JOURNAL OF VIROLOGY, June 1994, p. 3544–3549
0022-538X/94/$04.00+0
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Vol. 68, No. 6

Identification of Functional Regions in the Human T-Cell Leukemia Virus Type I SU Glycoprotein

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Received 21 October 1993/Accepted 1 March 1994

Single conservative and nonconservative amino acid substitutions were introduced into the gp45 external envelope protein (SU) of human T-cell leukemia virus type I (HTLV-I). The mutated amino acids were those identified as being conserved in HTLV-I, HTLV-II, and simian T-cell leukemia virus type I (but not in bovine leukemia virus). The mutated envelopes were tested for intracellular maturation and for function. Mutants with three major phenotypes could be defined: (i) 9 mutants with a wild-type phenotype, which included most of the conservative amino acid changes (five of seven) distributed throughout the SU protein; (ii) 8 mutants with affected intracellular maturation, 6 of which define a region in the central part of the SU protein essential for correct folding of the protein; and (iii) 13 mutants with normal intracellular maturation but impaired syncytium formation. These mutations likely affect the receptor binding step or postbinding events required for fusion. Five of these mutations are located between amino acids 75 and 101 of the SU protein, in the amino-terminal third of the molecule. The other mutations involve positions 170, 181, 195, 197, 208, 233, and 286, suggesting that two other domains, one central and one carboxy terminal, are involved in HTLV-I envelope functions.

Human T-cell leukemia virus type I (HTLV-I) is implicated in two different diseases: adult T-cell leukemia, a severe type of T-lymphocyte neoplasia, and a neuropathy, the tropical spastic paraparesis also called HTLV-I-associated myelopathy. HTLV-I is endemic in several parts of the world, including Japan (3), Africa (38), and the Caribbean region (3). One to four percent of infected individuals may develop one or the other disease in their lifetimes (25). The development of a vaccine against HTLV-I will probably be centered on the use of the external envelope glycoprotein of the virus, which exhibits a high degree of conservation among different strains (6, 7, 12, 16, 17, 24, 28, 39, 47).

The HTLV-I envelope glycoprotein, like the envelope glycoprotein of other retroviruses (for reviews, see references 15 and 49), is synthesized as a precursor product of 488 amino acids (13, 23). This glycoprotein is then cleaved by a cellular protease into two mature products, a surface protein (SU) of 313 amino acids and a transmembrane protein (TM) which allows anchoring of the SU-TM complex at the cell surface. Most sera from HTLV-I-infected people contain antibodies directed to peptides from the carboxy-terminal half of the envelope SU protein, whereas only a small percentage of sera react with peptides from the amino-terminal half of the SU (4, 14, 20, 30). In the TM protein, only the intracytoplasmic domain contains reactive linear epitopes (14). All sera from infected individuals, however, contain neutralizing antibodies. Linear peptide targets for neutralizing antibodies are found in two regions of the HTLV-I SU protein; the first region is localized around amino acids 187 to 199 of the SU protein (1, 21, 46), and the second target region, characterized more recently by using goat sera, is in the amino-terminal part of the SU, between amino acids 88 and 98 (29). It is not known whether neutralizing antibodies in human HTLV-I carriers recognize predominantly linear or conformational epitopes.

In the retrovirus life cycle, the envelope glycoproteins are implicated in viral entry into the target cell (reviewed in references 9, 15, and 49), including binding to a specific receptor, followed by postbinding events leading to fusion of the viral envelope with the plasma membrane of the target cell. Regions of the retroviral envelope proteins important for their function are now well documented in murine (2, 26, 27, 43) and avian (9) retroviruses. In human immunodeficiency virus, these regions are being defined with increasing precision (10, 18, 22, 34, 44, 50). However, there is little published information on the HTLV-I envelope proteins. Our previous findings suggested that a region of the SU protein, between amino acids 195 and 205, is important for function (33). The present study further defines, through the use of single amino acid substitutions, new regions important for envelope function.

MATERIALS AND METHODS

Cell lines. COS-1 cells (11) and HeLa cells were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The sarcoma virus-transformed XC cells (45) used as indicator cells in the syncytium formation assay were a generous gift from S. Gisaelbrecht (Hôpital Cochin, Paris, France). They were grown in minimum essential medium containing 10% fetal calf serum. All cell lines were maintained at 37°C in a 5% CO_2 atmosphere.

HTLV-I envelope expressor plasmid. The HTLV-I envelope expressor plasmid is the HTE-1 expressor previously described (8). Briefly, HTE-1 contains an HTLV-I promoter and all viral sequences corresponding to the *env*, *tax*, and *rex* genes. A negative control plasmid (HTE-24stop) was obtained by insertion of a nonsense codon at position 5251 in the *env* gene. In the envelope protein sequence, this is located just after the sequence coding for the signal peptide.

DNA mutagenesis and cloning. Site-directed mutagenesis

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was performed by the Kunkel method (19). A 448-bp *Sph*I-*Sal*I fragment and a 778-bp *Xho*I-*Xho*I envelope fragment were subcloned into plasmid pGEM7ZF+ (Promega). The presence of the desired mutations was confirmed by DNA sequencing. The envelope fragments bearing the desired mutation were then subcloned into the HTE-1 plasmid, and the mutated envelope expressor was resequenced in the envelope region before being used for transfection.

Transfection procedure. The plasmid (1 μg) was transfected into 5 × 10^5 COS-1 cells as described by Cullen (5), using DEAE-dextran and chloroquine (Sigma). At 48 h posttransfection, cells were labeled for immunoprecipitation or used for the syncytium formation assay.

Detection of the envelope proteins by radioimmunoprecipitation. Transfected COS-1 cells were labeled, and the envelope products were immunoprecipitated as previously described (33), using tropical spastic paraparesis patient serum (a gift from Y. Coste, CRTS, Montpellier, France), both from the transfected cell lysates (to assess envelope expression and maturation) and from the transfected cell supernatants (to assess SU-to-TM association). The envelope proteins were visualized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis under reducing conditions followed by autoradiography. Cleavage of the gp61 precursor into the mature SU gp45 and TM gp20 products in mutant envelope was compared with wild-type precursor cleavage by scanning the exposed films with a Chromoscan III apparatus (Joyce-Loebl Ltd.). The relative percentage of cleavage was calculated as (counts for TM gp20/counts for gp61 precursor for the mutated protein)/(counts for TM gp20/counts for gp61 precursor for the wild-type protein) × 100. In Table 1, this percentage is given as the mean percentage obtained from two to three independent experiments.

Syncytium formation assay. The syncytium formation assay was previously described (33) and consisted of coculture of COS-1 transfected envelope-expressing cells with indicator XC or HeLa cells. Numbers of syncytia per well were scored as follows: ++++, 40 or more; +++, 25 to 40; ++, 15 to 25; +, 10 to 15; and −, none.

RESULTS

Choice of point mutations and nomenclature of amino acid substitution mutants. To define the HTLV-I SU glycoprotein regions important for envelope functionality, 23 different amino acid positions were changed by site-directed mutagenesis in the HTLV-I *env* gene. These residues (indicated in Table 1) were selected on the basis of two main criteria. First, they were all conserved among the then published sequences of HTLV-I isolates (6, 7, 12, 16, 17, 24, 28, 39, 47). Second, most of them were also conserved among HTLV-I, HTLV-II (40, 41), and simian T-cell leukemia virus type I (STLV-I) (48), which share a common cell surface receptor, but not in bovine leukemia virus (BLV) (36, 37), which uses a different cell surface receptor on human cells (42). All 23 selected residues were changed nonconservatively, since this was more likely to affect envelope functions. For seven positions in regions in the SU defined as important for function (see below), a second change, consisting of a conservative substitution, was also performed. The mutated constructions were named HTE-X (Table 1), X referring to the position of the mutated amino acid (where Met, the initiation codon of the envelope, is residue 1). When a conservative mutation was made, the construct was named HTE-Xbis. The corresponding mutated proteins were named amino acid 1X→amino acid 2 (for instance, Ser-58→Leu), where amino acid 1 is the original wild-type amino acid and amino acid 2 is the substituted amino acid (Table 1).

The mutated constructions were transfected into COS-1 cells and compared with the plasmid expressing wild-type envelope proteins (HTE-1) with regard to envelope glycoprotein expression, processing, SU-to-TM association, and syncytium formation.

Intracellular maturation of the mutated envelope glycoproteins. To examine the effects of the mutations on envelope synthesis and intracellular maturation, transfected cells were metabolically labeled and analyzed by immunoprecipitation (Fig. 1 and Table 1). For each mutated protein, gp61 precursor cleavage into mature SU gp45 and TM gp20 was compared with wild-type precursor cleavage, and the relative percentage of cleavage was calculated. Figure 1 shows that none of the mutations had a significant effect on envelope product stability, since comparable amounts of the envelope precursor gp61 were detected in all cases (compare the amounts of wild-type and mutated envelope precursor products in the various gels). Most of the mutated proteins exhibited a mobility in SDS-polyacrylamide gels comparable to that of the wild-type precursor except for Asp-197→Val (Fig. 1, lane 37) and Thr-212→Ile (Fig. 1, lane 41). The slightly faster mobility consistently shown by these two mutated proteins might reflect disruption of precursor protein glycosylation. In particular, the mutation at position 212 (Thr-212→Ile) might affect O glycosylation of the precursor.

Among the 30 mutations, 22 had little or no effect on the gp61 precursor cleavage into the SU and TM products, whereas 8 mutations significantly affected precursor cleavage compared with that of the wild-type envelope (Table 1; less than 20% cleavage). These eight mutated proteins, shown in Fig. 1, are Ser-58→Leu (lane 4; wild type in lane 2), Ser-109→Phe (lane 18), Ser-119→Leu (lane 19), Ser-130→Ile (lane 20), Lys-156→Ile (lane 22), Ser-162→Phe (lane 23), Thr-178→Ala (lane 30), and Thr-212→Ile (lane 41). It is notable that six of these mutations cluster in the central portion of the SU protein, implicating this region as critical to the envelope maturation process.

The envelope products were also immunoprecipitated from the transfected cell supernatants to examine a possible disruption of the SU-to-TM association. Previous work from our group had shown that the two proteins are not covalently linked (31). One mutation, Tyr-170→Ser (lane 28), resulted in secretion of large amounts of SU protein into the supernatant (left part of the lane). The quantity of secreted SU in this mutant was comparable to that obtained with a truncated envelope having a nonsense codon proximal to the anchorage domain of the TM protein (Fig. 1; compare lane 27 with lane 28) (32). None of the other mutations disrupted the association between SU and TM proteins, since no product was detected in the supernatant.

Biological activity of the mutated envelopes. The effects of the mutations on HTLV-I envelope function were examined by scoring syncytium formation. In each case, two different indicator cell lines (XC cells and HeLa cells) were used, with identical results.

As shown in Table 1, nine mutations did not affect syncytium formation. These were Asn-95→Asp, Ser-105→Leu, Ser-105→Thr, Ser-181→Thr, Ser-194→Phe, Ser-194→Thr, Asn-195→Asp, Ser-203→Phe, and Ser-220→Ile. Note that most of these mutations produced a conservative amino acid substitution. One of these mutated proteins (Ser-194→Phe) exhibited normal syncytium formation despite a marked reduction of the precursor cleavage (34% of the value for the wild-type envelope). This indicates that at least in some cases, only a small

TABLE 1. HTLV-I envelope mutations and their effects on precursor cleavage, SU-to-TM association, and syncytium formation

Plasmid[a]	Amino acid conservation[b]	Mutated protein[c]	Precursor cleavage[d] (%)	SU in supernatant[e]	Syncytium formation[f]
HTE-1 (WT)			100	−	++++
HTE-58	H1 H2 S1	Ser-58→Leu	<20	−	−
HTE-75	H1	Ser-75→Phe	67	−	++
HTE-81	H1 H2 S1	Ser-81→Phe	47	−	++
HTE-95	H1 H2 S1	Asn-95→Ile	100	−	++
HTE-95bis	H1 H2 S1	Asn-95→Asp	98	−	++++
HTE-101	H1 H2 S1	Ser-101→Leu	73	−	+
HTE-101bis	H1 H2 S1	Ser-101→Thr	74	−	++
HTE-105	H1 S1 B	Ser-105→Leu	74	−	+++
HTE-105bis	H1 S1 B	Ser-105→Thr	91	−	++++
HTE-109	H1 H2 S1	Ser-109→Phe	<20	−	++
HTE-119	H1 S1	Ser-119→Leu	<20	−	−
HTE-130	H1 H2 S1 B	Ser-130→Ile	<20	−	−
HTE-156	H1 H2 S1	Lys-156→Ile	<20	−	−
HTE-162	H1 H2 S1 B	Ser-162→Phe	<20	−	−
HTE-170	H1 H2 S1 B	Tyr-170→Ser	51	+++	+
HTE-178	H1 H2 S1	Thr-178→Ala	<20	−	−
HTE-181	H1 H2 S1	Ser-181→Ile	43	−	+
HTE-181bis	H1 H2 S1	Ser-181→Thr	77	−	++++
HTE-194	H1 H2 S1	Ser-194→Phe	34	−	++++
HTE-194bis	H1 H2 S1	Ser-194→Thr	81	−	++++
HTE-195	H1 H2 S1 B	Asn-195→Ile	65	−	++
HTE-195bis	H1 H2 S1 B	Asn-195→Asp	100	−	++++
HTE-197	H1 H2 S1 B	Asp-197→Val	49	−	+
HTE-197bis	H1 H2 S1 B	Asp-197→Glu	79	−	+
HTE-203	H1 H2 S1 B	Ser-203→Phe	86	−	+++
HTE-208	H1 H2 S1	Ser-208→Leu	56	−	+
HTE-212	H1 S1	Thr-212→Ile	<20	−	+
HTE-220	H1 H2 S1	Ser-220→Ile	100	−	++++
HTE-233	H1 H2 S1	Ser-233→Ile	39	−	++
HTE-286	H1 H2 S1	Ser-286→Phe	44	−	+

[a] Numbers represent amino acid positions (the first residue is the initial methionine). WT, wild type.
[b] H1 indicates conservation in HTLV-I strains, H2 indicates conservation in HTLV-II strains (46, 47), S1 indicates conservation in STLV-I (54), and B indicates conservation in BLV (43).
[c] The first amino acid represents the wild-type amino acid; the second one represents the mutated amino acid.
[d] Percentage of precursor cleavage of the mutated protein relative to the wild-type glycoprotein (see Materials and Methods for the calculation procedure).
[e] Results of immunoprecipitation of the transfected cell supernatant.
[f] Numbers of syncytia per well were scored as described in Materials and Methods.

amount of cleaved products is needed to elicit normal syncytium formation.

Twenty-one mutants gave no syncytia or exhibited abnormal syncytium formation. These included the eight mutations which affected precursor cleavage as described above. The remaining 13 mutations which affected syncytium formation exhibited normal maturation or slightly affected precursor cleavage. Five of these mutations (Ser-75→Phe, Ser-81→Phe, Asn-95→Ile, Ser-101→Leu, and Ser-101→Thr) were clustered between amino acids 75 and 101. The others (Tyr-170→Ser, Ser-181→Ile, Asn-195→Ile, Asp-197→Val, Asp-197→Glu, Ser-208→Leu, Ser-233→Ile, and Ser-286→Phe), were distributed throughout the SU protein, although a second cluster can be detected between amino acids 170 and 233.

DISCUSSION

In this study, 30 single amino acid mutations of the HTLV-I SU glycoprotein were tested for intracellular maturation and function. The mutations involved 23 different amino acid positions in the SU protein, mostly chosen for their conservation among HTLV-I, HTLV-II, and STLV-I. In 16 cases, a serine residue was substituted, since this amino acid, despite its hydrophilic nature, is not usually important for the stability of protein secondary or tertiary structure. Such mutations are more likely to detect late functional steps rather than intracellular maturation.

The mutants could be divided into three different categories. The first includes mutations with normal or near normal maturation and syncytium formation. These are essentially conservative mutations (five of our seven conservative changes). Conversely, nonconservative amino acid substitutions led in most (19 of 23) cases to a nonfunctional protein. This finding suggests that only conservative mutations are tolerated by the SU in the positions conserved between HTLV-I, HTLV-II, and STLV-I, corroborating our previous work (33).

The second group consists of mutations which affect intracellular maturation (Fig. 2). Most of the mutations affecting the intracellular cleavage of the envelope precursor cluster in the central part of the HTLV-I SU protein, between positions Ser-109 and Thr-178. This region of the SU protein probably plays a critical role in the correct folding of the SU protein. Since a normal intracellular maturation is required for normal function, it is impossible from this study to determine whether amino acids in this central part of the SU molecule are also involved in subsequent envelope function.

We also showed that Tyr-170 is involved in the association between the SU and TM proteins, since its replacement by a Ser residue results in secretion of the SU protein in the supernatant. The intracellular maturation of Tyr-170→Ser is

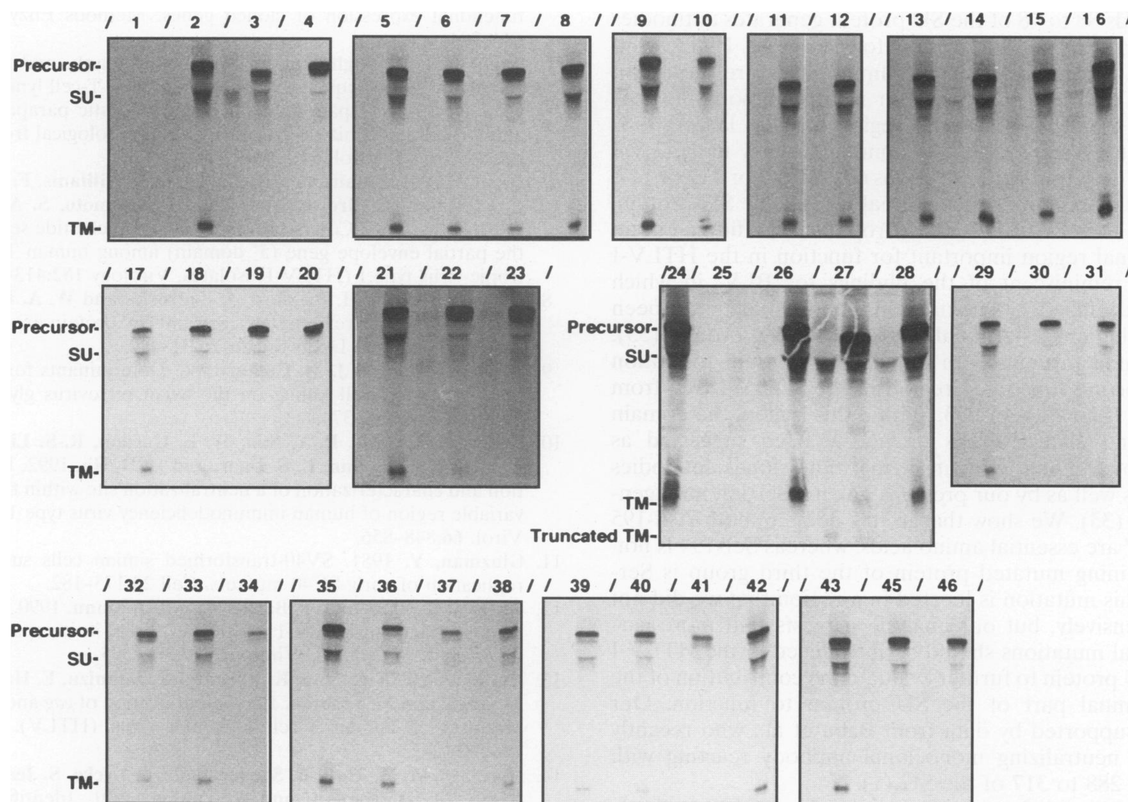

FIG. 1. Immunoprecipitation of wild-type and mutant envelope proteins. For each protein, the left part of the lane corresponds to immunoprecipitation of the transfected cell supernatant and the right part corresponds to immunoprecipitation of the cell lysate. Lanes: 1 and 25, HTE-24stop (negative control); 2, 5, 9, 11, 13, 17, 21, 26, 29, and 35, HTE-1 (wild-type envelope); 3 and 27: HTE-438 (truncated secreted envelope, described in reference 32); 4, HTE-58; 6, HTE-75; 7, HTE-81; 8, HTE-95; 10, HTE-95bis; 12, HTE-101; 14, HTE-101bis; 15, HTE-105; 16, HTE-105bis; 18, HTE-109; 19, HTE-119; 20, HTE-130; 22, HTE-156; 23, HTE-162; 24, C91/PL HTLV-I-infected cells; 28, HTE-170; 30, HTE-178; 31, HTE-181; 32, HTE-194; 33, HTE-194bis; 34, HTE-195; 36, HTE-195bis; 37, HTE-197; 38, HTE-197bis; 39, HTE-203; 40, HTE-208; 41, HTE-212; 42, HTE-181bis; 43, HTE-220; 45, HTE-233; 46, HTE-286.

unaffected (51% of the wild-type value), meaning that the global conformation of the mutated protein is probably unchanged. This Tyr is conserved in BLV (36, 37), in which it might play a similar role in the SU-to-TM association.

The third category of mutant displays normal intracellular maturation but altered syncytium formation (Fig. 2). Syncytium formation is the only functional test used in this study, since all of our attempts to design a binding assay for the wild-type HTLV-I envelope protein have been unsuccessful. Among the 13 mutated proteins with normal intracellular maturation but affected syncytium formation, 5 are located in the amino-terminal third of the SU protein and define a functional amino-terminal region of the SU protein. Previous work by Palker et al. (29) showed that a peptide corresponding

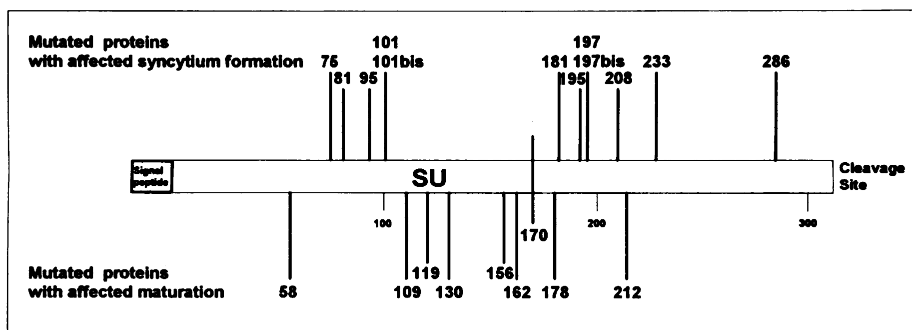

FIG. 2. Positions, along the HTLV-I SU protein, of the amino acid changes affecting maturation or syncytium formation of the envelope protein. The mutated envelope proteins are indicated by vertical bars. Each number indicates the position of the amino acid, 1 being the initial methionine. The mutation at position 170 affects SU-to-TM association.

to amino acids 88 to 98 of the SU protein generates antibodies capable of neutralizing syncytium formation by HTLV-I-infected cells, suggesting that these amino acids are important for HTLV-I envelope function. Our results support this view but suggest that this functional region is even larger. It is possible that covalent disulfide bonding occurs in HTLV-I between the Cys residues at positions 67 and 108 or 112 or 117, positioning this region at the external face of the SU protein, since amino acids 87 to 107 are hydrophilic. The finding of an amino-terminal region important for function in the HTLV-I envelope is reminiscent of the findings for BLV, in which epitopes recognized by neutralizing antibodies have been mapped in the amino-terminal region of the SU protein (35).

Our mutated proteins with impaired syncytium formation identify a second functional region in the HTLV-I SU, from amino acids 181 to 208 or 233. Within this region, the domain around amino acid 195 has previously been suggested as important, by binding of neutralizing monoclonal antibodies (1, 21, 46) as well as by our previous linker insertion mutagenesis analysis (33). We show that in this domain, both Asn-195 and Asp-197 are essential amino acids, whereas Ser-194 is not.

The remaining mutated protein of the third group is Ser-286→Phe; this mutation is located in a region that we did not explore extensively, but our analysis suggests that more carboxy-terminal mutations should be introduced in the HTLV-I envelope SU protein to further evaluate the contribution of the carboxy-terminal part of the SU protein to function. Our results are supported by data from Baba et al., who recently identified a neutralizing monoclonal antibody reacting with amino acids 288 to 317 of the SU (1).

Whether these defined amino-terminal, central, and carboxy-terminal domains function independently or are part of the same conformational structure is also unclear; in particular, we cannot distinguish between receptor binding and postbinding events. However, these studies further elucidate structure-function relationships in the HTLV-I envelope and will aid in the design of an effective vaccine.

ACKNOWLEDGMENTS

We thank A. H. Bellan and A. Lever for constant help and critical reading of the manuscript, I. Callebaut for help with alignment of the BLV envelope amino acid sequence, J. P. Mornon (Université Jussieu, Paris, France) for allowing the use of computer facilities in his laboratory, and Y. Coste (CRTS, Montpellier, France) for providing patient sera.

This work was supported by the Association Nationale de la Recherche sur le SIDA (Paris, France) and by the Association pour la Recherche contre le Cancer (Villejuif, France).

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