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Amino Acid Changes in the Fourth Conserved Region of Human Immunodeficiency Virus Type 2 Strain HIV-2_{ROD} Envelope Glycoprotein Modulate Fusion

REGINA KELLER,[1] KEITH PEDEN,[2] SYLVIE PAULOUS,[1] LUC MONTAGNIER,[1] AND AGNES CORDONNIER[1]†*

Unité d'Oncologie Virale (Centre National de la Recherche Scientifique UA1157), Institut Pasteur, 75724 Paris Cedex 15, France,[1] and Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892[2]

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The fourth conserved region (C4) of human immunodeficiency virus type 1 (HIV-1) surface glycoprotein has been shown to participate in CD4 binding and to influence viral tropism (A. Cordonnier, L. Montagnier, and M. Emerman, Nature [London] 340:571–574, 1989). To define the role of the corresponding region of HIV-2, we introduced single amino acid changes into the C4 sequence of HIV-2_{ROD}. The effects of these mutations on glycoprotein function and on virus infectivity have been examined. We have shown that the tryptophan residue at position 428 is necessary primarily for CD4 binding. The isoleucine residue at position 421 is necessary for the establishment of productive infection in the promonocytic cell line U937, while it is dispensable to some extent for infection of primary T lymphocytes or the lymphocytic cell line SUP-T1. This replication defect correlated with the failure of the Ile-421-to-Thr (Ile-421→Thr) mutant glycoprotein to form syncytia in U937 cells. DNA analysis of revertant viruses revealed that a strong selective pressure was exerted on residue 421 of the surface glycoprotein to allow HIV-2 infection of U937 cells. These results demonstrate that this region of HIV-2 plays an important role in determining fusion efficiency in a cell-dependent manner and consequently can influence viral tropism.

The tropism of human immunodeficiency virus (HIV) for CD4-positive cells results mainly from a specific interaction between CD4 and the envelope glycoprotein (3, 12, 14). However, different virus strains display various host ranges among CD4-positive cell types. All isolates infect human primary T lymphocytes, but they show differential tropism for macrophages or immortalized CD4-positive cell lines. Macrophage tropism of HIV type 1 (HIV-1) is determined at least in part by the V3 loop of gp120, the main neutralizing epitope of HIV-1 (1, 9, 15, 18) and a major determinant of fusion in HIV-1 (6) and in HIV-2 (5). The mechanism through which the V3 region determines macrophage tropism is unknown but probably involves a step that follows gp120-CD4 binding.

HIV strains vary also in their tropism for different CD4-positive cell lines. Replication-competent HIV-1 strains tolerate a considerable range of CD4-binding affinities (20). Thus, the requirements for viral entry after initial binding differ according to the cell line, and it is possible that the difference in tropism results from secondary interactions during fusion with the cell membrane or during uncoating of the virus core.

We have previously shown that point mutations in a region of HIV-1 envelope glycoprotein involved in the binding to CD4 did not affect the CD4-binding ability of soluble gp120 or the infectivity of viruses containing these mutations in T cells, either peripheral blood mononuclear cells (PBMC) or SUP-T1. However, these mutants were found to be unable to establish a productive infection in the promonocytic cell line U937 (2). To determine whether this finding is a general phenomenon or is restricted to the particular host-virus system used in an earlier work, we constructed a series of mutations in the corresponding region of HIV-2 (Fig. 1). Mutations were made by oligonucleotide-directed mutagenesis (13) in a *Pst*I fragment (from nucleotides 7319 to 8462) of HIV-2_{ROD} (8) and then introduced into a proviral clone, pROD23 (derived from pROD9 [7]). Virus derived from this clone is infectious in both U937 and SUP-T1 cell lines.

Expression, processing, and CD4-binding ability of mutant HIV-2 envelope glycoproteins. To analyze the effects of the Env mutations on envelope protein expression, HeLa cells were transfected with proviral DNA plasmids and metabolically labelled with [^{35}S]cysteine and [^{35}S]methionine. The envelope precursor (gp140) was immunoprecipitated from the labelled cell lysates with serum from an HIV-2-positive patient (Fig. 1). None of the mutations had a detectable effect on the expression of the HIV-2 envelope glycoprotein. Furthermore, they did not significantly affect processing and transport of the envelope glycoprotein to the cell surface, since gp125 was released into the medium at a level equivalent to that of the wild type (data not shown).

The CD4-binding ability of the envelope glycoprotein was assayed by incubation of the labelled cell lysates with an excess of unlabelled soluble CD4, followed by immunoprecipitation of the Env-CD4 complexes with the CD4-specific monoclonal antibody OKT4. Substitution of serine for tryptophan at position 428 (Trp-428→Ser) eliminated CD4 binding, while conservative amino acid substitutions (phenylalanine or tyrosine for tryptophan: Trp-428→Phe or Trp-428→Tyr) reduced the CD4-binding capacity. Changing the isoleucine residue at position 421 had a less dramatic effect on CD4 binding: substitution with lysine (Ile-421→Lys) reduced binding, while replacement with threonine (Ile-

* Corresponding author.
† Present address: Institut G. Roussy, Unite de Biochimie-Enzymologie et de Physicochimie Macromoleculaire, Rue Camille Desmoulins, 94805 Villejuif Cedex, France.

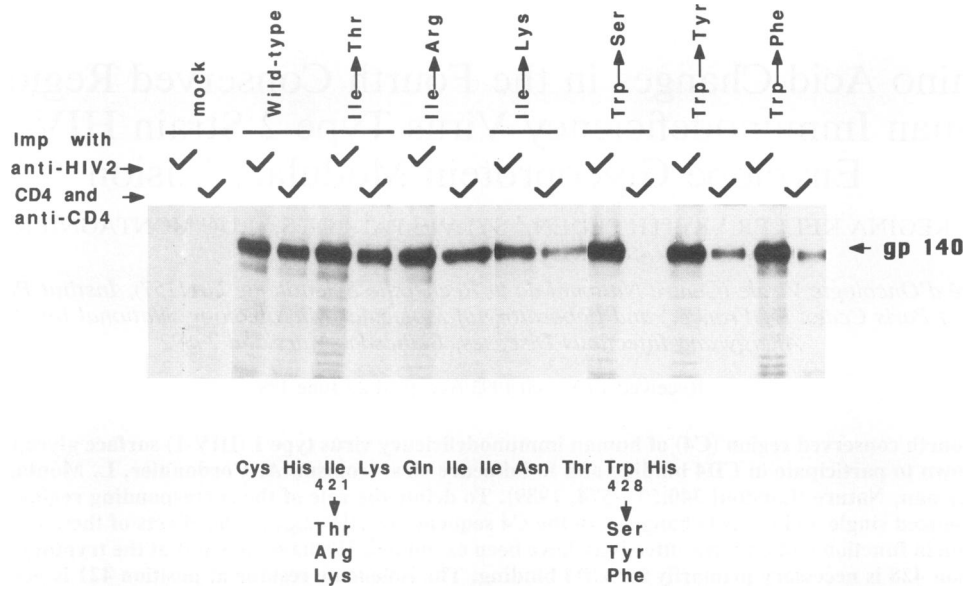

FIG. 1. Phenotype of the mutant envelope glycoproteins. Forty-eight hours after transfection of HeLa cells with a DNA proviral clone, ^{35}S-labelled protein extracts were immunoprecipitated with an HIV-2 patient serum or with antibody against CD4 (OKT4) after incubation with soluble CD4. Amino acid codon substitutions introduced by in vitro mutagenesis are shown at the bottom.

421→Thr) or arginine (Ile-421→Arg) had no detectable effect. These data confirm and extend previous studies of the corresponding region of HIV-1 gp120 (2, 16). Thus, mutation of analogous tryptophan and isoleucine residues had similar consequences on CD4 binding for both HIV-1 and HIV-2.

Effects of mutations on syncytium formation. To determine the ability of the mutant envelope glycoproteins to induce the formation of syncytia in the absence of a spreading infection, the mutated envelope genes were introduced into a vector that expresses HIV-2 envelope protein when cotransfected with a plasmid that expresses Tat (10). Twenty-four hours after transfection of COS-1 cells, the cells were washed and detached from the plate with trypsin and plated in 24-well plates (10^5 cells per well). The following day, CD4-positive cells from different cell lines (10^6 cells per well) were added to these monolayers of COS-1 cells, which were then scored for syncytium formation from 12 to 48 h. One well was used for labelling and immunoprecipitation as a control for the efficiency of transfection. The wild-type Env glycoprotein induced the formation of syncytia in all the target cells tested (Table 1 and Fig. 2). Those mutants that were unable to bind to soluble CD4 (Trp-428→Ser or Trp-428→Phe and Ile-421→Lys) not surprisingly failed to induce the formation of syncytia. The Ile-421→Thr mutant, which bound to soluble CD4, induced syncytia with T cells (SUP-T1, CEM, and Molt-4) at about 80% of the wild-type levels but failed to do so with U937 cells.

TABLE 1. Effects of mutations on syncytium formation

Wild type or mutant	Syncytium formation in cell line[a]:				No. of syncytia with CD4-LTR-β-Gal[b]
	SUP-T1	CEM	Molt-4	U937	
Wild type	++	++	++	++	732 (28)
Trp-428→Ser	−	−	−	−	<1
Trp-428→Phe	−	−	−	−	<1
Ile-421→Thr	+	+	+	−	250 (30)
Ile-421→Lys	−	−	−	−	<1

[a] ++, wild-type levels; +, 70 to 80% of wild-type levels; −, no syncytium formation.

[b] Assay with CD4-expressing HeLa cells containing an integrated copy of the β-galactosidase gene under the control of the HIV-1 LTR. Values are the averages of three assays. Standard deviations are indicated.

In order to quantify changes in the fusogenic properties of the Ile-421→Thr mutant envelope compared with the wild-type envelope, the MAGI cell assay (11) was used. MAGI cells are CD4-expressing HeLa cells that contain an integrated copy of the β-galactosidase gene under the control of the HIV-1 long terminal repeat (LTR). Upon fusion with cells expressing a functional gp125 and HIV-1 Tat, the LTR will be activated by Tat and blue cells will be evident after staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The results of these experiments are presented in Table 1 and Figure 2. Syncytia induced by coexpression of the Ile-421→Thr mutant glycoprotein and HIV-1 Tat protein were less abundant (30% of the wild-type) and smaller (4 or 5 nuclei per syncytium) than those induced by the wild-type glycoprotein (>15 nuclei per syncytium). Therefore, substitution of Thr for Ile at position 421 caused a significant reduction of the fusogenic potential of the HIV-2 gp125 glycoprotein. This alteration was much more marked with the promonocytic cell line U937 than with T cells, suggesting that the former cells have a more stringent requirement for a particular envelope glycoprotein structure to mediate the membrane fusion process. Differential levels of expression of CD4 and/or accessory factors on the cell surface could contribute to the absence of syncytium formation with the Ile-421→Thr mutant in these cells, although their susceptibility to syncytium formation with the wild-type protein was indistinguishable from that observed with T-cell lines.

Effects of mutations on HIV-2 replication. In order to compare the replication rates of the mutant viruses, the proviral DNA clones were transfected into HeLa cells and virus stocks were prepared. Comparable amounts of progeny virions (as measured by the amount of reverse tran-

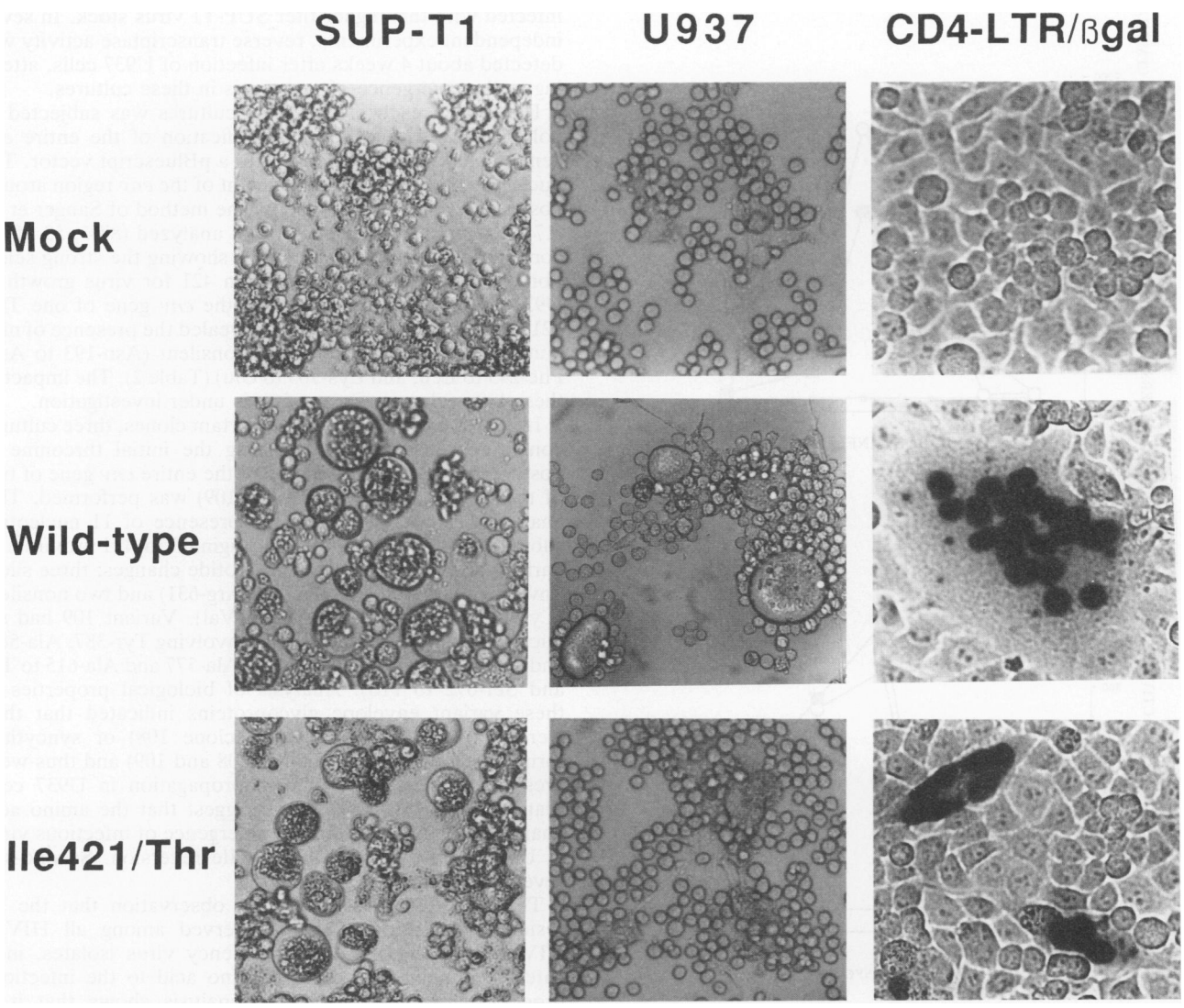

FIG. 2. Comparison of syncytium formation mediated by wild-type or Ile-421→Thr or Trp-428→Ser mutant glycoprotein. COS-1 cells transfected with wild-type or mutant envelope-expressing vectors were cocultured with SUP-T1, U937, or HeLa-CD4-LTR–β-Gal cells. Plates were photographed under a magnification of ×250.

scriptase activity) were used to infect three cell types: PBMC, the T-cell line SUP-T1, and the promonocytic cell line U937. Both the SUP-T1 and U937 cell lines were permissive for the wild-type ROD23 virus. Although ROD23 displayed similar growth kinetics on SUP-T1 and U937 cells, with a peak in virus production occurring about day 17, greater amounts of virus were routinely produced in U937 cells (Fig. 3A and B). Virus in which Trp-428 was replaced by Phe, Tyr, or Ser failed to initiate a productive infection in either PBMC or established cell lines. This was also true when Ile-421 was changed to either Arg or Lys (Fig. 3). However, when Thr was substituted for Ile-421, the mutant virus was able to infect both PBMC and SUP-T1 cells with a time course of infection comparable to that observed for the wild-type virus (Fig. 3A and B). In contrast, this mutant could not infect U937 cells, either by cell-free infection (Fig. 3C) or by using coculture of transfected cells with CD4-positive cells, which is a more efficient mode of virus spread (data not shown). Thus, when assayed for infectivity, Ile-421 mutants showed a phenotype that was consistent with that seen in the syncytium formation assay. Mutations that did not affect the binding of soluble CD4 to Env did nevertheless alter both cell line tropism and syncytium-forming ability in parallel.

Analysis of Ile-421→Thr revertants. Defective viruses that have adapted to grow in a particular cell have been useful to define regions of protein-protein interaction within the viral envelope (21). Therefore, we attempted to isolate revertants of the Thr-421 mutant that are capable of replicating in U937 cells. Initially, the Thr-421 mutant proviral molecular clone was introduced into U937 cells by electroporation. While the wild-type proviral clone began to produce virus on about day 12 posttransfection, no infectious virions emerged from cells transfected with Thr-421 mutant even after culture for 3 months. This was assumed to be due to the low levels of input virus. Therefore, we amplified the transient viral production obtained 48 h after electroporation of U937 cells by passage through SUP-T1 cells. U937 cells were then

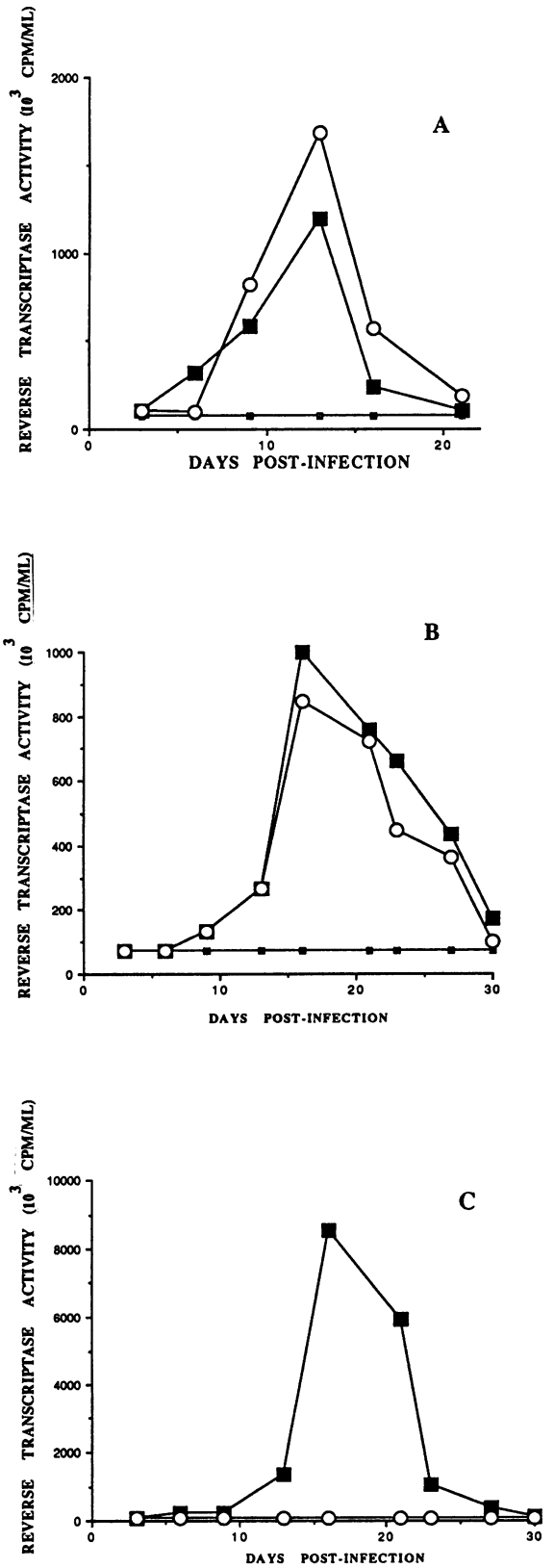

FIG. 3. Growth of pROD23 and Ile-421→Thr mutant on PBMC (A), SUP-T1 (B), and U937 (C) cells. Cell-free supernatants from transfected HeLa cells corresponding to 10^6 cpm of reverse transcriptase were used to infect 2×10^6 cells. Reverse transcriptase activities in culture supernatants were evaluated for each infection at the indicated time intervals. Symbols: -□-, mock; -■-, pROD23; and -○-, Ile-421→Thr mutant.

infected with this higher-titer SUP-T1 virus stock. In seven independent experiments, reverse transcriptase activity was detected about 4 weeks after infection of U937 cells, attesting to the emergence of revertants in these cultures.

DNA from each of the U937 cultures was subjected to polymerase chain reaction amplification of the entire *env* gene, which was then cloned into a pBluescript vector. The nucleotide sequence of the segment of the *env* region around position 421 was determined by the method of Sanger et al. (17). The great majority of clones analyzed (87%; 53 of 61) contained a T at nucleotide 7408, showing the strong selection for an Ile residue at position 421 for virus growth in U937 cells. DNA sequencing of the *env* gene of one Thr-421→Ile revertant (clone 4813) revealed the presence of nine mutations: six silent and three nonsilent (Asn-193 to Asp, Phe-245 to Leu, and Lys-705 to Glu) (Table 2). The impact of these latter changes on tropism is under investigation.

In addition to Thr-Ile→421 revertant clones, three cultures contained some clones harboring the initial threonine at position 421. DNA sequencing of the entire *env* gene of two of these clones (clones 108 and 109) was performed. This analysis (Table 2) revealed the presence of 11 nucleotide substitutions not present in the original Thr-421 provirus. In variant 108, there were five nucleotide changes: three silent (involving His-325, Ala-386, and Arg-631) and two nonsilent (Cys-105 to Tyr and Ala-531 to Val). Variant 109 had six nucleotide changes: three silent (involving Tyr-387, Ala-581, and Val-685) and three nonsilent (Ala-577 and Ala-615 to Thr and Ser-672 to Pro). Analysis of biological properties of these variant envelope glycoproteins indicated that they were defective for processing (clone 108) or syncytium formation in U937 cells (clones 108 and 109) and thus were presumably defective for viral propagation in U937 cells (data not shown). These data suggest that the amino acid change that accounted for the emergence of infectious virus in U937 cells was the Thr-421→Ile reversion. Second-site revertants were not found.

This result, together with the observation that the Ile residue at position 421 is conserved among all HIV-1, HIV-2, and simian immunodeficiency virus isolates, indicates the importance of this amino acid to the infectious process. However, mutational analysis shows that it is dispensable to some extent for infection of T-cell lines and activated PBMC in vitro for both HIV-1 (2, 16) and HIV-2. Biological differences between natural and in vitro infection may explain this paradox.

The biochemical mechanism by which alteration of the isoleucine residue affects the fusion activity of the surface (SU) protein is presently undefined. The binding of the HIV envelope to CD4-positive cells results in a conformational change in Env leading to the activation of the fusion process (4). This function involves several domains of the HIV-2 envelope glycoproteins, including the highly hydrophobic amino terminus of the transmembrane gp36 (19) and the V3 region of the SU glycoprotein (5). Mutation of Ile-421 may modify the interaction of the CD4-binding domain with one of these regions involved in syncytium-forming capacity. Recently, in HIV-1 gp120, the substitution of Arg for the Ile residue corresponding to Ile-421 in HIV-2 has been reported to increase the envelope protein recognition by an anti-V3

TABLE 2. Comparison of the envelope glycoprotein amino acid sequences of mutant Ile-421→Thr and clones 108, 109, and 4813

Domain and residue no.	Mutation in mutant or clone[a]:			
	Ile-421→Thr	108	109	4813
gp125				
34	Asn (AAT)			Asn (AAC)
89	His (CAT)			His (CAC)
105	Cys (TGT)	Tyr (TAT)		
193	Asn (AAT)			Asp (GAT)
231	Gly (GGT)			Gly (GGC)
245	Phe (TTT)			Leu (CTC)
325	His (CAC)	His (CAT)		
358	Ala (GCA)			Ala (GCG)
386	Ala (GCA)	Ala (GCG)		
387	Tyr (TAC)		Tyr (TAT)	
421	**Thr (ACA)**	**Thr (ACA)**	**Thr (ACA)**	**Ile (ATA)**
444	Leu (CTG)			Leu (CTG)
gp36				
500	Lys (AAA)			Arg (AGA)
531	Ala (GCG)	Val (GTG)		
577	Ala (GCA)		Thr (ACA)	
581	Ala (GCT)		Ala (GCC)	
615	Ala (GCA)		Thr (ACA)	
631	Arg (CGC)	Arg (CGA)		
672	Ser (TCC)		Pro (CCC)	
685	Val (GTA)		Val (GTG)	
705	Lys (AAG)			Glu (GAG)

[a] The codon corresponding to the amino acid change is in parentheses.

loop monoclonal antibody (22). This suggests that the structure of the CD4-binding site may influence the conformation of the V3 loop and that these two domains may interact during the establishment of a productive infection. Also, it has been reported recently that an amino acid change within the CD4-binding domain of the simian immunodeficiency virus strain SIV_{mac} could functionally compensate for a "V3" change influencing cell tropism (11a).

The data presented here demonstrate that the isoleucine residue at position 421 in the fourth conserved region of HIV-2 plays an important role in determining fusion efficiency and consequently viral tropism. This is in accordance with the hypothesis that HIV tropism is mediated by strain-dependent conformational change of the SU protein that governs the efficiency of fusion. Understanding the mechanisms by which the CD4-binding domain interacts with other regions of the SU protein, together with the identification of cell-specific factors involved in membrane fusion, may help to elucidate the HIV entry process in different target cells.

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