

## Functional Tolerance of the Human Immunodeficiency Virus Type 1 Envelope Signal Peptide to Mutations in the Amino-Terminal and Hydrophobic Regions

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**We demonstrated that the leader sequence of the human immunodeficiency virus type 1 envelope functions as signal peptide (SP) despite low scoring in a prediction program. As expected for SP, the hydrophobic core (HC) is essential, and no other sequence could compensate for HC deletion. Contrary to other SPs, major substitutions in the HC, such as introduction of basic, polar, or  $\alpha$ -helix-breaking residues, still allowed efficient translocation and glycosylation. Also, extensive deletions or substitutions of the charged residues at the N terminus had little if any inhibitory effect. This report, which is the first study of human immunodeficiency virus SP, describes the exceptional tolerance of this peptide to mutations.**

According to the signal hypothesis (3), targeting and translocation into the endoplasmic reticulum of retroviral envelope glycoproteins (Env) depends on an amino-terminal signal peptide (SP) (39). In general, SP contain approximately 15 to 30 amino acids organized in a tripartite structure comprising an amino-terminal (N<sub>term</sub>) region and a central hydrophobic core (HC) followed by small polar amino acids (35-37, 39). Most retroviral SPs, identified only by their HC located immediately upstream from the mature protein, score poorly in prediction programs such as PSIGNAL (Intelligenetics) (not shown). Except for a few mutations in the avian Rous sarcoma virus SP (41), no retroviral SP has been specifically studied. Because of the importance of human immunodeficiency virus type 1 (HIV-1) as a pathogen, we studied the HIV-1<sub>BRU</sub> SP (38). Since Env expression is submitted to complex regulations (42), we used an in vitro system with rabbit reticulocyte lysate, T7 RNA polymerase, and dog pancreatic microsomal membranes (Promega), as previously described for the study of SP functions (2, 3, 15, 23, 24, 34, 39).

**Tolerance of the HIV-1 Env hydrophobic core to mutations.** The T7-transcribed mRNA corresponding to the entire HIV-1<sub>BRU</sub> env coding region (Fig. 1A) translated in rabbit reticulocyte lysate in presence of microsomal membranes (6) yielded a complete gp160 envelope precursor (Fig. 1B) and a 120-kDa protein, gp120\*, resulting from a translational arrest occurring in the Rev-responsive element (6). Both products were sensitive to endoglycosidases F and H, confirming their extensive glycosylation (not shown). Oligonucleotide-directed mutagenesis of the wild-type env was performed by using a highly efficient method adapted from that of Kunkel (20) to Bluescript phagemid (Stratagene) (6). Mutated and wild-type env genes were transcribed in vitro, mRNAs were quantitated, identical amounts of mRNAs were translated in parallel, and aliquots were run on sodium dodecyl sulfate gels. All mutants were transcribed with similar efficiencies, and translation in the absence of microsomal membranes yielded similar amounts of the unglycosylated Pr90<sup>env</sup>, indi-

cating that the overall transcriptional and translational efficiencies were not altered by the different mutations. Although the prediction score for the potential SP of HIV-1 Env was very low (PSIGNAL, PC/Gene; Intelligenetics), deletion of the entire SP sequence with the exception of the AUG initiation codon (p $\Delta$ SP) yielded only the nonglycosylated precursor Pr90<sup>env</sup>, similar to the wild-type pE160 translated in absence of microsomal membranes (Fig. 1B, lane e). Therefore, no mechanism independent of an amino-terminal SP was used for translocation of Env, and as expected for an SP, deletion of the HC abolished protein glycosylation (p $\Delta$ Whc and p $\Delta$ hc; Fig. 2A).

General requirements for functional HC identified in vitro and in vivo seem to include overall hydrophobicity and  $\alpha$ -helical structure (15, 23, 27, 37). The HC of the HIV-1 SP is flanked at its N terminus by a unique Trp motif that includes hydrophobic and charged residues. Using a deletion mutant, we found that this Trp motif (p $\Delta$ W) was not essential for translocation (Fig. 2A). Introduction in the center of the HC of either an  $\alpha$ -helix-breaking proline or a charged amino acid blocks translocation of several SPs (2, 15, 23, 24, 27, 33, 37). Such mutations analyzed in vitro or in vivo result in complete or dramatic inhibition of glycosylation. We derived four mutants with single mutations at position -8 or -9, the center of the HC of the HIV-1 Env SP. Glycosylation was unaltered after either a charged (arginine in pER<sub>-8</sub>) or a polar (serine in pES<sub>-8</sub>) residue was substituted for Gly-8 (Fig. 2B). Substitution of arginine for Leu-9 (pER<sub>-9</sub>) resulted in substantial accumulation of the nonglycosylated Pr90<sup>env</sup>; however, glycosylation was not completely abolished in this mutant (Fig. 2B). Thus, the effect of the positive charge introduced into this HC appeared to be position dependent. It was not clear whether the alteration was due to the introduction of a charge per se or to the subsequent reduction of the overall hydrophobicity due to replacement of Leu-9. Alteration of glycosylation similar to that observed with pER<sub>-9</sub> was also observed with pEP<sub>-9</sub>, in which Leu-9 was replaced by the hydrophobic proline. Therefore, both secondary structure and hydrophobicity at this position might be important for a functional SP. Accordingly, introduction of a proline in the middle of the SP

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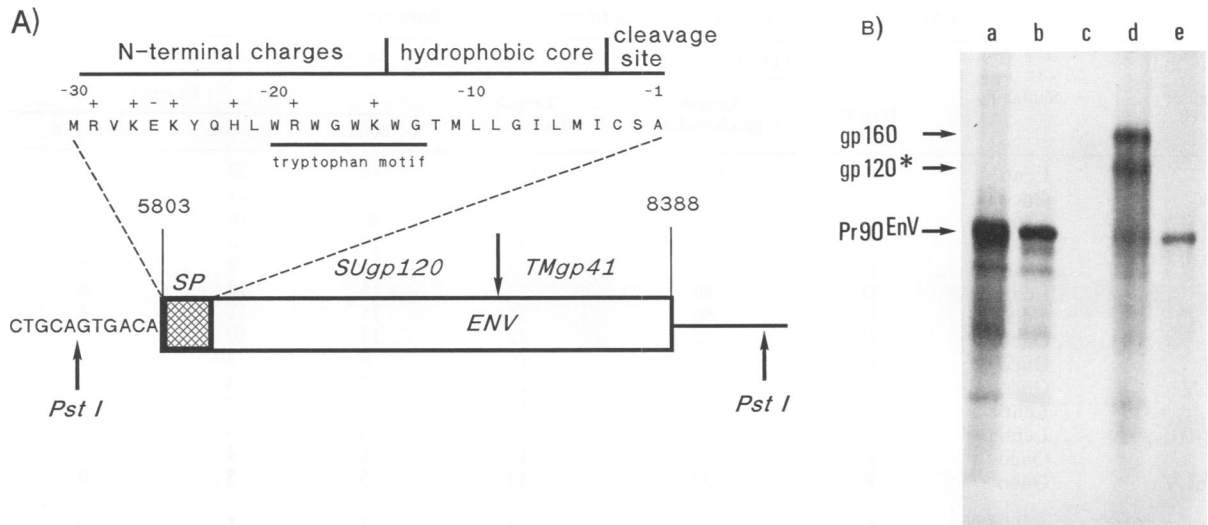


FIG. 1. The HIV-1<sub>BRU</sub> envelope signal peptide. (A) Schematic representation of the HIV-1 envelope construct used for in vitro transcription and translation. Only the HIV-derived insert is shown. Numbering is according to reference 38. By using the two *Pst*I sites, this region was introduced into the Bluescript vector. The region coding for the gp160 envelope precursor is drawn as a box. The surface protein SUgp120 and the transmembrane protein TMgp41 are indicated. The surface-transmembrane cleavage site between residues 516 and 517 is indicated with an arrow above the box. The SP is shaded, and the corresponding amino acid sequence is shown above. The three distinct SP regions are indicated above the sequence, and the tryptophan motif is underlined. The amino acid sequence is according to the one-letter code, and numbering indicates positions starting from the carboxy-terminal amino acid of the SP as -1. (B) Translation of an SP deletion mutant of HIV-1 *env*. Wild-type pE160 (lanes a and d) and pEΔSP (lanes b and e) were translated in vitro for 90 min in the absence (lanes a and b) or presence (lanes d and e) of microsomal rough membranes. Translation in the absence of specific mRNA is shown in lane c. Glycosylated and unglycosylated Env products are indicated.

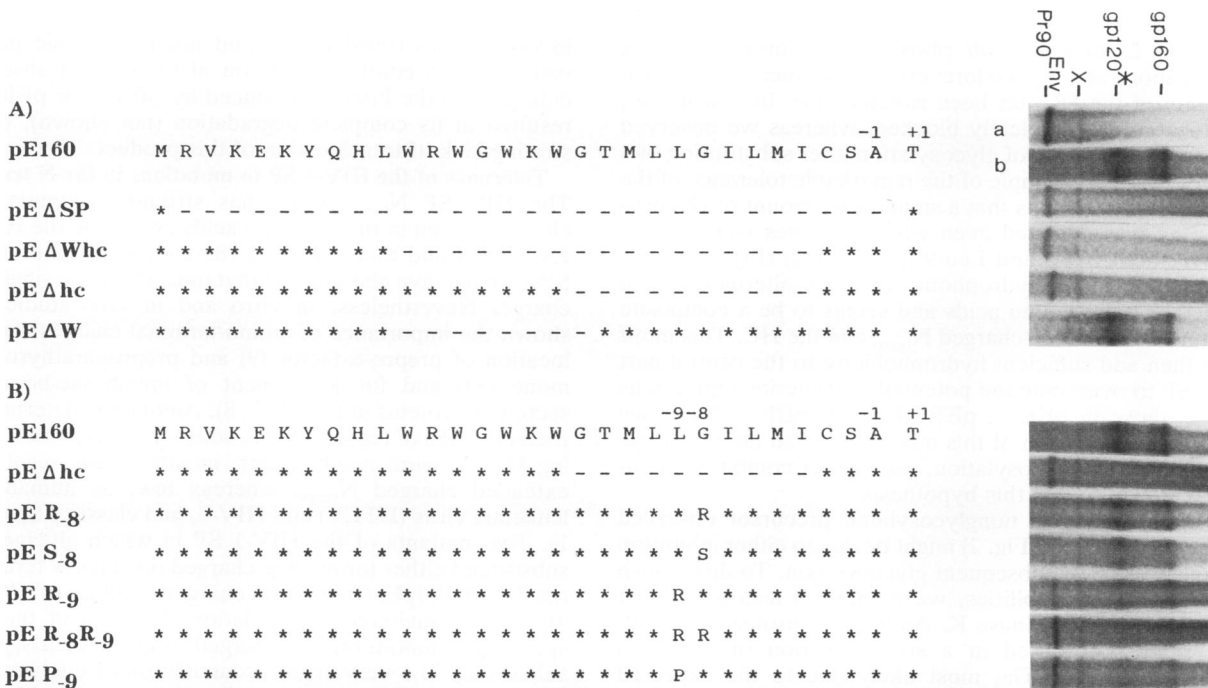


FIG. 2. Mutations in the HC of the SP. Translated proteins are shown on the right. Main products are indicated. X indicates a nonspecific protein also produced without addition of mRNA in the rat reticulocyte lysate. The sequence of SP of pE160 and schematic representations of the mutations are shown on the left. Dashed lines represent deletions; asterisks represent unchanged amino acids. Mutated residues are indicated. -1 and +1 indicate the carboxy terminus of the SP and the amino terminus of the mature protein, respectively. (A) Deletion mutants. For comparison, products of pE160 translated in the absence (a) or presence (b) of microsomal rough membranes are shown. (B) Point mutations in the HC. pE160 and pEΔHC are included as controls.

TABLE 1. Amino-terminal charges of retroviral envelope signal peptides<sup>a</sup>

Virus	Subfamily	Signal peptide		Amino-terminal charged region <sup>b</sup>				
		D or P	Length (nucleotides)	Length (nucleotides)	Charges			
					Total	Positive	Negative	Net
FIV	Lenti	P	187	154	48	23	25	-1
MMTV	Onco	D	98	72	28	21	7	+15
OMVV	Lenti	P	95	82	25	16	9	+8
HFV	Spuma	P	87	63	17	10	7	+4
SFV	Spuma	P	87	63	17	9	8	+2
EIAV	Lenti	D	86	73	16	10	6	+5
JSRV	Onco	P	80	54	15	11	4	+8
RSV	Onco	D	62	42	14	10	4	+7
BLV	Onco	P	33	16	8	7	1	+7
F-MuLV	Onco	P	34	18	7	4	3	+2
HIV-1 <sub>BRU</sub>	Lenti	P	30	16	7	6	1	+6
HTLV-III <sub>451</sub>	Lenti	D	33	17	6	6	0	+7
FeLV	Onco	P	33	13	6	4	2	+3
Mo-MuLV	Onco	P	33	17	5	5	0	+6
HTLV-I	Onco	P	20	3	1	1	0	+2
MPMV	Onco	P	25	6	1	1	0	+2
HTLV-II	Onco	P	20	0	0	0	0	+1
SIV <sub>MAC</sub>	Lenti	P	22	0	0	0	0	+1
HIV-2 <sub>ROD</sub>	Lenti	P	18	0	0	0	0	+1

<sup>a</sup> Retroviruses (references): FIV (feline immunodeficiency virus, PPR isolate) (25); MMTV (mouse mammary tumor virus) (13, 21); OMVV (ovine maedi-visna virus strain SA) (26); HFV (human foamy virus) (7); SFV (simian foamy virus) (22); EIAV (equine infectious anemia virus) (18); JSRV (jaagsiekte sheep retrovirus) (43); RSV (Rous sarcoma virus) (14); BLV (bovine leukemia virus) (28); F-MuLV (Friend murine leukemia virus) (19); HIV-1<sub>BRU</sub> (38); HTLV-III<sub>451</sub> (17); FeLV (feline leukemia virus strain B) (5); Mo-MuLV (Moloney murine leukemia virus) (31); HTLV-I (29); MPMV (Mason-Pfizer monkey virus) (32); HTLV-II (30); SIV<sub>MAC</sub> (simian immunodeficiency virus from macaque) (4); HIV-2<sub>ROD</sub> (10). The corresponding subfamilies, *Lentivirinae* (Lenti), *Oncovirinae* (Onco), and *Spumavirinae* (Spuma) are indicated. Also indicated is whether the cleavage site has been determined by amino acid sequencing (d) or predicted from the nucleotide sequence (p).

<sup>b</sup> The amino-terminal charged region is located between the initiation methionine and the last charged residue before the HC. The net charge includes the positively charged NH<sub>2</sub> group of the amino-terminal methionine.

HC of an *Escherichia coli* ribose-binding protein inhibits translocation, and a pseudorevertant with increased hydrophobicity of the HC has been isolated (16). In that model, translocation is completely blocked, whereas we observed only partial inhibition of glycosylation after substitution of a proline. Another example of the remarkable tolerance of the HIV HC to mutation is that a significant amount of glycosylated Env was detected even when arginines were substituted for both Gly-8 and Leu-9 (pER<sub>-8</sub>R<sub>-9</sub>) (Fig. 2B). The Trp motif includes hydrophobic residues alternating with charged or polar amino acids and seems to be a composite structure between the charged N<sub>term</sub> and the HC. This motif might then add sufficient hydrophobicity to the central part of the SP to overcome the potentially deleterious mutations such as those in pER<sub>-8</sub>, pER<sub>-8</sub>R<sub>-9</sub>, or pEP<sub>-9</sub>. Since we observed that deletion of this motif alone had no detectable effect on protein glycosylation, use further combined mutations will help to test this hypothesis.

Accumulation of a nonglycosylated precursor observed with certain mutants (Fig. 2) might be due to either inhibition of translocation or subsequent glycosylation. To distinguish between these possibilities, we monitored accessibility of the products to proteinase K. Addition of protease to wild-type products resulted in a size reduction of gp160 to approximately 135 kDa, most likely due to the expected proteolysis of 150 amino acids of the cytoplasmic domain (11) (not shown). The gp120\* was protected from protease because of the absence of the cytoplasmic domain due to a translational arrest in the Rev-responsive element (6). Addition of 0.5% Nonidet P-40 prior to proteolysis allowed degradation of both proteins, indicating that protection was

indeed due to translocation and not to intrinsic protease resistance. In contrast, addition of protease in absence of detergent to the Pr90<sup>env</sup> produced by pEASP or pER<sub>-8</sub>R<sub>-9</sub> resulted in its complete degradation (not shown), demonstrating lack of translocation of this product.

**Tolerance of the HIV-1 SP to mutations in the N terminus.** The HIV SP N<sub>term</sub> region has striking accumulation of charges: seven in the 16 amino acids preceding the HC (Fig. 1A). We found that only 1 of 185 eukaryotic nonretroviral SPs (40) had five charges and that over 150 had a single or no charge. Nevertheless, in vitro and in vivo studies have shown the importance of amino-terminal charges for translocation of prepro- $\alpha$ -factor (9) and preproparathyroid hormone (34) and for attachment of membrane-bound and secretory proteins in *E. coli* (1, 8). Among the determined or predicted SPs of representative retroviral Env, most, including HIV-1, were much longer, mostly as the result of an extended charged N<sub>term</sub>, whereas few, as human T-cell leukemia virus (HTLV) and HIV-2, had classical Sps (Table 1). Two mutants of the HIV-1 SP in which glycines were substituted either for the five charged residues N terminal of the Trp motif (pE5G) or for all charged residues (pE7G) (Fig. 3) allowed unaltered glycosylation. Deletion of the corresponding amino-terminal charged region (pEAN<sub>term</sub>) resulted only in a very slight accumulation of unglycosylated Pr90<sup>env</sup> (Fig. 3), confirming the tolerance of this SP to extensive mutations.

This report constitutes the first study of an HIV SP. Like most retroviral SPs, this SP scores poorly in a prediction program, most likely because of a striking accumulation of charged residues (Table 1). Conservation of this feature

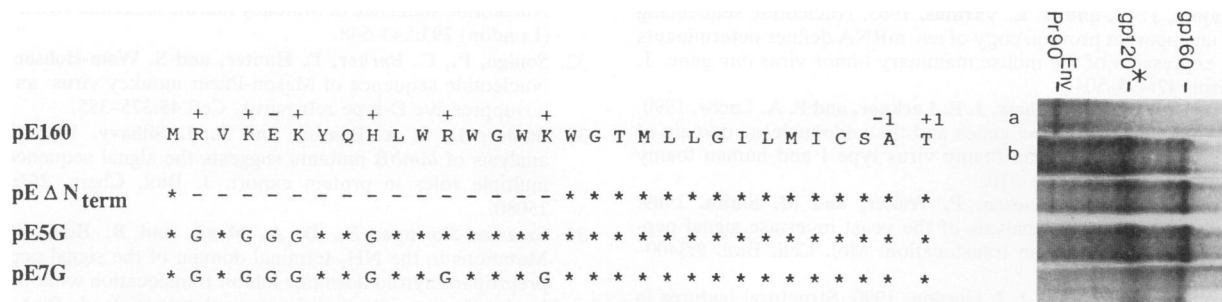


FIG. 3. Mutations in the amino-terminal charged region. Symbols are as described in the legend to Fig. 2. Either the amino-terminal charged region was deleted (pEΔN<sub>term</sub>) or charged residues were changed to glycines (pE5G and pE7G).

suggests some biological importance. The extended SP N<sub>term</sub> often overlaps with other genetic elements (integrase in oncoviruses and *vpu* in HIV-1) and might thus act as a buffer region during adaptive mutations. Accordingly and in contrast to HIV-1, HIV-2 has no *vpu* and no described function in the region of the SP. Since most retroviruses have exceptionally long Env SPs, it is tempting to speculate that, by analogy with oncoviruses and HIV-1, there are alternative functions for all of these regions. Recently, it has been shown that SP-derived peptides can associate with molecules of the major histocompatibility complex through an alternative antigen processing pathway (13). Interaction between this pathway and peculiar SP features as described above might have additional functions selected for the benefit of the virus. The study of SP mutations in different cellular contexts might then unmask new potential deleterious effects.

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