shioda *et al.*, 1991). Similarly, it has been demonstrated that the variable envelope regions V1 and V2 are also involved in these processes (Andeweg *et al.*, 1993; Groenink *et al.*, 1993; Freed & Martin, 1994; Koito *et al.*, 1994; Westervelt *et al.*, 1992; Boyd *et al.*, 1993). 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 (A. C. Andeweg *et al.*, unpublished results).

The V2 and V1 regions of the surface glycoprotein gp120 form a double loop structure through common disulphide bonds (Leonard *et al.*, 1990). In multimeric

* Author for correspondence. Fax +31 10 4365145. e-mail osterhaus@viro.fgg.eur.nl

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

‡ Present address: Regional Primate Research Center and Department of Pathobiology, University of Washington, Seattle, Washington, USA.

0001-3177

envelope proteins expressed at the surface of infected cells the V2 loop exposure is increased as a result of sCD4 binding (Sattentau *et al.*, 1993; Moore *et al.*, 1993), 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 (Sullivan *et al.*, 1993). 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 (Fung *et al.*, 1992; Davis *et al.*, 1993; Ho *et al.*, 1991; McKeating *et al.*, 1993; Gorny *et al.*, 1994). The screening of a large set of V2 regions from viruses with different syncytium-inducing (SI) capacities suggested a correlation between V2 region length and SI capacity (Groenink *et al.*, 1993). 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 (Bosch *et al.*, 1994).

We have demonstrated that variation in the V2 region alone is sufficient to change considerably the SI capacity of transiently expressed chimeric envelope proteins (Andeweg *et al.*, 1993). 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. 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.

Methods

Construction of mutant chimeric envelope genes. Previously we genetically and phenotypically characterized a set of envelope genes from HIV-1 clones obtained from a single donor at a single time point. These were designated 16.1 to 16.4 (Andeweg *et al.*, 1992). For the present study we have introduced a series of synthetic V2 regions into the 16.3 non-syncytium-inducing (NSI) envelope gene. The V2 regions were produced by cloning T7 DNA polymerase (Sequenase; USB)-extended products of two mutually priming long oligonucleotide pairs as depicted in Fig. 1 (*a*). The primers used to construct the wild-type 16.1 V2 region are shown in Fig. 1 (*b*). Mutant V2 regions were produced using similar primers containing the desired sequence changes and/or site-directed mutagenesis of constructed (mutant) V2 regions according to the method of Deng & Nickoloff (1992). 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; Andeweg *et al.*, 1992) 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 site and of the designed *Asp*I 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

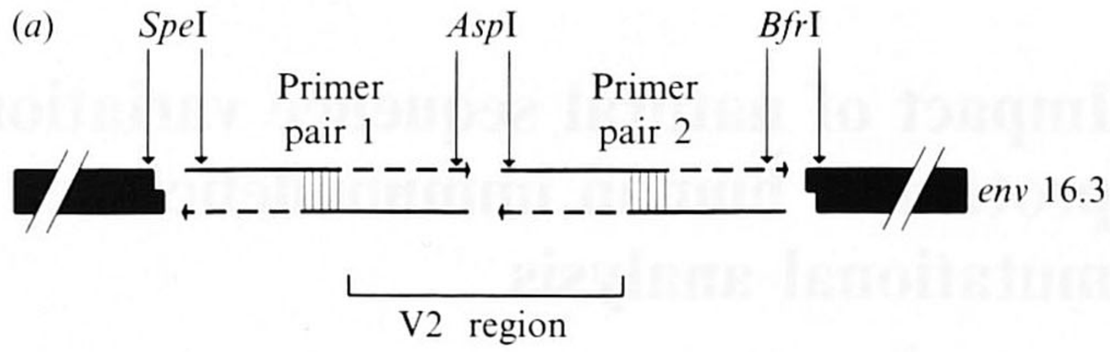

Fig. 1. (*a*) The cloning strategy of synthetic 16.1-based V2 regions into the NSI 16.3 envelope gene is illustrated. T7 DNA polymerase-extended products of two mutually priming long oligonucleotide pairs were ligated at a designed central *Asp*I site and subsequently inserted into the NSI 16.3 envelope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

Fig. 2. The entire cloned region of all generated chimeric mutant envelope genes was sequenced to verify correct assembling.

Transient envelope gene expression. All generated chimeric envelope genes were cloned in the simian virus 40-based expression vector pSRHS as previously described (Andeweg *et al.*, 1993; Dubay *et al.*, 1992). Subsequently these constructs were transfected into SupT1 cells by electroporation. Cells (5×10^6) were mixed with 20 μg DNA in 250 μl RPMI 1640 (Gibco) supplemented with 10% fetal calf serum in a 4 mm electroporation cuvette (Eurogentec). After electroporation (Bio-Rad Gene Pulser; 200 V, 960 μF) the cells were transferred to culture flasks with RPMI 1640 medium. One day post-transfection, viable cells were isolated on a Ficoll density gradient and cultured for an additional 4 days. At 3 days post-transfection the relative expression of envelope protein was determined in supernatants and cell lysates in an ELISA as previously described (Andeweg *et al.*, 1993). The catching antibody D7324 (Aalto BioReagents) used in this ELISA is directed to the conserved C-terminal 15 amino acids of gp120, indicating that both gp120 and gp160 will contribute to the signals obtained. Finally, envelope protein processing and expression kinetics were examined by radioimmunoprecipitation assay (RIPA) and by testing multiple cell

		Net positive charge	Helix 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	+
mutant1	.....V.....Y.....	3	–
mutant2	.....V.....R.....	4	–
mutant3	.....-.....N...T.....N.	2	+
mutant4	.....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. 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, the net positive charge of all listed V2 regions is indicated at the right. The last column lists whether an α-helical structure is predicted.

lysate and supernatant samples over time in ELISA. For RIPA, COS-7 cells transfected with the envelope constructs were metabolically labelled 48 h after electroporation for 30 min with a mixture of [^{35}S]methionine and [^{35}S]cysteine (Amersham). Cells were lysed and supernatants were collected at 16 and 40 h after labelling. Immunoprecipitation, using 2 μg of a pool of three different monoclonal antibodies, gp13 (Schutten *et al.*, 1993), 391/95-D (Gorny *et al.*, 1993) and K14 (Teeuwsen *et al.*, 1990), was performed as previously described (Andeweg *et al.*, 1992).

Syncytium-forming assay. Previously we defined the SI 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 (Andeweg *et al.*, 1993). Therefore, the transfected cell cultures were examined each day 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). To this end the cultures were independently examined at the respective time points by three different observers. Each envelope gene construct was tested for expression and SI 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 represented wild-type and mutant V2 sequences of the 16.1 SI envelope gene, which were mutated toward the 16.3 NSI sequence at single and multiple positions (Fig. 2). The transient expression of these 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.

Table 1. *Relative envelope gene expression in the syncytium-forming assay**

env code	Cell lysate	Supernatant	Ratio†
Wild-type 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
Wild-type 16.3	74	35	41

* Values given are relative A_{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 mutant 3 for *env* expression in cell lysate and supernatant, respectively).

† 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 mutant 3.

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 3 days after transfection are listed, both for cell-associated and

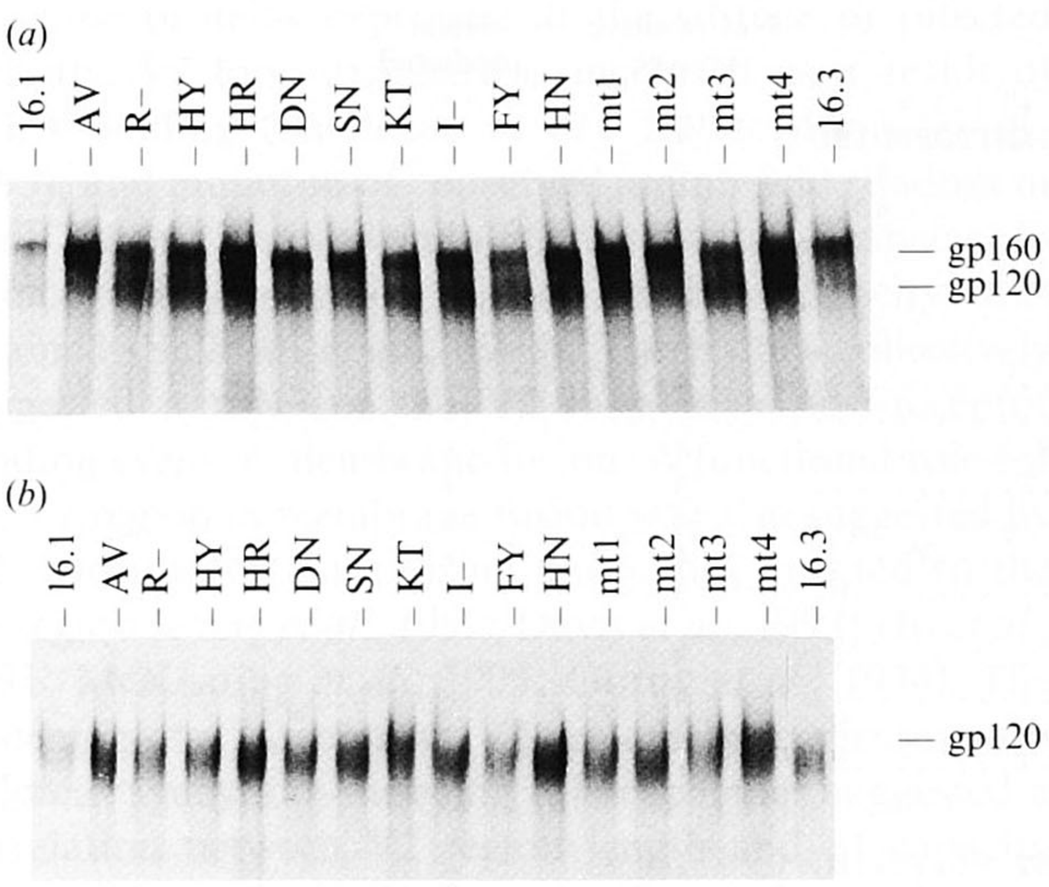

Fig. 3. Expression of envelope gene products by COS-7 cells transfected with the chimeric envelope gene constructs as demonstrated by RIPA (all V2 sequences indicated were expressed in the 16.3 NSI background). Pulse-labelled proteins from transfected cell lysates (*a*) or from culture medium (*b*) were immunoprecipitated 16 h after transfection with a mixture of monoclonal antibodies and analysed by SDS–PAGE. Arrows indicate the precursor glycoprotein gp160 and the surface glycoprotein gp120.

shed glycoproteins in 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 lysates for each construct is also presented. The relative amounts of envelope proteins present in the cell lysate and supernatant varied by two- to threefold. On average 35% of the envelope proteins present at day 3 were present in the culture medium (data not shown). The observed ratio varied twofold when comparing all expressed chimeric genes (Table 1). SupT1 cells transfected in parallel by electroporation with a β-galactosidase-encoding control plasmid (Pharmacia) demonstrated that under the conditions described 3 to 5% of the cells were successfully transfected (data not shown). Since we observed a considerable delay in syncytium formation for some of the constructs, envelope processing kinetics was 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 Fig. 3, gp160 expression, cleavage and gp120 shedding into the supernatant occurred within 16 h for all envelope constructs analysed.

Syncytium induction 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 SupT1 cells as shown

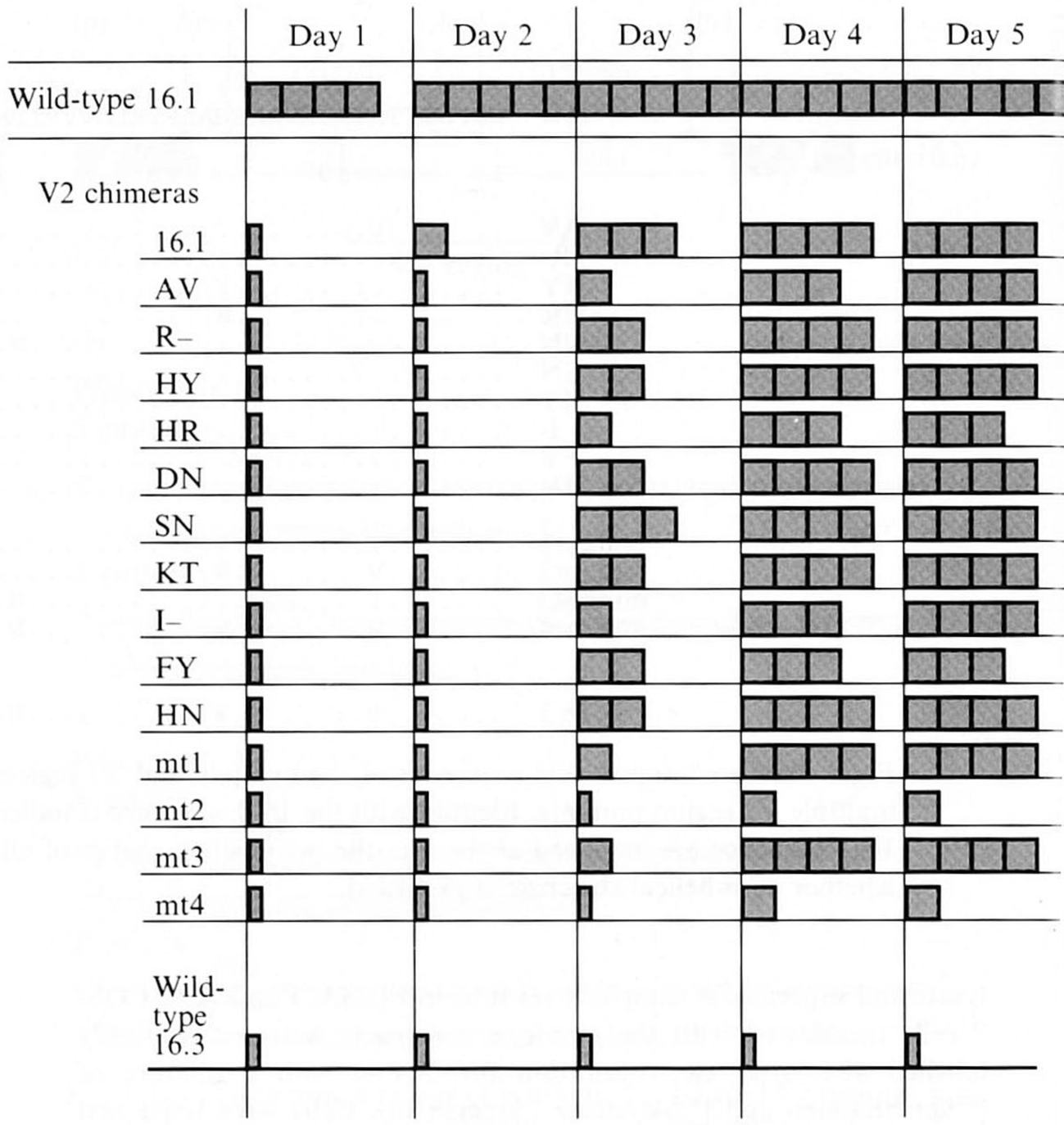

Fig. 4. Kinetics of syncytium size induced by envelope gene products. The size of the syncytia was quantified by classifying the observed syncytia in five arbitrarily chosen categories. The smallest syncytia were about five times the size of single cells and are indicated by a single square. The largest syncytia, which were estimated to contain over 100 fused cells and exceeding 250 μm in diameter, are indicated by a bar of five squares; intermediately sized syncytia are indicated by bars with a number of squares corresponding to their relative sizes. Wild-type 16.1 and wild-type 16.3 represent 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.

in Fig. 4, which confirmed data from previous studies (Andeweg *et al.*, 1993). 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. 4). 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 occurred only after prolonged times in culture (Fig. 4). The mutations

influenced either the overall net charge of the V2 region, the predicted secondary structure, or both. These changes are indicated in Fig. 2. Garnier–Robson analysis (Garnier *et al.*, 1978) predicted the formation of an α-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 and mutants 1 and 3, which differ in net positive charge from the 16.1 V2 region, did not significantly reduce the size of the induced syncytia. However, combination of multiple mutations which introduced differences in charge and two mutations which abolished the predicted secondary structure (mutant 4) almost completely abolished syncytium formation. Mutant 2 contained the histidine to arginine change that was also found in mutant HR. The introduction of arginine was different from all other changes made in that an arginine has never been 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 α-helix, whereas the natural HY variation at this position also affects the net charge of the V2 region. As shown in Fig. 4, 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

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 also that significant differences in kinetics and size were observed (Fig. 4). The envelope expression as demonstrated by ELISA 3 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 envelope protein expression was not a limiting factor in syncytium formation. The expression levels were at least eight times greater than those sufficient for immediate massive syncytium formation (data not shown). Finally all envelope-expressing constructs were assayed independently for their SI capacity at least three 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 SI 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. 4). 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, we performed RIPA and tested multiple cell lysate and supernatant samples over time in ELISA. The results presented in Fig. 3 demonstrate that gp160 expression, cleavage and gp120 shedding into the supernatant occurred within 16 h for all envelope constructs analysed, including mutant 2 and mutant 4. Moreover, cell lysates and supernatants from cultures transfected with mutant 2 or mutant 4, taken 24 h after electroporation, already showed similar levels of envelope expression to those obtained with the other constructs when tested in ELISA (data not shown). These results indicate that the delayed appearance of syncytia seen for mutants 2 and 4 is not due to reduced processing or expression kinetics of these envelope proteins. Therefore we concluded that the phenotypic differences observed are the direct consequence of the amino acid sequences of the expressed envelope glycoproteins. 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 is 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.* (1993).

Garnier–Robson computer analysis suggested that the V2 variation at residues 8 and 17 greatly affects the predicted secondary structure of the V2 loop (Andeweg *et al.*, 1993). 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 (Andeweg *et al.*, 1993).

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, 1 and 3, which differ in net positive charge from the 16.1 V2 region, did not display a significantly decreased capacity to induce syncytia. This observation demonstrates that the observed differences in SI 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 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 SI phenotype (Fouchier *et al.*, 1992). Several studies indicated physical or functional interactions of the V2 region with other envelope regions like C4 (Freed & Martin, 1994; Moore *et al.*, 1993; Andeweg *et al.*, 1993). Interestingly, in the chimeric envelope proteins expressed here, 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 (Andeweg *et al.*, 1993), functional interactions with other envelope regions in our chimeric envelopes are not seriously disturbed or lost. This is probably due to the high degree of sequence similarity in the parental envelope genes used in this study (Andeweg *et al.*, 1992). Apparently, the robust V2 organization allows the virus to accumulate mutations as a means of escape from immune selection pressure (Yoshiyama *et al.*, 1994; Lamers *et al.*, 1993), without necessarily having direct consequences for 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 hamper attempts to interfere with the biology of HIV-1 through immunological or other intervention strategies.

We thank Eric Hunter and Susan J. Roberts of the University of Alabama at Birmingham, Birmingham, USA, for providing the pSRHS expression plasmid, and Martin Schutten and Jos Karlas for technical assistance. This work was supported by a grant from the Advisory Council on Health Research [RGO 88–75/89039 (A.C.A and P.H.M.B.)].

References

ANDEWEG, A. C., GROENINK, M., LEEFLANG, P., DE GOEDE, R. E. Y., OSTERHAUS, A. D. M. E., TERSMETTE, M. & BOSCH, M. L. (1992). Genetic and functional analysis of a set of human immunodeficiency virus type 1 envelope genes obtained from biological clones with varying syncytium inducing capacities. *AIDS Research and Human Retroviruses* **8**, 1803–1813.

ANDEWEG, A. C., LEEFLANG, P., OSTERHAUS, A. D. M. E. & BOSCH, M. L. (1993). Both the V2 and V3 regions of the human immunodeficiency virus type 1 surface glycoprotein functionally interact with other envelope regions in syncytium formation. *Journal of Virology* **67**, 3232–3239.

BOSCH, M. L., ANDEWEG, A. C., SCHIPPER, R. & KENTER, M. (1994). Insertion of N-linked glycosylation sites in the variable regions of the human immunodeficiency virus type 1 surface glycoprotein through AAT triplet reiteration. *Journal of Virology* **68**, 7566–7569.

BOYD, M. T., SIMPSON, G. R., CANN, A. J., JOHNSON, M. A. & WEISS, R. A. (1993). A single amino acid substitution in the V1 loop of human immunodeficiency virus type 1 gp120 alters cellular tropism. *Journal of Virology* **67**, 3649–3652.

DALGLEISH, A. G., BEVERLEY, P. C. L., CLAPHAM, P. R., CRAWFORD, D. H., GREAVES, M. F. & WEISS, R. A. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312**, 763–767.

DAVIS, D., STEPHENS, D. M., CARNE, C. A. & LACHMANN, P. J. (1993). Antisera raised against the second variable region of the external envelope glycoprotein of human immunodeficiency virus type 1 cross-neutralize and show an increased neutralization index when they act together with antisera to the V3 neutralization epitope. *Journal of General Virology* **74**, 2609–2617.

DENG, W. P. & NICKOLOFF, J. A. (1992). Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Analytical Biochemistry* **200**, 81–88.

DUBAY, J. W., ROBERTS, S. J., HAHN, B. H. & HUNTER, E. H. (1992). Truncation of the human immunodeficiency virus type I transmembrane glycoprotein cytoplasmic domain blocks virus infectivity. *Journal of Virology* **66**, 6616–6625.

FOUCHIER, R. A. M., GROENINK, M., KOOTSTRA, N. A., TERSMETTE, M., HUISMAN, H. G., MIEDEMA, F. & SCHUITEMAKER, H. (1992). Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. *Journal of Virology* **66**, 3183–3187.

FREED, E. O. & MARTIN, M. A. (1994). Evidence for a functional interaction between the V1/V2 and C4 domains of human immunodeficiency virus type 1 envelope glycoprotein gp120. *Journal of Virology* **68**, 2503–2512.

FUNG, M. S. C., SUN, C. R. Y., GORDON, W. L., LIOU, R. S., CHANG, T. W., SUN, W. N. C., DAAR, E. S. & HO, D. D. (1992). Identification and characterization of a neutralization site within the second variable region of human immunodeficiency virus type-1 gp120. *Journal of Virology* **66**, 848–856.

GARNIER, J., OSGUTHORPE, D. & ROBSON, B. (1978). Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *Journal of Molecular Biology* **120**, 97–120.

GORNY, M. K., XU, J. Y., KARWOWSKA, S., BUCHBINDER, A. & ZOLLA-PAZNER, S. (1993). Repertoire of neutralizing human monoclonal antibodies specific for the V3 domain of HIV-1 gp120. *Journal of Immunology* **150**, 635–643.

GORNY, M. K., MOORE, J. P., CONLEY, A. J., KARWOWSKA, S., SODROSKI, J., WILLIAMS, C., BURDA, S., BOOTS, L. J. & ZOLLA-PAZNER, S. (1994). Human anti-V2 monoclonal antibody that neutralizes primary but not laboratory isolates of human immunodeficiency virus type 1. *Journal of Virology* **68**, 8312–8320.

GROENINK, M., FOUCHIER, R. A. M., BROERSEN, S., BAKER, C. H., KOOT, M., VAN'T WOUT, A. B., HUISMAN, H. G., MIEDEMA, F., TERSMETTE, M. & SCHUITEMAKER, H. (1993). Relation of phenotype evolution of HIV-1 to envelope V2 configuration. *Science* **260**, 1513–1516.

HART, T. K., KIRSH, R., ELLENS, H., SWEET, R. W., LAMBERT, D. M., PETTEWAY, S. R., JR., LEARY, J. & BUGELSKI, P. J. (1991). Binding of soluble CD4 proteins to human immunodeficiency virus type 1 and infected cells induces release of envelope glycoprotein gp120. *Proceedings of the National Academy of Sciences, USA* **88**, 2189–2193.

HO, D. D., FUNG, M. S. C., CAO, Y. Z., LI, X. L., SUN, C., CHANG, T. W. & SUN, N. C. (1991). Another discontinuous epitope on glycoprotein gp120 that is important in human immunodeficiency virus type-1 neutralization is identified by a monoclonal antibody. *Proceedings of the National Academy of Sciences, USA* **88**, 8949–8952.

HWANG, S. S., BOYLE, T. J., LYERLY, H. K. & CULLEN, B. R. (1991). Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. *Science* **253**, 71–74.

KOITO, A., HARROWE, G., LEVY, J. A. & CHENG-MAYER, C. (1994). Functional role of the V1/V2 region of human immunodeficiency virus type 1 envelope glycoprotein gp120 in infection of primary macrophages and soluble CD4 neutralization. *Journal of Virology* **68**, 2253–2259.

LAMERS, S. L., SLEASMAN, J. W., SHE, J. X., BARRIE, K. A., POMEROY, S. M., BARRETT, D. J. & GOODENOW, M. M. (1993). Independent variation and positive selection in env V1 and V2 domains within maternal-infant strains of human immunodeficiency virus type 1 in vivo. *Journal of Virology* **67**, 3951–3960.

LEONARD, C. K., SPELLMAN, M. W., RIDDLE, L., HARRIS, R. J., THOMAS, J. N. & GREGORY, T. J. (1990). Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immundeficiency virus envelope glycoprotein (gp120) expressed in chinese hamster ovary cells. *Journal of Biological Chemistry* **265**, 10373–10382.

LIFSON, J. D., FEINBERG, M. B., REYES, G. R., RABIN, L., BANAPOUR, B., CHAKRABARTI, S., MOSS, B., WONG STAAL, F., STEIMER, K. S. & ENGLEMAN, E. G. (1986*a*). Induction of CD4-dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. *Nature* **323**, 725–728.

LIFSON, J. D., REYES, G. R., MCGRATH, M. S., STEIN, B. S. & ENGLEMAN, E. G. (1986*b*). AIDS retrovirus induced cytopathology: giant cell formation and involvement of CD4 antigen. *Science* **232**, 1123–1126.

MCKEATING, J. A., SHOTTON, C., CORDELL, J., GRAHAM, S., BALFE, P., SULLIVAN, N., CHARLES, M., PAGE, M., BOLMSTEDT, A., OLOFSSON, S., KAYMAN, S. C., WU, Z., PINTER, A., DEAN, C., SODROSKI, J. & WEISS, R. A. (1993). Characterization of neutralizing monoclonal antibodies to linear and conformation-dependent epitopes within the first and second variable domains of human immunodeficiency virus type 1 gp120. *Journal of Virology* **67**, 4932–4944.

MOORE, J. P., MCKEATING, J. A., WEISS, R. A. & SATTENTAU, Q. J. (1990). Dissociation of gp120 from HIV-1 virions induced by soluble CD4. *Science* **250**, 1139–1142.

MOORE, J. P., SATTENTAU, Q. J., YOSHIYAMA, H., THALI, M., CHARLES, M., SULLIVAN, N., POON, S. W., FUNG, M. S., TRAINCARD, F., PINKUS, M. *et al.* (1993). Probing the structure of the V2 domain of human immunodeficiency virus type 1 surface glycoprotein gp120 with a panel of eight monoclonal antibodies: human immune response to the V1 and V2 domains. *Journal of Virology* **67**, 6136–6151.

POPOVIC, M., SARNGADHARAN, M. G., READ, E. & GALLO, R. C. (1984). Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* **224**, 497–500.

SATTENTAU, Q. J. & MOORE, J. P. (1991). Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. *Journal of Experimental Medicine* **174**, 407–415.

SATTENTAU, Q. J., MOORE, J. P., VIGNAUX, F., TRAINCARD, F. & POIGNARD, P. (1993). Conformational changes induced in the envelope glycoproteins of the human and simian immunodeficiency viruses by soluble receptor binding. *Journal of Virology* **67**, 7383–7393.

SCHUTTEN, M., MCKNIGHT, A., HUISMAN, R. C., THALI, M., MCKEATING, J. A., SODROSKI, J., GOUDSMIT, J. & OSTERHAUS, A. D. (1993). Further characterization of an antigenic site of HIV-1 gp120 recognized by virus neutralizing human monoclonal antibodies. *AIDS* **7**, 919–923.

SHIODA, T., LEVY, J. A. & CHENG MAYER, C. (1991). Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. *Nature* **349**, 167–169.

SODROSKI, J., GOH, W. G., ROSEN, C., CAMPBELL, K. & HASELTINE, W. A. (1986). Role of the HTLV-III/LAV envelope in syncytium formation and cytopathicity. *Nature* **322**, 470–474.

SULLIVAN, N., THALI, M., FURMAN, C., HO, D. D. & SODROSKI, J. (1993). Effect of amino acid changes in the V1/V2 region of the human immunodeficiency virus type 1 gp120 glycoprotein on subunit association, syncytium formation, and recognition by a neutralizing antibody. *Journal of Virology* **67**, 3674–3679.

TEEUWSEN, V. J. P., SIEBELINK, K. H. J., CRUSH STANTON, S., SWERDLOW, B., SCHALKEN, J. J., GOUDSMIT, J., VAN DE AKKER, R., STUKART, M. J., UYTDEHAAG, F. G. C. M. & OSTERHAUS, A. D. M. E. (1990). Production and characterization of a human monoclonal antibody reactive with a conserved epitope on gp41 of human immunodeficiency virus type I. *AIDS Research and Human Retroviruses* **6**, 381–392.

WESTERVELT, P., TROWBRIDGE, D. B., EPSTEIN, L. G., BLUMBERG, B. M., LI, Y., HAHN, B. H., SHAW, G. M., PRICE, R. W. & RATNER, L. (1992). Macrophage tropism determinants of human immunodeficiency virus type 1 in vivo. *Journal of Virology* **66**, 2577–2582.

YOSHIYAMA, H., MO, H., MOORE, J. P. & HO, D. D. (1994). Characterization of mutants of human immunodeficiency virus type 1 that have escaped neutralization by a monoclonal antibody to the gp120 V2 loop. *Journal of Virology* **68**, 974–978.

(*Received 3 February 1995; Accepted 20 March 1995*)