

Topogenic Analysis of the Human Immunodeficiency Virus Type 1 Envelope Glycoprotein, gp160, in Microsomal Membranes

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Abstract. The orientation in cellular membranes of the 856 amino acid envelope glycoprotein precursor, gp160, of human immunodeficiency virus type 1 was investigated *in vitro*. Variants of the *env* gene were transcribed using the bacteriophage SP6 promoter, translated using a rabbit reticulocyte lysate, and translocated into canine pancreatic microsomal membranes. Immunoprecipitation studies of gp160 variants using antibodies specific for various gp160-derived polypeptides provided evidence that the external (cell surface) domain of gp160 begins at the mature amino terminus of the protein and continues through amino acid 665.

A stop-transfer sequence (transmembrane domain) was identified in a hydrophobic region COOH-terminal to amino acid 665 and NH₂-terminal to amino acid 732. Protease protection experiments demonstrated that gp160 possesses a single cytoplasmic domain COOH-terminal to residue 707. Membrane extraction studies using carbonate buffer provided evidence that the 29 amino acid hydrophobic domain (residues 512–541) of gp160 was unable to serve as a stop-transfer sequence. Finally, we propose that the cytoplasmic tail of gp160 forms a secondary association with the microsomal membranes.

THE human immunodeficiency virus type 1 (HIV-1)¹ has been identified as the etiologic agent of the acquired immunodeficiency syndrome (AIDS; Barré-Sinoussi et al., 1983; Gallo et al., 1983; Curran et al., 1985; Weiss, 1986). In HIV-1-infected cells, the proviral envelope gene (*env*) directs the synthesis of a membrane-bound glycoprotein, gp160, that is modified by the attachment of numerous N-linked oligosaccharide side chains (Allan et al., 1985). Sometime during intracellular transport or upon reaching the cell surface, gp160 is proteolytically cleaved to give rise to the two mature viral envelope glycoproteins: gp120 derived from the amino-terminal 510 amino acid residues of the precursor, and gp41 derived from carboxy-terminal 346 amino acids of the glycoprotein (Allan et al., 1985; DiMarzo-Veronese et al., 1985). It is thought that gp41 is an integral membrane protein that forms a noncovalent complex with the gp120 on the surface of virions (Geldernblom et al., 1985). Hydrophobic analysis of gp160 revealed that other than the amino-terminal signal sequence, two additional regions of prominent hydrophobicity were present, both of which were located in gp41 and consisted of amino acid residues 512–541 and 684–707, respectively (Muesing et al., 1985). One or both of these regions might serve as a stop-transfer sequence (Blobel, 1980) by which the protein is stably incorporated into the lipid bilayer of either cellular or viral membranes. To date, two models have been presented to account for the orientation of gp160 in biological

membranes. The first model, based on computer analysis and epitope mapping, postulated that both of the hydrophobic domains in gp41 as well as sequences located between these domains were inserted into the viral membrane (Modrow et al., 1987). More recently, this model has been called into question by two independent studies of the expression of various deletion and truncation variants of the gp160 gene transfected into RAJI cells (Kowalski et al., 1987) or Chinese hamster ovary (CHO) cells (Berman et al., 1988), which provided evidence that variants that included the NH₂-terminal hydrophobic domain of gp41 in their sequence were secreted into the cell culture media.

To understand the role of gp160 in the assembly and infectivity of HIV-1, it is important to define the extracellular as well as the intracellular domains of the viral glycoprotein. We have therefore attempted to clarify this issue by analyzing the topogenesis of gp160 in microsomal membranes through the use of transcription-coupled translation and translocation systems (Milstein et al., 1972; Blobel and Dobberstein, 1975; Walter et al., 1984). In these experiments, mRNAs transcribed using the bacteriophage SP6 promoter were translated in rabbit reticulocyte lysates and the nascent proteins translocated across canine pancreatic microsomal membranes. This approach allows access to the glycoprotein being studied and is thus more suited than *in vivo* systems for direct experimental manipulations. The *in vitro* system has proved useful in defining the function of hydrophobic domains from a variety of viral envelope and cell surface glycoproteins and in elucidating their topology in biological membranes (Perara and Lingappa, 1985; Holland and Drickamer, 1986; Eble et al., 1986; Teixidó et al., 1987; Zerial et al.,

1. *Abbreviations used in this paper:* endo H, endo- β -N-acetylglucosaminidase H; gD-1, herpes simplex virus type 1 glycoprotein D; HIV-1, human immunodeficiency virus type 1; RIP, radioimmunoprecipitation; Staph A, *Staphylococcus aureus*.

1987). In this report we provide evidence that the extracellular domain of gp160 extends from the mature amino terminus (residue 31) through amino acid 665 to residue 683, and that the cytoplasmic tail consists of residues 708–856. We also present novel evidence that indicates that the cytoplasmic tail loops back to interact with the membrane bilayer.

Materials and Methods

Plasmids and Nomenclature

To enhance the expression of gp160 and gp160 variants in mammalian cells, the signal sequence of the *env* gene from the HIV-1 (IIIB isolate) was deleted and replaced with that of herpes simplex virus type 1 glycoprotein D (gD-1) as described previously (Lasky et al., 1986; Berman et al., 1988). This resulted in genes that encoded proteins containing the signal sequence and 27 amino acid residues of gD-1 fused to amino acid 61 of native gp160. The prefix "d" was used to designate chimeric genes containing the amino-terminal gD-1 sequences. The chimeric gp160 gene and its truncated variants are also assigned numerical designations that refer to the last amino acid residue of gp160 before the translational stop codon. Thus, the gene containing the gD-1 signal sequence and 27 amino-terminal residues fused to residues 61–856 of gp160 is designated d856. To maintain uniformity with the literature, all numeric references to amino acid residues in the text is with reference to the methionine residue at position 1 of the native gp160. The gene encoding the truncated variant d665 was constructed by mutagenesis of the d856 gene using a mismatched synthetic DNA primer as described previously (Berman et al., 1988). Construction of the truncated variant d531 has been described elsewhere (Lasky et al., 1986). The d41Ct gene encodes a protein that contains the signal sequence and amino-terminal 27 amino acids of herpes simplex virus gD-1 fused to the carboxy-terminal region of gp160 (amino acid residues 710–856). To assemble the d41Ct gene, the d856 gene was cleaved at the codon corresponding to amino acid residue 724 by digestion with the endonuclease *Ava* I and the 5' sequences up to the codon for residue 710 restored by in frame ligation of a synthetic DNA fragment. This introduced a *Kpn* I site at the 5' end which was used for fusion to the gD-1 sequences as described previously (Lasky et al., 1986).

In Vitro Transcription-coupled Translation

The genes d856, d665, d531, and d41Ct were inserted downstream of the bacteriophage SP6 promoter in a plasmid closely resembling that described by Eaton et al. (1986) and then transcribed in vitro according to a modified protocol of Melton et al. (1984). Briefly, linearized plasmid DNA (1 µg/ml) was transcribed in a 50-µl reaction volume at a final concentration of 0.1 µg/ml, containing 10 µl 5× transcription buffer (200 mM Tris-HCl, pH 7.5, 30 mM MgCl₂, 20 mM spermidine), 1.25 µl dithiothreitol (DTT; 0.2 mM), 5 µl ribonucleotides (2.5 mM of each rATP, rCTP, rUTP, and rGTP; P-L Biochemicals, Milwaukee, WI), 1.25 µl ribonuclease inhibitor (40 U/µl RNAsin; Promega Biotec, Madison, WI), 1 µl SP6 RNA polymerase (15 U/µl; Riboprobe; Promega Biotec) and 26.5 µl sterilized water. Transcription was allowed to proceed for 2 h at 40°C and then stopped by lowering the temperature on ice.

Aliquots of the transcription mixtures containing the appropriate mRNAs were translated in the presence of canine pancreatic microsomal membranes (Promega Biotec) in a rabbit reticulocyte lysate system (Promega Biotec) according to the manufacturer's protocol. Briefly, 2 µl of transcription mixture were added to 35 µl rabbit reticulocyte lysate, 7 µl L-[³⁵S]methionine (15 µCi/µl; Amersham Corp., Arlington Heights, IL) 1 µl amino acid mixture (1 mM minus methionine), 1 µl ribonuclease inhibitor, and 1 µl canine pancreatic microsomal membranes (1 eq/µl). The volume was supplemented to 50 µl with sterilized water. Translation was carried out at 30°C for 60 min and stopped by chilling on ice.

Antisera

The antisera used in this study were raised in rabbits against specific regions of gp160: PB33 was elicited against d531-encoded recombinant gp120 in a manner similar to that described previously (Lasky et al., 1986); PB12 was elicited against an *Escherichia coli* fusion protein consisting of amino acid 542–641 of gp160 fused to the Trp E protein (Cabradilla et al., 1986); RK22 (Kennedy et al., 1986, 1987) and RK773 antisera were kindly provided by Dr. Ronald Kennedy and were elicited against synthetic peptides corre-

sponding to residues 728–745 (DRPEGIEEGGERDRDRS)² and 839–853 (AIRHIPRRIRQGLER; Chanh et al., 1988; R. Kennedy, personal communication) of the cytoplasmic tail, respectively. The numbers of the amino acid residues as presented in this manuscript is based on the sequence of the H9 clone of the HIV-1 III B isolate (Meusing et al., 1985) and differs from the numbers provided by Chanh et al. (1988).

RIP and Gel Electrophoresis

To assay for HIV-1 proteins a specific radioimmunoprecipitation (RIP) procedure was used whereby RIP lysis buffer (1% NP-40, 0.5% deoxycholate, 0.1% SDS in PBS-solubilized L-[³⁵S]methionine-radiolabeled cells were reacted with the appropriate antisera for 30 min at room temperature (22°C). The antibody-antigen complexes were then incubated with 10% glutaraldehyde fixed *Staphylococcus aureus* (Staph A; Pansorbin; Calbiochem-Behring Corp., San Diego, CA), and incubated for 30 min at room temperature. The Staph A was preadsorbed with 2% heat inactivated FBS in PBS to reduce the nonspecific adsorption of proteins to the bacteria. The Staph A antibody-antigen complexes were sedimented by centrifugation and washed twice with RIP wash buffer (1% NP-40, 0.1% SDS in PBS). The resulting pellets were solubilized with SDS-PAGE sample buffer (Laemmli, 1970) and fractionated, unless otherwise specified in 7% polyacrylamide gels.

Endo H Digestion

Immunoprecipitated HIV-1 proteins were released from the Staph A pellets by incubation in 1% SDS/PBS solution for 10 min at 22°C (Platt et al., 1986; John and Firestone, 1986). The supernatants were collected and treated with endo-β-N-acetylglucosaminidase H (endo H) as described by Haffar et al. (1986). After digestion, the HIV-1 proteins were immunoprecipitated again and fractionated by SDS-PAGE.

Protease Protection Assay

In vitro translation samples were adjusted to 10 mM CaCl₂ and incubated in the presence or absence of 0.1 mg/ml proteinase K as described previously (Eble et al., 1986). Representative samples of d856, d665, and d531 translation reactions were solubilized with 1% NP-40 before addition of the protease. After an incubation period of 1 h on ice, all samples received 5 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Co., St. Louis, MO) protease inhibitor (final concentration) in DMSO (Sigma Chemical Co.) and directly transferred into tubes containing boiling 1% SDS in 100 mM Tris-HCl, pH 9.0, buffer and allowed to incubate for 5 min. Samples were then placed on ice, solubilized with RIP lysis buffer, and assayed for HIV-1 proteins by RIP and SDS-PAGE.

Carbonate Extraction

Membranes from in vitro-translated fractions were sedimented by centrifugation of the translation mixtures for 10 min in an airfuge (Beckman Instruments Inc., Palo Alto, CA) at 20 psi air pressure. The resulting pellets were resuspended in either PBS containing 2% heat inactivated FBS or 0.1 M Na₂CO₃, pH 11.4 (Fujiki et al., 1982) and allowed to incubate for 30 min on ice. Membranes were sedimented again by centrifugation and both the membrane pellet and supernatant fractions were collected, solubilized in RIP lysis buffer, immunoprecipitated with PB33 antiserum, and fractionated by SDS-PAGE. Similar analysis of the d41Ct protein was conducted using RK773 antiserum and fractionation in 15% polyacrylamide gels.

Sequence-specific Antibody Binding

At the end of translation-translocation reaction of d856 protein, intact vesicles were isolated by centrifugation in an airfuge, and aliquots of the resulting membrane fraction reacted with the various sequence specific antisera for 1 h on ice. Vesicles were then washed free of unconjugated antibody, solubilized in RIP lysis buffer, and the antigen-antibody complexes were bound to Staph A. Unbound proteins were assayed from the post-Staph A supernatants by the further addition of antisera and Staph A. The adjunct experiment to test the efficiency of RK773 antiserum to react with its epitope was as follows: translation samples were solubilized in 20 mM Tris-HCl, pH 7.4, 5 mM EDTA buffer supplemented with either 1, 0.5, or 0.1% Triton

2. The sequences presented are homologous to the sequences in the gp160 analyzed in this study with the exception of a single amino acid residue substitution at position 737 of G to D.

X-100 detergent, vortexed vigorously, and then immunoprecipitated with either PB33 or RK773 antisera as mentioned previously.

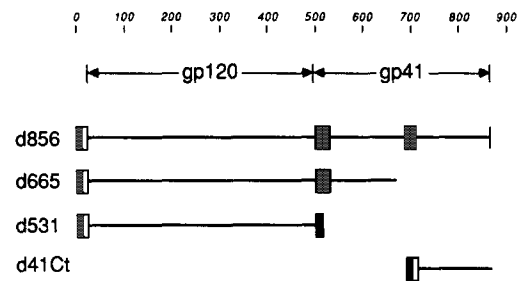
Results

Expression of HIV-1 Antigens *In Vitro*

To identify the domains of the HIV-1 envelope glycoprotein that are associated with cellular membranes, the translocation of recombinant gp160 (d856 gene) was investigated along with that of two truncated variants that terminated at amino acid residues 665 and 531, respectively (Fig. 1 A). The gene encoding the first variant, d665, was generated by insertion of a translational stop codon immediately downstream of the codon corresponding to amino acid residue 665 of the d856 gene. This allowed for the deletion of all amino acids downstream of that site that included the second hydrophobic domain of gp41 (Berman et al., 1988). The second truncated variant, d531, was generated by introduction of a translational stop codon immediately after the codon corresponding to amino acid residue 531 located within the first hydrophobic domain of gp41 (Lasky et al., 1986). mRNAs from the three genes, d856, d665, and d531, were transcribed using the bacteriophage SP6 promoter system and translated in rabbit reticulocyte lysates. To analyze the translation products, four polyclonal antisera were obtained from rabbits immunized with various gp160 derived peptides (Fig. 1 B). Briefly, PB33 reacts with recombinant gp120 (Lasky et al., 1986), PB12 reacts with peptide 542–641 of gp41 (Cabrada et al., 1986), and RK22 and RK773 recognize residues 728–745 and 839–853, respectively (Kennedy et al., 1986, 1987; Chanh et al., 1988; R. Kennedy, personal communication).

The mRNA transcribed from the d856 gene, encoded primarily a protein that migrated in SDS-PAGE with an apparent molecular mass of 88 kD (Fig. 2 A, lane A). This protein was processed in the presence of exogenous microsomal membranes to yield a major product of 150 kD (lane B, top band). Endo H digestion studies (lane D) demonstrated that this difference in molecular masses was due to the incorporation of the high mannose type of N-linked carbohydrate side chains which are known to be attached cotranslationally to nascent polypeptides as they are translocated into the lumen of the microsomal membranes (Hubbard and Ivatt, 1981). The disparity in size of the endo H-digested species relative to the unglycosylated proteins (lane D vs. lanes A or C) can be attributed to residual N-acetylglucosamine residues bound to the asparagines in the polypeptide backbone (Tarentino et al., 1974). This was confirmed by digestion of the translocated species with N-glycanase (data not shown). A second glycosylated species of 115 kD was detected as a minor band upon translation of the d856 mRNA in the presence of microsomes (lane B, lower band). As seen in Fig. 2, both of the glycosylated species could be specifically immunoprecipitated with PB 33 (lane B) and with PB12 (lane F) antisera, indicating that they had the same polypeptide backbone up to amino acid residue 641 (see epitope for PB12). However, only the larger species could be immunoprecipitated with the RK22 (lane H) and RK773 (lane J) antisera raised against peptides located in the carboxy-terminal domain of gp41, indicating that the smaller species lacked the sequences that included these two epitopes. Thus, the 115 kD protein appears

A HIV-1 Recombinant Envelope Glycoprotein and Truncated Variants



B Antisera and Corresponding Epitopes

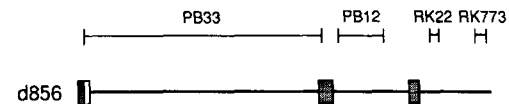


Figure 1. Schematic presentation of recombinant HIV-1 envelope glycoprotein and truncated variants. (A) The fragment of the herpes simplex virus gD-1 gene encoding the signal sequence and 27 amino acid residues of the mature amino terminus were fused to the *env* gene of HIV-1 at a position corresponding to amino acid 61 as described previously (Lasky et al., 1986; Berman et al., 1988) to generate a chimeric gp160 gene termed d856. Truncated variants, d665 and d531, were generated by introduction of stop translation codons immediately after the codons for amino acid residues 665 and 531, respectively (Berman et al., 1988; Lasky et al., 1986, respectively). The d41Ct gene was engineered by fusion of the carboxy-terminal sequences of gp160 (710–856) to the gD-1 sequences as described previously for the d856 construction. (B) Schematic representation of the specificity of the antisera used in the present studies. □, gD-1 signal and amino-terminal sequences. ■, hydrophobic regions.

to be generated by cleavage of the 150-kD species within the gp41 region at a site NH₂-terminal to residue 752.

In vitro biosynthesis of proteins encoded by the truncated gp160 variants, d665 and d531, showed that they were similarly translocated across the microsomal membranes. Thus, the protein encoded by the d665 gene migrated at ~67 kD in the absence of membranes (Fig. 2 B, lane A) and was modified to 126 kD in the presence of membranes (lane B, top band). The protein encoded by the d531 gene was synthesized as a 50-kD protein in the absence of membranes and as a 110-kD protein in the presence of membranes (Fig. 2 C, lanes A and B, respectively). Both the 126-kD d665 protein as well as the 110-kD d531 protein were sensitive to endo H digestion (lane D vs. lane B, in B and C, respectively), in contrast to their untranslocated counterparts (lane C vs. lane A, in B and C, respectively). Proteolytic processing of the mature d665 gave rise to a smaller glycosylated species that migrated at ~115 kD (B, lane B, lower band; more clearly evident in Figs. 3 and 4). As expected, none of the proteins encoded by the d665 gene or the d531 gene could be immunoprecipitated with either RK22 or RK773 antisera (lanes G–J in Fig. 2, B and C, respectively). Moreover, PB12 antiserum specifically immunoprecipitated only the larger d665 glycoprotein suggesting that the proteolytic processing was occurring upstream of the PB12 epitope (Fig. 2 B, lane

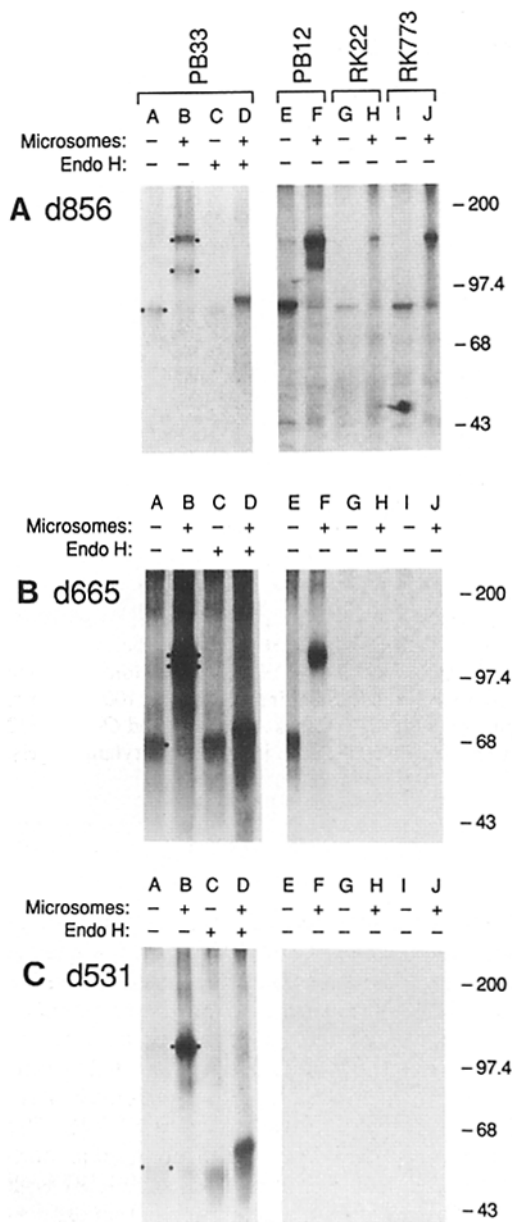


Figure 2. Polypeptides encoded by in vitro-transcribed mRNA. In vitro transcription reaction mixes containing the appropriate mRNAs were added to rabbit reticulocyte lysates supplemented with 15 $\mu\text{Ci}/\mu\text{l}$ L-[^{35}S]methionine. Translation reactions were carried out in the absence (lanes A, C, E, G, and I) and presence (lanes B, D, F, H, and J) of exogenous microsomal membranes. To confirm translocation across the membranes parallel samples were assayed for N-linked oligosaccharide side chain addition by treatment with endo H (lanes C and D). At the end of translation, the reaction mixtures were analyzed for HIV-1 polypeptides by RIP using the sequence-specific antisera (PB33 [lanes A-D], PB12 [lanes E and F], RK22 [lanes G and H], and RK773 [lanes I and J]) and fractionation by SDS-PAGE in 7% polyacrylamide gels. A presents the d856 encoded species. B presents the truncated d665 encoded species. C presents the truncated d531 encoded species. Molecular mass standards (shown on the right side of the figure) are as follows: myosin H chain, 200 kD; phosphorylase b, 97.4 kD; BSA, 68 kD; and ovalbumin, 43 kD.

F). Thus, the transcription-coupled translation system allowed for the expression and identification of several forms of the HIV-1 envelope glycoprotein and permitted direct analysis of its translocation and membrane topology.

Analysis of the Translocated Glycoproteins by Protection from Exogenous Protease Digestion

Previously it has been shown that the system employed in the present studies directs the vectorial translocation of nascent membrane-bound and secreted proteins into the interior of the microsomal vesicles (Blobel and Dobberstein, 1975; Walter et al., 1984). Proteins that lack stop-transfer sequences (e.g., secreted proteins) are completely translocated into the interior of the microsomal vesicles and become resistant to digestion with exogenous protease by virtue of their compartmentalization (Eble et al., 1986). The biosynthesis of proteins that possess stop-transfer sequences (e.g., membrane-bound proteins) differs from that of secreted proteins in that they are incompletely translocated into microsomal vesicles. In this case, translocation is arrested at the stop-transfer sequence, leaving regions COOH-terminal to it exposed on the outside of the vesicles (cytoplasmic surface) and susceptible to digestion by exogenous protease. Thus, addition of protease to the reaction mixture after completion of the translocation reaction, in principle, allows one to distinguish between completely translocated, protease-resistant secreted proteins and incompletely translocated, protease-sensitive membrane proteins. Moreover, the binding of sequence specific antibodies to incompletely translocated proteins permits the localization of specific domains on either side of the microsomal membrane. Fig. 3 illustrates a protease protection experiment in which the translation-translocation products were incubated with or without proteinase K in the presence or absence of NP-40. At the end of digestion, the vesicles were disrupted with RIP lysis buffer and their contents analyzed by RIP. In this study, the 150-kD d856 protein was readily cleaved by proteinase K in the absence of detergent as was evident by a shift in mobility on SDS-PAGE relative to control undigested protein equivalent to ~ 16 kD (lane D vs. lane C, arrows). This novel 134-kD species (lane D, lower arrow) was similar to untreated d856 protein (lane C, upper arrow) in that it could be immunoprecipitated with PB33 and PB12 antiserum (lanes D and F, respectively), but differed in that it was not reactive with either the RK22 or RK773 antisera (lanes G and H, respectively). These results suggested that proteinase K treatment of the translocated d856-derived protein destroyed the epitopes recognized by the RK22 and RK773 antisera. In contrast to the 150-kD species, the smaller glycosylated (115-kD) protein derived from d856 was sensitive to protease digestion only when the vesicles were permeabilized with NP-40 (lane E vs. lanes C and D) suggesting that it was translocated across the membrane into the lumen of the vesicles.

In similar studies, both glycosylated species encoded by the d665 gene, which lack the second hydrophobic domain of gp41, were found to be resistant to protease digestion (lane L vs. lane K) unless the vesicles were first permeabilized with NP-40 (lane M). Moreover, the PB12 antiserum immunoprecipitated the same size protein from protease treated d665 translation-translocation product as did the PB33 antiserum with control undigested samples (lane N vs. lane K). Together these studies suggested that amino acid residues

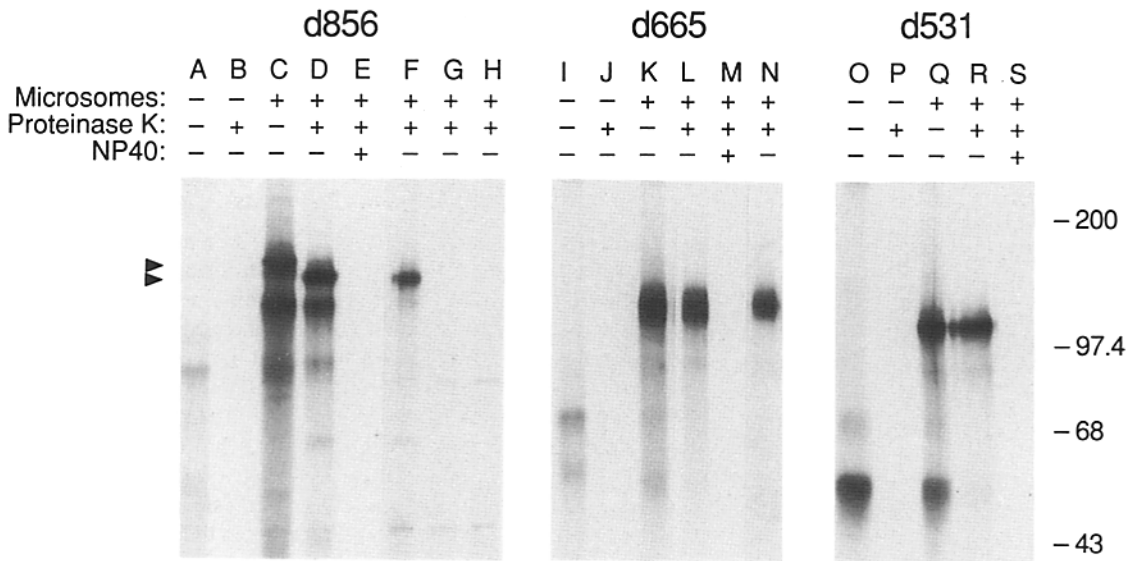


Figure 3. Protection of the translated proteins from protease digestion. Translation of mRNAs encoding d856 (lanes A-H), d665 (lanes I-N), and d531 (lanes O-S) proteins were carried out in the absence (lanes A and B, I and J, and O and P) and presence (lanes C-H, K-N, and Q-S) of added microsomal membranes. At the end of translation, samples were adjusted to 10 mM CaCl₂ and treated with or without 0.1 mg/ml proteinase K for 1 h on ice. Certain samples (lanes E, M, S) were solubilized with 1% NP-40 before digestion. Digestion was arrested by the addition of 5 mM PMSF/DMSO and reaction mixtures were rapidly transferred into boiling 1% SDS in 100 mM Tris, pH 9.0. Radiolabeled samples were then assayed for HIV-1 proteins by immunoprecipitation with PB33 (lanes A-E, I-M, and O-S), PB12 (lanes F and N), RK22 and RK773 (lanes G and H, respectively) antisera, and fractionation by SDS-PAGE in 7% polyacrylamide gels. Molecular mass standards (to the right of the figure) are as described in Fig. 2.

542-665, which are located between the two hydrophobic domains of gp41 and included the epitope for PB 12, were protected from protease digestion and thus, like authentic secretory proteins, were completely translocated into the lumen of the microsomal vesicles. The d531-encoded protein which lacked all of the second and most of the first hydrophobic domains of gp41, was also completely protected from protease digestion in the absence of detergent (lane R vs. lane S). Control studies conducted in the absence of microsomal membranes showed that the untranslocated d856, d665, and d531 proteins were all susceptible to proteolytic digestion (lane B vs. lane A, lane J vs. lane I, and lane P vs. lane O, respectively). Together these results suggested that while the first hydrophobic domain (residues 512-541) of the HIV-1 envelope antigen was not sufficient for arresting the protein in the membrane, a region located COOH-terminal to residue 665, and NH₂-terminal to epitope recognized by the RK22 antisera (728-745) functioned as a conventional stop-transfer sequence.

Carbonate Extraction of Translocated Proteins

To further explore the function of the first hydrophobic domain in gp41, carbonate extraction studies were conducted to determine whether species that contained only this domain might be peripherally associated with membranes by virtue of their interaction with the lipid bilayer. Previous studies have shown that treatment of microsomal vesicles with a basic solution of sodium carbonate lyses the vesicles and releases peripheral membrane proteins while preserving the integrity of the lipid bilayer and any associated integral membrane species (Fujiki et al., 1982). Thus, treatment with high

pH carbonate interferes with ionic noncovalent protein-protein interactions but preserves hydrophobic protein-lipid interactions. In the present studies, proteins derived from the d856, d665, and d531 genes were translated in the presence of microsomal membranes. At the end of the translation-translocation reaction, the membranes were isolated by centrifugation and resuspended in either PBS containing heat inactivated FBS or with sodium carbonate (pH 11.4). The membranes were reisolated by centrifugation, and both membrane and supernatant fractions were solubilized with RIP lysis buffer. The membrane-bound species (nonextractable) as well as supernatant-derived species (extractable) were analyzed by immunoprecipitation with PB 33 antiserum. Fig. 4 (lanes A, B, E, F, I, and J) showed that in the absence of carbonate all of the expressed proteins were detected in the sedimented membrane pellet (M) which is expected for a preparation of unbroken vesicles. However, after carbonate extraction it was observed that virtually all the full length d856 glycoprotein remained associated with the membrane fraction (lane C), while the smaller proteolytic fragment of d856 protein (115-kD species) was now detected mainly in the supernatant (S) fraction (lane D vs. lane C). Similarly, the smaller d665 protein (lane H vs. lane G) and the d531 protein (lane L vs. lane K) were detected in the supernatant fractions after extraction. These results are consistent with the idea that these three proteins were soluble within the vesicles due to complete translocation across the membranes. Interestingly however, the larger 126-kD d665 glycoprotein was present in both the M and S fractions with the greater portion of it being in the S fraction (lane H vs. lane G).

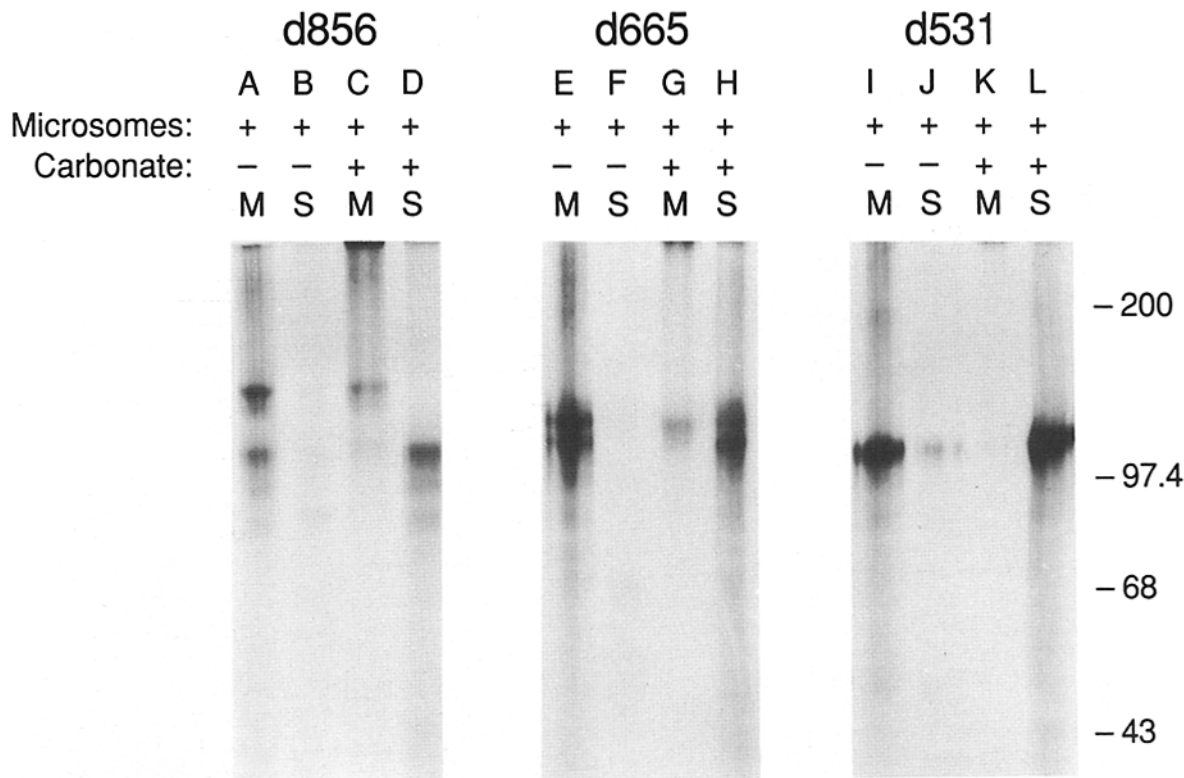


Figure 4. Identification of membrane-bound proteins by carbonate extraction. This figure depicts d856 (lanes C and D), d665 (lanes G and H), and d531 (lanes K and L) encoded proteins extracted from microsomal membrane pellets with 0.1 M sodium carbonate pH 11.4. After treatment with carbonate, the membranes were sedimented by centrifugation, and the supernatants (S) were isolated from the membrane pellets (M). Membrane-bound proteins, i.e., not extracted with carbonate, are shown in lanes C, G, and K, while soluble and extracted proteins are shown in lanes D, H, and L. Control samples (lanes A and B, E and F, and I and J) in which the initial membrane pellets were not solubilized with carbonate were run in parallel. All samples were immunoprecipitated with PB33 antisera and fractionated in 7% polyacrylamide gels. Molecular mass standards (to the right of the right of the figure) are as described in Fig. 2.

Binding of Antibodies Specific for the Carboxy Terminus of gp160 Suggests that it Associates with Microsomal Membranes

The results obtained thus far were consistent with the hypothesis that the HIV-1 envelope glycoprotein precursor, gp160, was a bitopic integral membrane protein that contained a single stop-transfer sequence. As such, upon translocation, discrete domains of the glycoprotein would be oriented either intravesicularly or extravesicularly. These domains could be defined by binding of sequence-specific antibodies to intact vesicles. To explore this hypothesis, intact vesicles containing translocated d856-derived protein were isolated by centrifugation and reacted with PB33, PB12, RK22, and RK773 antisera. Membranes were then washed free of unbound antibody, solubilized with RIP lysis buffer, and antibody-antigen complexes were sedimented after adsorption to Staph A. Antigens not bound by antibody were thus localized in the post-Staph A supernatant fractions, and were assayed by the further addition of antisera. Immunoprecipitated HIV-1 proteins from the supernatants (S) and the initial Staph A pellets (P) are presented in Fig. 5. In those samples that were reacted with either PB33 or PB12 antisera, the proteins were preferentially found in the supernatants (lane B vs. lane A, and lane D vs. lane C, respectively). In contrast, RK22 reacted with d856 glyco-

protein in the initial membrane pellets (lane E vs. lane F). These results revealed that whereas the epitope for RK22 was readily accessible for binding while the protein was still associated with the membrane, those of PB33 and PB12 were protected by virtue of their luminal disposition. These findings are consistent with a bitopic orientation revolving around the second hydrophobic domain in the carboxy-terminal region gp41.

An intriguing result however was the observation that the RK773 antiserum (directed against amino acid residues 839-853) did not react with vesicle-bound d856 protein, as evident by its localization in the supernatant fraction (lane H vs. lane G). Since RK773 was generated against a synthetic peptide it is conceivable that the antiserum reacts preferentially with the linearized epitope and does not recognize the secondary structure assumed when part of the whole protein. Moreover, this result could be due to masking of the antigenic epitope by its folding into the interior of the protein. To address this question, we replaced our standard RIP lysis buffer that contained NP-40 and low concentrations of the denaturants SDS and deoxycholate with another buffer supplemented only with low concentrations of the nonionic detergent Triton X-100 and no SDS or deoxycholate. This experiment allowed us to test directly whether the RK773 antibodies were able to react with their epitope simply by permeabilizing the vesicle membranes without denaturing

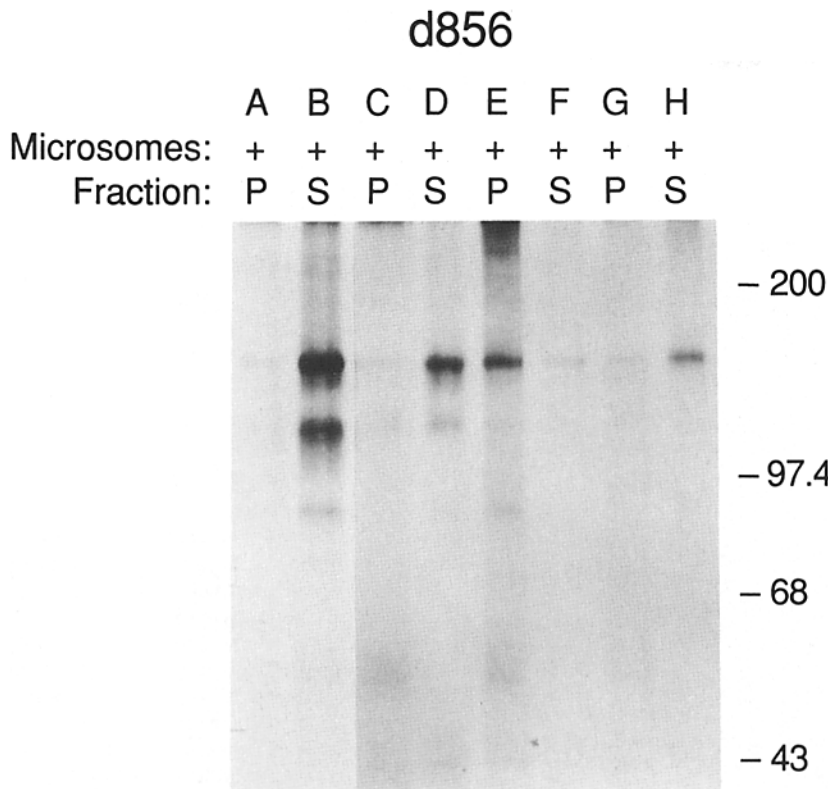


Figure 5. Sequence-specific antibody binding to translocated d856. After translation and translocation of d856, microsomal membranes were sedimented by centrifugation in an air-fuge. Membranes were then resuspended in PBS containing 2% heat inactivated FBS and incubated with the appropriate antisera for 1 h on ice then washed free of unconjugated antibody. After lysis of the vesicles, antibody-antigen complexes were adsorbed to Staph A, sedimented by centrifugation, and represented the pellet (*P*) fractions. Unbound antigens were assayed from the post-Staph A supernatants, *S* fractions, by the standard RIP analysis. HIV-1 antigens were fractionated in 7% polyacrylamide gels. Molecular mass standards (to the right of the figure) are as described in Fig. 2. PB33, lanes *A* and *B*; PB12, lanes *C* and *D*; RK22, lanes *E* and *F*; and RK773, lanes *G* and *H*.

the protein product. Thus, after translating the d856 mRNA in the presence of exogenous membranes, we treated the translation mixtures with 20 mM Tris-HCl, pH 7.4, 5 mM EDTA buffer containing various concentrations (1, 0.5, or 0.1%) of Triton X-100. As seen in Fig. 6 (lanes *D-F*) antiserum RK773 was capable of immunoprecipitating both the undenatured translocated as well as untranslocated d856 species in all three detergent concentrations (see *upper* and *lower arrows*, respectively). The experimental conditions used clearly permeabilized the vesicle membranes since PB33 antiserum was able to react with both glycosylated d856 species (Fig. 6, lanes *A-C*, *upper* and *middle arrows*). The uncommon background present in Fig. 6, lanes *D-F* was also present in lanes *A-C* at similar exposures of the autoradiogram and was mainly due to the buffer used since parallel experiments with the normal RIP buffer did not generate this background. The results of this experiment suggested that in the absence of known denaturants SDS and deoxycholate normally found in our RIP lysis buffer, RK773 reacted with its epitope efficiently. Thus, protection of this epitope from its antibody in intact vesicles (Fig. 5) could not be accounted for by either the secondary structure or the folding of the protein under nonreducing conditions. We therefore considered the alternative possibility that masking of the epitope was due to interaction with the membrane bilayer. To analyze this putative interaction, the carboxy-terminal domain of gp41, starting at the codon for amino acid residue 710 and ending at the codon for residue 856 was fused in frame and downstream of the herpes gD-1 signal sequence (Fig. 1 *A*). When this chimeric gene (d41Ct) was transcribed and translated in vitro, it encoded a protein of 22 kD which became modified to 26.5 kD in the presence of microsomes (Fig. 7

A, lanes *A* and *B*, *middle* and *top arrow*, respectively). This shift in size is consistent with attachment of high mannose core carbohydrate at the two potential N-linked glycosylation sites (Asn-X-Ser/Thr) at residues 750 and 816. Digestion with endo H generated a species smaller than the native untranslocated protein (19.5 kD), consistent with the shift in size due to cleavage of the signal sequence (Fig. 7 *A*, lane *C* vs. lane *A*, *bottom arrow* vs. *middle arrow*). The 22-kD species in lane *C* represents the residual untranslocated protein, the relative amount of which was not surprising since we had noted that the efficiency of translocation of proteins was directly related to the age of the microsomal preparation. When translation mixtures were subjected to carbonate extraction as described earlier, the translocated d41Ct protein remained completely associated with the membrane and not the supernatant fraction (Fig. 7 *B*, lane *C* vs. lane *D*), similar to the unextracted control samples (Fig. 7 *B*, lane *A* vs. lane *B*). This result, together with cleavage of the signal sequence, suggested that the peptide COOH-terminal to the second hydrophobic domain of gp41, which does not contain sequences typical of stop transfer sequences, was nevertheless associated with the lipid bilayer. The apparent reduction in size of the protein after treatment with carbonate (Fig. 7 *B*, lane *C* vs. lane *A*) was not due to aberrant proteolysis since the same size species could be radioimmunoprecipitated with PB33 antiserum which reacts with the amino terminal gD-1 sequences (Fig. 1 *B*), as well as with the carboxy-terminal-specific RK773 antiserum (data not shown).

Discussion

In this study, we report our findings on the topogenesis of

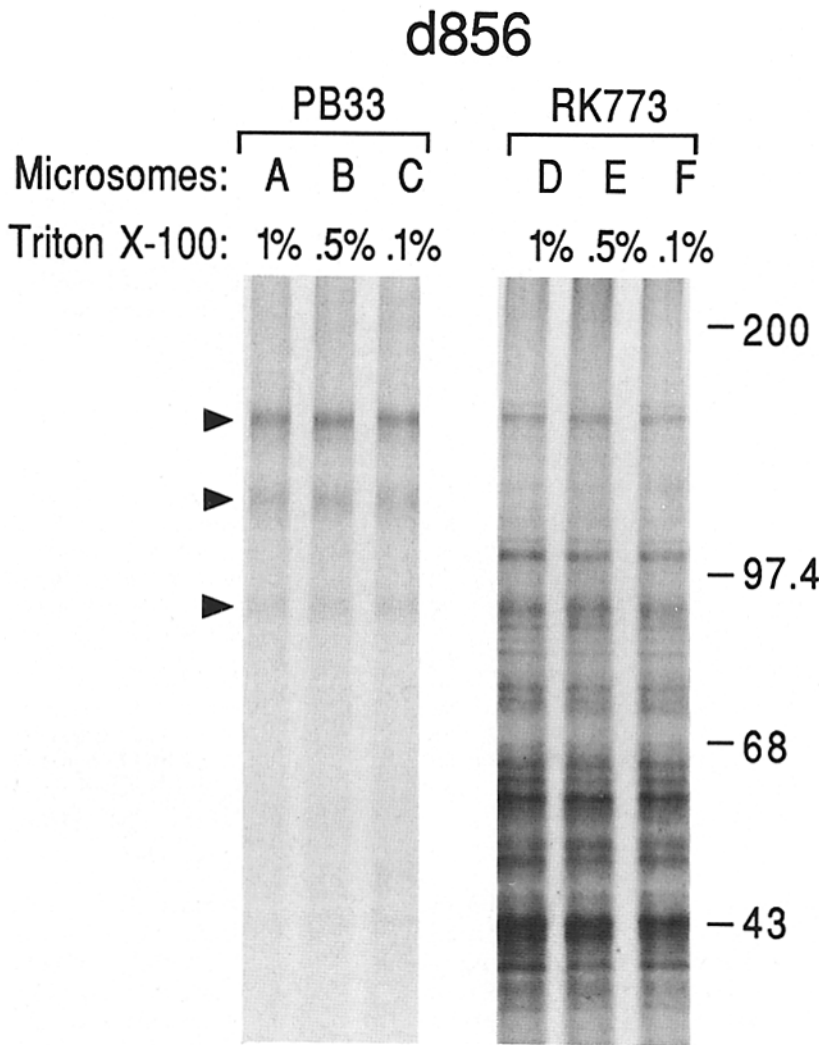


Figure 6. Microsomal membranes protect RK773 epitope from binding by the antibody in intact vesicles. Translation mixtures containing microsomes and translocated proteins were solubilized in 20 mM Tris-HCl, pH 7.4, 5 mM EDTA buffer supplemented with either 1, 0.5, or 0.1% Triton X-100 detergent (lanes *A* and *D*, *B* and *E*, and *C* and *F*, respectively). The solubilized samples were vortexed vigorously and then immunoprecipitated with either PB33 antiserum (lanes *A-C*) or RK773 antiserum (lanes *D-F*). The antibody-antigen complexes were reacted with Staph A and sedimented by centrifugation. The Staph A pellets were washed several times with the 1% Triton X-100 buffer before washing twice with the standard RIP wash buffer. The protein species were then fractionated by SDS-PAGE in 7% polyacrylamide gels and visualized by autoradiography. Molecular mass markers (to the right of the figure) are as described in Fig. 2.

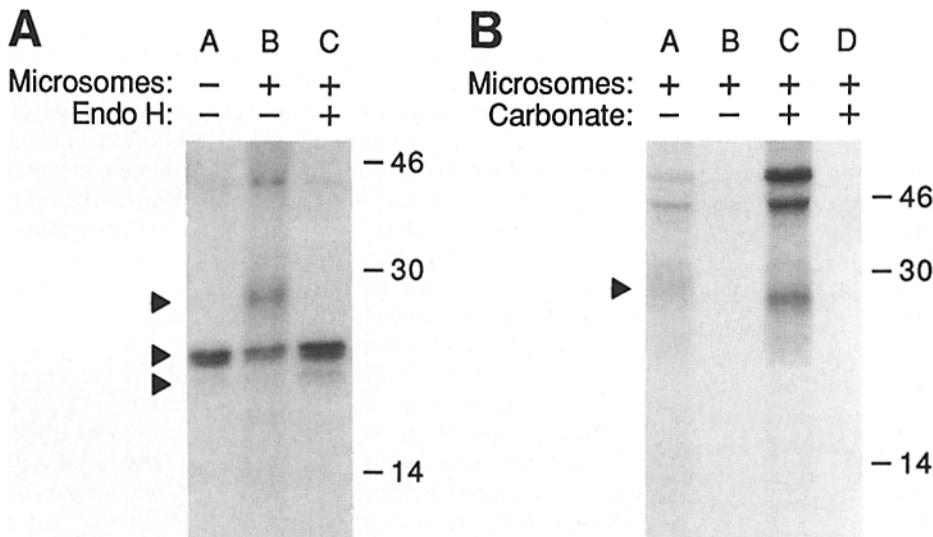


Figure 7. Association of gp41 carboxy-terminal sequences with microsomal membranes. Translation of d41Ct mRNA was conducted in the absence (*A*, lane *A*) and presence (*A*, lanes *B* and *C*, and *B*, all lanes) of exogenous microsomes. The translation products were analyzed directly by RIP using the PB33 antiserum (*A*, -). To show cleavage of the signal sequence concomitant with translocation across the membranes, the translocated species was subjected to endo H digestion to remove the carbohydrate moieties and expose the peptide backbone (*A*, lane *C* vs. lanes *A* and *B*, bottom arrow vs. middle and top arrows). *B* presents the extraction of translocated d41Ct protein from

isolated microsomal membranes with basic carbonate buffer as described in Fig. 4. After extraction, the membrane pellet (lanes *A* and *C*) and supernatant (lanes *B* and *D*) fractions of control samples and treated samples (lanes *A* and *B* and lanes *C* and *D*, respectively) were analyzed for d41Ct species by RIP with RK 773 antiserum. Proteins depicted in *A* and *B* were fractionated in 15% polyacrylamide gels. Molecular mass standards are as follows: carbonic anhydrase, 30 kD; and lysozyme, 14 kD.

HIV-1 envelope glycoprotein across microsomal membranes. Differential antibody-binding studies (Fig. 5) and protease protection analysis (Fig. 3) demonstrated that the 150-kD d856 protein was incompletely translocated across the microsomal membranes. The fact that PB33 and PB12 antisera reacted with the translocated glycoprotein only after detergent lysis of the microsomal membranes, suggested that the epitopes recognized by PB33 and PB12 antisera (61–510 fused to the 27 amino terminal gpD-1 residues and 542–641, respectively) were oriented to the lumen of the microsomal vesicles (Fig. 8), which corresponds to the extracellular compartment on mature virions or virus-infected cells. Conversely, RK22 antiserum recognized membrane-bound d856 before detergent lysis, suggesting that its epitope (residues 728–745) was located on the exterior of the microsomal vesicles, corresponding to the internal or cytoplasmic compartment of virions and virus-infected cells (Fig. 8). Inspection of the amino acid sequence of gp160 revealed that peptide 684–707 delineated the only sequence COOH-terminal to residue 641 and NH₂-terminal to residue 735 that possessed features typical of transmembrane domains or stop-transfer sequences (Sabatini et al., 1982; Adams and Rose, 1985; Davis et al., 1985). Protease digestion studies revealed a 16-kD decrease in electrophoretic mobility of d856 after proteolytic digestion, consistent with the predicted change in molecular mass due to the loss of ~148 amino acids located downstream of the second hydrophobic domain of gp41. In contrast to the 150-kD d856 protein, both the smaller (115 kD) d856 species, as well as the d665 protein, which contained the first hydrophobic domain of gp41 (residues 512–541), were fully protected from protease digestion and were completely translocated across the membranes. The incomplete extraction of the 126-kD d665 protein from membranes by basic carbonate treatment argued that at least a portion of the truncated protein was able to interact with the lipid bilayer, albeit to a lesser extent than the 150-kD d856 species (Fig. 4). This interaction did not appear to be mediated by the first hydrophobic domain of gp41, since the smaller 115-kD d856, which also contained this domain, was quantitatively extracted with carbonate treatment. Moreover, phase separation analysis in Triton X-114 (Bordier, 1981; Alcaraz et al., 1984) revealed that both the d665 and d531 variants partitioned completely into the aqueous phase, a characteristic of secreted soluble proteins (data not shown). Therefore, this finding could represent either limited solubility of the larger d665 protein in the presence of alkali, or might be a reflection of the presence of two conformationally distinct populations of d665: one with the first hydrophobic domain of gp41 (residues 512–541) folded into the interior of the molecule (supernatant material) and the other an unfolded molecule with the hydrophobic domain exposed to the aqueous environment (residual membrane fraction). Results from *in vivo* studies of the expression of gp 160 variants terminating at residues 665 in mammalian cells (Kowalski et al., 1987; Berman et al., 1988) agreed with the *in vitro* studies in that the truncated proteins were secreted into the cell culture medium and were not detected on the surface of transfected cells. More recently, it has been found that a gp160 truncated variant terminating at amino acid residue 683 was similarly secreted into the cell culture medium of transfected CHO cells (Nakamura G., W. Nunes, O. Haffar, L. Riddle, T. Gregory, and P. W. Berman, manuscript in preparation).

Since the first hydrophobic domain of gp41 (residues 512–541) failed to function as a stop-transfer sequence, this region must serve another role in the native molecule. One characteristic of HIV-1 infection is the formation of large syncytia between infected and uninfected CD4⁺T lymphocytes (Barré-Sinoussi et al., 1983; Popovic et al., 1984; Lifson et al., 1986a, b; Sodoroski et al., 1986) that is mediated through the specific interaction of gp120 with the CD4 protein (McDougal et al., 1986a, b; Lasky et al., 1987). Mutations in the first hydrophobic region of gp41 reduced the fusogenic activity of the variant proteins as determined by the absence of syncytia formation in transfected cell cultures (Kowalski et al., 1987). Recently, it has been reported that the 512–541 domain contained a tandem repeat of a consensus sequence (Phe-X-Gly) found in the amino terminus of fusion proteins of several paramyxoviruses (Gallaher, 1987; Gonzalez-Scarano et al., 1987). Thus by direct mutational analysis and by analogy to paramyxoviruses and other retroviruses such as mouse mammary tumor virus (Redmond et al., 1984), the first hydrophobic region of gp41 transmembrane protein appears to be the fusion domain of HIV-1.

The apparent inaccessibility of RK773 antiserum to its epitope when gp160 was integrated in the membrane (Fig. 5), and the finding that this observation could not be accounted for by either the secondary or tertiary structure of the protein but was due to the intervening lipid bilayer (Fig. 6) raised the question regarding the association of the cytoplasmic tail of gp160 with the membrane. To investigate this possibility, we expressed the peptide 710–856 as a separate fusion protein, d41Ct (Fig. 1 A and Fig. 7). Carbonate treatment of the translocated d41Ct failed to extract the protein from the membrane fraction, suggesting that it was integrated in the lipid bilayer. This interaction was not mediated by the amino-terminal signal sequence since endo H digestion analysis suggested that the signal sequence was cleaved from the translocated species (Fig. 7). Recently, we have also expressed the d41Ct gene in a transient mammalian cell culture system and have shown by various criteria that the encoded glycoprotein was exported to the cell surface as well as secreted from the cells (Haffar, O. K., D. J. Dowbenko, and P. W. Berman, manuscript in preparation). At the present time we do not know whether the cytoplasmic tail of gp41 actually traverses the bilayer or is associated peripherally with it. Inspection of the amino acid sequence of d856 failed to reveal any region typical of membrane binding domains (Sabatini et al., 1982; Adams and Rose, 1985; Davis et al., 1985) downstream of the defined membrane anchor region (residues 684–707). Interestingly however, it had been noted by D. Eisenberg (personal communication) that an amphipathic α -helix (Eisenberg et al., 1984) was formed by residues 833–849 which overlap the RK773 epitope (839–853). This helix was recently reported to extend from residue 824–856 (F. Carson, personal communication). Computer homology matching of this COOH-terminal region of gp41 from 10 different HIV-1 isolates revealed a remarkable conservation of the amphipathic α -helical secondary structure (data not shown). Given the polar nature of amphipathic α -helices, it is thermodynamically unlikely that such a structure crosses the membrane independently. For it to cross the membrane, it most likely would require an interaction with another amphipathic α -helix of complementary polarity similar to that described for ion channel proteins (Finer-

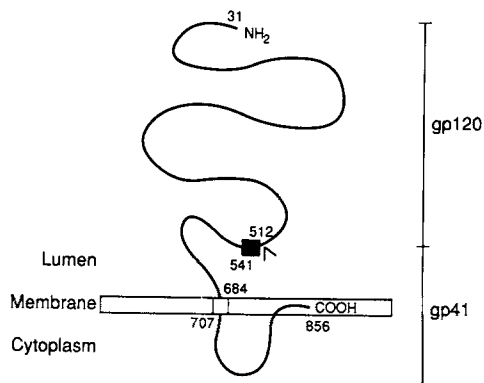


Figure 8. Model for the orientation of HIV-1 recombinant gp160 in microsomal membranes. The present studies suggested that gp160 spans microsomal membranes once using the second hydrophobic domain in the gp41 (amino acid residues 684–707 designated by an open box) as a stop-transfer sequence. All sequences NH₂-terminal to residue 684 which include the first hydrophobic domain in gp41 (residues 512–541 designated by the colored box) and extend through the NH₂-terminus of mature native gp160 (residue 31) are thus oriented to the luminal surface of intracellular vesicles (extracellular surface). Conversely, sequences COOH-terminal to residue 707 are oriented to the outside of the vesicles (cytoplasmic surface). In this model, we also propose that the carboxy terminus of gp160 interacts with the membrane, although the specific sequences involved in this interaction have not been directly identified yet. The open arrowhead symbol points to the cleavage site (residues 510 and 511) between gp120 and gp41 regions of the envelope glycoprotein.

Moore and Stroud, 1984). More recently, a second amphipathic α -helix was identified that was composed of amino acid residues 770–794 (F. Carson, personal communication). Similar to the previously described helix this second structure was also conserved in the 10 HIV-1 isolates examined (data not shown). It was suggested by F. Carson³ (personal communication) that the two helices may form a stable interaction in the molecule by orienting themselves in a way that allows the formation of salt bridges between the opposing charged residues. Although we do not have any direct evidence to show whether either or both of these domains are responsible for the association of the cytoplasmic tail with the bilayer, it is nevertheless surprising that they are so highly conserved. Recently, this region of gp160 has been the focus of much interest regarding its contribution to the structure and function of the virus. For instance, mutant virions generated by deletion of 18 amino acid residues from the carboxy-terminal end of their gp160 were found to be noninfectious and not to form syncytia (E. Hunter, personal communication).

In conclusion, the use of in vitro transcription-coupled translation and translocation systems has allowed us to analyze the orientation of gp160 in microsomal membranes. These studies suggest that the mature amino terminus of gp160 and all residues upstream of amino acid 665 are exposed on the surface of virions and virus-infected cells. The hydrophobic region delineated approximately by residues 684–707 appears to function as a transmembrane domain

3. This was presented at the Fourth International Congress on AIDS, Stockholm, Sweden, June 12–16, abstract No. 1516.

(stop-transfer sequence), and residues COOH-terminal to this sequence comprise the cytoplasmic domain of the protein. These results are inconsistent with the model for gp160 orientation in membranes previously proposed by Modrow et al. (1987) which suggested that gp160 spanned the bilayer three times by virtue of the two hydrophobic domains of gp41 and a region located between the two domains, all three of which were theorized to function as transmembrane domains. Because our results demonstrated that the epitope recognized by the RK22 antiserum (residues 728–745) was oriented towards the cytoplasmic side of the microsomal membranes (Fig. 8), we cannot account for the reports that antisera specific to the sequence delineated by residues 722–752 were able to neutralize HIV-1 infectivity in vitro (Chanh et al., 1986; Ho et al., 1987). Although the data presented is consistent with the simplest model of gp160 orientation (i.e., that it is an integral membrane protein that contains a single stop-transfer sequence) our results suggest that gp160 forms a second association with the membrane bilayer near its COOH terminus. We are currently in the process of elucidating the exact nature of this unique interaction.

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