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Analysis of the structure and function of the HIV-I envelope glycoprotein.

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ANALYSIS OF THE STRUCTURE AND FUNCTION OF THE HIV-1 ENVELOPE GLYCOPROTEIN

by

KARL DAVID SALZWEDEL

A DISSERTATION

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ABSTRACT OF DISSERTATION
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It has now been 13 years since the causative agent of Aquired Immunodeficiency Syndrome (AIDS) was identified to be the Human Immunodeficiency Virus (HIV). During this time, HIV has become arguably the most studied and best characterized virus to date. Despite this wealth of knowledge, however, a safe and effective vaccine against HIV has been elusive, and strategies for preventing the onset of disease in infected individuals are only now beginning to show promise. In light of the continuing need for effective antiviral therapies, it is crucial to elucidate the remaining features of the HIV lifecycle which are still poorly understood. This dissertation focuses on the earliest steps in the virus lifecycle which culminate in the irreversible infection of the host cell, as well as late steps in the production of virus from cells which have already been infected.

The first three sections of the dissertation address the roles of specific domains of the HIV envelope glycoprotein (Env) in the membrane fusion process during virus entry. The first two sections examine the role of the membrane-spanning domain in the fusion mechanism. The membrane-spanning and cytoplasmic domains of Env were replaced with a glycolipid membrane anchor. The resulting proteins were found to be incapable of mediating membrane fusion, thus demonstrating for the first time that Env must interact with both leaflets of the viral envelope via its membrane-spanning domain in order to complete the fusion reaction. The third section addresses the role of the membrane-proximal region of HIV Env in the membrane fusion reaction. Deletion, substitution, and insertion mutagenesis were utilized to probe the structural and sequence requirements for this region in the function of Env. A conserved tryptophan-rich motif was identified which is critical for efficient virus entry. Finally, the fourth section focuses on the intermolecular

interactions involved in HIV envelope assembly. Env was engineered to contain a functional endoplasmic reticulum-retrieval signal. Yet virus cores continued to assemble and bud normally from the plasma membrane despite the localization of Env to the ER. Thus, no evidence was found for a functional interaction between Env and the virus core proteins at the ER membrane during virus assembly. These new insights into the mechanisms of virus entry and virus assembly may lead to future studies which could prove useful in the design of new strategies to block virus replication as well as in the development of modified viral vectors for targeted gene therapy.

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

Figure | Page

DISCUSSION

LIST OF ABBREVIATIONS

AIDS	aquired immunodeficiency syndrome
CA	capsid protein
CAT	chloramphenicol acetyltransferase
CKR	chemokine receptor
C-terminal	carboxy-terminal
DAF	decay accelerating factor
DMEM	Dulbecco's modified Eagle medium
EM	electron microscopy
endo H	endoglycosidase H
Env	envelope glycoprotein
ER	endoplasmic reticulum
Gal Cer	galactosyl ceramide
GPI	glycosyl phosphatidylinositol
HA	hemagglutinin
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
IN	integrase
LTR	long terminal repeat
MA	matrix protein
MAGI	multinuclear activation of a galactosidase indicator
MuLV	murine leukemia virus
MW	molecular weight
NC	nucleocapsid protein

ND	not determined
N-linked	asparagine-linked
N-terminal	amino-terminal
PBL	peripheral blood lymphocyte
PBS	phosphate-buffered saline
PBS-C/M	PBS containing calcium and magnesium
PCR	polymerase chain reaction
PI-PLC	phosphatidylinositol-specific phospholipase C
Pol	retroviral polymerase precursor protein
RSV	Rous sarcoma virus
RT	reverse transcriptase
sCD4	soluble CD4
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SIV	simian immunodeficiency virus
sMAGI	SIV-infectable MAGI cell line
SU	surface glycoprotein
TM	transmembrane glycoprotein
VSV	vesicular stomatitis virus

INTRODUCTION

Aquired immunodeficiency syndrome (AIDS) continues to be a serious health threat in the United States as well as worldwide. Human immunodeficiency virus (HIV), the virus that causes AIDS, has proven to be a formidable pathogen, escaping all attempts thus far to permanently block its replication and pathogenesis in vivo through drug therapy and vaccination. Combinatorial drug therapy using inhibitors of viral enzymatic functions has at least prolonged the lives of infected individuals by slowing virus replication and, thus, the progression to disease. Thirteen years of intensive research has made HIV arguably the most studied virus in history, primarily due to the emergence of this lethal pathogen in the developed world during a revolution in molecular biological techniques. Yet, while much is known about the replication cycle of the virus, surprisingly little has been learned about the mechanism by which the virus causes disease. Nonetheless, our understanding of the life cycle of HIV facilitates analysis of the effectiveness of existing drug intervention and permits the design of new inhibitors to intervene at various points in the viral life cycle.

The work described herein focuses on two of the more poorly understood steps of the virus life cycle: the initial step, virus entry, and the final step, virus assembly. HIV incorporates its genome into that of the host cell upon infection. Because this step, termed integration, is, so far, irreversible, an infected cell cannot be cured of its infection. Therefore, it would be desirable to prevent infection altogether by blocking entry of the virus into the cell. Virus entry is mediated by the envelope glycoprotein (Env) after it binds receptor molecules on the surface of the target cell. The Env protein drives the fusion of the viral and target cell membranes. Little is known about the process by which retroviral glycoproteins effect membrane fusion, and that will be the primary focus of this

dissertation. Understanding the fundamentals of virus entry is also important in the development of modified viral vectors for targeted gene therapy.

An alternative antiviral strategy to blocking virus entry would be to block the production of infectious virus from cells which have already been infected. One way this could be done would be to prevent the incorporation of Env into the viral envelope during virus assembly. The nature of the intermolecular interactions which mediate envelope assembly have, again, not been well characterized. This will be the focus of the fourth section of the dissertation.

The following introduction provides a brief summary of what has been learned about HIV so far, with a more detailed overview of the current state of knowledge concerning the topic of this dissertation, the structure and function of the HIV envelope glycoprotein.

THE HUMAN IMMUNODEFICIENCY VIRUS

In the summer of 1981, the first cases of an emerging disease affecting homosexual males in the United States were reported to the Centers for Disease Control (33). This disease, later named acquired immunodeficiency syndrome, or AIDS, exhibits as its hallmark a slowly progressing depletion of CD4-positive helper T-cells, leading to severe immunodeficiency. Immunosuppresion is accompanied by the appearance of various opportunistic infections and cancers which are unique to immunocompromised patients and, frequently, neurologic disorders (60, 86, 92, 159, 174). This disease was later found to be associated with infection by a novel retrovirus, now termed HIV (5, 73, 117). HIV is transmitted through blood and genital secretions. Individuals at high risk for infection include intravenous drug users who share needles, health care workers exposed to infectious blood through needle sticks, hemophiliacs treated with contaminated blood products, infants born to infected mothers, and partners of infected individuals engaging in unprotected sexual intercourse. The mechanism by which HIV causes T-cell depletion and disease is still not clear, although disease progression appears to correlate well with virus

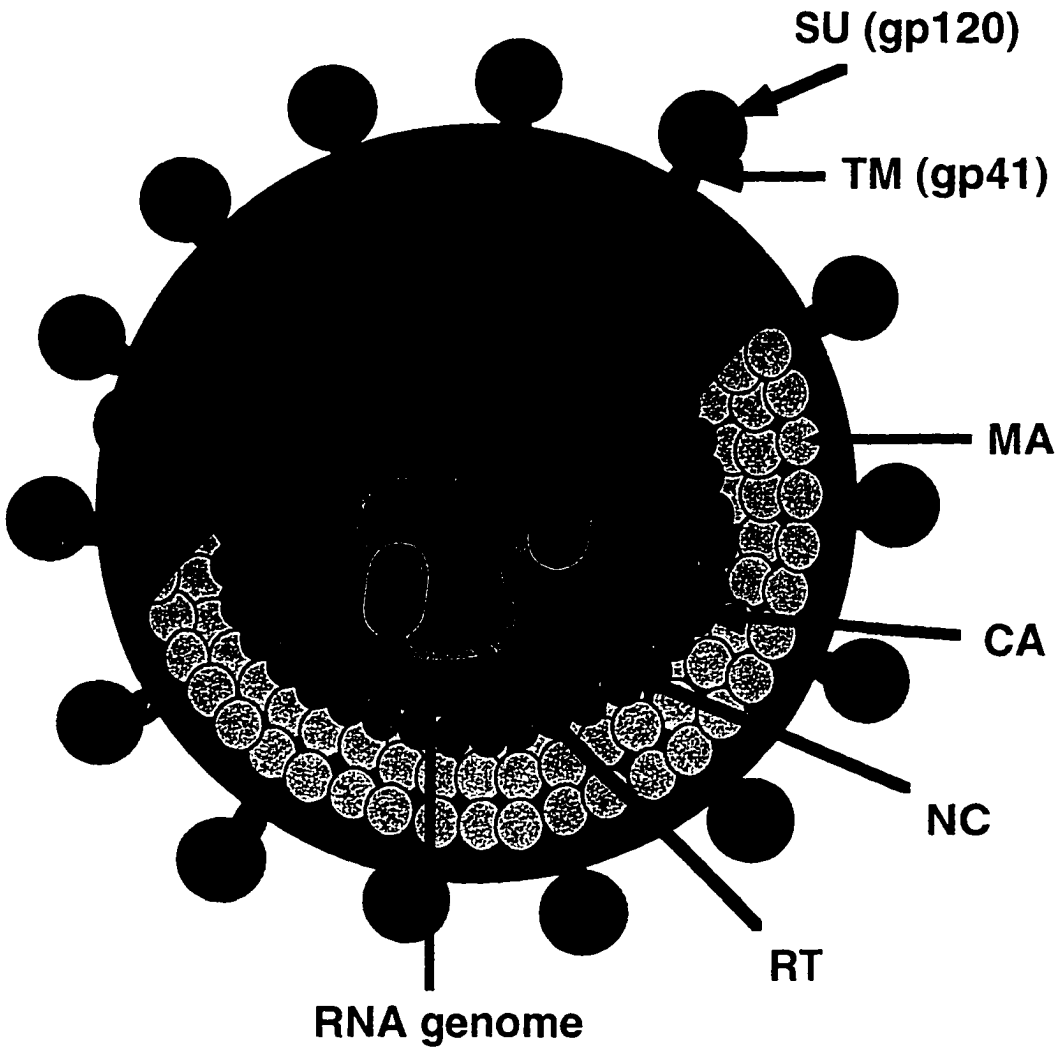
replication (32). Progress in the treatment of HIV infection, though relatively stagnant for several years, has been fueled by the recent introduction of inhibitors of the viral protease into the arsenal of antiviral drugs. Early trial results demonstrate that combined treatment with three drugs, including a protease inhibitor, initiated soon after the initial exposure to virus reduces the level of circulating virus below detectable levels and maintains virus suppression for over a year. Time will tell if such therapy is sufficient to rid the patient of virus by continuing treatment until all cells harboring infectious virus have died and then discontinuing treatment. It may be that virus will eventually be able to develop resistance to all three drugs in some patients and again replicate to high levels of virus and cause disease.

Nature of the HIV Virion

Human immunodeficiency virus type 1 (HIV-1) is a member of a family of viruses known as retroviruses (190). Retroviruses are enveloped viruses containing two copies of a single-stranded, non-segmented positive-sense RNA genome which replicate via a DNA intermediate (142). It is this nucleic acid replication strategy, specifically the synthesis of DNA from RNA by the viral reverse transcriptase, which is the hallmark of retroviruses. More specifically, HIV-1 is a member of the lentivirus genus of retroviruses. Lentiviruses are characterized by causing a late onset of disease in their host, up to several years after infection in the case of HIV (79). The other known lentiviruses which infect primates are human immunodeficiency virus type 2 (HIV-2) and simian immunodeficiency virus (SIV).

HIV is a spherical virus approximately 100 nm in diameter (see Fig. 1). Viewed by electron microscopy (EM), the mature, infectious HIV particle contains a central, cone-shaped condensed core. Surrounding the core is a spherical inner shell underlying a lipid bilayer outer shell. Embedded in this outer membrane shell, or "envelope," are numerous knobbed spikes protruding from the surface of the virion.

FIG. 1. Structure of a retrovirus. Envelope glycoprotein oligomers constitute knob-like structures protruding from the lipid bilayer envelope. The surface subunit (SU), or gp120, makes up the globular head of the structure; whereas the transmembrane subunit (TM), or gp41, forms the stalk which anchors SU to the envelope. The matrix protein, MA, underlies the envelope, and the capsid protein, CA, forms the viral core. Within the core, the nucleocapsid protein, NC, associates with the viral RNA genome along with the viral reverse transcriptase enzyme.



HIV Gene Products

As with all replication-competent retroviruses, the HIV genome consists of three principal open reading frames (see Fig. 2). They are, in order from 5' to 3', the *gag*, *pol*, and *env* genes and encode the key viral proteins necessary for replication. The *gag* gene encodes the structural proteins of the viral core and inner shell, the *pol* gene encodes the enzymes necessary for nucleic acid replication and virus assembly, and the *env* gene encodes the envelope glycoproteins, which form the knobbed spikes embedded in the viral envelope. All three genes are initially transcribed together as a single, genome-length mRNA from a promotor located within the 5' long terminal repeat (LTR) region of the viral genome. The *gag* gene product is translated from the unspliced, full-length mRNA. The *pol* gene product is translated as a Gag-Pol fusion protein resulting from a ribosomal frameshift which occurs during approximately 5% of all Gag translations. As a result, only about one Gag-Pol fusion protein is synthesized for every 20 Gag precursor proteins. The *env* gene product is translated from an mRNA which has been spliced to remove the intervening *gag* and *pol* open reading frames.

Each of the three primary translation products, Gag, Gag-Pol, and Env, is a precursor polyprotein from which the individual protein components of the virus are cleaved. The *pol* gene encodes an aspartyl protease which becomes activated by an unknown mechanism concomitant with or immediately following virus budding (104). Once activated, the protease cleaves itself out of the Gag-Pol fusion protein, thus releasing the Gag precursor protein and the other constituents of the Pol product, the reverse transcriptase (RT) and integrase (IN).

The protease also processes the Gag precursor into its constitutive proteins. The three major Gag proteins common to all retroviruses are, in order from amino- (N-) to carboxy- (C-) terminus within the precursor, matrix (MA), capsid (CA), and nucleocapsid (NC). The matrix protein is myristylated and associates with the underside of the viral envelope, forming the inner shell. Capsid protein forms the central core structure of the

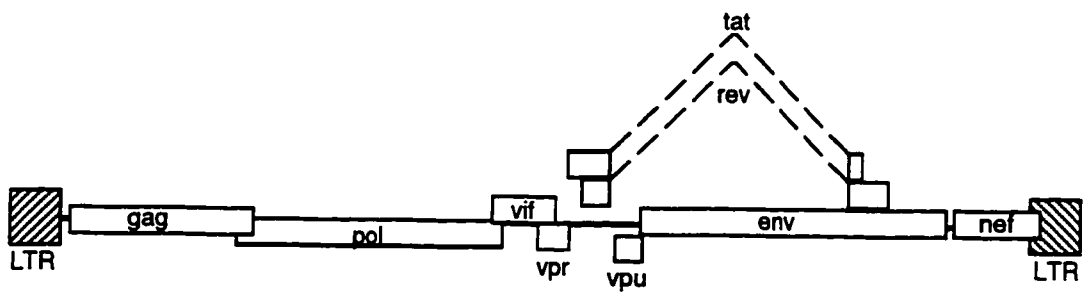


FIG. 2. The HIV-1 genome.

virion. Nucleocapsid associates with the RNA genome within the virus core. Thus, the order of these proteins within the Gag precursor reflects their placement within the spherical virion (see Figures 1 and 2). Another product of Gag processing, the proline-rich C-terminal peptide p6, appears to be important for the final membrane fusion event which releases budding virus (77).

The env gene encodes the membrane-spanning envelope glycoprotein precursor, which is synthesized as a homo-oligomer via the secretory pathway. During transport through the Golgi apparatus, each Env precursor monomer is cleaved by a cellular protease to form a noncovalently linked heterooligomer consisting of the surface glycoprotein (SU) subunit and the transmembrane glycoprotein (TM) subunit. SU, or gp120, forms the globular knob portion of the Env complex and TM, or gp41, forms the stalk which anchors the complex in the membrane. A more detailed description of the Env complex and its function follows in a later section.

HIV and the other lentiviruses are considered complex retroviruses. This is in reference to the fact that their genomes include, in addition to the primary *gag-pol-env* genome organization, multiple interspersed and often discontinuous and overlapping open reading frames (see Fig. 2). These open reading frames are translated from spliced mRNAs to produce accessory proteins, so called because, in most cases, their expression is not absolutely essential for virus replication. Two exceptions are the Rev and Tat proteins. The Tat protein interacts with the transcriptional promoter in the LTR, enhancing both transcriptional initiation and elongation. The Rev protein facilitates the export of incompletely spliced and unspliced mRNAs out of the nucleus for translation. Thus, both of these proteins are critical for the expression of high levels of viral proteins required for replication. The other accessory proteins of HIV-1 are Vpr, Vpu, Vif, and Nef. Vpr is incorporated into virions and appears to be important for the targeting of the reverse transcribed viral DNA into the nucleus of the infected cell (29, 30, 88, 214). Vpu has been shown to have two separate functions, the down-regulation of the CD4 receptor from the

plasma membrane and the enhancement of virus release from the cell at a late stage of budding (31, 110, 182, 185, 204). Vif is also associated with the virion and is important for virus infectivity. It appears to affect a post-entry step preceding or during viral DNA reverse transcription (12, 70, 93). However, the requirement for Vif is not absolute but rather is dependent upon the cell type from which virus is produced (162). Nef is found in the nucleus, the cytosol, and is associated with cellular membranes, and a subpopulation of the protein is myristylated. Nef appears to play an important role in the replication and pathogenesis of the virus in vivo, although how it functions in these roles remains elusive. Like Vpu, Nef has also been shown to down-regulate CD4 levels (24, 106).

The Life Cycle of HIV

In the first step of the HIV-1 life cycle, the virus enters the cell by fusing its lipid envelope with the plasma membrane of the target cell, depositing the viral core into the cytoplasm. This membrane fusion event is mediated by the Env protein and will be discussed in greater detail later in this dissertation.

Following partial uncoating of the viral core in the cytoplasm, the viral RNA genome undergoes an elaborate process of replication in which the viral RT enzyme uses tRNAs packaged into the assembling virion from the previous cell to transcribe a double-stranded genome, referred to as the provirus, from the single-stranded, positive-sense RNA template (191). It is this process for which retroviruses are named. Because of the relatively low fidelity of RT and lack of a proofreading function, reverse transcription also gives rise to the extensive heterogeneity seen within the population of a retrovirus (28).

The amino terminus of the MA subdomain of the Gag precursor protein, Pr55, is myristylated and contains a positively charged region, both of which are important for targeting Pr55 to the plasma membrane during virus assembly. However, following processing of Pr55 to p17 (MA) in the mature virion, this membrane-targeting signal becomes sequestered. Following virus entry, the mature MA protein dissociates from the

membrane and facilitates the targeting of the reverse-transcribed provirus into the nucleus of the infected cell (215).

The viral IN enzyme catalyzes the integration of the provirus into the host genome through nonhomologous recombination involving the ends of each of the LTRs. The integrated provirus then uses the cellular machinery to transcribe and translate its viral proteins as if they were encoded by the cell's own genes.

In the final step of the HIV life cycle, virus assembles and buds from the plasma membrane of the infected cell to seek out the next target cell (96). HIV is classified as a C-type retrovirus based on its assembly pathway. The Gag and Gag-Pol precursor proteins are synthesized in the cytoplasm of the cell and are transported by an unknown mechanism to the underside of the plasma membrane where they drive the assembly of the viral core containing the RNA genome (see Fig. 3). This is in contrast to the B/D-type retroviruses which preassemble viral cores in the cytoplasm prior to transport to the plasma membrane. The Env protein is synthesized via the secretory pathway and is transported independently to the plasma membrane. Concomitant with the assembly of the viral core, a portion of the plasma membrane enriched with the viral envelope is extruded from the cell to become the viral envelope. How the Gag precursor selectively incorporates viral glycoprotein into the envelope while excluding other cellular membrane proteins will be discussed in more detail in a later section.

THE ENVELOPE GLYCOPROTEIN OF HIV

The envelope glycoprotein of HIV projects from the surface of the virion, forming the knobbed spikes visible by electron microscopy. These glycoprotein spikes are what allow the virus to bind to the surface of the target cell and initiate infection. The Env glycoprotein precursor, gp160, is approximately 856 amino acids in length (HXB2 isolate) prior to signal peptide removal, with almost half of its molecular weight being due to extensive glycosylation. The SU subunit, gp120, is approximately 500 amino acids long and is processed from the amino-terminal amino acids of the Env precursor, while the TM

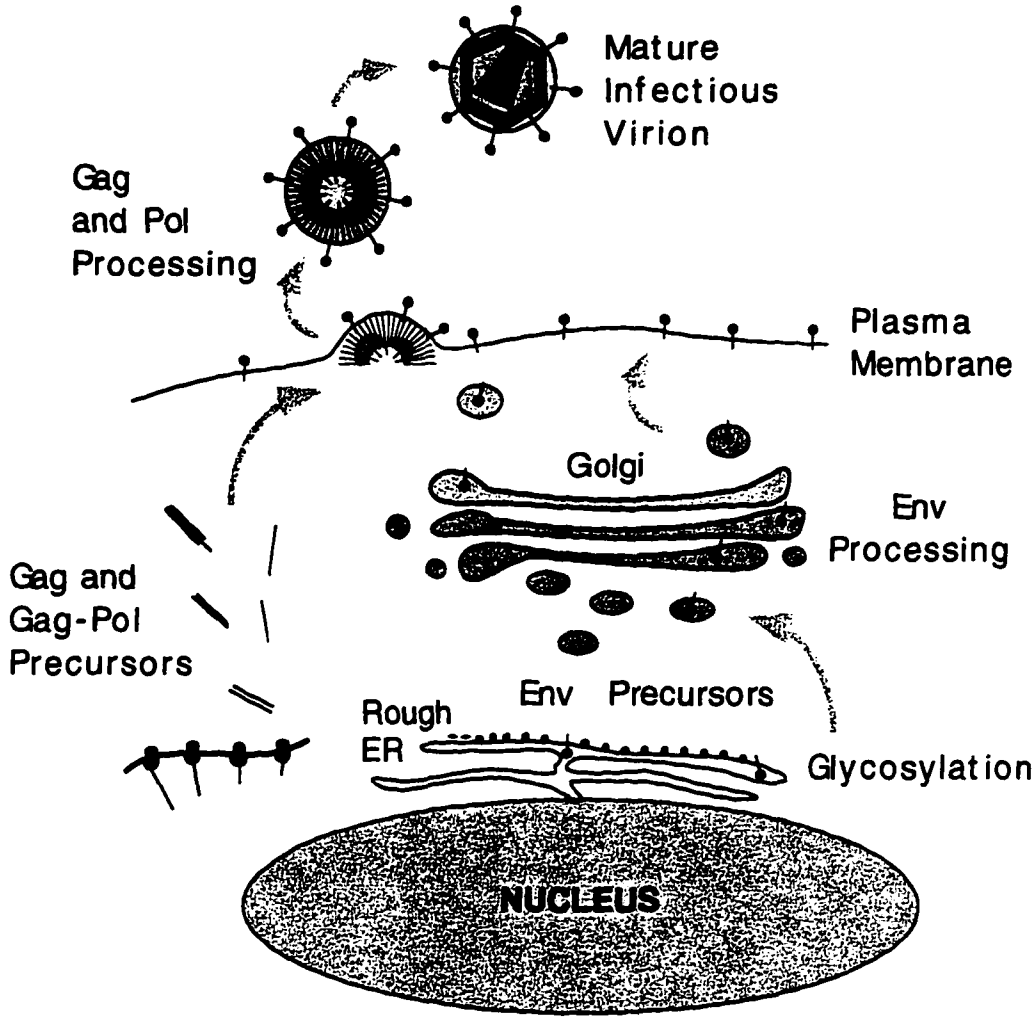


FIG. 3. Assembly of a C-type retrovirus. The membrane-spanning envelope glycoprotein is synthesized via the secretory pathway and accumulates on the cell surface. Gag and Gag-Pol precusor proteins are synthesized separately within the cytoplasm and are transported to the underside of the plasma membrane, where they drive the assembly of the viral core and the budding of the virion from a portion of the plasma membrane enriched with envelope glycoprotein. Following, or concomitant with, budding, the Gag and Gag-Pol precursor proteins become processed by the viral protease to yield the condensed core of the infectious particle.

subunit, gp41, constitutes approximately the C-terminal 350 amino acids. The association between the two noncovalently linked subunits following processing of the precursor is quite labile, since a significant amount of gp120 is shed into the culture medium of Env-expressing cells.

Biosynthesis and Maturation

Env is targeted to the secretory pathway of the cell by its amino-terminal signal peptide. The Env precursor is translated by membrane-associated ribosomes of the rough endoplasmic reticulum (ER) concomitant with its translocation into the lumen of the ER. The Env glycoprotein is a type 1 transmembrane protein, with its amino-terminus in the lumen of the ER and its carboxy-terminus exposed to the cytoplasm. Env appears to have a single translocation stop signal and, thus, is believed to span the membrane only once. The resulting cytoplasmic tail of HIV Env is unusually long for a retroviral glycoprotein, at approximately 150 amino acids, but is similar in length to the cytoplasmic tails of other lentiviral glycoproteins.

Oligomerization. Rapidly following translocation and signal peptide cleavage, the Env precursor oligomerizes (56). There have been conflicting reports as to the degree of oligomerization of Env. Most reports for HIV-1 and HIV-2 agree with a relatively loose dimeric association between two more tightly associated dimers to form a tetramer (21, 48, 53, 155, 158, 170, 187). However, other reports have supported a trimeric association of monomers (74, 195). The existence of a dimeric association is assured, since dimers have been detected in all of the biochemical analyses and appear to be the most prominent and stable species. Discrepancies in the identification of higher order species may be due to differences in experimental design or difficulties in the resolution of high molecular weight complexes. The only other retroviral Env proteins for which the quaternary structure has been determined are those of Rous Sarcoma Virus (RSV) and Murine Leukemia Virus (MuLV), both of which form trimers (58, 103). In addition, disulfide-linked HIV Env oligomers have been reported to occur in cells expressing high levels of protein, but these

structures are believed to be aberrations of the functional glycoprotein (150). Oligomerization of viral glycoproteins is believed to be a prerequisite for their proper folding and transport out of the ER.

The cytoplasmic tail and membrane-spanning domain of Env are dispensible for oligomerization, since both a soluble truncation mutant and a GPI-anchored Env ectodomain oligomerize (52, 54, 81, 196). However, it appears from these studies and others that the membrane-spanning domain, or at least membrane association, tends to stabilize the oligomeric complex (194). A region in the TM ectodomain has been implicated as a critical oligomerization domain (55). This domain and perhaps others are functionally conserved among the Env proteins of HIV-1, HIV-2, and SIV, since these proteins can form mixed oligomers with one another (47).

Glycosylation. Concomitant with translocation, the nascent Env peptide chain is extensively glycosylated by the addition of asparagine-linked oligosaccharides (reviewed in 95). gp120, alone, contains 25 asparagine-linked glycosylation sites. Glycosylation is important for the proper folding of Env into its three-dimensional structure. Use of tunicamycin to inhibit glycosylation results in a misfolded Env protein incapable of binding receptor, whereas deglycosylation of mature Env has little affect on CD4 binding (62, 119). Many individual glycosylation sites have been shown to be critical for the proper folding and function of Env as well. In addition, extensive glycosylation is believed to aid in the masking of potential epitopes on the exposed surfaces of Env. There have been reports of O-linked glycosylation of HIV Env as well (10, 82, 154), although this appears to occur only on a subset of molecules, and the biological significance of the modification, if any, is unclear.

Following oligomerization and transport of Env out of the ER, a subset of the oligosaccharide sidechains are processed as Env is transported through the Golgi apparatus (95). Mannosidases in the cis-Golgi first trim back the high-mannose sidechains. Then, beginning in the medial-Golgi and continuing into the trans-Golgi, glycosyl transferases

elongate the branches of the sidechain to form complex oligosaccharides. A biochemical analysis of the oligosaccharide sidechains of mature gp120 showed that 13 out of the 24 asparagine-linked sidechains had been processed to form complex oligosaccharides (116). The remaining 11 sidechains retained their high-mannose content from the ER, presumably due to differences in their accessibility to the modification enzymes. The biological significance of this heterogeneous glycosylation pattern is unknown.

Proteolytic processing. Also within the Golgi, the Env precursor is proteolytically processed by a cellular enzyme into its constituent subunits, SU and TM. The cleavage site contains the consensus recognition sequence R-X-K/R-R, which is highly conserved among retroviruses. The cellular enzyme(s) responsible for Env cleavage appear to be subtilisin-like proteases. Furin has been shown to be one such protease capable of processing HIV Env (80). However, it appears that other cellular proteases can also cleave Env in the absence of furin expression (40, 108, 147). Indeed, furin is unlikely to be responsible for cleavage in vivo, since it is not expressed efficiently in T cells. Other candidate proteases include members of the PC family of proteases (135). It is unclear in exactly which compartment of the Golgi the precursor is cleaved. The fact that only terminally glycosylated gp120 can be detected in most cells expressing the wild-type protein suggests that most of the cleavage occurs in the *trans*-Golgi (134, 164). However, an underglycosylated form of gp120 could be detected when an ER-retrieval signal was introduced into the protein, indicating that a subset of Env can be cleaved in the *cis*-Golgi if further transport of the protein is blocked (164). It has been reported that, during export from the Golgi, a subset of the oligosaccharide sidechains of Env become sulfated in the *trans*-Golgi network (9), although the significance of this modification is also unknown.

Envelope Assembly

In the final step of the secretory pathway, the mature Env proteins are transported in secretory vesicles to the plasma membrane, where they await incorporation into the envelopes of budding virus cores (see Fig. 3). For most nonretroviral enveloped RNA

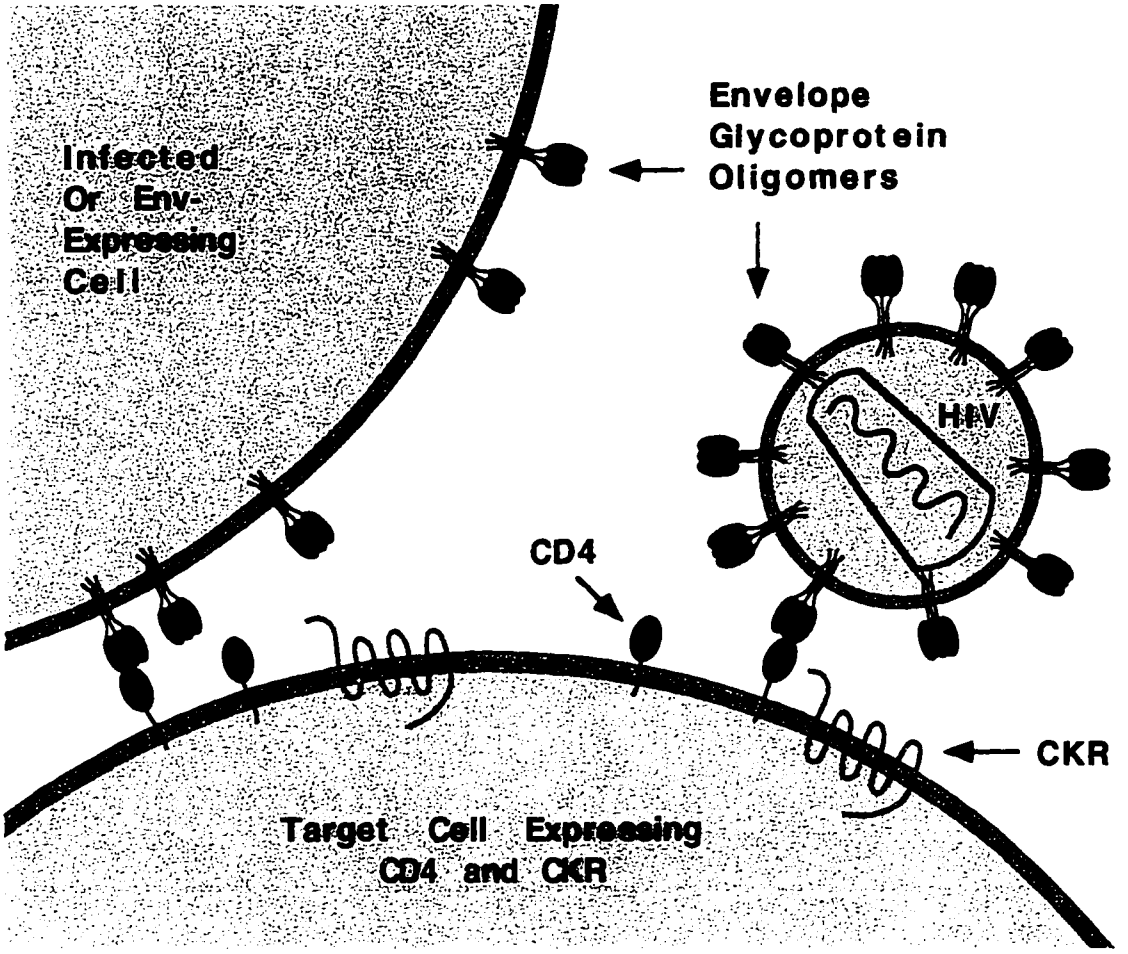
viruses, the viral glycoprotein is required for particle formation and release. The HIV envelope glycoprotein, however, is not required for the efficient budding of enveloped HIV particles from the host cell. The Gag precursor protein alone is sufficient for the extrusion of the plasma membrane as well as the membrane-fusion event that completes budding. However, virus particles produced in the absence of envelope glycoprotein are non-infectious. Therefore it was critical for the virus to have evolved a mechanism for the efficient incorporation of viral glycoproteins into its envelope. Exactly how Env is preferentially incorporated into virus while most cellular proteins are excluded is still under investigation. However, recent clues suggest that a strategy of active Env incorporation mediated by the Gag precursor is utilized by HIV. Evidence for such a mechanism will be discussed in detail in the third paper as well as in the Discussion section of this dissertation.

Functional Domains

The primary functions of the envelope glycoprotein are to mediate both attachment of the virus particle to the target cell and entry of the virus core into the cell's cytoplasm. Attachment is mediated by binding of Env to a receptor molecule, CD4, on the cell surface (see Fig. 4). The characteristics of the CD4 receptor will be discussed in detail in a later section. Entry occurs as the result of a membrane fusion reaction driven by Env between the viral envelope and the plasma membrane of the target cell. Probing and characterization of the functional domains of Env has proven to be critical in beginning to understand these processes. The primary functional domains of gp120 are reviewed below, while the functional domains of gp41 will be discussed in the context of the membrane fusion mechanism in later sections.

The V3 loop. Early comparisons of the amino acid sequences of multiple HIV isolates identified five highly variable regions, designated V1-V5, and four relatively conserved, or constant, regions, designated C1-C4 (177). These variable and constant regions are interspersed on the linear peptide sequence in the order C1-V1-V2-C2-V3-V4-C3-V5-C4. gp120 contains 18 cysteine residues, all of which form intrachain disulfide

FIG. 4. Molecular interactions leading to HIV-1 envelope glycoprotein-mediated membrane fusion and virus entry. Envelope glycoprotein oligomers on the surface of the virion or an Env-expressing cell (such as an infected cell or a cell transfected with an Env-expression plasmid) mediate membrane fusion via interactions with cell surface receptors to mediate virus entry or cell-cell fusion (syncytium formation), respectively. gp120 initially binds to CD4 which exposes a high affinity binding site for chemokine receptor (CKR) on gp120. The gp120-CD4 complex then binds CKR, inducing further conformational changes which result in insertion of the fusion peptide of gp41 into the target membrane and fusion of the opposing membranes.



bridges which have been mapped (116). These disulfide linkages, in many cases, delimit variable regions, forming what appears, at least in two dimensions, to be a "loop" of variable sequence. The most notable of these variable loops is that formed by the V3 region. This V3 loop has been implicated as the primary immunodominant region of Env, as well as a major determinant of virus tropism (97, 98, 139, 152). It is generally assumed that the variable loops are exposed on the surface of the Env oligomer and that the variability of their sequence allows the virus population to escape immune recognition. Indeed, monoclonal antibodies which recognize linear epitopes within the variable regions bind to oligomeric Env, whereas monoclonals which recognize linear epitopes within conserved regions do not (140).

The V3 loop of gp120 was identified early on as a principal neutralization domain of HIV Env (100, 128, 160). Antibodies to this linear epitope block Env-mediated membrane fusion independent of CD4 binding (173). Similarly, mutations within the V3-loop have since been shown to inhibit fusion, also independent of CD4 binding (8, 67, 98, 152). However, mutations within V3 which do affect CD4 binding or the binding of antibodies to the CD4-binding domain have also been described, suggesting that mutations in V3 can alter the conformation of this region. Presumably, mutations or the binding of antibodies in the V3 loop which do not affect CD4 binding interfere with subsequent conformational changes in Env required for membrane fusion (see section on chemokine receptors).

A major problem realized very early in trying to initially isolate and characterize HIV was that primary virus isolates do not grow well in immortalized T-cell lines. Primary virus isolates do, however, grow in peripheral blood lymphocytes (PBLs) and, often, in macrophages. Virus which was selected for growth in T-cell lines by repeated passage was later found to have lost the ability to replicate in macrophages. By constructing chimeras between the Env proteins of these so-called T-cell-tropic (T-tropic) and macrophage-tropic (M-tropic) viruses, it was eventually shown that transfer of the V3-loop was sufficient to

confer tropism for some isolates (97). Subsequent mutational analysis demonstrated that individual amino acid substitutions within the V3-loop were sufficient to alter virus tropism (184). Although it is clear that the V3 loop, in some cases, is sufficient to confer selective tropism from one virus to another, it is also clear in certain cases that other domains are involved in defining tropism. However, these determinants appear to be complex and have yet to be fully mapped, and thus the nature of their involvement in tropism is still not understood.

The CD4-binding domain. As might be expected, the conserved regions of gp120 have been shown to be important in the ability of Env to bind CD4. Residues in C2, C3, and C4 all affect receptor binding (113, 115, 148), suggesting a discontinuous binding site with these regions interacting in three dimensions. This binding site is, however, relatively refractory to mutations affecting other regions of gp120. For example, deletion of the V1, V2, and V3 regions, together, failed to abrogate receptor binding (209).

The conformational context of the CD4 binding site on gp120 has been probed further immunologically. Monoclonal antibodies which neutralize virus by blocking CD4 binding have been shown to interact with residues outside, as well as within, the CD4 binding site (186). The outlying residues have, thus, been proposed to constitute the region surrounding the binding site which are exposed on one face of gp120. In a more extensive mapping of exposed epitopes, a model of the gp120 surface has been proposed in which the epitopes for neutralizing antibodies and the CD4 binding site map to one face and the epitopes for nonneutralizing antibodies map to an opposite face, which is inaccessible in the oligomeric Env complex (140).

Binding of HIV Env to CD4 within an infected cell appears to cause down-regulation of CD4 expression at the cell surface (36, 37, 99). Down-regulation of receptor by Env is a common feature of retroviral infection and prevents superinfection of the infected cell with multiple viruses. In addition, intracellular interactions between Env and

CD4 have been proposed to contribute to the cytopathic effect of the virus by initiating subcellular membrane fusion events which may disrupt normal cell function (111).

ENVELOPE GLYCOPROTEIN-MEDIATED MEMBRANE FUSION AND VIRAL ENTRY

The process of HIV entry is initiated by the binding of the SU subunit of Env to the CD4 receptor on the surface of host lymphocyte. However, it is clear from the analysis of countless Env mutations that binding of SU to receptor, in itself, is not sufficient for fusion of the viral envelope with the plasma membrane. The binding of SU to receptor is known to induce a conformational change in SU which, in turn, alters the exposure of epitopes on TM (169). This CD4-induced conformational change has long been thought to activate the membrane fusion activity of TM, resulting in the fusion of the viral and cellular membranes and the introduction of the viral core into the cytoplasm. However, a family of fusion cofactors has just been discovered which not only is required for completion of the membrane fusion reaction, but also governs the tropism of different virus isolates. This discovery opens another chapter on the HIV entry process.

HIV Env mediates at least two independent membrane fusion events during virus replication in vitro: fusion of the virus envelope with the plasma membrane during virus entry and fusion of the plasma membrane of an infected cell with that of a neighboring infected or uninfected cell (see Fig. 4). The latter event results in the formation of multinucleated giant cells, or syncytia, throughout the infected culture. The occurrence of HIV-induced syncytia in vivo has not been well documented, so its role in pathogenesis is unclear. Nevertheless, syncytium formation in infected cultures provides a useful assay for the membrane fusion activity of HIV Env. Moreover, since Env expression is sufficient for syncytium formation, Env function can be studied independently of virus replication. Although the mechanism of membrane fusion is generally thought to be similar, if not identical, during virus entry and syncytium formation, the relative sensitivities of the two assays in detecting fusion can differ. This may be due to differences in the concentration of Env protein on the virion versus the cell surface, differences in the lipid content of virus as

compared to the plasma membrane, differences in the underlying architecture of the plasma membrane versus the viral envelope, differences in the contact distances between opposing membranes, or perhaps most importantly, differences in the number of Env-receptor interactions required for completion of each fusion reaction.

Finally, HIV Env function in the virus entry event, as in syncytium formation, does not appear to be dependent on the underlying HIV Gag proteins or accessory proteins, since vesicular stomatitis virus (VSV) virions pseudotyped with HIV Env are also infectious.

The CD4 receptor

The CD4 molecule found on the surface of helper T cells, monocytes, macrophages, and a few other cells (197) was first identified immunologically as a receptor for HIV in 1984 (39, 109), the same year in which HIV was identified as the causative agent in AIDS. Genetic evidence followed in 1986, confirming the finding (122). CD4 had originally been identified as an antigenic marker for the helper T cell population of lymphocytes and is involved in T-cell activation. The molecule was an obvious candidate for a potential receptor, since depletion of helper T cells is a hallmark of HIV-induced immunodeficiency. CD4 is an approximately 60-kDa type 1 transmembrane glycoprotein and is a member of the immunoglobulin superfamily. It consisits of four immunoglobulin-like domains, D1-D4, with the outermost domain, D1, containing the gp120 binding domain (132, 166). The three-dimensional structure of the D1-D2 fragment of CD4 has also been determined (161, 193). gp120 has a high affinity for CD4, with a dissociation constant on the order of 10-9 (115), and the two molecules can be coimmunoprecipitated following binding (131). CD4 is the receptor for both HIV types 1 and 2 as well as SIV (165).

The membrane-spanning and cytoplasmic domains of CD4 are not required to mediate HIV entry, since GPI-anchored CD4 acts as a functional receptor (112, 124). In contrast, the first paper of this dissertation shows that GPI-anchored Env cannot mediate

virus entry. This suggests that CD4 is probably not directly involved in disruption of the plasma membrane during virus entry and probably does not contribute directly to the formation of a "fusion pore" (discussed in a later section). In addition, most of the extracellular domain of CD4 can be deleted without abrogating virus entry (7). This further suggests that CD4 is probably not involved in the membrane fusion event beyond inducing a conformational change in Env.

Alternate Receptors

Several cases have been reported of HIV infection of cells which do not express CD4 (25, 197), suggesting the ability of HIV to use alternate receptor(s). In these cases, however, infection is very inefficient. Yet infection of these cells cannot be explained by low-level CD4 expression, since antibodies which block gp120-CD4 interaction fail to neutralize virus. One such receptor appears to be the glycolipid galactosyl ceramide (Gal Cer). gp120 binds Gal Cer tightly, and antibodies to Gal Cer neutralize CD4-independent infection (83). In addition, the efficiency of CD4 independent infection correlates with the level of Gal Cer expression on the surface of cells (59, 210).

The ability of HIV to use Gal Cer as a substitute receptor in the absence of CD4 does not, however, explain the ability of some HIV-2 isolates to infect CD4-negative cells. The ROD/B isolate of HIV-2, for example, infects CD4-negative B-cells very efficiently, while other isolates are unable to infect these cells unless incubated in the presence of soluble CD4 (sCD4) (26). One possible explanation for this result is that these cells express a molecule which can function as a receptor for most HIV-2 isolates, provided that their Env proteins are pretriggered to form a fusogenic conformation by binding to sCD4. In this scenario, the Env protein of the ROD/B isolate may have adapted to be either synthesized already in this conformation or triggered to adopt this conformation by binding to the cell-surface molecule. This unique case will be discussed again in a later section.

Route of Virus Entry

Influenza virus, another enveloped RNA virus, is endocytosed following attachment to sialic acid residues on components of the plasma membrane of the target cell. Following acidification of the endosome, the viral envelope fuses with the endosomal membrane, resulting in entry of the viral nucleocapsid into the cytoplasm (198). Influenza virus entry is thus pH dependent. An early report suggested that HIV also entered cells via endocytosis following binding to CD4 (122). While CD4 can be efficiently endocytosed, mutations within the cytoplasmic tail of CD4 which inhibit endocytosis were later shown to have no effect on HIV infectivity (6, 123). This agrees with the finding, mentioned previously, that GPI-anchored CD4 also mediated virus infection. It has also been shown that HIV infection is pH independent (130, 180) and thus does not require acidification within the endosome. Thus, HIV differs from influenza virus in that it enters cells directly through the plasma membrane.

CD4-Induced gp120 Dissociation and Conformational Change

A soluble form of the CD4 receptor molecule (sCD4) has been shown to induce the dissociation of gp120 from gp41 in a temperature-dependent manner (68, 136, 138). This dissociation occurs more efficiently with HIV isolates which have been adapted to replicate in T-cell line cultures than with primary virus isolates (137), suggesting differences in the stability of the Env oligomer. Consequently, sCD4 treatment efficiently neutralizes lab-adapted strains of virus but not primary isolates (38). Moreover, infection by certain primary isolates of HIV has actually been shown to be enhanced by low levels of sCD4 (183). As will be discussed in a later section, this may be due to an enhancement of the interaction between Env and a secondary receptor molecule by sCD4. Interestingly, the increased dissociation of gp120 from lab-adapted virus by CD4 correlates with a higher affinity of Env for CD4 (205). Perhaps the tighter association of Env with its receptor at the expense of increased gp120 dissociation is favorable for replication in vitro but is detrimental to the virus in the context of the host immune system.

Binding of sCD4 to Env at 4°C, which prevents gp120 dissociation, results in the exposure of previously masked epitopes on gp41 (169). sCD4 binding also induces the increased exposure of certain epitopes on gp120 as well as a thrombin cleavage site within the V3-loop (167, 168). This suggests that CD4 binding is accompanied by a conformational change in gp120 which results in the exposure of regions of gp41 that were previously masked by gp120. It is possible that the exposure of gp41 epitopes is due to a conformational change within the gp41 molecule, itself, although this cannot be discerned from the above experiments. A model for the role of these conformational changes in the interaction of Env with a secondary receptor will be discussed later in this Introduction.

The Influenza Virus HA Model System

Viral membrane fusion proteins provide a convenient system for studying protein-mediated membrane fusion. The best understood protein-mediated membrane fusion reaction is that driven by the hemagglutinin (HA) glycoprotein of influenza virus. While there appear to be structural similarities between HA of influenza and retroviral glycoproteins, the initial steps of viral entry differ. Nonetheless, the influenza system provides valuable insight into the process of virus entry and will be compared to what is known and what is being learned about retrovirus entry in this dissertation.

Although the three-dimensional structure of the influenza virus HA protein ectodomain was determined over 15 years ago (207), no retroviral glycoproteins have yet been crystallized. In the absence of three-dimensional structure information for HIV Env, the influenza HA structure has been useful in composing a conjectural model for probable Env structure (72). Like HIV Env, HA is proteolytically processed from a precursor protein to form a hetero-oligomer. The mature HA protein consists of two subunits, HA1, analogous in primary structure and function to SU, and HA2, analogous to TM. Unlike HIV Env, however, the two subunits of HA are disulfide-linked. As mentioned previously, while it is unclear whether HIV Env is trimeric or tetrameric, the HA heterodimers are known to associate as a trimer. HA and Env also appear to contain

structurally similar functional domains with fusion peptides at the N-terminus of their transmembrane subunits.

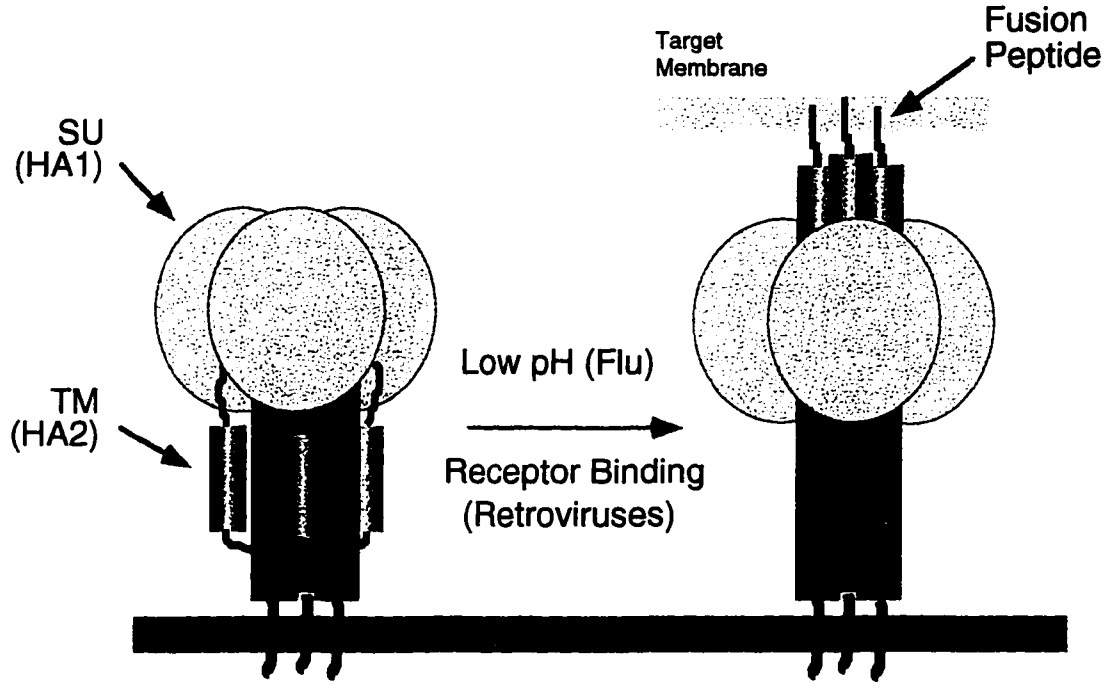
Coiled-Coil Formation

The requirement for acidification of the endosome in influenza virus is known to be due to the dependence of the membrane fusion event on a conformational change in the viral HA envelope glycoprotein which is induced by low pH (46, 198). Similarly, it has been shown that, upon binding to CD4, HIV Env undergoes a conformational change which correlates with the ability of Env mutants to mediate membrane fusion (167, 168). Thus the pH activation of HA appears to be analogous to the receptor activation of Env.

In order to develop a model for how the conformational change in Env induces membrane fusion, correlations have been derived from what is known about the analogous event in HA-mediated fusion. The most valuable information in this regard has been the solution of the three-dimensional structure of a modified HA2 fragment at the pH of activation (16). While this modified fragment has been removed from the context of the remaining HA molecule, its divergence from the structure of HA2 at neutral pH suggests that it may represent the pH-acitvated form of the molecule.

What has been proposed for the HA confomational change based on the two crystal structures is that upon pH activation, the HA2 subunit transitions from a hairpin-like structure, with its amino-terminal fusion peptide folded back near the viral membrane, to an extended α-helix, with the fusion peptide at the distal end (20). In this conformation, the extended helices of the three trimers associate to form an extended triple-helix coiled-coil. Thus, the conformational change catapults the three fusion peptides of the HA trimer out and into the target cell's plasma membrane by a sort of spring-loaded mechanism (see Fig. 5). In addition, the membrane-proximal portion of HA2 flips up to associate with the extended helix, forming an inverted hairpin. This, in turn, may bring the viral membrane sufficiently close to interact with the target cell's plasma membrane, initiating membrane fusion.

FIG. 5. Conformational changes in viral fusion proteins. The oligomeric envelope glycoprotein complex undergoes conformational changes induced by receptor binding (both CD4 and chemokine receptor) which result in the formation of a helical coiled-coil structure in the gp41 ectodomain. This coiled-coil formation causes the fusion peptide to be catapulted out and into the target membrane. This conformational change is based on analogies to the acid-induced conformational change and membrane fusion mechanism of the hemagglutinin of influenza virus.



What makes this mechansim of HA so attractive in proposing a model for HIV Env fusion is that the TM subunit of HIV Env also contains a region which is capable of forming an extended coiled-coil and which is important for fusion. This region contains a leucine-zipper-like heptad repeat motif which is critical for the fusion activity of the protein (22, 23, 51). A synthetic peptide corresponding to this region is capable of forming a stable coiled-coil oligomer (201), and the same mutations in the heptad-repeat motif which abrogate fusion also prevent this structure from forming in the context of the peptide (199). Moreover, fusion of this peptide with nonoligomeric proteins is sufficient to mediate their oligomerization, and mutations affecting fusion also reduce the efficiency of oligomerization (11, 171). Although this heptad repeat region has been proposed to be important in the oligomerization of Env during biosynthesis, only relatively severe mutations have been reported to affect Env assembly, whereas even relatively conservative single amino acid substitutions affect the fusogenicity of the protein (51). Also in support of the role of this motif in a receptor-triggered conformational change as opposed to assembly oligomerization is the ability of the above-mentioned synthetic peptide to potently inhibit Env-mediated fusion (201). Mutations in the heptad-repeat motif which block fusion in the context of the native protein and which inhibit the corresponding synthetic peptide from forming a coiled-coil also reduce dramatically the potency of the peptide in neutralizing fusion.

The Fusion Peptide

An amino terminal peptide of gp41 was originally identified as the so-called "fusion peptide" by its similarity to the fusion peptides of the paramyxoviruses (71). The HIV Env fusion peptide is approximately 22 amino acids long and is predicted to form an amphipathic α-helix. Generally, substitution of hydrophilic residues for hydrophobic residues within the fusion peptide is deleterious to the fusogenicity of the protein, while substituting more hydrophobic residues at these same positions tends to enhance fusogenicity (14, 66, 178). The current dogma, based on analogy to the influenza virus

system, is that receptor binding induces conformational changes in Env which allow the fusion peptide, previously buried within the Env complex, to penetrate the lipid bilayer of the target cell (see Fig. 5). But while CD4-binding does alter the conformation of gp120 and unmask epitopes within gp41, exposure of the fusion peptide has yet to be demonstrated for HIV.

Although it is generally assumed that membrane fusion occurs following insertion of the fusion peptide into the lipid bilayer, the only evidence for this with HIV comes from the interaction of synthetic fusion peptides with membranes, an assay far removed from the native event. Two such studies suggest that the α-helical fusion peptide inserts into the lipid bilayer of the target cell at an oblique angle (125, 126). Interestingly, though, mutations which abrogate fusion also inhibited the ability of the peptide to demonstrate this property (126). An alternative model has been proposed for HIV in which the fusion peptide interacts with a membrane-associated protein to trigger membrane fusion. In one such model, the HIV coreceptors, discussed in the following section, have been proposed to act as fusion peptide receptors. The most convincing evidence for fusion peptide insertion into the lipid bilayer during fusion, independent of proteins in the target membrane, comes from the influenza virus system. Following activation of soluble flu HA by pH in the presence of liposomes, the fusion peptide can be labeled with the lipid-soluble photoactivatable label 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl) diazirine, or [^{125}I]TID. Such fusion peptide labeling has never been demonstrated with the HIV fusion peptide in any system. However, studies with soluble Env from RSV have shown that, in the presence of a soluble form of its receptor, the transmembrane subunit binds to liposomes in a flotation sucrose gradient (90), suggesting that fusion peptide membrane insertion can occur with a retrovirus and that a fusion peptide receptor protein is not required for this interaction.

As with other retroviruses as well as influenza HA, cleavage of the HIV Env precursor is required for fusion, presumably because it frees the fusion peptide for

insertion into the target membrane following receptor binding. Interestingly, however, the fusion peptide of some viral fusion proteins, such as that of RSV, is located "internally" at a site several amino acid residues from the amino-terminus. Nevertheless, processing of the precursor protein is still required for the function of these proteins. It may be that proteolytic processing, following the normal folding of the glycoprotein oligomer, acts to preset a spring-loaded mechanism which is activated upon binding receptor to propel the fusion peptide out and into the target membrane (20).

Chemokine Receptors and HIV Entry

For over a decade, it has been known that cellular factor(s) other than CD4 are required for the Env-mediated membrane fusion event. Although CD4 can function as a receptor in most human cell lines, certain human cell lines and most nonhuman cell lines are resistant to HIV infection when transfected with CD4 cDNA. This finding led to a search for a secondary HIV receptor molecule, or fusion cofactor, present in permissive human cells. After years of searching by several laboratories, these elusive factors were finally identified last year as members of a family of seven-transmembrane G protein-coupled chemokine receptors (CKRs) (see Fig. 4) (61).

This discovery has triggered a dramatic refocusing of HIV research which has already led to the unraveling of additional long-standing, fundamental mysteries of HIV biology and pathogenesis. The molecular basis for virus tropism, the resistance of certain individuals to virus infection, and the slower progression to disease in some patients have all been explained in relation to this finding. The tropism of a virus isolate corresponds to its utilization of specific members of the CKR family, and changes in the V3 loop can define CKR usage. CKRs thus far identified as fusion cofactors for various HIV isolates include CXCR4, CCR5, CCR3, and CCR2b. In general, M-tropic isolates utilize CCR5 to gain entry into the cell, while T-tropic isolates utilize CXCR4. Dual-tropic virus isolates are able to utilize both of these receptors. As might be expected, macrophages have been shown to express CCR5 but not CXCR4, whereas T cells express CXCR4 but not CCR5.

A mutation in both alleles of the gene encoding CCR5 was identified in individuals who have resisted viral infection despite being at high risk for virus exposure. This homozygous mutation was further shown to reside in approximately 1% of caucasians of European descent in the general population. Moreover, individuals that are heterozygous for this mutation appear to exhibit a reduced frequency of infection and a slower than average progression to disease. The results of these studies provide valuable insight into the mechanisms of virus transmission, virus evolution, and viral pathogenesis and lay the foundation for the design of new antiviral compounds as well as the development of a small animal model for HIV. This work has profound implications toward the treatment and perhaps prevention of HIV infection.

The results of early studies, before the identification of the fusion cofactors, suggest that HIV Env interacts with both CD4 and CKR in a complex stable enough to undergo phorbol ester-induced endocytosis (76). More recently, CKR and Env have been shown to coimmunoprecipitate, although the Env-CKR complex appeared to be somewhat labile (114). Other recent reports have suggested that gp120 interacts with both CD4 and CCR5 to form a high-affinity complex (188, 208). V3-loop-neutralizing antibodies or deletion of the V3 loop prevented gp120-sCD4 binding to CCR5. Yet variability of the V3 loop sequence among different isolates which all utilize CCR5 is not consistent with the V3 loop, itself, being the primary CCR5 binding site. Other neutralizing antibodies that bind discontinuous gp120 epitopes on the exposed surface of the Env oligomer which, like the V3 loop, become more exposed following CD4-binding also block CCR5 binding. Interestingly, low affinity binding of gp120 to CCR5 in the absence of CD4 was also inferred in these reports. It is not known if such a CD4-independent interaction would result in fusion peptide exposure for HIV-1. One isolate of HIV-2, however, has been shown to utilize CKR directly to mediate fusion in the absence of CD4. The Env protein of this isolate may have adapted to assemble into a pretriggered conformation that mimics the conformational change typically induced by binding to CD4. The realization of the

fundamental requirement of CKRs in fusion, the direct interaction of Env with CKRs, and the ability for some virus isolates to circumvent the requirement for CD4 has led to the general reference to CKRs as true coreceptors for HIV, rather than merely as fusion cofactors or accessory molecules.

While these preliminary reports have already demonstrated that CKRs can interact with both CD4 and gp120, almost nothing is known about how these interactions contribute to membrane fusion. CD4-binding, alone, does not appear to be sufficient to expose the fusion peptide. Therefore, it may be that binding of Env to CKR, following an initial CD4-induced conformational change in gp120 (which may expose a high affinity CKR binding site), is required to induce a secondary conformational change in gp41, resulting in the insertion of the fusion peptide into the lipid bilayer of the target membrane. The above studies are all consisitent with such a model. Under these circumstances, binding of Env to liposomes or labeling of the fusion peptide with [^{125}I]TID could not have been demonstrated previously for HIV because the critical coreceptors had not yet been identified.

The identification of CKRs as HIV coreceptors provides new insight into the mechanism of virus entry. A host of mutations have been generated and characterized by numerous laboratories which affect an unknown post-CD4-binding step in fusion. Many such mutations in gp120 might be expected to be defective in CD4-induced conformational changes or CKR binding. However, many mutations, particularly those within gp41, may inhibit a subsequent post-CKR-binding step of the fusion event. Similarly, some mutations in CKRs might permit Env binding but inhibit fusion. Such studies in the future may begin to test the current model for CKR function during fusion.

Understanding the mechanism by which HIV Env functions in concert with CD4 and CKRs to fuse membranes may allow novel strategies to be developed to inhibit virus entry. The functional redundancy for CCR5 seen in ΔCCR5 individuals suggests that therapies designed to inhibit CCR5 function should be tolerable in patients. Based on what

has been learned so far about tropism, transmission, and disease progression, strategies designed to block HIV utilization of CCR5 would be expected to inhibit virus transmission, whereas blocking CXCR4 utilization, if similarly tolerable, might be expected to inhibit disease progression in those already infected.

Membrane Fusion

Membrane fusion events in biological systems would obviously be catastrophic if they were not tightly regulated. In the case of influenza virus entry, HA induces fusion only after the virus reaches the late endosome. In the case of HIV, Env must interact with both CD4 and a specific CKR before initiating fusion. The role of viral fusion proteins is essentially to overcome the forces which separate two membranes. As discussed above, the fusion peptide appears to associate with both membranes, forming a bridge between the two. Multiple oligomers appear to be required to initiate fusion, perhaps by associating together to form a fusion pore, overcoming the primary barriers to membrane fusion by dehydrating a localized region between the two membranes (198). Insertion of the fusion peptide into the target membrane may further facilitate lipid mixing by disrupting the packing of phospholipids in such a way as to perturb the integrity of the membrane.

Kinetics. The HIV Env-mediated membrane fusion event is temperature dependent. Virus can bind CD4 receptor efficiently at 4°C, but warming to 20°C is required to initiate membrane fusion (68, 172). Unlike influenza virus, which fuses efficiently at 20°C, HIV fuses with cells much more efficiently at 37°C than at 20°C. Upon warming to 37°C, virus entry proceeds rapidly, on the order of minutes (78), although the rate of entry appears to be dependent on cell type (176). Env-mediated cell-cell fusion, however, proceeds more slowly than virus-cell fusion, on the order of hours following cell-cell adhesion (43, 45). The formation of large syncytia involves multiple, independent interactions between fused cells and adjacent cells and, thus, requires several hours (43).

Composition of the lipid bilayer. While the presence of cholesterol in the target membrane has been reported to be a requirement for fusion by Semliki Forest virus

and Sendai virus, influenza virus fusion is relatively independent of target membrane composition. Very limited studies on the lipid requirements of HIV infection have suggested that higher levels of cholesterol in cell membranes facilitate HIV replication (2). However, how cholesterol plays a role in replication is unknown. In addition, it is clear that HIV, like other enveloped viruses, contains a higher cholesterol to phospholipid ratio in its viral membrane than is found in most cell membranes (2). But the role of cholesterol in the viral membrane on infectivity has also been difficult to study because the virus is too fragile to permit reconstitution of its envelope (34).

Accessory factors. The prerequisite step in virus entry is the attachment or adsorption of the virus onto the target cell surface. Cationic molecules such as the poly-amines polybrene and DEAE-dextran have long been known to facilitate this interaction for enveloped viruses, presumably by countering the net negative charge repulsion between the viral and cellular membranes. The net charge is due in large part to the phospholipid head groups and sialylated proteins of each membrane. Reduction of charge repulsion by desialylation of the virion surface has similarly been shown to facilitate infection of HIV (94). The HIV envelope glycoprotein has also been reported to interact with proteoglycans, which may facilitate virus infection by a similar mechanism (129). In addition, the adhesion molecule LFA-1 (CD18) appears to be involved in the prerequisite cell-cell binding which precedes the membrane fusion event in syncytium formation (91, 189).

In addition to attachment, charge neutralization is also an important step in overcoming the barriers to membrane fusion. Calcium has long been known to play an important role in the fusion of membranes, apparently through interactions of the cation with the negatively charged phospholipid headgroups of apposed lipid bilayers, which may act to stabilize intermediate stages of membrane fusion (reviewed in 206). Indeed, calcium ions, alone, are sufficient to cause fusion of synthetic membranes in vitro. Calcium has been shown to be required for exocytosis, but not for other intracellular fusion events.

Calcium has been shown to be required for HIV Env-mediated membrane fusion (44). Interestingly, though, calcium is not required for fusion mediated by influenza HA.

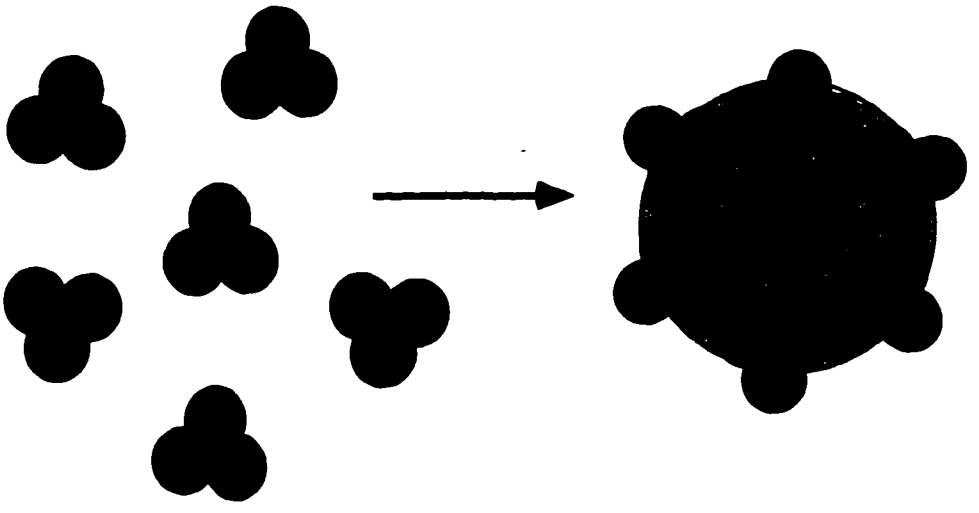
Tryptase TL2, a membrane-associated serine esterase, has also been reported to specifically bind and cleave the V3 loop (27, 87, 107). V3 loop exposure does increase following CD4-binding, although whether the cleavage reaction is relevent in vivo is not clear. One study has reported that the fusion reaction can be inhibited by protease inhibitors (84). Interestingly, proteolytic activation of the Env protein of ecotropic MuLV has also been proposed.

Fusion pore formation. Viral protein-mediated fusion is believed to initiate at relatively miniscule junctions defined by oligomers of the viral protein between the opposing membranes. These junctions, referred to as fusion pores, are hypothesized to consist of multiple fusion-competent oligomers that associate together to form higher-order multimers which bridge the two membranes in a proteinaceous pore-like structure (179, 198) (see Fig. 6). Although there is not yet direct evidence that such a structure indeed forms, biophysical studies of the earliest steps in fusion are consisitent with such a model. Once again, all of the information available on fusion pore formation comes from studies with myxovirus fusion proteins, and, in this case, correlations with HIV Env function are purely conjecture.

It is possible that the individual Env oligomers undergo conformational changes or even monomer reassortment to favor higher-order multimerization leading to fusion pore formation. As noted previously, most studies favor the formation of dimers and tetramers during Env assembly. Yet peptide studies which suggest the formation of coiled-coil structures by Env favor a trimeric association of helices during fusion. Interestingly, the envelope glycoprotein of tick-borne encephalitis virus undergoes just such a reassortment from dimers to trimers following pH activation (1, 181).

The following sections represent a series of papers published or submitted for publication in peer-reviewed journals as a result of original research on the structure and

FIG. 6. Fusion pore formation. This figure represents the hypothetical view of the glycoprotein-containing membrane, or donor membrane, from the plasma membrane of the target cell. Based on analogy to the influenza virus system, multiple Env oligomers aggregate together to form super oligomers at some point prior to membrane fusion. Upon transition of the glycoproteins to their fusogenic conformation and insertion of the fusion peptide into the membrane, these super oligomers form pore-like structures, or fusion pores, between the opposing membranes, which facilitate the mixing of both lipids and soluble contents between the two membrane compartments. Dilation of the fusion pores results in the merging of the two membranes and completion of the fusion reaction.



Envelope Glycoprotein Oligomers

Formation of Fusion Pore

function of the HIV envelope glycoprotein. The first two papers examine the role of the membrane-spanning domain in the maturation and function of HIV Env. The third paper describes the identification of a novel peptide sequence motif which is involved in Env-mediated membrane fusion and virus entry. Finally, the fourth paper describes a unique approach to the study of HIV envelope assembly.

EXPRESSION AND CHARACTERIZATION OF GLYCOPHOSPHOLIPID-ANCHORED HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ENVELOPE GLYCOPROTEINS

by

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ABSTRACT

Four chimeric human immunodeficiency virus type 1 (HIV-1) *env* genes were constructed which encoded the extracellular domain of either the wild-type or a cleavage-defective HIV-1 envelope glycoprotein (gp160) fused at one of two different positions in *env* to a C-terminal glycosyl-phosphatidylinositol (GPI) attachment signal from the mouse Thy-1.1 glycoprotein. All four of the constructs encoded glycoproteins that were efficiently expressed when Rev was supplied in *trans*, and the two cleavable forms were processed normally to gp120 and a chimeric "gp41." The chimeric glycoproteins, in contrast to the wild-type glycoprotein, could be cleaved from the surface of transfected cells by treatment with phosphatidylinositol-specific phospholipase C, indicating that they were anchored in the plasma membrane by a GPI moiety. These GPI-anchored glycoproteins were transported intracellularly at a rate only slightly lower than that of the full-length HIV-1 glycoprotein and were present on the cell surface in equivalent amounts. Nevertheless, all four glycoproteins were defective in mediating both cell-cell and virus-cell fusion as determined by syncytium formation in COS-1-HeLa-T4 cell mixtures and *trans* complementation of an *env*-defective HIV-1 genome.

INTRODUCTION

The *env* gene product of human immunodeficiency virus type 1 (HIV-1) is synthesized as a glycosylated polypeptide precursor, gp160, which undergoes cleavage during transport to the plasma membrane to yield two noncovalently linked subunits, gp41, the transmembrane glycoprotein (TM), and gp120, the surface glycoprotein (SU). The envelope glycoprotein of HIV-1 is essential for attachment of the virus particle to the target cell, fusion of the viral envelope with the plasma membrane, and formation of syncytia in infected cultures. Each of these functions involves an interaction between the glycoprotein and the CD4 receptor (see references 14 and 23 for reviews).

The TM glycoprotein contains an unusually long putative cytoplasmic domain which appears to be required for productive infection in most cell lines but not for

syncytium formation (6, 10, 32). Mutations have defined the region from residue 684 to 705 as being necessary to anchor the protein in the membrane (1, 6, 15). This putative membrane-spanning domain is unusual in that it contains a highly conserved, charged basic residue in the middle of the long uncharged hydrophobic domain (26). Site-directed mutagenesis of this charged residue (arginine 696) and the uncharged residues from position 684 to 705 has demonstrated that the membrane-spanning domain is apparently not involved in the fusion mechanism beyond serving as a membrane anchor (9, 12). However, mutagenesis of a flanking charged residue (lysine 683) or substitution of four foreign residues for the arginine at position 696 within the putative membrane-spanning domain has been shown to dramatically affect syncytium formation and to block virus entry (12). Therefore, the role of the membrane-spanning anchor domain of the HIV-1 glycoprotein in the fusion event remains an intriguing question.

Many cellular glycoproteins are anchored in the plasma membrane through a glycosyl-phosphatidylinositol (GPI) moiety, rather than a membrane-spanning protein anchor. Thy-1.1, a glycoprotein of murine thymocytes and neurons, is a well-studied example of such a protein. The C-terminal 53 amino acids of the Thy-1.1 precursor protein have been shown to be sufficient to mediate the attachment of a GPI anchor to a chimeric protein (3), a process that involves the specific removal of a 31-amino-acid C-terminal peptide and the formation of an amide linkage between a C-terminal cysteine and an ethanolamine residue (22, 31). The GPI linkage can be cleaved at the cell surface by using phosphoinositol-specific phospholipase C (PI-PLC), which releases the protein in a soluble form into the medium (see references 8 and 21 for reviews).

In order to further define the polypeptide domains and membrane anchorage requirements involved in the oligomerization, transport, processing, and fusion properties of the HIV-1 glycoprotein, as well as the incorporation of glycoprotein into virions, we have expressed four chimeric HIV-1-Thy-1.1 glycoproteins which are GPI anchored. Such molecules might also facilitate the isolation of a soluble, oligomeric form of the HIV-

1 glycoprotein suitable for structural analyses. In order to define the sequences proximal to the membrane-spanning domain that are important in the above processes, we replaced by gene fusion either 191 (HT-1) or 173 (HT-2) C-terminal amino acids of the wild-type HIV-1 glycoprotein with the 53-amino-acid Thy-1.1 GPI attachment signal. In addition, we made analogous constructs (HTC-1 and HTC-2, respectively) by using an *env* gene that encoded three amino acid substitutions (K-502→E [K502E], R504S, and K510E) near the C terminus of gp120 which prevent the processing of gp160 to gp120 and gp41 (7). The products of each of these constructs would be expected to retain 22 amino acid residues from the Thy-1.1 signal following GPI attachment. The added sequence includes an additional site for N glycosylation.

These chimeric glycoproteins were expressed in COS-1 cells at levels comparable to wild-type levels when a complete *rev* gene was supplied in *trans* by cotransfection and could be efficiently cleaved from the cell surface by treatment with PI-PLC. The rate of intracellular transport of these GPI-anchored HIV-1 glycoproteins was only slightly less than that of the full-length glycoprotein. Although the GPI-anchored glycoproteins could be detected on the cell surface in amounts comparable to wild-type glycoprotein, all were completely defective in mediating fusion as determined by assays of both syncytium formation and virus entry.

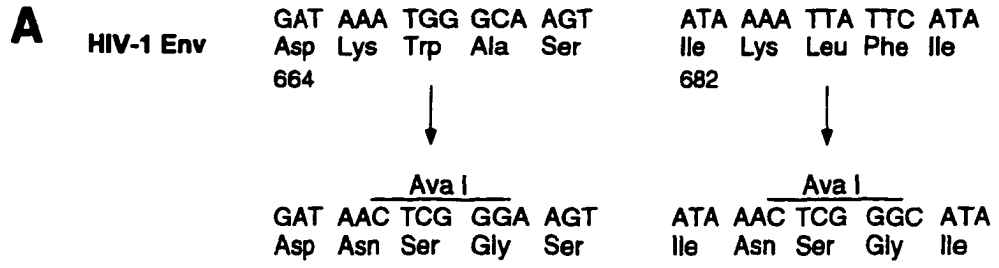
MATERIALS AND METHODS

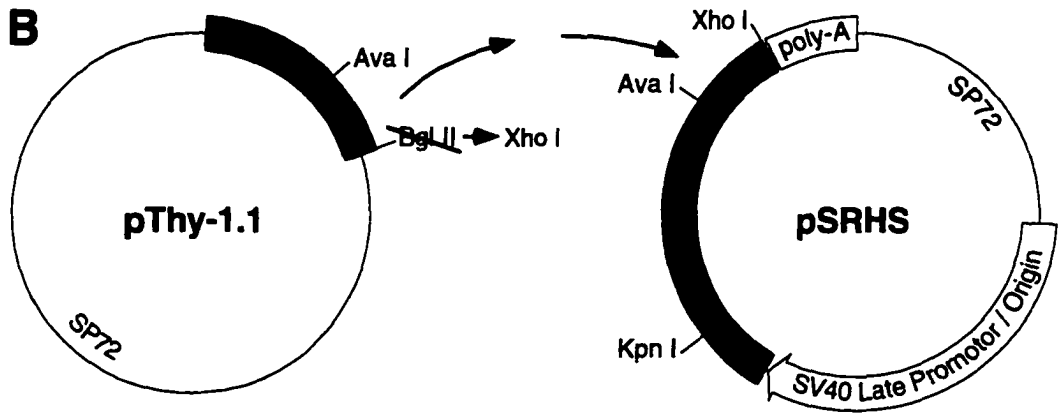
Cell culture. COS-1 cells were obtained from the American Type Culture Collection. HeLa-T4 and H9 cells were obtained through the AIDS Reference and Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, and were originally contributed by Richard Axel and Robert Gallo, respectively. COS-1 and HeLa-T4 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum. H9 cells were maintained in RPMI 1640 medium containing 15% fetal bovine serum.

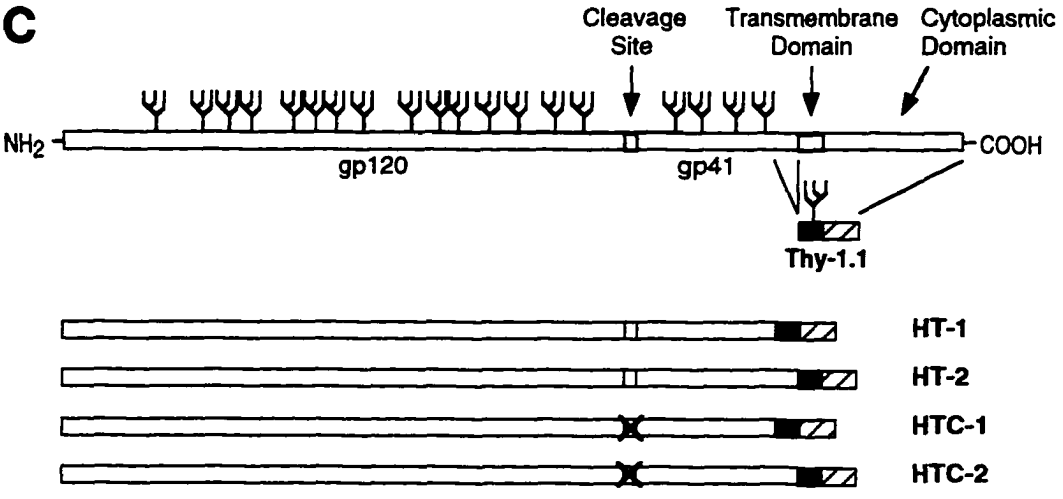
Construction of plasmids. In order to create the chimeric HIV-1 *env*-Thy-1.1 genes, an *Ava*I site was introduced into the 3' end of the *env* gene of the HXB2 strain of HIV-1 (27) at nucleotide 7796 (pHT-1 and pHTC-1) or 7850 (pHT-2 and pHTC-2) (Fig. 1A) by oligonucleotide-directed mutagenesis using the Altered Sites system from Promega as described previously (5). pThy-1.1, a cDNA clone encoding Thy-1.1 (16), was a gift from Ihor Lemischka (Princeton University). An *Xho*I linker (Boehringer Mannheim) was inserted into the 3' flanking polylinker region downstream of the Thy-1.1 cDNA of pThy-1.1 following cleavage with *Bgl*II and blunt ending with the Klenow fragment of *Escherichia coli* DNA polymerase I (Fig. 1B). The resulting *Ava*I-*Xho*I fragment from pThy-1.1 encoding the 53 C-terminal amino acids of Thy-1.1 was substituted into both wild-type and cleavage-defective HXB2 *env* genes between the engineered *Ava*I sites and a unique *Xho*I site. Chimeric *Kpn*I-*Xho*I *env* gene fragments were then substituted into pSRHS, an HIV-1 *env* expression plasmid utilizing the late promotor and origin of replication from Simian Virus 40 and the poly(A) addition signal from the Mason-Pfizer monkey virus (6), for expression in COS-1 cells. The resulting constructs (pHT-1 and pHT-2, and pHTC-1 and pHTC-2, respectively) encode the ectodomain of gp160 fused at the C terminus to the GPI attachment signal from Thy-1.1 (Fig.+ 1C).

Glycoprotein expression and radioimmunoprecipitation. Each of the chimeric constructs was cotransfected with pRev1 (18, a gift from Marie-Louise Hammarskjold) into COS-1 cells by a modified calcium phosphate-mediated method (2). At 48 h posttransfection, the cells were starved in leucine-free DMEM for 1 h and pulsed for 30 min in leucine-free DMEM supplemented with [^{3}H]leucine (500 μCi/ml, 145 Ci/mmol; DuPont-NEN). The labeled cells were then either lysed or chased for 3 h in complete DMEM. The chase medium was collected, and the cells were lysed by a 10-min incubation on ice in lysis buffer (1% Triton X-100, 25 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1% sodium deoxycholate). Cellular debris was removed by centrifugation in a microcentrifuge for 5 min at 4°C. Lysates and chase media were immunoprecipitated by

FIG. 1. Construction of chimeric HIV-1-Thy-1.1 glycoprotein mutants. (A) The nucleotide sequences encoding the indicated amino acids of the wild-type HIV-1 Env glycoprotein are shown at top; the mutations introduced to form the *Ava*I sites used for introducing the Thy-1.1 GPI-attachment signal sequence and the resulting amino acid changes are indicated below. (B) Basic cloning strategy for pHT-1. The *Bgl*II site in pThy-1.1 was converted to an *Xho*I site. The resulting *Ava*I-*Xho*I fragment from pThy-1.1 was ligated into HIV-1 *env*, replacing the fragment between the new *Ava*I site created by site-directed mutagenesis and an *Xho*I site, using an intermediate vector (not shown). The *Kpn*I-*Xho*I fragment from the chimeric *env* was then substituted into pSRHS. (C) The wild-type glycoprotein is diagrammed at top with representative glycosylation sites. The transmembrane and cytoplasmic domains of gp41 were replaced with a 53-amino-acid, C-terminal GPI attachment signal from Thy-1.1 by gene fusion at each of the two new *Ava*I sites created in *env*. The resulting two constructs, HT-1 and HT-2, and their cleavage-defective sister constructs, HTC-1 and HTC-2, are diagrammed below. The hatched region represents the 31-amino-acid peptide which is cleaved concomitant with GPI attachment. The shaded region represents the 22 amino acids of Thy-1.1 which remain fused to the HIV-1 glycoprotein.







addition of HIV-1-positive human serum and incubation at 4°C for 1 h. The immune complexes were incubated for 30 min at room temperature with fixed *Staphylococcus aureus* and pelleted in a microcentrifuge. The pellets were washed three times in lysis buffer containing 0.1% sodium dodecyl sulfate (SDS) and once in 20 mM Tris-HCl (pH 6.8) prior to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

PI-PLC treatment. PI-PLC treatment was performed essentially as described previously by Crise et al. (3). Following labeling as described above, cells that had been chased for 3 h were incubated for 15 min at room temperature in Ca^{2+}-Mg^{2+}-deficient phosphate-buffered saline (PBS) containing 25 mM EDTA to remove them from the dish. Cells were then washed and resuspended in DMEM containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid) (pH 7.3). PI-PLC was added to the samples at a concentration of 17 U/ml (1 U = 1 μmol/min); this was followed by a 1-h incubation at 37°C. PI-PLC was generously provided by Martin Low (Columbia University). Both cells and media were collected following centrifugation and prepared as described above for immunoprecipitation and analysis by SDS-PAGE.

Endo H treatment. COS-1 cells were transfected and labeled as described above, except that they were starved in cysteine- and methionine-free DMEM for 30 min and labeled in cysteine- and methionine-free DMEM supplemented with [^{35}S]cysteine-[^{35}S]methionine (500 μCi/ml, 1,200 Ci/mmol; Expre^{35}S^{35}S; DuPont-NEN). Cells were lysed immediately following the pulse or were chased for 1, 2, or 4 h. Lysates were immunoprecipitated as described above, and the resulting washed pellets were boiled for 5 min in 0.04% SDS-200mM 2-mercaptoethanol. The *S. aureus* was removed by pelleting at room temperature in a microcentrifuge, and the supernatant was divided into two tubes containing equal volumes of either 100 mM sodium citrate (pH 5.3)—2 mM EDTA—1 mM phenylmethylsulfonyl fluoride—200 μg of soybean trypsin inhibitor per ml—2 μg of leupeptin per ml—2 μg of pepstatin A per ml (mock treated) or the same buffer containing endoglycosidase H (endo H) (Boehringer-Mannheim) at a concentration of 50 U/ml (endo

H treated). The samples were incubated for 16 h at 37°C, adjusted to 10 mM Tris-HCl (pH 6.8)—2% SDS—10% glycerol—0.1% bromophenol blue, and reboiled for 2 min prior to being loaded onto SDS-PAGE gels.

Syncytium assay and total cell immunofluorescence. COS-1 cells were trypsinized following transfection as described above, mixed with untransfected HeLa-T4 cells at a ratio of 1:10, and replated. At 48 to 72 h posttransfection, the cells were stained by the May-Grunwald-Giemsa technique as described previously (5), and the number and size of syncytia were quantitated.

For immunofluorescence staining, parallel cultures were seeded onto glass coverslips following mixing and were fixed at 48 to 72 h posttransfection with 95% ethanol—5% acetic acid at -20°C. These cells were stained for gp120 and gp160 expression by using a 1:25 dilution of a monoclonal antibody to the V3 loop (monoclonal 9284 from DuPont-NEN), followed after washing by a 1:25 dilution of Texas red-conjugated goat anti-mouse antibody (Southern Biotechnology Associates) as described previously (5).

Biotinylation of glycoproteins on the cell surface. COS-1 cells transfected as described above were treated with Sulfo-NHS-biotin (Pierce) essentially as described previously by Lisanti et al. (20). Briefly, cells were washed five times at 48 to 72 h posttransfection with ice-cold PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-C/M) and then incubated with 0.5 mg of sulfo-NHS-biotin per ml in PBS-C/M for 30 min on ice. The cells were then incubated with DMEM for 10 min on ice to quench the reaction and were washed four times with PBS-C/M containing 20 mM glycine prior to lysis, immunoprecipitation, and SDS-PAGE as described above. The proteins were blotted onto nitrocellulose, and the membrane was blocked with 5% nonfat dry milk in PBS containing 0.1% Tween 20 (PBS-T) for 1 h at room temperature. The blot was washed with PBS-T and probed with a 1:2,500 dilution of horseradish peroxidase-conjugated streptavidin (Fisher Scientific) in PBS-T for 45 min at room temperature. The blot was

washed again in PBS-T prior to enhanced chemiluminescence detection as described by the manufacturer (Amersham).

PHXBΔenvCAT complementation assay and incorporation of glycoproteins into virions. The pHXBΔenvCAT-complementation assays were performed essentially as described previously by Helseth et al. (11). Briefly, COS-1 cells were cotransfected with pHXBΔenvCAT, pRev1, and each of the chimeric *env* constructs by using DEAE-dextran (1 mg/ml). At 60 h posttransfection, the supernatant was collected and passed through a 0.45-μm filter. A portion of the filtered supernatant was assayed for reverse transcriptase activity as described previously (5). The remaining filtered supernatant was used to infect H9 cells with equivalent amounts of virus as determined from the reverse transcriptase assays. At 60 h postinfection, cells were lysed, and a portion of the lysate was assayed for chloramphenicol acetyltransferase (CAT) activity by incubation with [^{14}C]chloramphenicol and acetyl coenzyme A, followed by thin-layer chromatography. The percent acetylation was determined by quantitating the radioacitvity in each of the resulting spots with an AMBIS radioanalytic imaging system (AMBIS Systems). pHXBΔenvCAT was a gift from Joseph Sodroski.

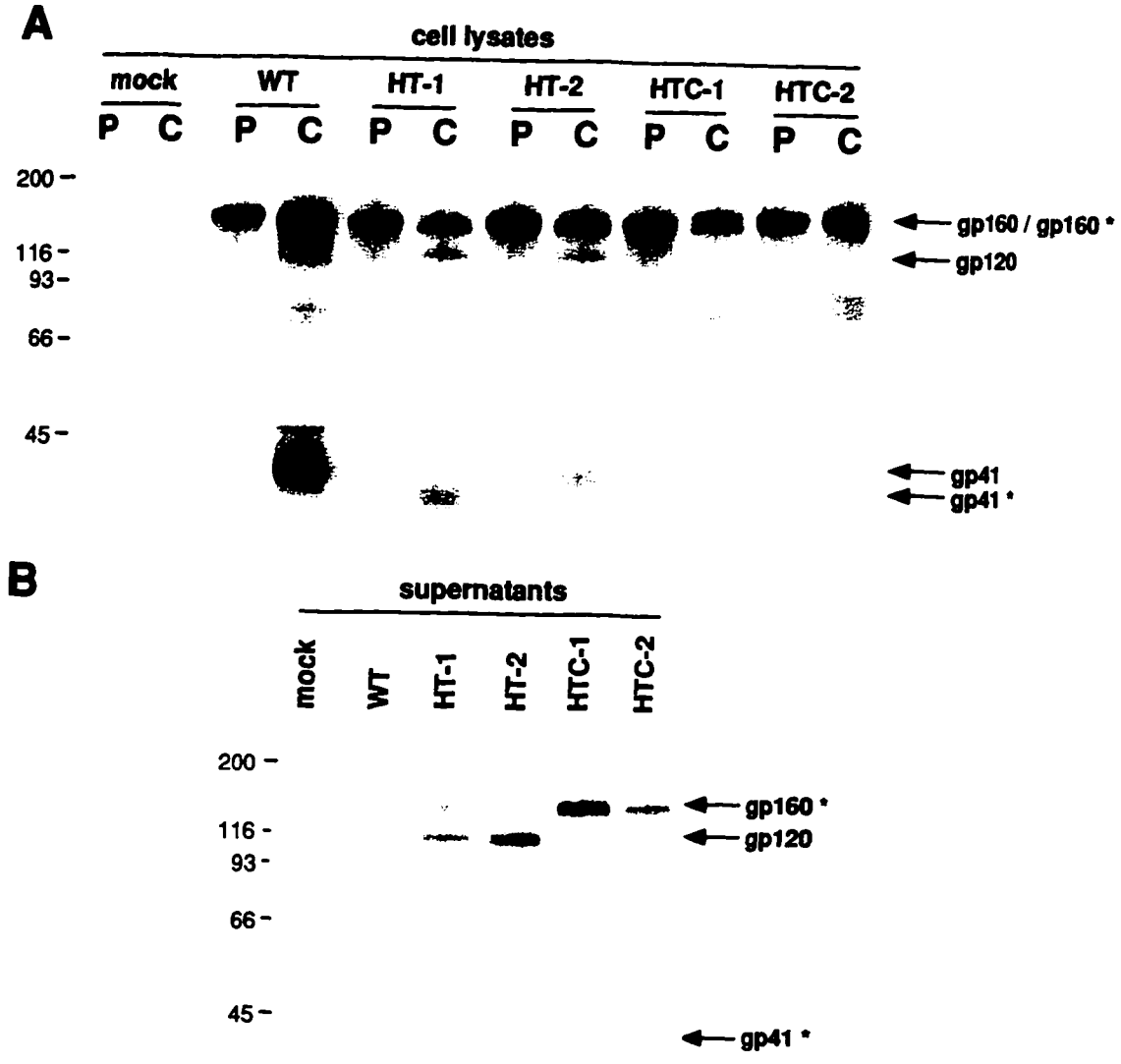
For the detection of glycoproteins in virions, COS-1 cells were either cotransfected with pFNΔenvCAT, a construct identical to pHXBΔenvCAT except for an ~300-bp deletion in the *pol* gene (4), and pSRHS (wild type) or cotransfected with pFNΔenvCAT, pRev1, and the HTC-1 *env* construct by using calcium phosphate. At 60 h posttransfection, the cells were labeled for 2 h with [^{35}S]cysteine-[^{35}S]methionine (1 mCi/ml) as described above. Following a 10-h chase, the supernatant was filtered through a 0.45-μm filter. The filtered supernatant was then layered onto a 1-ml sucrose cushion (15% sucrose and 10 mM HEPES prepared in PBS) and centrifuged at 25,000 rpm for 2 h at 18°C in an SW41 rotor (Beckman Instruments). The resulting virus pellet was resuspended in lysis buffer and prepared as described above for immunoprecipitation and SDS-PAGE.

RESULTS

Expression of HIV-1-Thy-1.1 chimeric glycoproteins. The chimeric *env* constructs described above have the second coding exon of the *rev* overlapping reading frame deleted. Since the *rev* gene product is required for efficient expression of the glycoprotein (30), it was necessary to cotransfect cells with both the chimeric pSRHS-based constructs and pRev1, a complete *rev* cDNA clone under the transcriptional control of the cytomegalovirus immediate early promoter. Cotransfection mixtures were titrated against pRev1 to ensure that glycoprotein expression was optimal (data not shown). While the chimeric HIV-1 *env*-Thy-1.1 genes are still not expressed as highly as wild-type *env* in the pSRHS expression system, their products appear to be processed and transported normally in a pulse-chase analysis (Fig. 2A). Following a pulse-label, a band migrating slightly faster than the wild-type gp160 was seen for each of the chimeric proteins (gp160*). During the chase, HT-1 and HT-2 were cleaved to gp120 and a gp41-Thy-1.1 chimeric molecule (gp41*). The gp41* proteins from HT-1 and HT-2 showed the approximately 2-kDa size difference expected from the different fusion points (18 amino acids apart) within the HIV-1 *env* gene sequence. However, both migrated more slowly than might have been expected from the substitution of the shorter Thy-1.1 sequences (22 amino acids and an additional oligosaccharide side chain, following addition of the GPI anchor) for the longer HIV coding sequences (191 and 173 amino acids, respectively). Nevertheless, as addressed in more detail in the Discussion, the observed molecular weights (MWs) of the gp41* proteins from HT-1 (38,000) and HT-2 (40,000) are consistent with those predicted for the constructs, whereas that of wild-type gp41 (which migrates with an MW of 43,000 in these experiments) is significantly less than would be predicted.

The products of the HTC-1 and HTC-2 constructs were not cleaved to gp120 and gp41* following the chase, although a nonspecific cleavage of the V3 loop was observed (see below). A second band, migrating slightly slower than gp160*, was also

FIG. 2. Pulse-chase analysis of HIV-1-Thy-1.1 chimeric glycoprotein expression in COS-1 cells. COS-1 cells were transfected with simian virus 40-based expression plasmids encoding the wild-type HIV-1 glycoprotein (WT) and each of the chimeric products (HT-1, HT-2, HTC-1, and HTC-2). The chimeric constructs were cotransfected with pRev1, a cytomegalovirus-based Rev cDNA expression plasmid. The cells were metabolically pulse-labeled for 30 min with [^{3}H]leucine and chased for 3 h. Cell lysates (A) from both the pulse (P) and the chase (C) and the culture medium supernatants from the chase (B) were collected and immunoprecipitated with HIV-positive human serum prior to SDS-PAGE (8%) and fluorography. gp160, gp120, and gp41 denote the positions of the Env precursor, SU, and TM cleavage products, respectively. gp160* and gp41* indicate the analogous HIV-1-Thy-1.1 chimeric products. Molecular weight marker positions (in thousands) are shown at left. Mock-transfected cells were included as a negative control.



immunoprecipitated from these cells. This band, most clearly visible in Fig. 2A, lane HTC-1 C, presumably represents the terminally glycosylated, uncleaved form of the chimeric gp160 present on the surface of expressing cells (see below).

Immunoprecipitation of the culture medium harvested from the chase plates showed that gp120 was shed into the medium from the wild-type, pSRHS-transfected cells (Fig. 2B, lane WT), consistent with the absence of a covalent linkage between gp120 and gp41. Similarly, primarily gp120 was found in the supernatant fraction of HT-2 (Fig. 2B, lane HT-2). In the case of HT-1, however, gp160*, gp120 and a trace of gp41* were found to be released from the cells during the chase (Fig. 2B, lane HT-1). We suspected that some membrane-bound chimeric proteins might be released by contaminating phospholipase activity from the fetal bovine serum in the culture medium, since a GPI-specific phospholipase D has been purified from mammalian serum (see reference 21 for a review). However, the HT-1 protein appears to be less stably associated with the plasma membrane than the HT-2 protein. Similarly, a significantly higher level of the uncleaved product from the HTC-1-transfected cells was shed into the medium than was observed with HTC-2 (Fig. 2B, lanes HTC-1 and HTC-2). It is possible, therefore, that following truncation of the external domain of gp41, the GPI anchor either is less able to secure the protein in the membrane or is more accessible to contaminating phospholipase.

It should also be noted that two bands migrating at MWs of approximately 85,000 and 70,000 appear predominantly in lanes corresponding to the cleavage-defective mutants HTC-1 and HTC-2. The sizes of these products are consistent with a proteolytic cleavage occurring within the V3 loop domain of the chimeric glycoprotein. We have previously observed heightened sensitivity to cleavage in this domain with full-length cleavage-defective mutants of gp160 (29).

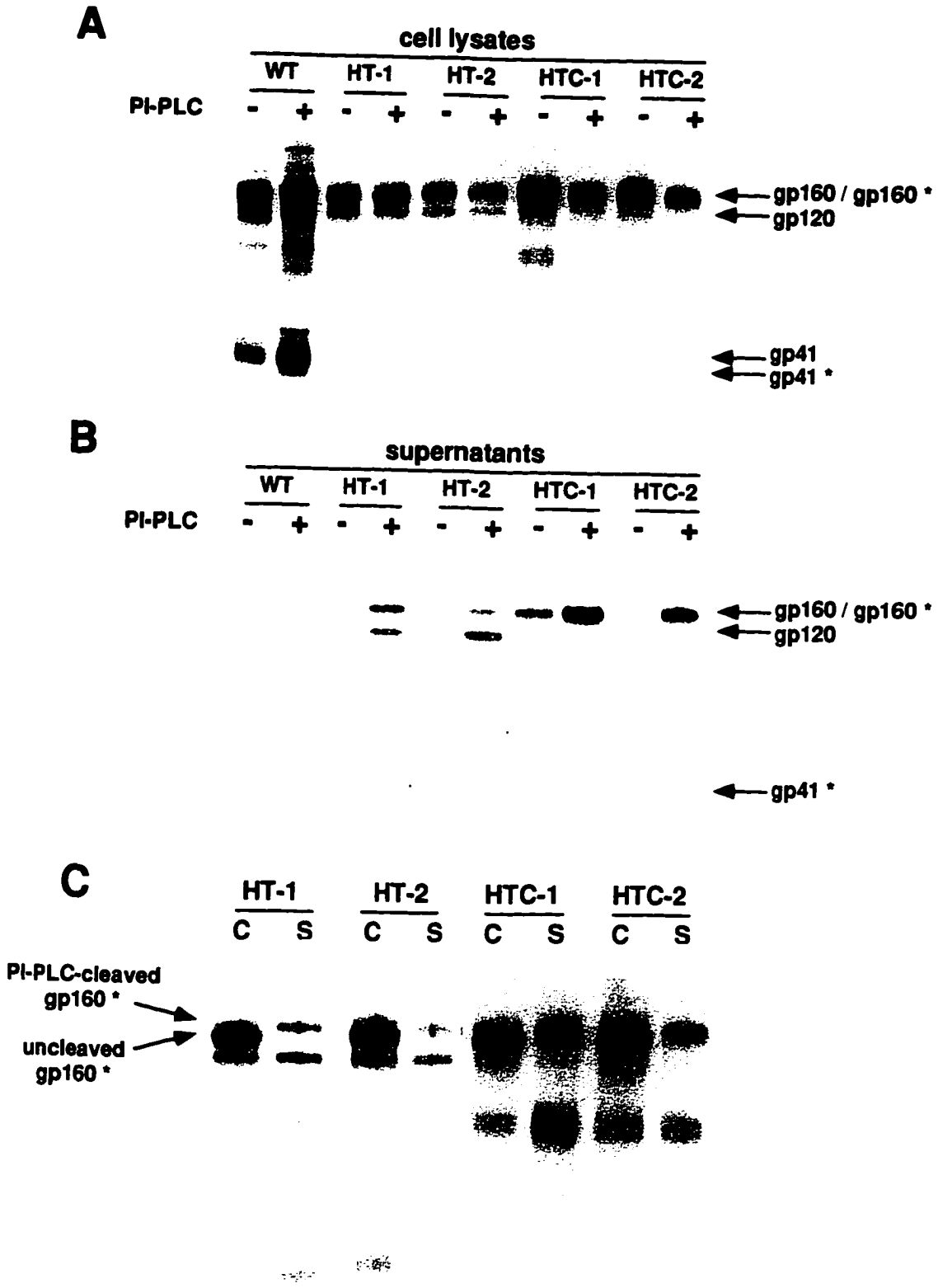
The chimeric glycoproteins are GPI anchored and are transported to the cell surface. In order to determine whether the HIV-1-Thy-1.1 chimeric glycoproteins were GPI anchored and were transported to the cell surface, we determined

whether these proteins could be cleaved by PI-PLC from the surface of cells cotransfected with each of the chimeric constructs and pRev1. PI-PLC has been used previously to show that up to 50% of the wild-type GPI-anchored Thy-1 glycoprotein produced by a cell can be cleaved from the cell surface (22).

The glycoprotein-expressing cells were pulse-labeled with [^{3}H]leucine and then chased for 3 h, at which time they were removed from the dish and either treated with PI-PLC or mock digested for 1 h at 37°C. Following removal of the solubilized proteins and lysis of the cells, both the cell lysates and supernatants were immunoprecipitated (Fig. 3A and B, respectively). A significant decrease in the intensities of both the gp120 and the gp41* bands was observed following digestion of cells expressing either HT-1 or HT-2, (Fig. 3A; compare HT-1, - and +, and HT-2, - and +, lanes). This decrease in cell-associated protein was accompanied by the appearance of gp120 and gp41* in the supernatant (Fig. 3B, lanes HT-1 [+] and HT-2 [+]), while during the 1-h mock digestion little protein was spontaneously shed into the medium (Fig. 3B, lanes HT-1 [-] and HT-2 [-]). Similar results were obtained with the cleavage-defective constructs HTC-1 and HTC-2. In each instance the amount of cell-associated protein decreased following digestion, and significant amounts of the solubilized gp160 ectodomain were released (Fig. 3A and B, lanes HTC-1 and HTC-2). In contrast, treatment of wild-type gp160-expressing cells with PI-PLC did not result in any release of the labeled protein into the medium (Fig. 3B, lane WT [+]).

It is interesting that a band corresponding to the gp160 ectodomain (gp160*) was also cleaved from the surface of both the HT-1- and HT-2-expressing cells. A more detailed analysis of this protein, as well as that released from HTC-1- and HTC-2-expressing cells, revealed that it migrated more slowly than the bulk of the protein that remained cell associated after PI-PLC digestion (Fig. 3C; compare the cell-associated [C] and solubilized [S] lanes) and corresponded to the upper band of the doublet described in the previous section. We interpret this result to mean that only the terminally glycosylated

FIG. 3. Detection of GPI-anchored chimeric glycoproteins on the cell surface by cleavage with PI-PLC. COS-1 cells labeled with [^{3}H]leucine for 30 min were suspended in PBS following a 3-h chase, washed with serum-free medium, and incubated for 1 h at 37°C in serum-free medium (-) or serum-free medium containing PI-PLC (+). Both the cell lysates (A) and the culture medium supernatants (B) were collected and immunoprecipitated for SDS-PAGE (8%) and fluorography. WT, wild type. (C) Side-by-side comparison of products immunoprecipitated from the cell lysates (C) and the supernatants (S) following PI-PLC treatment, showing the differential mobilities of the cell-associated and solubilized products.



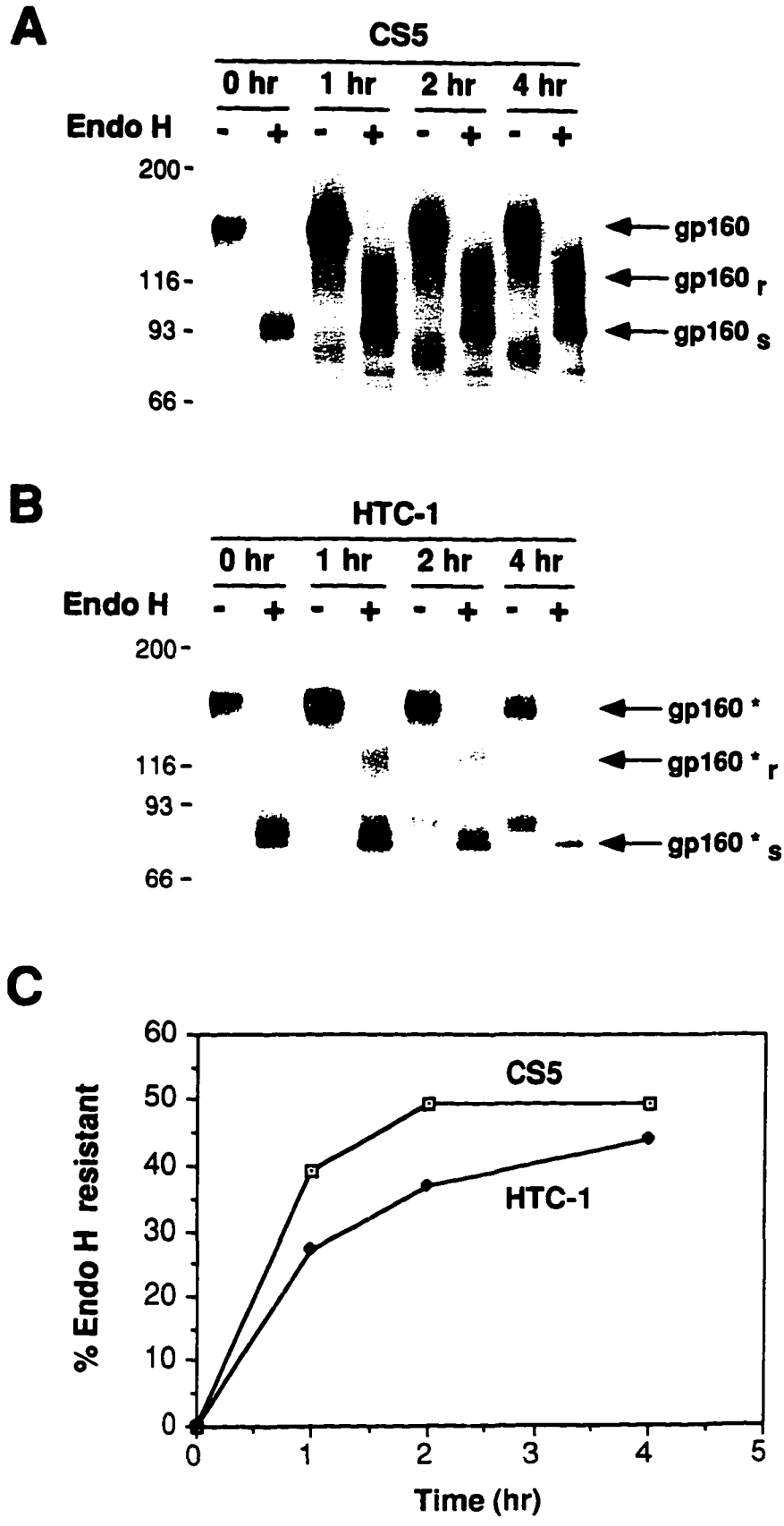
(higher-MW), surface-expressed gp160 is accessible to PI-PLC cleavage and that the bulk of this species is cleaved from the cell (compare, for example, lanes HTC-1, C and S in Fig. 3C). Moreover, this result indicates that a significant fraction of the HT-1 and HT-2 glycoprotein product is transported to the cell surface without being proteolytically cleaved to gp120 and gp41* in the Golgi.

The data presented in Fig. 3C also show that following cleavage, the cell-associated and solubilized gp41* proteins comigrate on SDS-PAGE. This indicates that removal of the lipid moiety from the GPI anchor does not significantly affect the apparent MW of gp41* in SDS-PAGE.

Rate of transport of the GPI-anchored HIV-1 glycoproteins. In order to determine whether the GPI anchor influenced the rate of intracellular transport of the chimeric glycoproteins relative to that of full-length gp160, we determined the rate at which they became resistant to endo H cleavage. This enzyme is unable to cleave from proteins oligosaccharide chains that have been trimmed by the mannosidases located in the *cis* and medial Golgi and thus provides a means to determine the rate of transport to this organelle (reviewed in reference 13). For this experiment, we compared the transport of natively and GPI-anchored cleavage-defective HIV-1 glycoproteins in order to simplify interpretation of the results. Cells expressing a full-length cleavage-defective (CS5) glycoprotein or the HTC-1 construct were pulse-labeled and then chased for 1, 2, or 4 h prior to lysis. Each of the cell lysates was then immunoprecipitated, divided into two tubes, and either treated with endo H or mock treated prior to SDS-PAGE.

As can be seen in Fig. 4, after a pulse-label, the full-length CS5 mutant HIV-1 glycoprotein was completely sensitive to endo H treatment, with the product of digestion migrating at approximately 90 kDa because of the removal of its high-mannose oligosaccharide side chains (Fig. 4A, lane 0 hr [+]). After a chase period of 1 h or more, an endo H-resistant band which migrated at approximately 116 kDa was present, consistent with the modification of 13 of the 24 N-linked oligosaccharide side chains of gp120 to

FIG. 4. Acquisition of endo H resistance by the GPI-anchored glycoprotein. Cells expressing either full-length cleavage-defective HIV-1 glycoprotein (CS5) (A) or HTC-1 (B) were lysed following a 30-min pulse (0 hr) with [^{35}S]cysteine-[^{35}S]methionine or following a 1-, 2-, or 4-h chase. The lysates were immunoprecipitated, boiled in buffer containing 0.02% SDS and 200 mM 2-mercaptoethanol, and incubated for 16 h at 37°C in the absence (-) or presence (+) of endo H. The samples were adjusted to 2% SDS and reboiled prior to SDS-PAGE (6%). $gp160_r$ and $gp160^*_r$, endo H-resistant species; $gp160_s$ and $gp160^*_s$, endo H-sensitive species. Molecular weight marker positions (in thousands) are shown at left. (C) The percent endo H resistance was determined by quantitating the radioactivity in each band with an AMBIS radioanalytic imaging system.



endo H-resistant, complex oligosaccharides (17) (Fig. 4A, lanes 1 hr [+], 2 hr [+], and 4 hr [+]). Similar sensitive and resistant species could be seen following digestion of the GPI-anchored glycoprotein with endo H (Fig. 4B, + lanes). It is interesting that the endo H-sensitive, deglycosylated forms of the CS5 and HTC-1 precursors (Fig. 4, lanes 0 hr [+]) migrate with apparent MWs (90,000 and 75,000 respectively) that are consistent with the MWs calculated from their amino acid sequences.

In order to compare the rates at which endo H resistance was acquired for the CS5 and HTC-1 glycoproteins, the percent endo H resistance was calculated at each chase time point by quantitating the radioactivity in each resistant and sensitive band with an AMBIS radioanalytic imaging system. These results, summarized in Fig. 4C, showed that the CS5 glycoprotein reached 50% endo H resistance after a 2-h chase. The GPI-anchored glycoprotein was transported at a similar rate, reaching 37% endo H resistance after a 2-h chase. Thus, the rate of transport of GPI-anchored HIV-1 glycoprotein was reduced only 25 to 30% relative to that of the full-length glycoprotein. Similar results were obtained with the HTC-2 construct (data not shown).

The GPI-anchored HIV-1 glycoproteins are nonfusogenic. In order to determine whether the GPI-anchored glycoproteins were functional in mediating cell-cell or virus-cell fusion events, constructs were assayed for their abilities both to mediate syncytium formation in COS-1-HeLa-T4 cell mixtures and to complement the entry-single-round replication of an *env*-deficient HIV-1 provirus in H9 cells. For the syncytium assay, COS-1 cells cotransfected with each of the HIV-1-Thy-1.1 chimeric constructs and pRev1 were mixed at a ratio of 1:10 with untransfected HeLa-T4 cells. The resulting cell mixtures were examined for the presence of syncytia 48 to 72 h later. No syncytia were seen with any of the GPI-anchored constructs, while numerous large syncytia were present in cells transfected with wild-type *env* (Table 1). Moreover, cells cotransfected with pRev1 and pSRHBΔSX, a construct expressing the *env* gene of the BH10 strain of HIV-1 that has the upstream coding region of *rev* deleted (4), produced a similar number and average size of

syncytia (Table 1, ΔSX). Therefore, the lack of syncytia in the cultures expressing the GPI-anchored glycoproteins should not be due to decreased expression resulting from the cotransfection.

TABLE 1. Fusogenicity of GPI-anchored HIV-1 glycoproteins

Mutant[a]	Syncytium formation		Replication complementation	
	No. of syncytia/ plate[b]	No. of nuclei/ syncytium[c]	% acetylation[d]	Relative CAT activity[e]
Mock	0	-	0.4	0
pHXBΔenvCAT	ND[f]	ND	0.5	1
WT	299	38	10.5	100
ΔSX	190	31	ND	ND
HT-1	0	-	0.4	0
HT-2	0	-	0.6	2
HTC-1	0	-	0.6	2
HTC-2	0	-	0.5	1

[a] For replication complementation, chimeric *env* constructs were cotransfected with both pRev1 and pHXBΔenvCAT. Mock, pRev1 transfected alone; pHXBΔenvCAT, pRev1 cotransfected with pHXBΔenvCAT; WT, wild-type.

[b] Determined as the average number of syncytia per microscopic field (15 non-overlapping, random fields counted) multiplied by the approximate number of fields per tissue culture plate (determined by using a grid).

[c] Determined as the average from15 syncytia per construct.

[d] Percentage of [^{14}C]choramphenicol that was acetylated upon incubation with the lysates of H9 target cells that had been infected 60 h previously with the filtered supernatants of transfected COS-1 cells.

[e] Determined as the percentage of the wild-type value after subtraction of the mock transfection value.

[f] ND, not determined.

We further examined glycoprotein expression in this syncytium assay by staining parallel cultures for immunofluorescence with a monoclonal antibody specific for the V3 loop region of gp120 and gp160. The wild-type- and ΔSX-expressing cultures both contained numerous large, brightly fluorescing syncytia (Fig. 5, WT and ΔSX) with no brightly fluorescing single cells. This indicates that every COS cell expressing a significant level of wild-type glycoprotein, whether *rev* is provided in *cis* or in *trans*, is able to fuse adjacent cells. Conversely, the cultures expressing GPI-anchored glycoproteins contained

numerous brightly fluorescing single and dividing cells (Fig. 5, HT-1, HT-2, HTC-1, and HTC-2), but again no syncytia were observed. Thus, even though the GPI-anchored glycoproteins were being efficiently expressed, they were unable to mediate syncytium formation.

The results of the syncytium assay are dependent not only on the relative levels of total glycoprotein expression by the cells but also on the relative levels of surface expression of the different forms of the glycoprotein. In order to determine the approximate amount of each mutant glycoprotein relative to wild-type glycoprotein present on the cell surface, we selectively biotinylated proteins on the surface of COS-1 cells expressing each of the glycoproteins used in the syncytium assay. Following biotinylation and cell lysis, the glycoproteins were immunoprecipitated, separated by SDS-PAGE, blotted onto nitrocellulose, and detected by probing with horseradish peroxidase-conjugated Streptavidin followed by enhanced chemiluminescence.

As can be seen in Fig. 6, all four GPI-anchored glycoproteins were present on the cell surface at levels equivalent to that of wild-type glycoprotein. In addition to gp120, we also detected a considerable amount of uncleaved wild-type glycoprotein precursor (gp160), as well as uncleaved GPI-anchored glycoprotein precursor (gp160*) on the surface of cells expressing cleavable forms of the glycoprotein (Fig. 6, lanes WT and ΔSX,. and HT-1 and HT-2, respectively). This result is consistent with that of the PI-PLC treatment experiment (see above) and demonstrates further that a significant fraction of the wild-type glycoprotein, as well as the HT-1 and HT-2 glycoproteins, is transported to the cell surface without being proteolytically cleaved to gp120 and gp41. The surface-expressed GPI-anchored gp160* detected in this experiment migrates as a single band. This again is consistent with the PI-PLC treatment experiment, which showed that only the terminally glycosylated (higher-MW) form of the glycoprotein is present on the cell surface (see above). The results of the syncytium quantitation, immunofluorescence staining, and

FIG. 5. Total-cell immunofluorescence of cells used in syncytium assay. COS-1 cells were transfected with pSRHS alone (WT) or cotransfected with pRev1 and pSRHBΔSX (ΔSX) or pRev1 and each of the chimeric constructs (HT-1, HT-2, HTC-1, and HTC-2). Immediately following transfection, the COS-1 cells were trypsinized and mixed at a ratio of approximately 1:10 with untransfected HeLa-T4 cells. At 48 h later, the cells were fixed with 95% ethanol-5% acetic acid and stained for immunofluorescence with a monoclonal antibody to the V3 loop and a Texas red-conjugated secondary antibody.

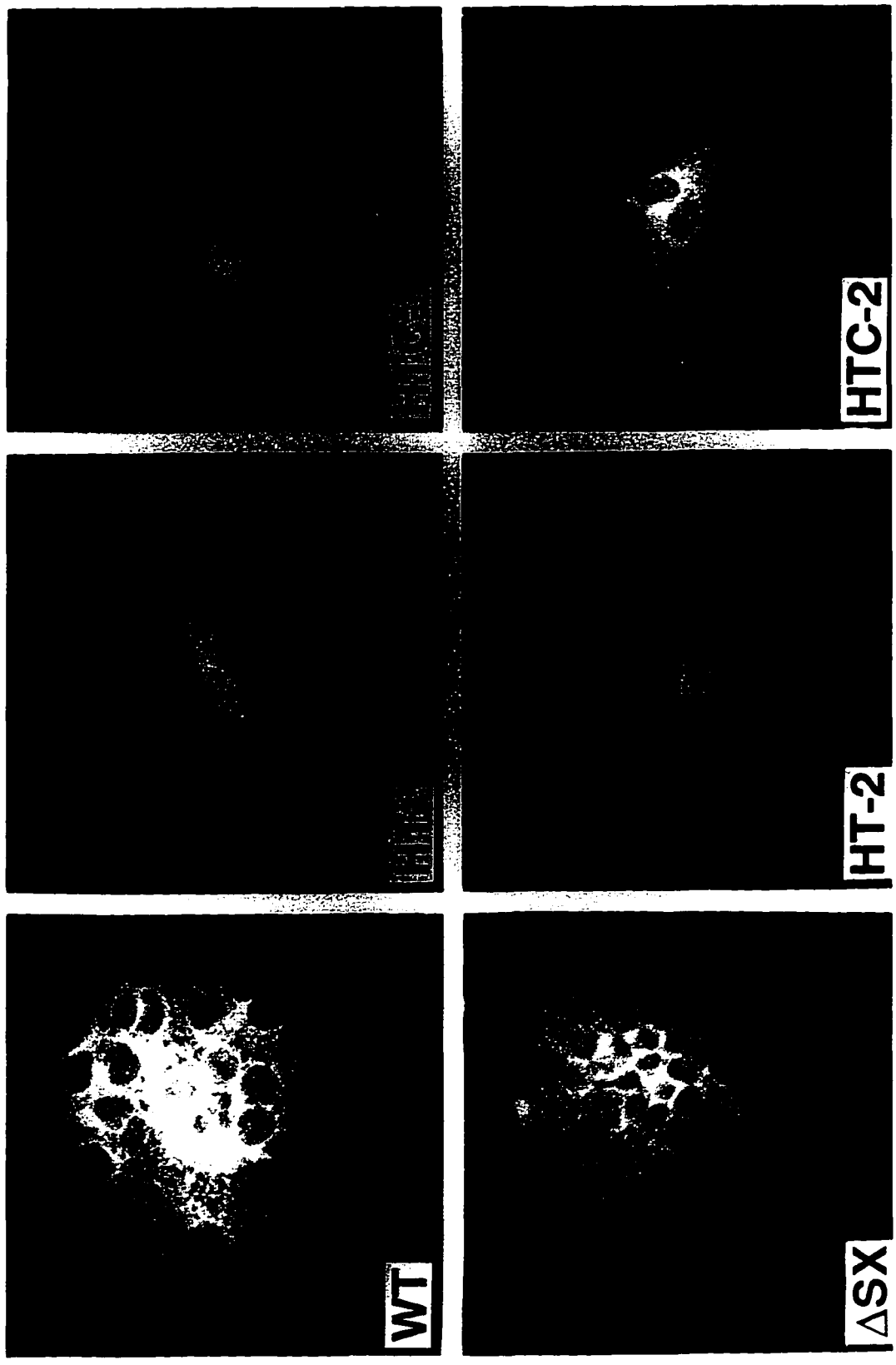
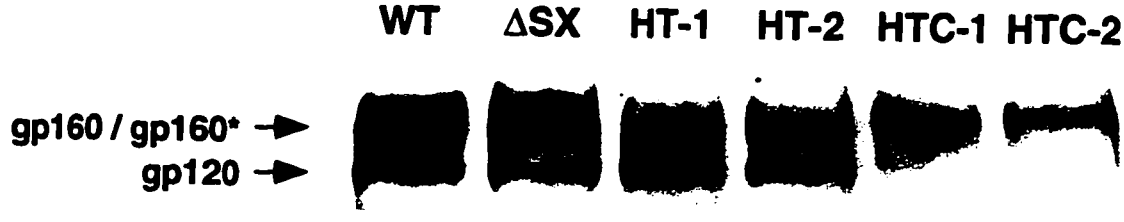


FIG. 6. Cell surface biotinylation. COS-1 cells transfected as for the syncytium assay were biotinylated at 48 h posttransfection by using sulfo-NHS-biotin. The cells were then lysed, and the glycoprotein was immunoprecipitated with HIV-positive human serum. The immunoprecipitates were run on an SDS-PAGE gel (8%) and blotted onto nitrocellulose. The biotinylated proteins were detected by probing the membrane with horseradish peroxidase-conjugated streptavidin, followed by ehhanced chemiluminescence.



surface biotinylation assays together demonstrate that the GPI-anchored glycoproteins are completely blocked in their ability to mediate cell-cell fusion between these two cell types.

In order to determine whether the GPI-anchored glycoproteins could mediate entry of HIV-1 virions into T cells, we included the constructs in an *env* complementation assay described previously by Helseth et al. (11). This assay involves cotransfection of the *env* construct of interest with an HIV-1 proviral clone (pHXBΔenvCAT) containing a partially deleted *env* gene and a CAT reporter gene in place of the *nef* gene. The approximately 110-kDa product of this defective *env* gene is not incorporated into virions (data not shown). The virus produced by cotransfected COS-1 cells is used to infect $CD4^+$ target cells, which are then assayed for CAT activity 48 to 72 h later. Expression of CAT activity by the infected cells thus indicates that the glycoprotein encoded by the *env* construct is capable of mediating virus entry.

All four of our GPI-anchored mutants were incapable of complementing pHXBΔenvCAT in infecting H9 cells. Each yielded less than 2% of the CAT conversion obtained with a wild-type *env* control, and no significant difference between the cleavage-deficient (HTC-1 and -2) and the cleaved (HT-1 and -2) GPI-anchored constructs was observed (Table 1). These values were essentially identical to the levels of background conversion observed in the control transfection of pHXBΔenvCAT alone. Thus, the GPI-anchored HIV-1 glycoproteins are apparently defective in mediating virus-cell as well as cell-cell fusion.

The GPI-anchored HIV-1 glycoprotein is efficiently incorporated into virions. The results of the pHXBΔenvCAT complementation assay are dependent both on the ability of the mutant glycoprotein to mediate fusion of the viral and cell membranes and on the efficiency with which the mutant glycoproteins are incorporated into virus particles. We therefore analyzed labeled virus pelleted from the filtered COS-1 supernatants of a similar experiment for glycoprotein content. As shown in Fig. 7, gp120 can be seen associated with virus released from cells cotransfected with the wild-type *env*

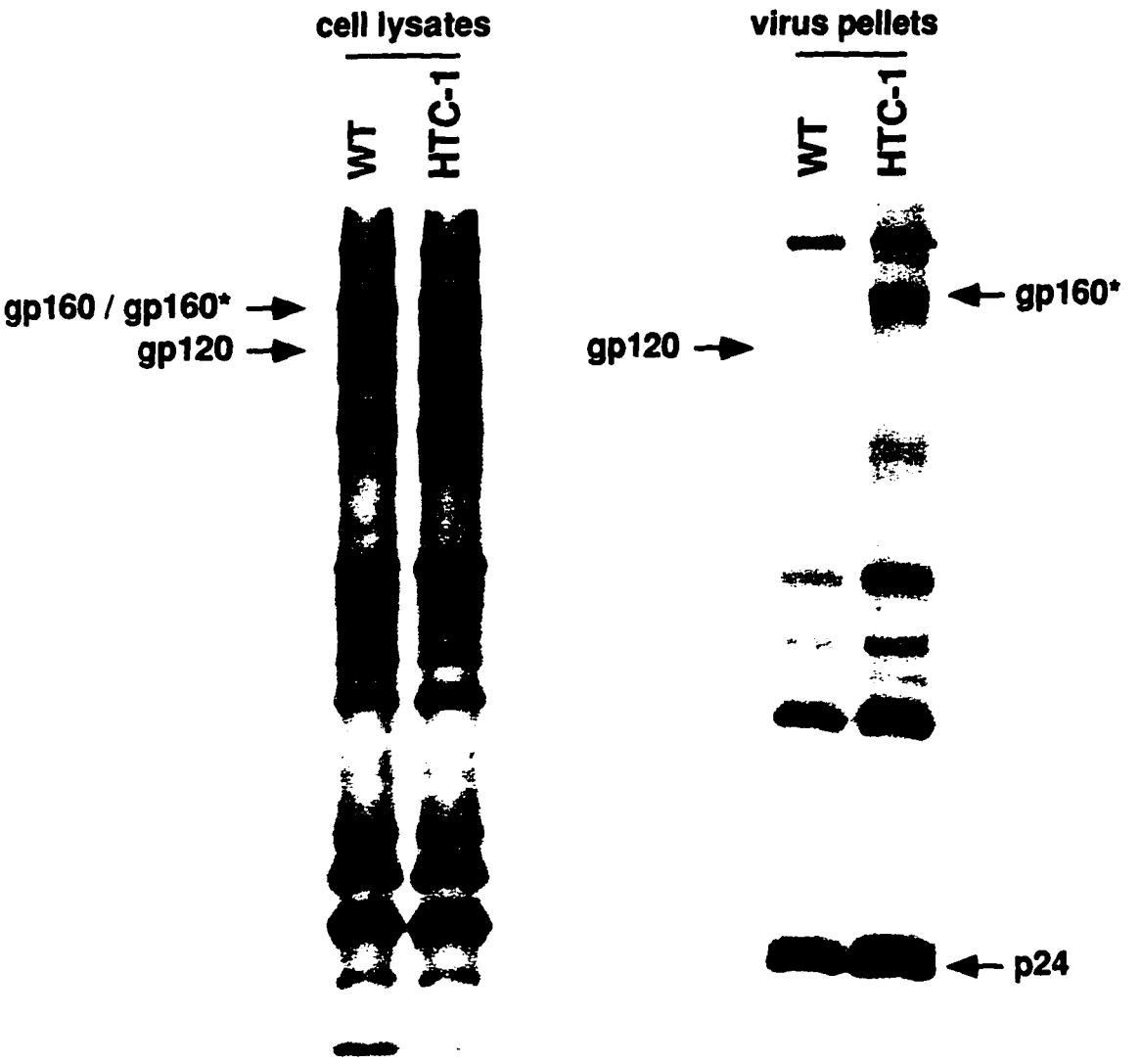
gene construct and pFNΔenvCAT (Fig. 7B, lane WT). The virions released from cells expressing the GPI-anchored HTC-1 product (gp160*) contained at least as much viral glycoprotein relative to the level of p24 *gag* core protein present (Fig. 7B, lane HTC-1). The cell lysates were also immunoprecipitated to ensure that equivalent amounts of glycoprotein were expressed in each culture (Fig. 7A). Thus, it appears that the GPI-anchored protein is incorporated into HIV-1 virions as efficiently as natively anchored glycoprotein.

DISCUSSION

In this study we have constructed four *env* constructs expressing chimeric HIV-1 glycoproteins in which the normal transmembrane and cytoplasmic domains were replaced by a GPI addition signal from Thy-1.1. These constructs express the glycoprotein efficiently when *rev* is supplied in *trans*. The resulting glycoproteins are anchored in the plasma membrane by a GPI anchor and can be solubilized from the cell surface by treatment with PI-PLC. This PI-PLC cleavage was highly efficient, since almost all of the terminally glycosylated glycoprotein species (presumably the only species present at the cell surface) was released into the supernatant by PI-PLC treatment.

A surprising result of these studies was the observation that the GPI-anchored gp41* proteins of HT-1 and HT-2 migrated on SDS-PAGE with a mobility similar to that of the wild-type gp41, despite the fact that sum totals of 169 and 151 amino acids (+22/-191 and +22/-173, respectively) had been deleted from the HT-1 and HT-2 gp41* proteins. Because the membrane-anchored and PI-PLC-solubilized gp41* proteins migrated with similar mobilities, this high apparent MW could not be attributed to the lipid anchor itself. Moreover, the additional glycosylation site present in the Thy-1.1 sequences, together with the oligosaccharide component of the anchor, would not be expected to increase the MW by 15,000. In the studies of Crise et al. (3), it was pointed out that the slightly larger apparent size of the GPI-linked, as compared with the wild-type, vesicular stomatitis virus G protein was consistent with the MW calculated following the replacement

FIG. 7. Incorporation of GPI-anchored glycoproteins into HIV-1 virions. COS-1 cells were cotransfected either with pFNΔenvCAT and pSRHS (WT) or with pFNΔenvCAT, pRev1, and pHTC-2 (HTC-2). At 60 h posttransfection, the cells were labeled for 2 h with [^{35}S]cysteine-[^{35}S]methionine and chased for 10 h. The virus was pelleted from the filtered cell supernatants through a 1-ml 15% sucrose cushion, resuspended in lysis buffer, and immunoprecipitated for analysis by SDS-PAGE (9%). p24 denotes the position of the major capsid (CA) protein encoded by the *gag* gene.



of 46 native amino acids with the 22-amino-acid-long, glycosylated Thy-1.1 sequence and GPI anchor. When the molecular masses of the HT-1 and HT-2 gp41* proteins were calculated from their amino acid sequences (176 and 194 amino acids, respectively) together with the additional mass contributed by five glycosylation sites (approximately 15 kDa) and the GPI anchor sequence (approximately 2 kDa), they were 37.1 and 39.5 kDa, respectively. These calculated values are very close to the 38- and 40-kDa values determined empirically from SDS-PAGE gels. In contrast, when the MW of wild-type gp41 was calculated in a similar fashion, a value of 51,600 was obtained. This is significantly greater than the empirically determined 43,000 MW for gp41 in these experiments. The basis for this discrepancy appears to be an effect on SDS-PAGE mobility imposed by the membrane-spanning domain and the long cytoplasmic domain of gp41, since a truncated "gp41" protein produced from Δ192-"gp160"-expressing cells has a mobility consistent with a molecular mass of approximately 31 kDa (4)—again, very close to the 29.2 kDa calculated from its amino acid sequence (17.2 kDa) and four oligosaccharide chains (12 kDa). Thus, it appears that the C-terminal region of gp41 that is deleted in the GPI-anchored proteins causes the protein to assume a more compact structure in SDS-PAGE that allows a more rapid, anomalous migration.

Analysis of the intracellular transport kinetics of these GPI-anchored glycoproteins indicated that their transport was only slightly reduced relative to that of the natively anchored glycoprotein. These findings are somewhat different from the results obtained when the same GPI attachment signal was fused to the vesicular stomatitis virus G glycoprotein. In this latter case, the effect on transport was much more significant, with the GPI-anchored protein being transported approximately eight times slower than the wild-type G protein (3). The altered transport kinetics of the GPI-anchored G protein are similar to those observed with G proteins lacking a cytoplasmic domain (28) and are consistent with this domain playing a role in efficient intracellular transport. In contrast, deletion of the entire cytoplasmic domain of the HIV-1 glycoprotein appeared to have no effect on

intracellular transport of the protein in COS-1 cells (6, 10, 32), and the results we present here for the GPI-anchored "gp160" are consistent with both this domain and the membrane-spanning anchor domain being dispensable for efficient intracellular transport.

We have assessed the functionality of the chimeric GPI-anchored glycoproteins by assaying their abilities to mediate both cell-cell and virus-cell fusion. First, the GPI-anchored glycoproteins were assayed for their abilities to mediate syncytium formation in COS-1-HeLa-T4 cell mixtures. No syncytia were seen with any of the constructs when they were expressed in COS-1 cells that were then mixed with the permissive HeLa-T4 cells. Some mutations in gp160 that affect membrane fusion events were shown by Helseth et al. to affect syncytium formation more dramatically than virus entry (11). Therefore, we used the same pHXBΔenvCAT complementation system of Helseth et al. (11) to assay for virus entry mediated by the chimeric glycoproteins. The GPI-anchored glycoproteins proved to be completely defective in mediating virus-cell fusion in this assay, yielding acetylation values essentially identical to those obtained with the pHXBΔenvCAT vector alone. Thus, the GPI-anchored glycoproteins appeared to be completely nonfusogenic. When we assayed the efficiency with which GPI-anchored glycoprotein was incorporated into virions, we found the HTC-1 glycoprotein to be incorporated into virions at least as efficiently as wild-type glycoprotein. Therefore, the 18- to 26-fold lower acetylation values for the GPI-anchored glycoproteins as compared with wild-type glycoprotein in the complementation assay could not be due to a low efficiency of incorporation into virions for these proteins.

The basis for the lack of HT-1 and HT-2 glycoprotein fusogenicity is not clear at present. While HT-1 lacks 18 amino acids from the putative extracellular domain of the HIV-1 glycoprotein which could be involved in the fusion event, this domain is intact in HT-2. In addition, preliminary experiments have demonstrated that both of these GPI-anchored glycoproteins are oligomeric (data not shown), suggesting that the conformation of the extracellular domain may not be significantly affected by the addition of the Thy-1.1

signal or the GPI anchor. Since previous studies have demonstrated that the cytoplasmic domain of gp41 is dispensable for intracellular transport of gp160 and for syncytium formation, it is possible that the membrane-spanning domain itself plays a critical role in membrane fusion. Indeed, Helseth et al. showed that a mutation of lysine 683 to an isoleucine residue inhibited both syncytium formation and complementation of pHXBΔenvCAT (12). Alternatively, the GPI anchor may not provide a sufficiently stable membrane association for the HIV-1 glycoprotein to function in bringing the two membranes sufficiently close together for membrane fusion to be initiated. It is clear from the pulse-chase experiments that the HT-1 and HTC-1 glycoproteins are shed into the medium of expressing cells at quite high levels. While the HT-2 and HTC-2 proteins are more stable, they also appear to be shed from the membrane more readily than the wild-type anchored protein, which cannot be detected in the culture medium. However, this "shedding" could be due to glycoprotein release by a contaminating phospholipase in the culture medium containing fetal bovine serum.

It is also possible that the 22 amino acid residues and additional N-glycosylation site from Thy-1.1 which remain fused to the extracellular domain of the HIV-1 glycoprotein following addition of the GPI anchor, and/or the GPI anchor itself, may interfere with the fusion event. This interference could occur by blocking a conformational change that presumably must occur during fusion, by steric hindrance, or by positioning the glycoprotein complex too far outside the membrane bilayer, since there may be spatial constraints involved in bringing the two lipid bilayers together or since the fusion peptide may need to interact directly with the host membrane. Experiments are currently under way to address this issue by constructing analogous GPI-anchored HIV-1 glycoproteins which utilize a recently described minimal GPI attachment signal (19, 24, 25), thus eliminating any exogenous amino acid sequences.

While the GPI-anchored glycoproteins described above are nonfusogenic, we anticipate that these constructions will be useful in the production of quantities of a soluble

form of HIV-1 glycoprotein suitable for structural analyses. Preliminary experiments have demonstrated that the GPI-anchored glycoprotein forms oligomers with sizes comparable to those of the full-length glycoprotein and that these oligomers can be detected in the culture supernatant after treatment with PI-PLC (data not shown). These constructs should be particularly useful in obtaining pure glycoprotein because they allow the molecule to be specifically cleaved from the cell surface by PI-PLC.

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EXPRESSION AND CHARACTERIZATION OF PRIMATE LENTIVIRAL ENVELOPE GLYCOPROTEINS CONTAINING A MINIMAL GPI-ADDITION SIGNAL

by

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ABSTRACT

Previously we described the expression of chimeric glycosyl-phosphatidylinositol (GPI)-anchored glycoproteins containing the ectodomain of the the HXB2 strain of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein fused to 22 amino acid residues from the murine Thy-1.1 glycoprotein. These proteins did not retain the ability to mediate membrane fusion. To determine whether the Thy-1.1 sequence was responsible for this lack of fusogenicity, we describe here the expression of analogous constructs with a minimal GPI-addition signal which lacks these residues. In addition, we expressed HIV-1/YU-2 and SIV/mac239 envelope glycoprotein constructs using the same signal. The minimal signal was functional when attached to the full-length SIV glycoprotein ectodomain; whereas, truncation of the HIV-1 glycoprotein was required for recognition. All of the GPI-anchored glycoproteins were found to be incapable of mediating complete fusion, further demonstrating that the function of glycoproteins from diverse primate lentiviruses requires a membrane-spanning peptide anchor.

INTRODUCTION

Glycosyl-phosphatidylinositol (GPI)-anchored forms of several viral glycoproteins have now been constructed and characterized (2, 5, 8, 11, 16, 19, 21). We previously used a 53-amino acid C-terminal peptide of the murine Thy-1 glycoprotein to supply the signal necessary for GPI attachment in constructing GPI-anchored human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins (19). The envelope glycoprotein of HIV-1 mediates attachment of the virus to the target cell surface via an interaction with CD4 and subsequently induces virus entry via a membrane fusion event between the viral envelope and the plasma membrane (reviewed in 20). Processing of the GPI-addition signal and attachment of the GPI moiety resulted in a chimeric HIV-1 glycoprotein ectodomain which retained at its C-terminus 22 amino acids from Thy-1.1. In addition, this 22-amino acid sequence included a potential site for N-glycosylation. Another group later reported the expression of GPI-anchored HIV-1 envelope glycoprotein

by using a C-terminal peptide from the human decay accelerating factor (DAF) glycoprotein to provide the signal for GPI attachment (21). Using this peptide, only nine amino acids from the DAF sequence remained fused to the ectodomain of the glycoprotein following GPI addition. In both of these reports, the GPI-anchored glycoproteins were incapable of mediating membrane fusion in a cell-cell fusion (syncytium-forming) assay. In addition, we demonstrated that the HIV-1/Thy-1.1-chimeric glycoproteins were also unable to mediate virus entry, despite being incorporated efficiently into virions.

It is possible that the 22-amino acid Thy-1.1 peptide and the nine-amino-acid DAF peptide interfered with the ability of the HIV-1 envelope glycoprotein to induce membrane fusion. Interference could be due to an alteration of the normal structure of the glycoprotein ectodomain. Indeed, differences in the carbohydrate modifications and receptor-binding ability of a GPI-anchored ectodomain of the hemagglutinin (HA) glycoprotein of influenza virus as compared to the wild-type protein have been observed when the DAF peptide was used for a GPI addition signal (5). However, the GPI-anchored HIV-1 envelope glycoproteins did oligomerize, were processed normally, and retained the ability to bind the CD4 receptor, suggesting that there were no major structural aberrations. Alternatively, the peptides could interfere sterically with a conformational change required during fusion. Such conformational changes have been demonstrated for the HIV-1 envelope glycoprotein following binding to the CD4 receptor (20). Finally, since the peptides are not derived from membrane fusion proteins and contain polar amino acids, they could disrupt the flow of lipids between membranes or the formation of a fusion pore which has been proposed to occur during membrane fusion mediated by myxovirus glycoproteins (22).

Following synthesis in the presence of an inhibitor of carbohydrate side-chain processing, GPI-anchored influenza virus HA has been shown to initiate "hemifusion," or the intermixing of lipids between bilayers in the absence of the mixing of the soluble contents of each membrane (4, 13). Thus, the presence of the DAF peptide in this protein

did not interfere with the initiation of fusion by this protein. It was further demonstrated that the same peptide in the context of the wild-type glycoprotein did not interfere with the ability of the protein to complete the fusion reaction (4). The lack of complete fusion of the GPI-anchored molecule was, therefore, concluded to be due to the lack of a membrane-spanning peptide anchor. However, we have found that the insertion of the same DAF peptide into the analogous position of the HIV-1 envelope glycoprotein abrogated both cell-cell fusion and virus entry, thus demonstrating that this peptide can interfere with membrane fusion in the context of the HIV-1 protein (18).

MATERIALS AND METHODS

The details of protein expression and characterization are essentially as described previously (19) unless otherwise noted. The constructs were expressed by transient DEAE-dextran-mediated transfection of SV40-based plasmids into COS-1 cells. Cells expressing the constructs were metabolically radiolabeled with [^{35}S]cysteine-[^{35}S]methionine for 30 min and chased in complete medium for 3 h. Following the chase, the culture supernatants were collected, and the adherent cells were either treated with phosphatidylinositol-specific phospholipase C (PI-PLC) (0.5 U/ml) for 1 h at 37°C or mock treated. Following incubation, the cell supernatants were collected, and the cells were lysed. Proteins were immunoprecipitated from all samples for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography.

RESULTS

Construction of minimal GPI-anchored HIV-1 envelope glycoproteins. In order to determine whether the lack of fusion exhibited by previous GPI-anchored HIV-1 glycoproteins was due solely to interference by the Thy-1.1 and DAF peptides and whether the elimination of these peptides from the protein ectodomain might be sufficient to permit fusion, we have constructed analogous HIV-1 glycoproteins which lack these nonviral sequences by using a minimal GPI-addition signal. The minimal sequence requirement for the GPI-addition signal in DAF has been described and consists

simply of two small amino acids spaced 10 to 12 amino acids from a C-terminal hydrophobic anchor sequence approximately 20 amino acids in length (9, 14, 15). We, thus, used the polymerase chain reaction (PCR) to amplify the analogous minimal coding sequence for Thy-1.1, incorporating convenient restriction sites into the primers to permit substitution of the fragment into our original HIV-1/Thy-1.1 chimeric constructs in place of the coding sequence for the longer 53-amino acid Thy-1.1 peptide (see Fig. 1). The newly generated constructs encoded either the full-length ectodomain of the HIV-1 envelope glycoprotein (HG-2) or an ectodomain lacking the 18 C-terminal amino acids adjacent to the membrane-spanning domain in the wild-type protein (HG-1), both linked to the minimal GPI-attachment site. The two different sites for the insertion of the GPI-addition signal were available, because at the time that the original work was initiated the N-terminal boundary of the membrane-spanning domain was not clearly defined. In addition, analogous constructs (HGC-1 and HGC-2) were generated that contained mutations in the proteolytic cleavage site of the envelope glycoprotein precursor, gp160. These constructs served as controls, since proteolytic processing is required for the function of the glycoprotein (12).

Expression and PI-PLC analysis of minimal GPI constructs. As can be seen in Fig. 2, HG-1 was efficiently processed and could be released from the cell surface with PI-PLC, as evidenced by the appearance of unprocessed glycoprotein precursor in the supernatant upon treatment with the enzyme. As with the wild-type cultures, both mock- and PI-PLC-treated HG-1 cultures contained gp120 in the media, due to spontaneous shedding as a result of the labile association of the two glycoprotein subunits. However, PI-PLC treatment resulted in an increase in the amount of gp120 in the media, consistent with the specific release of mature, processed glycoprotein from the cell surface.

Unlike HG-1, HG-2 was not processed efficiently and neither gp120 nor unprocessed precursor could be detected in the cell culture supernatants, even after a 3-h

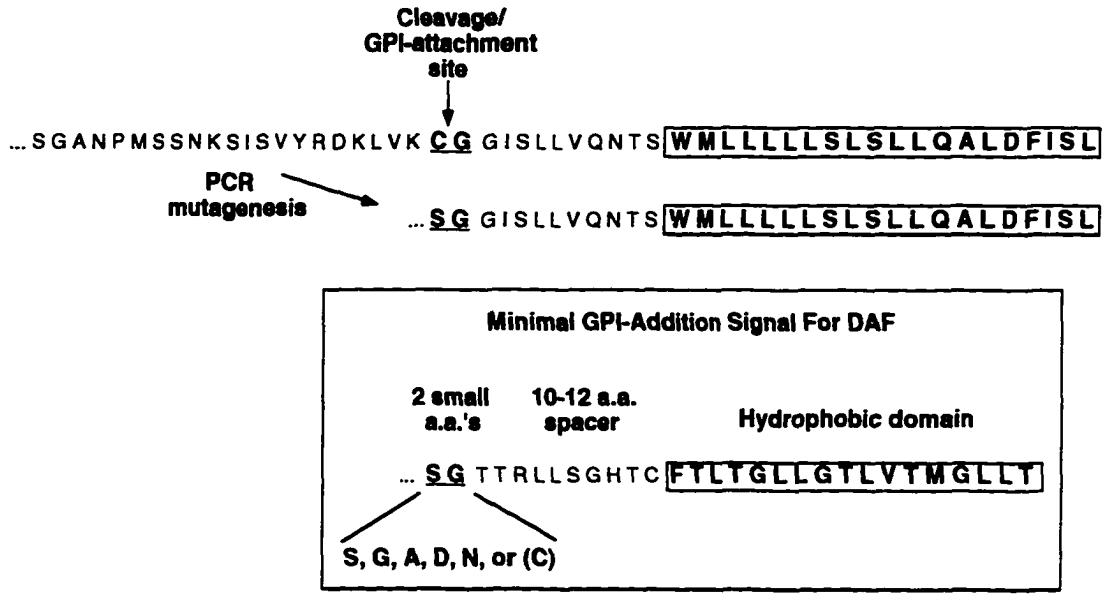
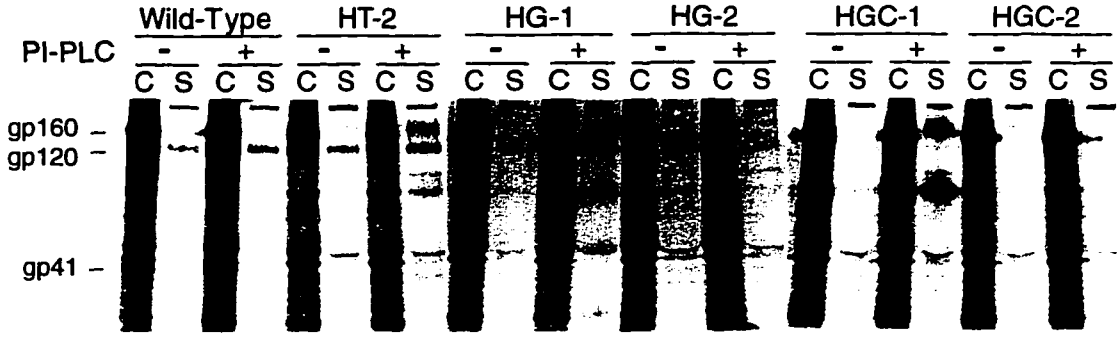


FIG. 1. Generation of a minimal signal for GPI-addition. A 53-amino acid C-terminal peptide from murine Thy-1.1 (shown at top) was reduced to a 32-residue signal sequence (bottom) by PCR amplification of the corresponding coding sequence. The cysteine residue to which the GPI anchor is attached in Thy-1.1 was converted to a serine residue to mimic the attachment site in DAF. Following processing of the signal, the glycoprotein ectodomain to which it is fused will retain the C-terminal serine residue, covalently linked to the GPI-moiety.

FIG. 2. PI-PLC release of glycoprotein ectodomains. COS-1 cells transiently expressing mutant HIV-1 envelope glycoproteins from an SV40-based plasmid expression vector were metabolically radiolabeled and treated after a 3-h chase with 0.5 U/ml PI-PLC for 1 h. Parallel cultures were mock treated. Supernatants and cell lysates were collected and immunoprecipitated for analysis by SDS-PAGE (8%) and detection of proteins by fluorography. HT-2 has been described previously.

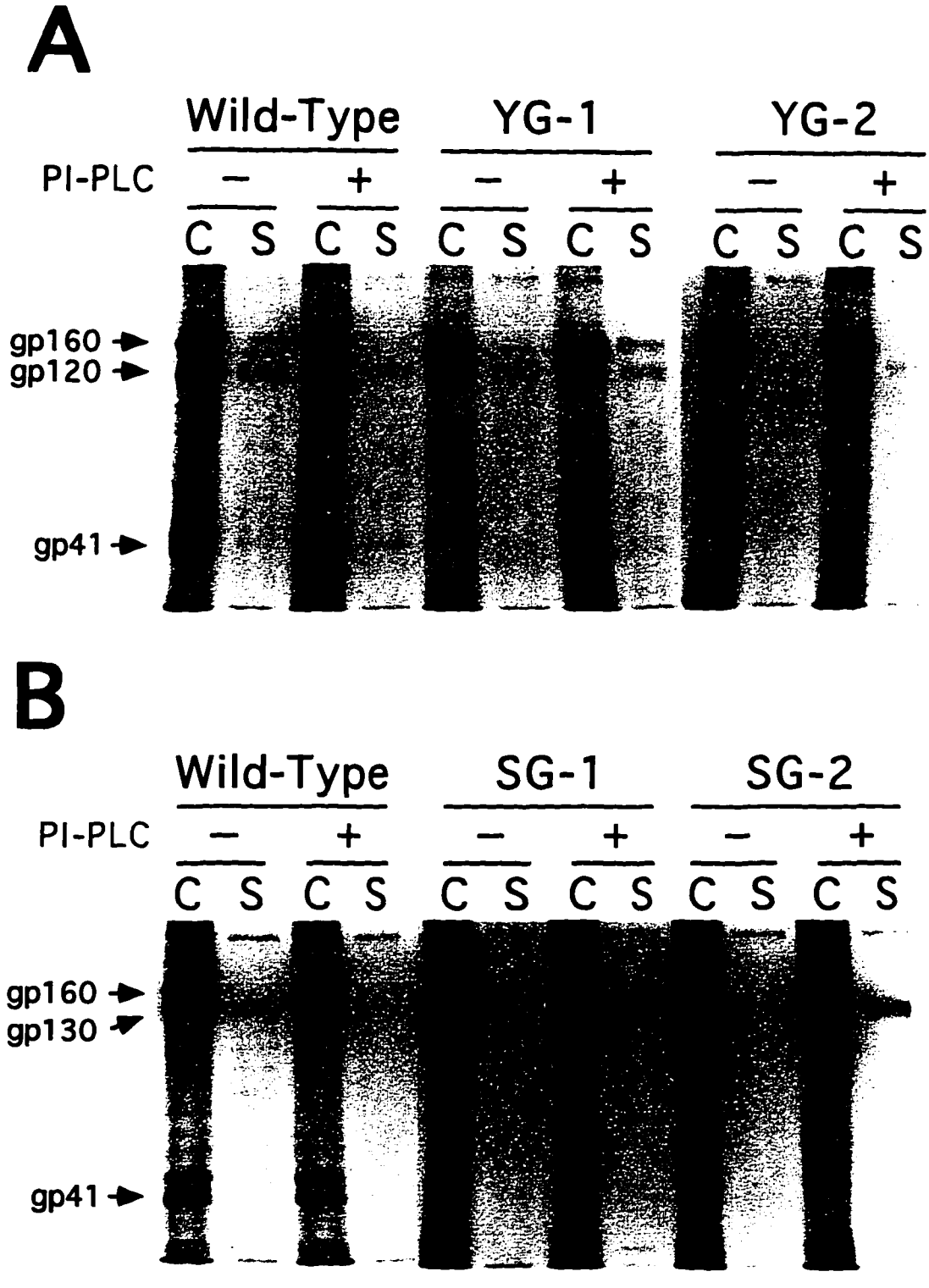


chase. Similarly, HGC-1, but not HGC-2, was efficiently released into the supernatant upon PI-PLC treatment. Therefore, the 18 amino acids at the C-terminus of the HIV-1 glycoprotein ectodomain which are present in HG-2 and HGC-2, but not in HG-1 and HGC-1, somehow interfere with the processing and transport of these proteins. Since this region contains several hydrophobic residues, including five highly conserved tryptophans, and is immediately adjacent to the cleavage site for GPI attachment, the most likely explanation is that these residues interfere with the accessibility of the GPI addition signal. Since the wild-type Thy-1.1 protein is not transported unless the GPI anchor is added, lack of GPI addition to the chimeric proteins would also be expected to prevent transport (10).

Generation of minimal GPI-anchored HIV-1/YU-2 and SIV envelope glycoproteins. Unfortunately, we have found that the deletion of these same 18 residues from the wild-type glycoprotein abrogates the function of the protein (18). Therefore, the GPI-anchored HG-1 protein was not a desirable candidate for testing the fusogenicity of minimal GPI-anchored envelope glycoprotein because it would be expected to be nonfusogenic. In order to determine whether the interference of GPI attachment was specific for the HXB2 strain of HIV-1 or was a feature of HIV-1 envelope glycoproteins in general, the minimal GPI-addition signal was substituted into the sequence of the envelope glycoprotein of the YU-2 strain of HIV-1. Unlike the lab-adapted strain, HXB2, the molecular clone of YU-2 was derived directly from a clinical isolate without passage in vitro and replicates in macrophages as opposed to T-cell lines (7). As can be seen in Fig. 3, the resulting proteins, YG-1 and YG-2, behaved identically to their respective counterparts, HG-1 and HG-2. Therefore, the interference of the 18 C-terminal residues of the HIV-1 glycoprotein ectodomain with GPI addition appeared to be unavoidable.

Since this 18-amino-acid stretch of the HIV-1 glycoprotein is relatively well conserved among different strains of HIV-1 but differs significantly from the analogous peptide in SIV, we fused the minimal GPI addition signal to the glycoprotein ectodomain of the mac239 strain of SIV. The SIV peptide is less hydrophobic than the HIV-1 peptide,

FIG. 3. PI-PLC release of HIV-1/YU-2 and SIV/mac239 envelope glycoproteins. Cells were treated as described in the legend for Fig. 2. (A) Wild-Type, wild-type HIV-1/YU-2 envelope glycoprotein. (B) Wild-Type, wild-type SIV/mac239 envelope glycoprotein.



containing two aspartic acid residues absent in HIV-1 and two less tryptophan residues. As can be seen in Fig. 3, the resulting proteins, SG-1 and SG-2, were both efficiently processed, and a similar amount of gp130 as compared to wild-type SIV glycoprotein could be detected in the supernatants of mock-treated cells expressing both proteins. Moreover, a significant increase in the amount of gp130 in the culture supernatants was seen for both constructs upon treatment with PI-PLC, indicating that the C-terminus of the complete SIV glycoprotein ectodomain did not interfere with GPI addition.

While the cell-associated, unprocessed precursors of these SIV ectodomains migrated closely to gp130 on the gel, a larger, terminally glycosylated form of the precursor was clearly evident in the supernatant of cells expressing SG-1. The proportion of precursor protein released by PI-PLC relative to gp130 was much less for SG-1 than for either HG-1 or YG-1, suggesting more efficient processing of the SIV protein. The amount of precursor released by PI-PLC was also much greater for SG-1 than for SG-2, suggesting that the processing of the truncated ectodomain was more efficient than that of the full-length ectodomain. Alternatively, the C-terminus of the full-length ectodomain might be less efficient at directing GPI attachment, such that less GPI-linked SG-2 protein is transported throught the Golgi and, consequently, less SG-2 protein escapes processing. Interestingly, a similar difference was seen previously in the processing of GPI-anchored, truncated and full-length HIV-1 glycoprotein ectodomain/Thy-1.1 chimeras (19), suggesting that the negative effect of the envelope glycoprotein C-terminus on GPI addition is not dependent on its proximity to the site of GPI attachment.

Fusogenicity of minimal GPI-anchored constructs. Since the effect of truncation of the ectodomain on the fusogenicity of the wild-type SIV envelope glycoprotein is not known, the full-length GPI-anchored ectodomain of SG-2, lacking any nonviral sequence, is the most appropriate candidate generated thus far with which to assay the fusogenicity of a GPI-anchored lentiviral glycoprotein. Cell-cell fusion was assayed by mixing glycoprotein-expressing COS-1 cells with either HeLa-CD4/LTR- ß-gal (MAGI)

indicator cells (6) (HIV-1 glycoproteins) or CMMT-CD4/LTR-ß-gal (sMAGI) indicator cells (contributed by Julie Overbaugh, University of Washington) (SIV glycoproteins) at a ratio of approximately 1:10. Since the glycoprotein expression vectors also express the viral Tat transcriptional activator protein, fusion of the COS-1 cells with the indicator cells induces expression of nuclear-targeted ß-galactosidase from the viral LTR promotor. Thus cell-cell fusion can be scored microscopically by staining the cultures in situ with X-gal. This assay is more specific than those used previously to assay GPI-anchored HIV-1 glycoproteins and is capable of detecting even rare fusion events between as few as two cells (data not shown). The results of these assays are summarized in Tables 1 and 2. The YU-2 glycoproteins are not included because wild-type YU-2 does not fuse MAGI cells (data not shown). None of the GPI-anchored glycoproteins, including SG-2, were able to mediate any level of cell-cell fusion in this highly sensitive assay.

Virus entry was also assayed for the processed, GPI-anchored glycoproteins by coexpressing them either in COS-1 cells with an *env*-defective provirus, pHXBΔenvCAT, derived from the HXB2 strain of HIV-1 (3) (HIV glycoproteins) or in 293T cells with an *env*-defective provirus derived from the NL4-3 strain of HIV-1 (SIV glycoproteins). Cell supernatants containing virus were filtered through a 0.45-µm filter and normalized for reverse transcriptase activity as previously described (1). Normalized supernatants were then used to infect the appropriate indicator cell line (MAGI or sMAGI) as described by the contributors. The results of these assays are shown in Tables 1 and 2. As predicted by the cell-cell fuson assays, none of the processed, GPI-anchored glycoproteins, including SG-2, were capable of mediating any detectable virus entry in what is, again, a very sensitive assay.

Binding of minimal GPI-anchored SIV envelope glycoprotein to CD4. Previously generated GPI-anchored HIV-1 glycoproteins have been shown to efficiently bind CD4 (17, 21). To confirm that the same was true for the GPI-anchored SIV glycoproteins, each was immunoprecipitated with CD4-IgG in the absence of sodium

TABLE 1. Fusogenicity of minimal GPI-anchored HIV-1 glycoproteins

Construct	Syncytium formation		Virus entry
	No. of syncytia/ well[a]	No. of nuclei/ syncytium[b]	No. of blue cells/ well[c]
Mock	0	-	7
pHXBΔenvCAT	ND[d]	ND	8
Wild-type	2.2×10^3	49	183
ΔSX[e]	7.7×10^2	35	ND
HG-1	0	-	15
HG-2	0	-	ND
HGC-1	0	-	ND
HGC-2	0	-	ND

[a] Determined as the average number of blue syncytia per microscopic field (15 nonoverlapping, random fields counted) multiplied by the number of fields per tissue culture plate (determined by using a grid).

[b] Determined as the average from 25 syncytia per construct.

[c] Determined as the average from duplicate wells.

[d] Not determined.

[e] ΔSX encodes the wild-type glycoprotein, but does not express Rev. HG-1, HG-2, HGC-1, and HGC-2 also do not express Rev. The expression vector for these constructs contains a constitutive transport element which complements Rev function.

TABLE 2. Fusogenicity of minimal GPI-anchored SIV glycoproteins

Construct	Syncytium formation		Virus entry
	No. of syncytia/ well[a]	No. of nuclei/ syncytium[b]	No. of blue cells/ field[c]
Mock	0	-	5
pNL4-3Δenv	ND[d]	-	5
Wild-type	2.1×10^3	12	20
SG-1	0	-	3
SG-2	0	-	4

[a] Determined as the average number of blue syncytia per microscopic field (15 nonoverlapping, random fields counted) multiplied by the number of fields per tissue culture plate (determined by using a grid).

[b] Determined as the average from 25 syncytia.

[c] Determined as the average from 15 nonoverlapping, random fields in each of two duplicate wells.

[d] Not determined.

dodecyl sulfate in parallel with serum from SIV-infected monkeys. Fig. 4 demonstrates that both of the GPI-anchored SIV glycoproteins efficiently bound CD4-IgG, indicating that the lack of fusion seen with these proteins was not due to an inability to bind the CD4 receptor.

DISCUSSION

In conclusion, the lack of fusion seen with the SG-2 protein demonstrates for the first time that complete primate lentiviral envelope glycoprotein ectodomains, lacking any nonviral sequences, that are anchored in the membrane via a GPI moiety are incapable of mediating either cell-cell fusion or virus entry. Therefore, the block in fusion seen with this construct is due solely to the GPI moiety. Since hemifusion has been observed with a GPI-anchored influenza HA (4, 13), it will be interesting in the future to determine whether the SG-2 protein can also induce hemifusion.

ACKNOWLEDGMENTS

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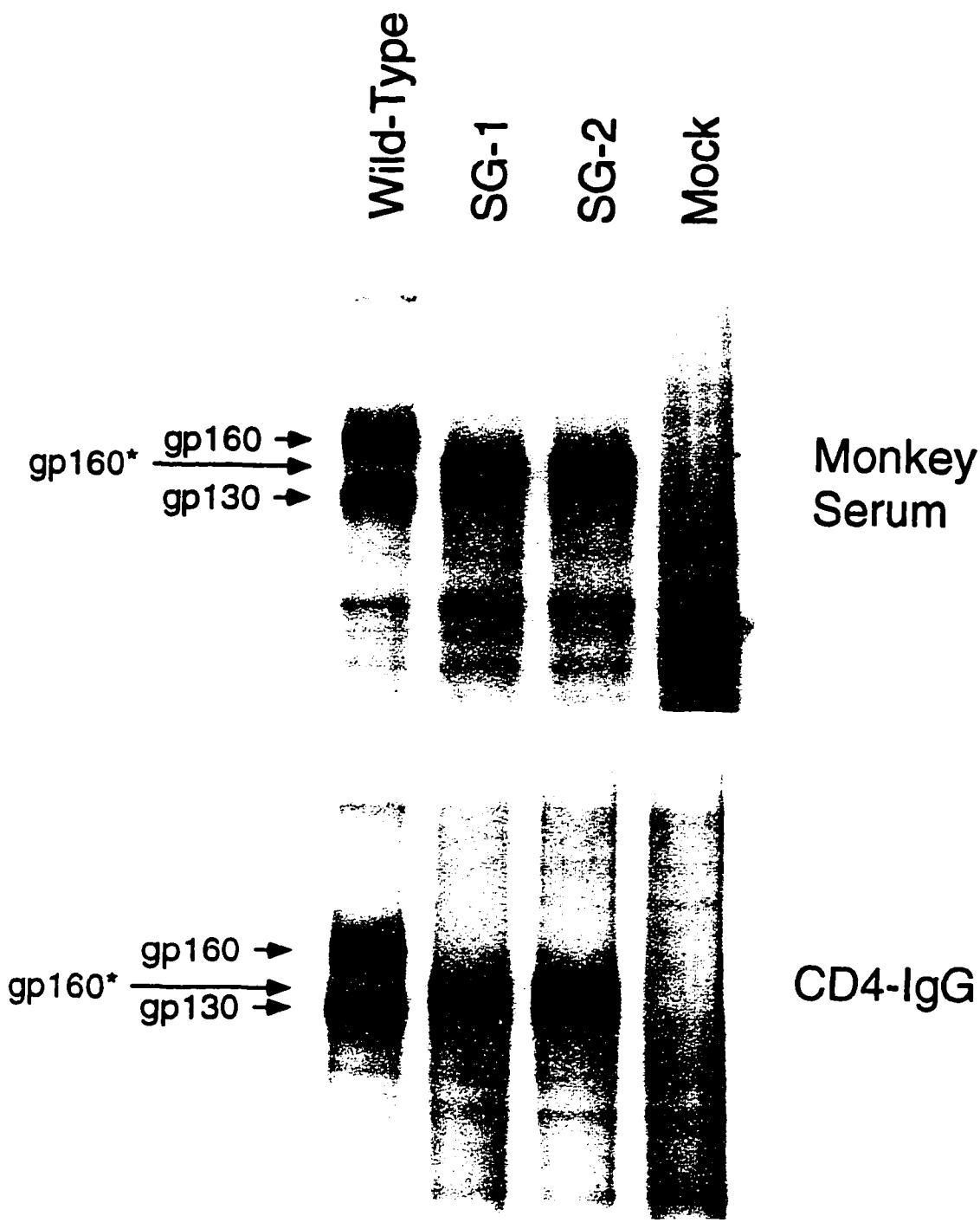
FIG. 4. CD4 binding ability of GPI-anchored SIV glycoprotein ectodomains. Cell lysates were split into two tubes and immunoprecipitated with either serum from an infected monkey (Monkey Serum) or 0.5 μg/ml CD4-IgG (CD4-IgG). gp160* indicates the unproteolytically processed GPI-anchored glycoprotein product.



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A CONSERVED TRYPTOPHAN-RICH MOTIF IN THE MEMBRANE-PROXIMAL REGION OF THE HUMAN IMMUNODEFICIENCY VIRUS TYPE I gp41 ECTODOMAIN IS IMPORTANT FOR VIRUS ENTRY

by

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ABSTRACT

Mutations were introduced into the ectodomain of the human immunodeficiency virus type 1 (HIV-1) transmembrane envelope glycoprotein, gp41, within a region immediately adjacent to the membrane-spanning domain. This region, which is predicted to form an α-helix, contains highly conserved hydrophobic residues and is unusually rich in tryptophan residues. In addition, this domain overlaps the epitope of a neutralizing monoclonal antibody, 2F5, as well as the sequence corresponding to a peptide, DP-178, shown to potently neutralize virus. Site-directed mutagenesis was used to create deletions, substitutions, and insertions centered around a stretch of 17 hydrophobic and uncharged amino acids (residues 666-682 of the HXB2 strain of HIV-1) in order to determine the role of this region in the maturation and function of the envelope glycoprotein. Deletion of the entire stretch of 17 amino acids abrogated the ability of the envelope glycoprotein to mediate both cell-cell fusion and virus entry without affecting the normal maturation, transport, or CD4-binding ability of the protein. This phenotype was also demonstrated by substituting alanine residues for three of the five tryptophan residues within this sequence. Smaller deletions, as well as multiple amino acid substitutions, were also found to inhibit but not block cell-cell fusion. Surprisingly, even the substitution of phenylalanine for a single tryptophan residue was sufficient to reduce the efficiency of virus entry approximately 10-fold, despite the fact that the same mutation had no significant effect on syncytium formation. Finally, the insertion of a nine-amino-acid peptide from the human decay accelerating factor (DAF) between this tryptophan-rich region and the membrane-spanning domain also abrogated fusion, despite the fact that the same peptide inserted into the analogous position of the influenza virus hemagglutinin (HA), which lacks a similar tryptophan-rich region, failed to inhibit membrane fusion. These results demonstrate the crucial role of a tryptophan-rich motif in gp41 during a post-CD4-binding step of virus entry, possibly involving an interaction with the viral membrane.

INTRODUCTION

The process of viral entry is a key step in the initiation of HIV-1 infection. Attachment of the HIV-1 virion to the target cell is mediated by binding of the viral envelope glycoprotein complex to the CD4 receptor on the cell surface (reviewed in reference 42). The envelope glycoprotein is processed from an oligomeric precursor protein, gp160, into two noncovalently linked subunits, the surface subunit, gp120, which is responsible for binding to CD4, and the transmembrane subunit, gp41, which initiates membrane fusion. In addition to CD4, HIV-1 has also been shown to require an interaction between gp120 and a coreceptor, members of a family of seven-transmembrane G-protein-coupled chemokine receptors, on the cell surface in order to mediate membrane fusion (13). Many viruses, such as the influenza virus, are endocytosed following binding to their receptor (27). Their viral glycoproteins then undergo a pH-activated conformational change to a fusion-competent form in the acidic environment of the endosome (8, 18). HIV, however, fuses directly with the plasma membrane at the surface of the target cell in a pH-independent (31, 32, 46). In the case of HIV, it has been shown that binding to CD4 induces conformational changes in gp120 and increases the exposure of gp41 epitopes independent of gp120 dissociation (41, 43, 44). Thus, it has been proposed that the envelope glycoprotein of HIV undergoes receptor-induced fusion activation analogous to the pH-induced activation of other viral glycoproteins.

The amino-terminal fusion peptide of gp41 was originally identified by its similarity to functional domains of paramyxovirus glycoproteins (17) and has since been extensively characterized through mutagenesis as the primary fusion domain (1, 14, 45). Based on analogy to similar rearrangements in the glycoproteins of influenza virus (8) and Rous sarcoma virus (19, 22), the CD4-induced conformational change in the HIV-1 glycoprotein is believed ultimately to lead to the insertion of the fusion peptide into the target cell membrane. The details of this conformational change and how the gp120-coreceptor interaction contributes to it is unclear. However, recent studies have suggested that

domains of gp41 may also be involved in a conformational change leading to fusion. Mutagenesis of a heptad repeat, leucine zipper-like motif in the ectodomain of gp41 demonstrated that the hydrophobicity of position 573 in the center of the repeat dictated the efficiency with which gp41 mediated fusion (5, 6, 11). It has been hypothesized, based on peptide studies (46, 48), that this motif is involved in forming a coiled-coil structure in the glycoprotein oligomer, similar to that formed by an acid-activated fragment of HA (2), following receptor binding. Two peptides, DP-178 and SJ-2176, derived from this membrane-proximal region of gp41 have been shown to potently inhibit HIV-mediated fusion (23, 54). Conflicting evidence exists, however, for the interaction of these peptides with either the fusion peptide or heptad-repeat regions of the glycoprotein (24, 35, 52). A neutralizing human monoclonal antibody, 2F5, has also been described whose epitope overlaps the C-terminus of the DP-178 peptide (33, 34). Interestingly, this epitope has been shown to become inaccessible following CD4-binding, suggesting that this region may undergo a change in conformation (44).

It is unknown how the envelope glycoprotein overcomes the hydration forces and charge repulsion separating the viral envelope from the plasma membrane of the target cell to cause membrane fusion. Studies of the interaction of a synthetic fusion peptide with membranes suggest that the fusion peptide inserts into the membrane at an oblique angle and may disrupt the bilayer by perturbing the packing of phospholipid molecules (27, 29). In addition, evidence from sudies with the hemagglutinin of influenza virus suggest that the formation of a higher order glycoprotein oligomer or fusion pore may be involved (50).

While it has been shown that the cytoplasmic tail of gp41 is not required for fusion (12, 16, 55, 58), we and others have shown that the fusion event does require that gp41 contain a membrane-spanning peptide anchor, since substitution of a covalently linked lipid anchor for the membrane-spanning domain of gp41 did not support fusion (39, 49). The structural requirements of this membrane-spanning domain, however, are less clear. Truncation of the gp41carboxy-terminus beyond the lysine residue at postion 683 (HXB2

strain) is sufficient to cause a loss of membrane anchoring and secretion of the glycoprotein in a soluble form (12, 37), whereas truncation to the arginine at position 696 results in a stably anchored protein that is expressed on the cell surface (37), suggesting that the minimal membrane-spanning domain could be residues 684-695. However, this truncated glycoprotein is nonfunctional, and residues C-terminal to this minimal anchor domain are important for the function of the glycoprotein (37). These results suggest that while the glycoprotein can be forced to use an abreviated membrane-spanning domain, such a domain is not sufficient for the completion of the fusion reaction. Therefore, the functional membrane-spanning domain in the context of the full-length protein most likely consists approximately of residues 684-706.

The sequence requirements of the membrane-spanning domain appear to be flexible, since the transmembrane domains of both CD4 (48) and CD22 (56) are sufficient for the function of the glycoprotein. Interestingly, however, substitution of a hydrophobic residue for the arginine at position 696 of the HIV-1 glycoprotein has been reported to abrogate fusion (37). This charged residue is hypothesized to reside within the membrane-spanning domain, with its charge perhaps masked in the context of the oligomeric structure. Since the CD4 and CD22 transmembrane domains lack a central charged residue, this requirement appears to be specific for the HIV-1 glycoprotein transmembrane sequence.

The studies described here focus on a membrane-proximal region in the ectodomain of gp41 that is unusually rich in tryptophan residues, several of which are conserved in both primate and ungulate lentiviruses (see Fig. 1A and B). This region overlaps a biologically important domain of gp41. In order to examine the role of the tryptophan-rich membrane-proximal domain of the HIV-1 gp41 ectodomain in the structure and function of the envelope glycoprotein, we have created deletion, substitution, and insertion mutations in this region by site-directed mutagenesis. Our analyses of the resulting mutant glycoproteins indicate that a stretch of 17 hydrophobic and uncharged amino acids

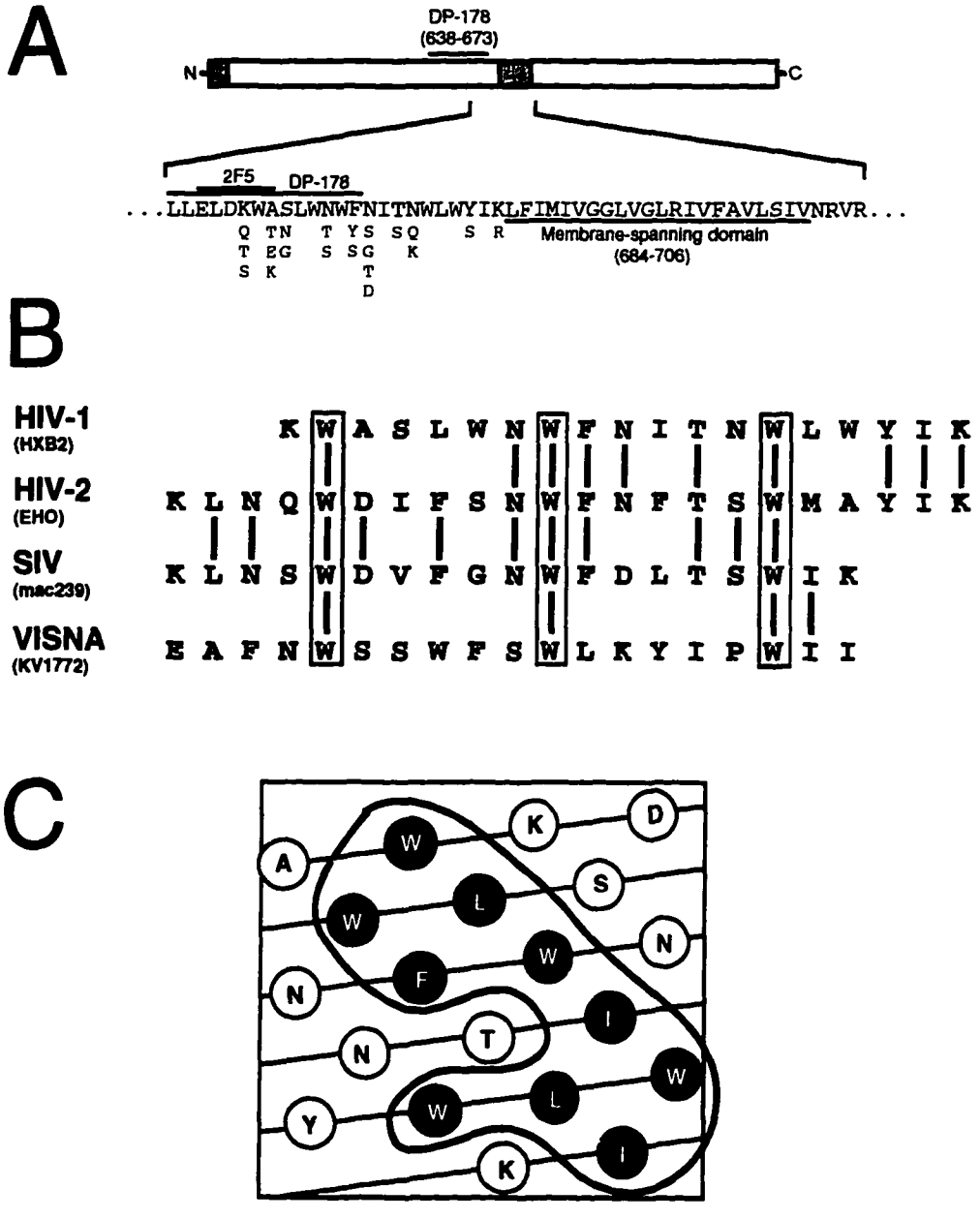


FIG. 1. Sequence conservation and predicted structure of the tryptophan-rich region. (A) Schematic representation of gp41, showing the location and sequence variability of the tryptophan-rich region and the overlapping sequences of the 2F5 epitope and DP-178 peptide. The amino acids in the HIV-1 sequence database which occur at each position are denoted underneath the respective amino acid in the HXB2 strain. (B) Alignment of the tryptophan-rich sequence of HIV-1/HXB2 gp41 with homologous regions of HIV-2, SIV, and VISNA glycoproteins. (C) Helical net projection of the tryptophan-rich region. The outlined region indicates the cluster of conserved hydrophobic residues shown in black.

immediately adjacent to the border of the membrane-spanning domain is dispensible for the normal maturation, transport, and CD4-binding ability of the protein. However, even subtle mutations affecting the conserved tryptophan residues had a profound effect on the ability of the glycoprotein to mediate virus entry. Features of the structure and function of this tryptophan-rich motif are discussed in relation to its possible function in the fusion mechanism.

MATERIALS AND METHODS

Cell culture. COS-1 and 293T cells were obtained from the American Type Culture Collection. HeLa-CD4/LTR-ß-gal cell lines were obtained through the AIDS Reference and Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, and were originally contributed by Michael Emerman. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum. HeLa-CD4/LTR-ß-gal cells were additionally maintained in medium containing Hygromycin and G418 (Geneticin) as recommended by the contributor.

Mutagenesis and construction of plasmids. Construction of the AK1 and AK2 mutations has been described previously (39). To create the AΔK1-K2 mutation, constructs containing either the AK1 or AK2 mutation were digested with AvaI, and the resulting fragments of the two constructs were ligated to one another in such a way as to delete the intervening sequence between the two mutations. The +FLAG and +DAF mutations were created by site-directed mutagenesis using the Altered Sites system from Promega.

To create simpler mutations in the tryptophan-rich region, an *env* expression plasmid was first modified by site-directed mutagenesis to encode two unique restriction enzyme sites within or adjacent to the coding sequence for the tryptophan-rich region. pSRHS, an SV40-based expression vector, was first digested with XbaI, filled in using the Klenow fragment of DNA polymerase to create a blunt end, and religated, destroying an XbaI site in the polylinker. An oligonucleotide was then designed which encoded both an

XbaI site and an NheI site for use in the Altered Sites system to create an expression plasmid, pSRHS-XUN, containing a unique XbaI site adjacent to and an NheI site within the coding sequence for the tryptophan-rich region while retaining the wild-type peptide sequence. The Altered Sites system was then used to destroy a second NheI site in the HXB2 env gene without affecting the peptide sequence. The final plasmid, pSRHS-XNU, and the corresponding env fragment served as the wild-type control used in this study.

A reverse PCR primer which overlapped a unique 3' BamHI site in the gp41 coding sequence was designed to amplify the noncoding strand. This primer was used in combination with mutagenic primers overlapping either the unique XbaI or NheI site to create XbaI-BamHI or NheI-BamHI fragments, respectively, which were then substituted into the pSRHS-XNU plasmid. Certain constructs containing multiple mutations were generated by using a primer encoding a secondary mutation to prime PCR from a previously mutated construct. Likewise, some mutated PCR products were substituted via the NheI and BamHI sites into constructs containing upstream mutations to create multiple mutations. A primer overlapping the unique XbaI site was used with the BamHI reverse primer to amplify the mutated fragment for insertion into pSRHS-XNU. All mutations were confirmed by DNA sequencing with a primer approximately 100 bp upstream of the coding sequence for the tryptophan-rich region to read from upstream of the mutagenic primer through the coding sequence of the membrane-spanning domain. The phenotypes of a majority of the mutated constructs were confirmed by comparison of two indepedent clones.

For construction of proviral clones, a partially digested NheI-BamHI HXB2 *env* fragment from the original pSRHS-XUN construct, containing the engineered XbaI and NheI sites, was substituted into the *env* gene of the pNL4-3 proviral clone. The resulting clone, pNL4-3/XUN, was used as a positive control. XbaI-BamHI fragments derived from pSRHS-XNU mutant env constructs were then substituted into pNL4-3/XUN. All proviral constructs were confirmed by sequencing as described above.

Protein expression and radioimmunoprecipitation. SV40-based *env* expression plasmids were transfected into COS-1 cells in 60 mm plates by using DEAE-dextran (1 mg/ml). At 36-48 h posttransfection, the cells were starved in methionine/cysteine-free DMEM for 20 min and pulse-labeled in methionine/cysteine-free DMEM supplemented with [^{35}S]methionine/cysteine (357 μCi/ml; Dupont-NEN) for 30 min. The labeled cells were then chased in complete DMEM for 3 h prior to harvest of the medium and lysis of the cells. Cells were lysed by a 10-min incubation on ice in lysis buffer. Cellular debris was removed by centrifugation in a microcentrifuge for 5 min at 4°C. HIV-1 glycoproteins were immunoprecipitated from the cell lysate (C) and medium (M) by incubation for 1 h at 4°C with AIDS patient serum. The immune complexes were incubated for 30 min at room temperature with fixed *Staphylococcus aureus* and pelleted in a microcentrifuge. The pellets were washed three times in lysis buffer containing 0.1% sodium dodecyl sulfate (SDS) and once in 20 mM Tris-HCl (pH 6.8) and were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

CD4 binding assay. Following pulse-chase labeling of transfected COS-1 cells as described above, cells were lysed in lysis buffer lacking SDS. The cell lysate was clarified by centrifugation and divided into two tubes. One half of the lysate was incubated with patient serum as described above. The other half was incubated overnight with 0.5 μg/ml CD4-IgG (a gift from Mark Mulligan, UAB) at 4°C. Both samples were immunoprecipitated using Staph A and analyzed as described above.

Cell surface biotinylation. Following pulse-chase radiolabeling as described above, transfected COS-1 cells were placed on ice in a cold room and washed three times with ice-cold PBS containing calcium and magnesium (PBS-C/M). The cells were then incubated with PBS-C/M containing 0.5 mg/ml NHS-SS-biotin for 30 min on ice. The reaction was quenched by incubation of the cells in ice-cold serum-free DMEM for 10 min on ice. The cells were then washed twice with ice-cold PBS-C/M containing 20 mM Glycine and lysed in lysis buffer containing 20 mM Glycine. Following

immunoprecipitation with patient serum as described above, the Staph A pellet was resuspended in PBS containing 2% SDS and boiled for 5 min. The sample was then diluted with PBS to a final concentration of 0.05% SDS and incubated with 40 µl of a 50% slurry of streptavidin-agarose beads (Pierce) at 4°C overnight. The beads were then pelleted, washed, and prepared for SDS-PAGE as described above for immunoprecipitates.

Cell-cell fusion assays. 24 h post-transfection, COS-1 cells in a six-well plate were trypsinized, mixed approximately 1:10 with untransfected HeLa-CD4/LTR-ß-gal indicator cells, and replated in a six-well plate. 24 h after replating, fusion of the two cell types was detected microscopically following staining in situ with X-gal as described previously (26).

Virus expression and pelleting. Virus expression was assayed by labeling COS-1 cells transfected with pNL4-3 proviral clones, followed by lysis, immunoprecipitation, and SDS-PAGE as described above for the analysis of glycoprotein expression.

For virus pelleting, 293T cells in 35-mm plates were transfected with pNL4-3 proviral clones by using a modified calcium phosphate technique (4). 48 h after adding DNA to the cells, the cells were metabolically radiolabeled as described above for 30 min and chased for 20 h in 1 ml of complete growth medium. Culture supernatants were then collected and filtered through a 0.45-µm filter to remove cellular debris. The filtered supernatants were then transferred to a microfuge tube, underlayed with a 20-µl sucrose cushion (10% sucrose in PBS), and centrifuged for 4 h at room temperature to pellet virions. The supernatant was then carefully removed, and the virus pellets were lysed for 10 min at room temperature prior to immunoprecipitation with patient serum as described above and SDS-PAGE.

Virus entry assay. 60-72 h after transfecting 293T cells as described for virus pelleting, culture supernatants were collected and filtered through a 0.45-µm filter. Relative levels of reverse transcriptase activity were determined for each sample, and the

volumes were normalized by dilution with complete medium. The normalized supernatants were used to infect HeLa-CD4/LTR-ß-gal cells in duplicate as described previously by the contributor. Supernatants were diluted as necessary to remain within the quantitatable range of the assay.

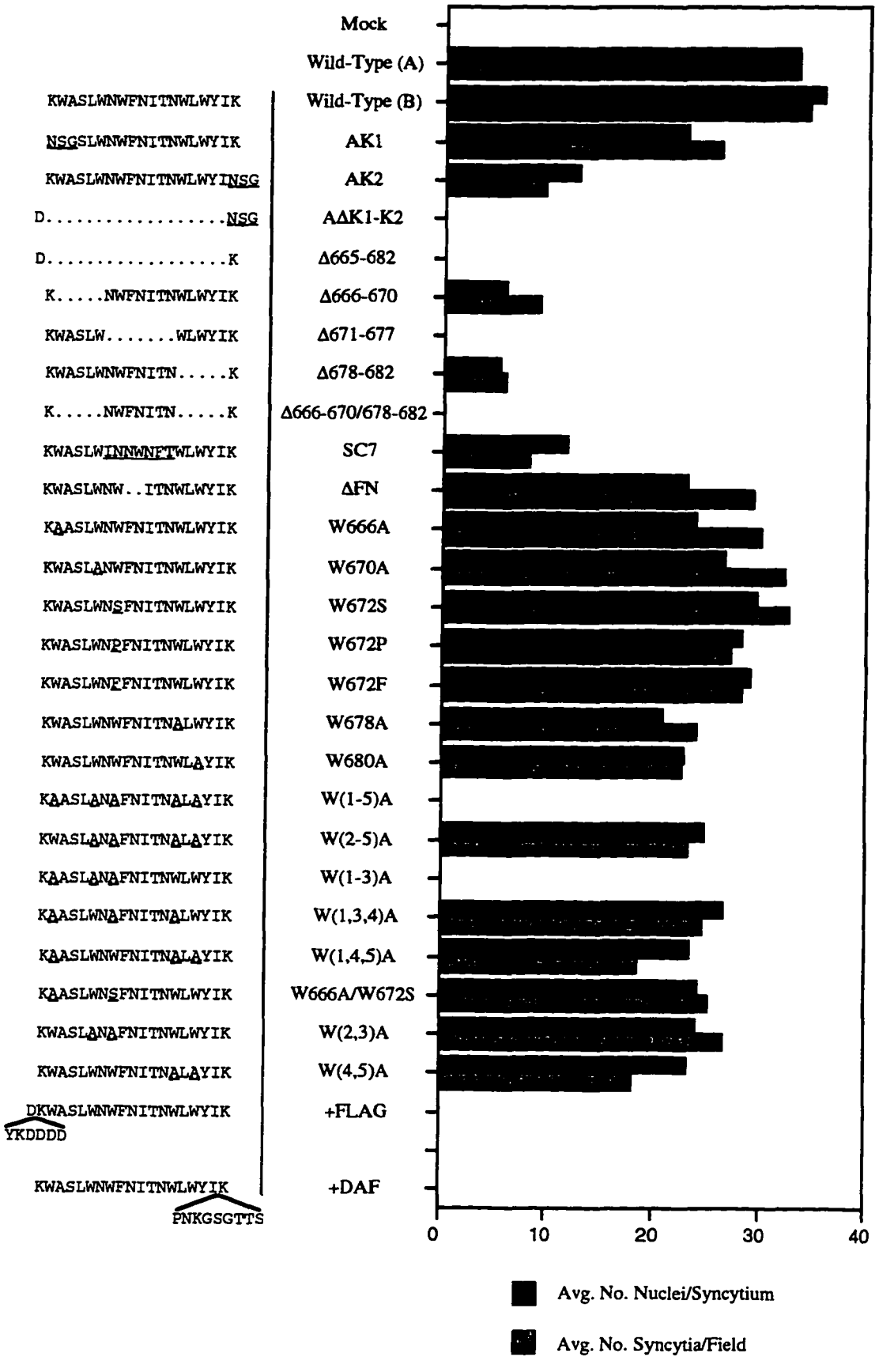
RESULTS

Mutagenesis of the membrane-proximal region of the HIV-1 transmembrane glycoprotein. To examine the functional role of the tryptophan-rich membrane-proximal region of gp41, deletion, substitution, and insertion mutations were created (see Fig. 2, left panel). Two mutations, AK1 and AK2, were designed previously which created AvaI sites at the coding sequences for the lysines at either the N-terminal (AK1) or C-terminal (AK2) border of the 17 amino acid tryptophan-rich region (39). These mutations substitute an Asn-Ser-Gly peptide sequence at either end of the tryptophan-rich sequence. In order to determine if the tryptophan-rich region of gp41 was necessary for fusion, a preliminary mutant, AΔK1-K2, was constructed which deletes the 18 residues, including the entire tryptophan-rich sequence.

More specific deletions (Δ665-682, Δ666-670, Δ671-677, Δ678-682, and Δ666-670/678-682) were then constructed to probe for critical subdomains within this region. In addition, two mutations were specifically designed to probe the structural requirements of the region. SC7 scrambles the central seven residues, 671-677, in a nonconservative substitution pattern, switching the highly conserved hydrophobic positions with the non-conserved neutral and polar positions (see Fig. 1A). ΔFN deletes two central non-conserved residues, Phe 673 and Asn 674, in an attempt to disrupt the alignment of a potential α-helical structure (see Fig. 1C).

Single amino acid substitutions were made for each of the conserved tryptophan residues to determine their roles in the function of the membrane-proximal region. Alanine was substituted for the first (W66A), second (W670A), fourth (W678A), and fifth (W680A) tryptophan residue within this 17-amino acid stretch. Serine, proline, and

FIG. 2. Diagram of mutations and cell-cell fusion assay. The mutations are diagrammed in the left panel juxtaposed to the corresponding cell-cell fusion data for each mutant. Amino acid changes are underlined, and deletions are denoted by periods. COS-1 cells expressing glycoprotein were mixed 1:10 with HeLa-CD4/LTR-ß-gal cells and replated. The cells were stained 24 h later with X-gal, and blue syncytia were quantitated microscopically. The average number of syncytia per low power field was determined by counting six nonoverlapping fields per well for each of two wells and averaging the total. The average number of nuclei per syncytium was determined by quantitating 15 syncytia in each of two wells and averaging the total.



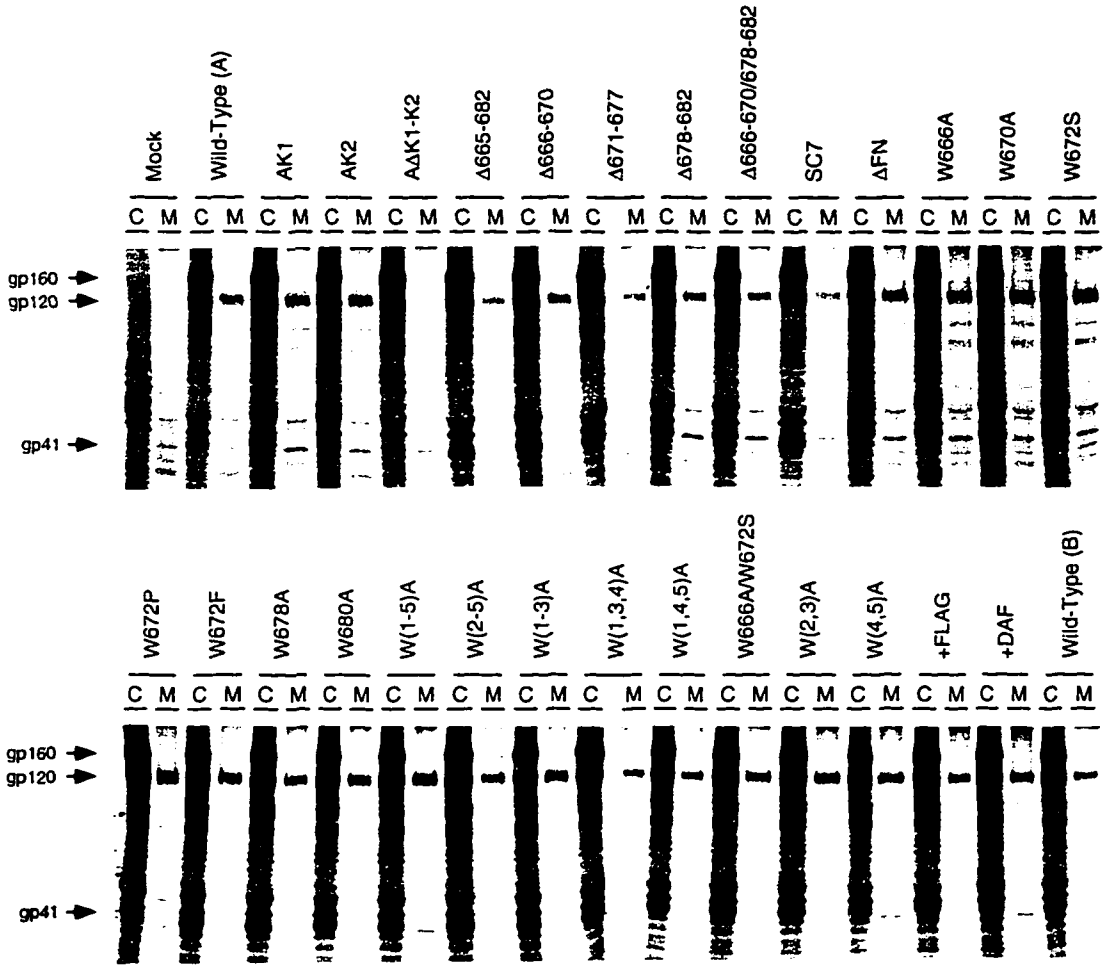
phenylalanine were all substituted for the third tryptophan (W672S, W672P, and W672F, respectively). In addition, multiple substitutions were combined to further define the sequence requirements of the region. Multiple substitutions of alanine for tryptophan residues are denoted by parenthetically listing the relative positions of the changed tryptophans within the membrane-proximal region. For example, W(2,3)A changes the second and third tryptophan residues to alanines. One of these multiple substitutions, W(1,3,4)A, changes the three tryptophan residues that are conserved in HIV-2 and SIV (see Fig. 1B).

Finally two insertion mutants were generated. +FLAG introduces six highly charged residues into the epitope of the 2F5 neutralizing monoclonal antibody in such a way as to create the eight-amino-acid FLAG epitope (Kodak-IBI). The other insertion mutant, +DAF, was designed to introduce the coding sequence of the nine membrane-proximal residues of human red blood cell decay accelerating factor (DAF) between the tryptophan-rich region and the membrane-spanning domain.

Expression of envelope glycoprotein mutants. Each of the plasmids containing mutated *env* genes was transiently expressed in transfected COS-1 cells. The proteins were metabolically labeled in a pulse-chase experiment, and the resulting products were immunoprecipitated from both the cell lysates and the cell medium (Fig. 3). All of the mutant glycoprotein products were expressed at levels similar to the wild-type protein and were processed normally to gp120 and gp41, with the sole exception of the initial deletion, AΔK1-K2. This mutation was found to significantly reduce the efficiency with which the glycoprotein precursor was proteolytically processed (Fig. 3, AΔK1-K2, C and M). Processing of the Δ665-682 mutant glycoprotein, however, was not significantly affected (Fig. 3, Δ665-682, C and M).

All of the protein products were of the predicted size based on the presence or absence of deleted residues, suggesting that the mutant glycoproteins were glycosylated normally. One exception, the gp41 subunit of the SC7 mutant, migrated more rapidly than

FIG. 3. Expression and maturation of envelope glycoprotein mutants. SV40-based *env* expression plasmids were transfected into COS-1 cells. Cells were labeled with [^{35}S]methionine/cysteine, and HIV-1 glycoproteins were immunoprecipitated as described in Materials and Methods from the cell lysate (C) or culture medium (M) using AIDS patient serum for analysis by SDS-PAGE (8%). Mock, transfected with wild-type *env* plasmid lacking a eukaryotic transcriptional promotor.



was expected (Fig. 3, SC7, C). Since this mutation did not delete any residues, it appears that the scrambling of the peptide sequence was sufficient to alter the mobility of the subunit in the SDS gel. Similar departures from the predicted mobility have been noted previously with gp41 when predicted amphipathic helical regions within the cytoplasmic tail were lost upon truncation of the protein (12, 39). Since the SC7 mutation was designed specifically to disrupt the central predicted amphipahic helical region of the tryptophan-rich region (Fig. 1C), it seems reasonable that the predicted migration of this mutant was altered in a similar fashion as the truncation mutants.

Previous reports have demonstrated that the tryptophan-rich region is not sufficient to anchor the glycoprotein in the membrane (12, 37). All of the cell cultures expressing mutant glycoproteins, including the AΔK1-K2 and Δ665-682 mutants, released gp120, but not gp41 or gp160, into the culture medium (Fig. 3, M lanes), indicating that the mutant glycoprotein precursors and transmembrane subunits were stably anchored in the membrane. This demonstrates further that the membrane-proximal region is not only insufficient but also unnecessary for the stable association of the glycoprotein with the membrane. The above results demonstrate that the tryptophan rich region is not required for the normal biosynthesis and maturation of the glycoprotein.

Effects of the mutations on cell-cell fusion. Most reports quantitate the fusogenicity of HIV-1 envelope glycoproteins solely by counting the number of syncytia which result from the coculture of *env*-transfected cells with CD4+ fusion-permissive cells. While this method of quantitation should, in theory, reveal the relative abilities of different glycoproteins to induce cell-cell fusion events, in practice, the outcome often varies significantly among glycoproteins which would be expected to have similar phenotypes and among repeated experiments with the same constructs yielding different relative phenotypes. This variation can be due to differences in the purity or concentration of the DNAs used to transfect cells, differences in relative cell culture densities, or other differences in the transfection efficiencies due to experimental error. In order to assess the

fusogenicity of different constructs, we have found that quantitation of the average number of nuclei per syncytium, rather than the number of syncytia, typically provides a more consistent and reproducible measure of this biological activity.

For this study we used a highly sensitive approach to assay cell-cell fusion. COS-1 cells expressing the mutant glycoproteins as demonstrated in the previous experiments were mixed with HeLa-CD4/LTR-ß-gal (MAGI) cells at a ratio of approximately 1:10. Because the *env*-expression plasmids also express the viral Tat transcriptional activator protein, fusion of the two cell types induces the expression of a ß-galactosidase reporter gene under control of the viral LTR promotor (26). Because the ß-galactosidase has been modified to contain a nuclear targeting signal, the nuclei of the resulting syncytium stain a dark blue with X-gal in situ (26). This simplifies the microscopic quantitation of both the average number of syncytia in a low power field and the average number of nuclei per syncytium. Immunofluorescent staining of selected cultures scoring negative for fusion in this assay demonstrated that individual COS-1 cells were indeed expressing high levels of glycoprotein but were failing to fuse with the MAGI cells (data not shown).

The AK1 mutant was found to reduce fusogenicity approximately 25%, despite the substitution of three residues within the epitope for the neutralizing monoclonal antibody, 2F5 (Fig. 2, right panel). This suggests that the neutralizing ability of 2F5 is not due to the masking of critical residues within its epitope. AK2, however, had a more significant effect on fusion, yielding only about 30% the wild-type level of fusion. This suggests that Lys 683 is somehow important in fusion, since substitution of the hydrophobic residues at positions 684 and 685 has been shown previously not to affect fusion (15). This agrees with a previous study which found that substitution of an isoleucine for this arginine at the border of the membrane-spanning domain essentially abrogated fusion (21). The deletion mutant AΔK1-K2 was found to be completely defective in mediating fusion. Although this mutant was processed less efficiently than the other mutants, it is unlikely that the reduced level of processed glycoprotein, alone, was sufficient to block fusion in this assay.

A more precise deletion of the entire tryptophan-rich region (Δ665-682) as well as a deletion of the central seven amino acid residues, alone (Δ671-677), was sufficient to abrogate cell-cell fusion, demonstrating conclusively that the tryptophan-rich region is indeed critical for the fusion function of the glycoprotein. Deletion of either the first or last five residues of the region (Δ666-670 or Δ678-682, respectively) resulted in an approximately 20% reduction in fusogenicity as measured by both the average number of syncytia and the average number of nuclei per syncytium. Since the central seven residues were found to be necessary for membrane fusion to occur, the deletions of the first and last five residues were combined to address whether the central seven residues were also sufficient for fusion. The resulting mutation, Δ666-670/678-682, also abrogated fusion, indicating that the central seven residues of this sequence are necessary but not sufficient to mediate the function of this region in fusion. This suggested that multiple residues contribute to the function of this region.

To distinguish between a requirement for the specific sequence of the central portion of this region and simply a requirement for a certain number of residues, the central seven amino acid residues were scrambled. Because this sequence is predicted to form an amphipathic α-helix involving highly conserved hydrophobic residues (Fig. 1C), particular effort was made to scramble the sequence such that the positions of hydrophobic and polar residues were exchanged. The resulting mutation, SC7, resulted in an almost four-fold reduction in fusogenicity, indicating that there was some specificity for the sequence of residues, but that this specificity was not absolute for the functional role of the region.

To further test the potential role of an α-helical structure in the function of this region, two central nonconserved residues were deleted. The rationale was that such a deletion in the middle of a helix would skew the structure such that residues that previously aligned on one face of the helix would become displaced. This mutation, ΔFN, was found to have no significant effect on fusion. While this result provides no evidence for a helical structure, it also does not necessarily preclude the existence of such a structure. Indeed,

when the mutated sequence was modeled as a helical net, the conserved hydrophobic residues still tended clustered together on one face of the helix, though in a somewhat distorted arrangement (data not shown).

Because the most striking feature of this region of the glycoprotein is the unusually high density of tryptophan residues, all of which are highly conserved in all HIV-1 isolates, single amino acid substitutions were made at each of these five positions. Surprisingly, none of these substitutions had any significant effect on fusion. None were as fusogenic as wild-type, with the greatest effect being only a 20% reduction in fusion (W680A). While most of the tryptophans were replaced only with alanine, the central tryptophan was substituted with serine, proline, and phenylalanine. The lack of major effect caused by the proline substitution provided further evidence against the requirement for a rigid α-helical structure in this region, though still not precluding the existence of a relatively refractory helical structure.

Since single amino acid substitutions had only limited effect on fusion, multiple mutations were combined and assayed for an effect. Substitution of alanine for all five of the tryptophan residues together [W(1-5)A] was sufficient to abrogate fusion, demonstrating that the function of this region is indeed dependent on the highly conserved tryptophan residues. Surprisingly, however, the replacement of even a single tryptophan [W(2-5)A] was sufficient to return fusion to approximately 70% of the wild-type level. Even more surprising was the abrogation of fusion when just the first three tryptophans were replaced [W(1-3)A]. The other combinations of substitutions were only slightly less fusogenic than the single amino acid substitutions. This includes the W(1,3,4)A mutation, which changed the three tryptophans that are also conserved in HIV-2 and SIV (Fig. 1B). These results demonstrate both the unusual resiliency and intracacy of the function of this region during fusion.

Finally, two insertion mutations, +FLAG and +DAF, were found to completely abrogate fusion. The +FLAG mutation inserts six residues, five of which are charged,

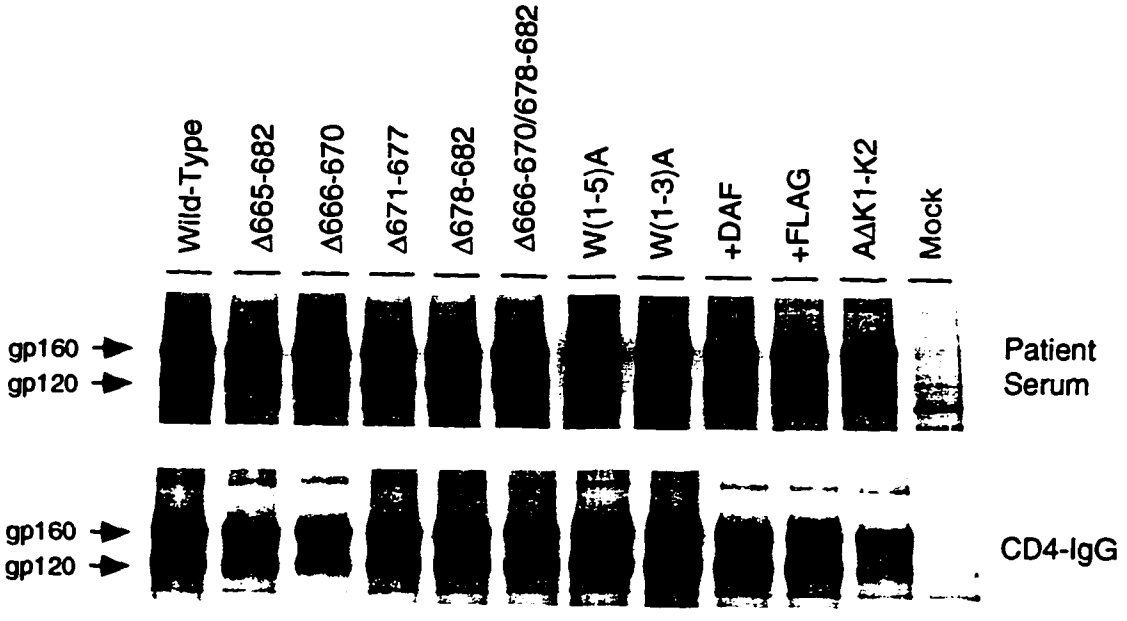
between two charged residues within the epitope for 2F5. Since this region is accessible to antibody, it seemed possible that this epitope could accommodate the additional hydrophilic residues and perhaps allow the use of the commercially available 2F5 monoclonal antibody to neutralize virus. But, although this region is accessible to the 2F5 antibody and is relatively refractory to substitution mutations (AK1 and W666A) as well as deletions (Δ666-670), these results indicate that it cannot accommodate the amino acid insertions and still maintain function. It may be that the insertion acts in the same way as the bound antibody in preventing fusion.

The +DAF mutation, which inserts nine residues from the human DAF protein between the tryptophan-rich region and the membrane-spanning domain, also completely abrogated fusion. The same insertion into the analogous position in influenza virus HA did not affect fusion (25). Since HA does not have a tryptophan-rich or hydrophobic region at the same position as the HIV-1 glycoprotein, it is likely that the tryptophan-rich region must function in close proximity to the membrane.

Ability of the mutant glycoproteins to bind CD4. To determine whether mutations in the tryptophan-rich region affected the ability of the glycoprotein to bind CD4, selected mutant glycoproteins which exhibited reduced levels of fusion or a lack of fusion were immunoprecipitated from metabolically radiolabeled COS-1 cells with CD4-IgG in parallel with patient serum (Fig. 4). All of the mutant glycoproteins bound CD4-IgG at similar levels to wild-type glycoprotein, indicating that the membrane-proximal region was dispensible for the efficient binding of the glycoprotein to its primary receptor.

Transport of the mutant glycoproteins to the cell surface. Because the ability of the mutant glycoproteins to form syncytia could be affected by reduced cell-surface expression, it was necessary to determine the relative levels of each of the fusion-defective mutant glycoproteins on the surface of transfected cells as compared to wild-type glycoprotein. Metabolically radiolabeled cells expressing mutant glycoproteins were treated on ice with the membrane-impermeable, thiol-cleavable biotinylation reagent, NHS-SS-

FIG. 4. CD4 binding capability of envelope glycoprotein mutants. Radiolabelled HIV-1 glycoproteins were immunoprecipitated from one half of the cell lysate using CD4-IgG and from the other half of the lysate using patient serum. The immunoprecipitates were analyzed by SDS-PAGE (8%).

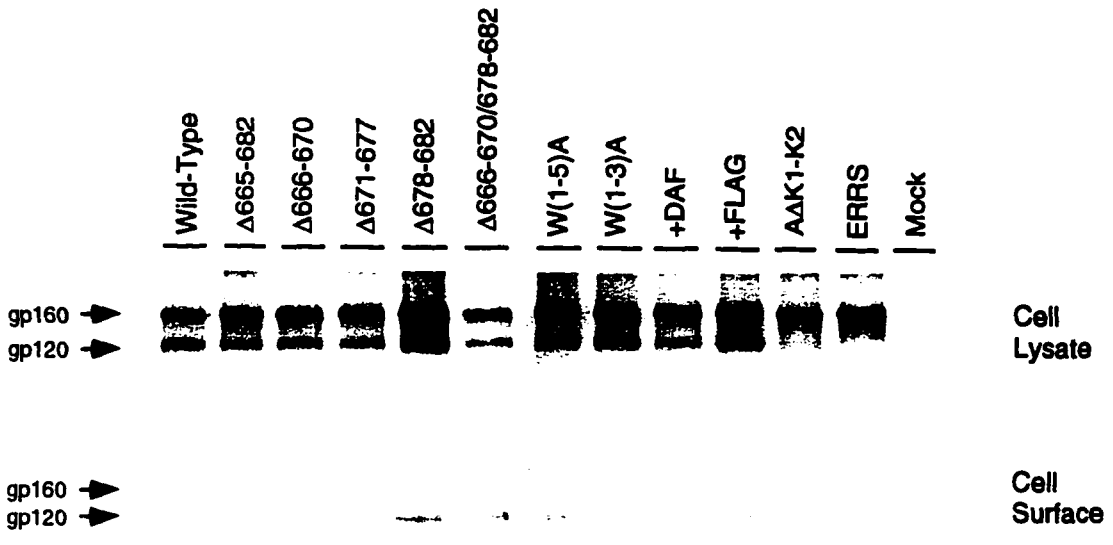


biotin. Viral glycoproteins were initially immunoprecipitated from the culture medium of transfected COS-1 cells by using HIV-positive patient serum to isolate the viral protein from cellular proteins. The immune complexes were then denatured by boiling in SDS, and a portion of this immunoprecipitate was reserved to analyze in parallel with subsequent samples. The remaining sample was incubated a second time with streptavidin-agarose to isolate just the subpopulation of viral glycoproteins which were exposed to biotinylation reagent on the cell surface.

As can be seen in Fig. 5, all of the fusion-defective glycoprotein mutants except AΔK1-K2, which was also found to be processed inefficiently, were detected on the cell surface at levels similar to wild-type glycoprotein. To control for biotinylation of only glycoproteins that were expressed on the cell surface, a modified HIV-1 glycoprotein containing an endoplasmic reticulum retrieval signal attached to the C-terminus of its cytoplasmic tail (36) was included as a negative control. This mutant glycoprotein, ERRS, is efficiently retrieved from the Golgi and, thus, is not processed to gp120 or expressed on the cell surface (40). Little or no ERRS gp160 could be detected with the biotinylation reagent (Fig. 5, panel Cell Surface, lane ERRS), despite the efficient expression of the glycoprotein intracellularly. Therefore, the assay was specific for proteins on the surface of the cell. Cell-surface immunofluorescent staining performed on the same subset of fusion-defective mutants was found to be consistent with the results presented here (data not shown). Thus, the effect of these mutations on fusion appears to be independent of the relative levels of glycoprotein on the cell surface.

Expression of the envelope glycoprotein mutants in the context of virus. The hydrophobic residues within the 17-amino-acid membrane-proximal region are all highly conserved, including the five tryptophan residues, which are present in every isolate of HIV-1 sequenced to date (Fig. 1A). Thus, it was surprising that only relatively severe mutations affecting these residues abrogated fusion in the cell-cell assay. In order to determine the effects of these mutations in a more biologically relevant functional assay,

FIG. 5. Biotinylation of envelope glycoprotein mutants expressed on the cell surface. Proteins on the surface of metabolically radiolabeled glycoprotein-expressing cells were biotinylated with NHS-SS-biotin. Viral proteins were isolated by immunoprecipitation with patient serum, and a portion of the immunoprecipitate was analyzed by SDS-PAGE. The remaining immunoprecipitate was boiled to denature antibodies and incubated with streptavidin-agarose. Following washing, biotinylated proteins were released by reducing agent and analyzed by SDS-PAGE (8%).



representative mutations were cloned into the pNL4-3 proviral construct for analysis of their effects on virus entry.

Fig. 6 shows a pulse-chase analysis of viral protein expression for selected proviral constructs with varying fusion phenotypes. All of the constructs express similar levels of normally processed glycoprotein as well as gag structural proteins, the Pr55 precursor and p24 capsid protein, as compared to the wild-type construct. The other proviral constructs not depicted on this gel also expressed similar levels of properly processed viral proteins (data not shown).

Effects of the mutations on virus entry. To determine the effect of the mutations within the tryptophan-rich region on virus entry, virus was harvested from cells expressing the mutant proviral constructs and filtered to remove cellular debris. The relative levels of reverse transcriptase activity was determined for each of the virus stocks as a measure of relative virus concentrations. The viral stocks were then normalized by appropriate dilution with media and used to infect MAGI cells. As with the cell-cell fusion assay above, the use of MAGI cells provides a highly sensitive assay for virus entry. Upon infection of the MAGI cell and expression of viral proteins, Tat induces expression of the ß-galactosidase reporter gene. After staining in situ 48 h after virus adsorption, individual virus entry events can be scored by the presence of either single blue cells or blue syncytia, both containing intensely stained nuclei. Titration of virus stocks was shown to give linear results in this assay over a range of at least three logs, and in situ staining and microscopic quantitation has been shown to be much more sensitive than staining of cell lysates for colorimetric quantitation (26).

Under conditions where approximately 10^4 infectious units of wild-type virus was added to cells, it was possible to detect low levels of virus entry only with mutants which were permissive for fusion in the cell-cell assay (Fig. 7A). Two of these mutants, W672P and W672F, were dramatically more efficient at mediating entry than the other mutants. Yet even these mutants yielded only about 10% of the wild-type level of entry. Mutants

FIG. 6. Expression of envelope glycoprotein mutants in the context of virus. Radiolabeled viral proteins were immunoprecipitated from COS-1 cells expressing pNL4-3 proviral constructs using patient serum and analyzed by SDS-PAGE (9%).

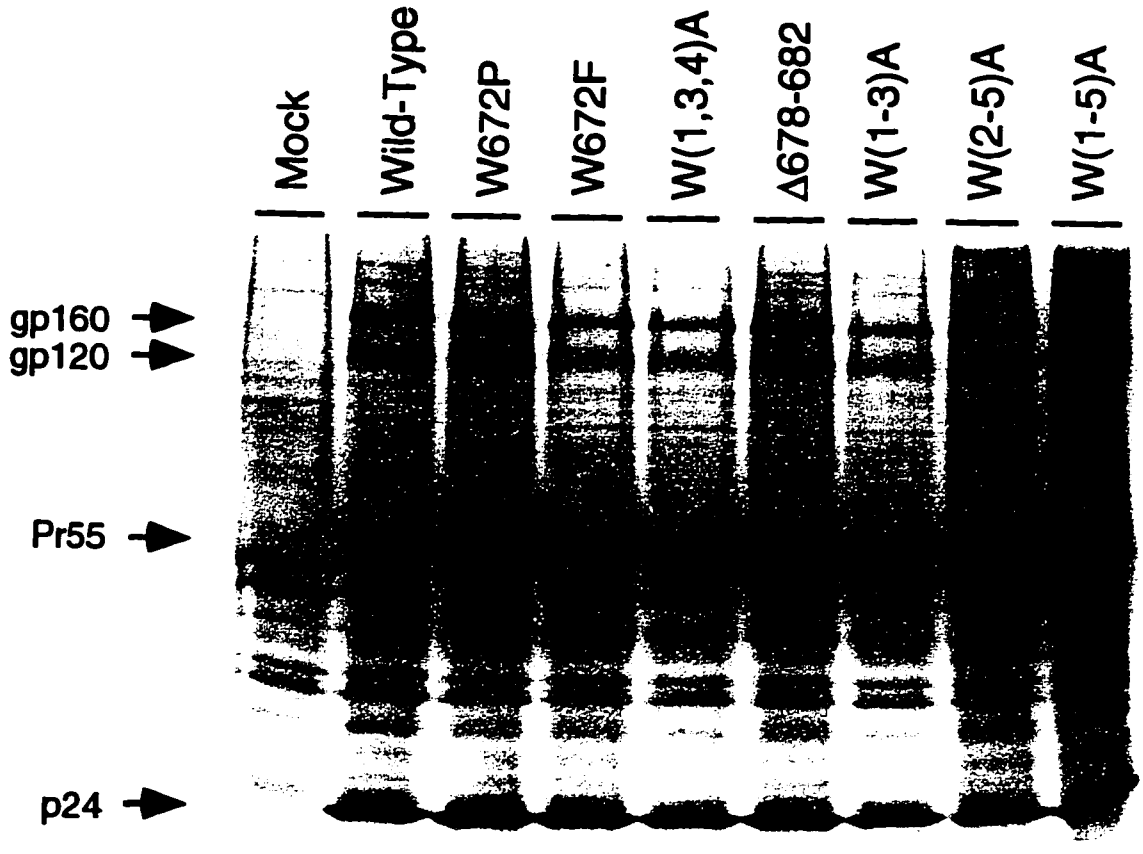
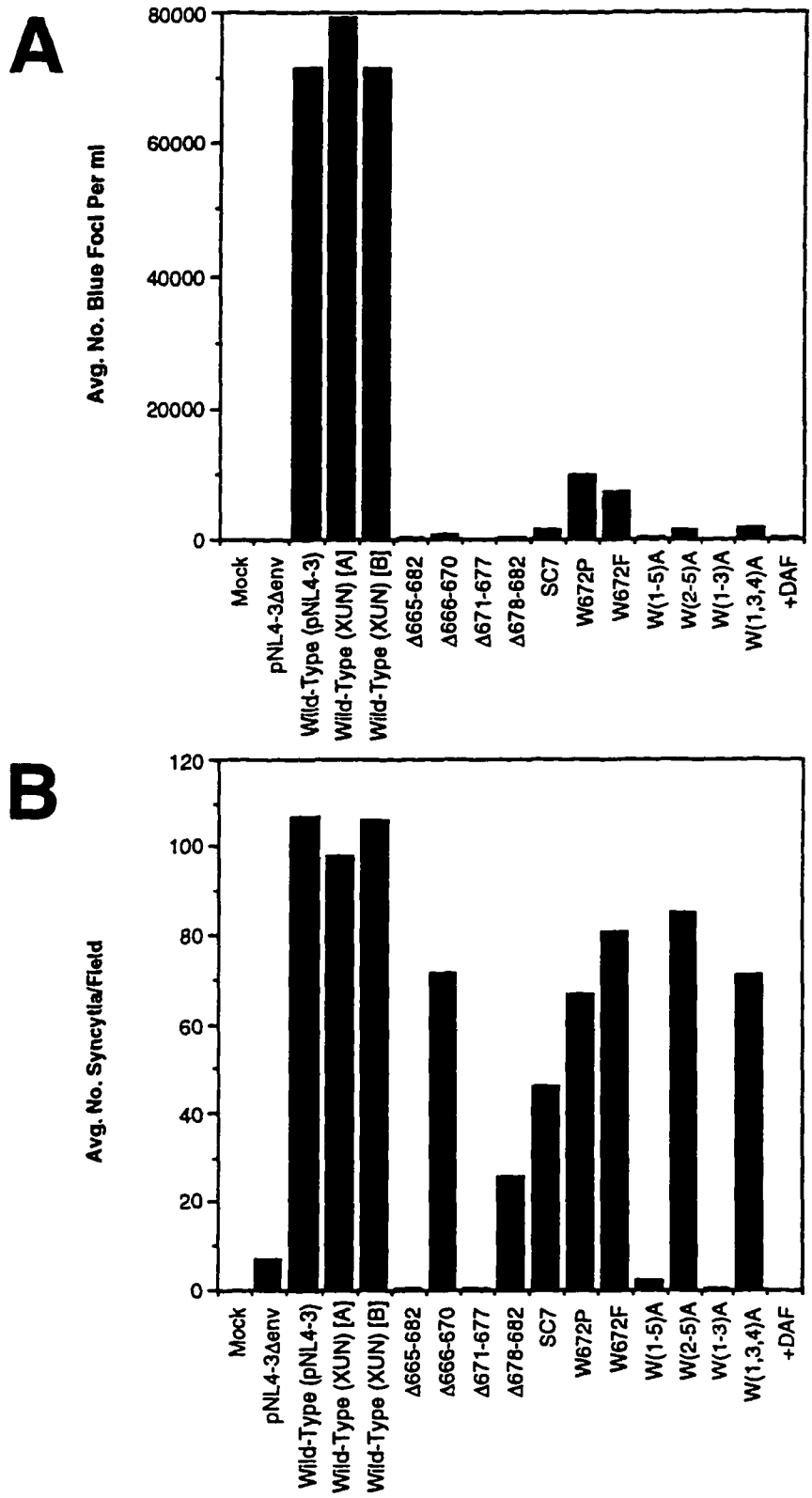


FIG. 7. Virus entry and cell-cell fusion in the context of virus. (A) Culture medium from cells expressing virus was filtered and normalized for RT activity. The normalized media was used to infect duplicate wells (12-well plates) of HeLa-CD4/LTR-ß-gal cells. Cells were stained with X-gal in situ and blue syncytia and single cells (foci) were quantitated microscopically. Virus entry was quantitated as the average number of blue foci per ml by initially counting the the number of blue foci per 16 mm^2 field (low power) for six non-overlapping fields in each of two wells. The average number of blue foci was then multiplied by the area of the well and the dilution factor. (B) Cell-cell fusion was determined as in the legend for Fig. 3, except that proviral constructs were expressed in 293T cells and mixed with HeLa-CD4/LTR-ß-gal cells.



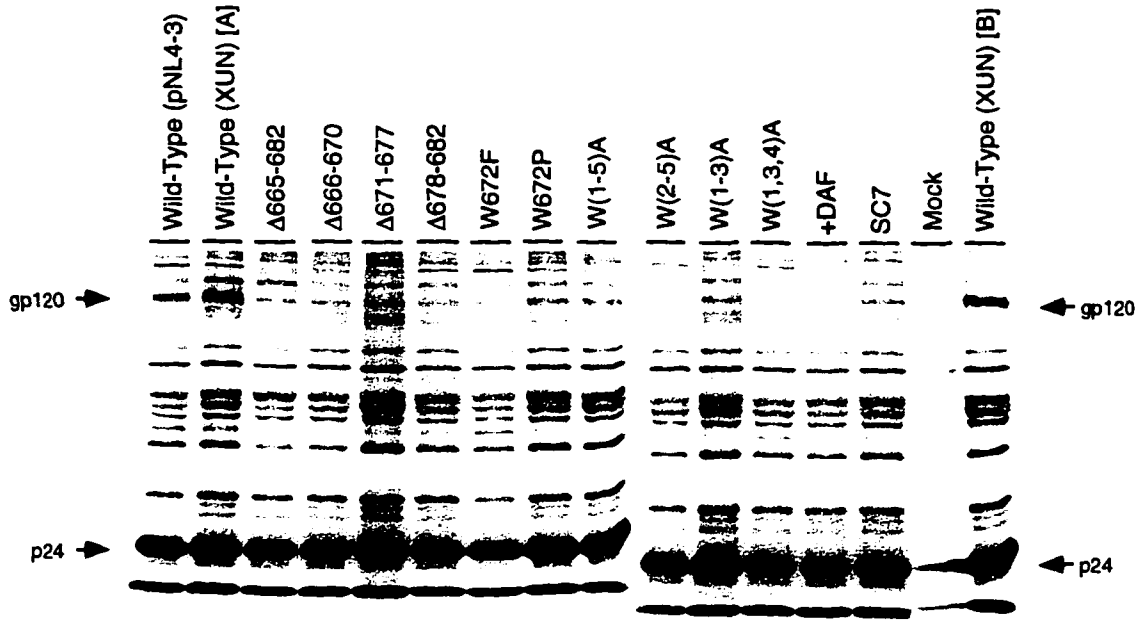
which were also markedly reduced in cell-cell fusion ability scored just barely above background levels of blue cells (Fig. 7A, Δ666-670 and Δ678-682). Since these results did not parallel the results of the cell-cell fusion assay, it was important to determine whether these proviral constructs were capable of mediating cell-cell fusion comparably to the glycoprotein expression constructs. When cells expressing each of the viral constructs were mixed 1:10 with MAGI cells, the levels of fusion seen with each construct paralleled the results of the COS-1 cell-mediated fusion assays (compare Fig. 7B with Fig. 3, right panel). Moreover, in the virus entry assays, those mutant constructs which showed limited infectivity and which were permissive in the cell-cell fusion assay yielded small syncytia of similar size to those seen with the wild-type clones (data not shown). These results demonstrate that the dramatic decrease in virus entry seen with the permissive mutant viral constructs was not due to a general defect in fusion, but rather to a specific inhibition of virus entry.

Incorporation of the envelope glycoprotein mutants into virions. In order to determine whether defects in infectivity reflected a difference in the efficiency with which the mutant glycoproteins were incorporated into virions, the composition of released virions was assessed following metabolic labeling. Supernatants were collected from cells expressing the proviral constructs and filtered to remove cellular debris. Virus was pelleted from the supernatants through a sucrose cushion and solubilized for immunoprecipitation and SDS-PAGE. As can be seen in Fig. 8, the mutant glycoproteins were all detected in the viral pellets at levels much lower than those of wild-type glycoprotein. This demonstrates that changes in the tryptophan-rich region can dramatically affect incorporation of glycoprotein into virions.

DISCUSSION

The region proximal to the N-terminal border of the membrane spanning domain of the HIV-1 glycoprotein is unusually rich in tryptophan residues, containing five that are conserved in all sequenced strains of HIV-1, and is predicted by computer algorithms to

FIG. 8. Incorporation of envelope glycoprotein mutants into virions. Supernatants were collected from metabolically radiolabeled 293T cells expressing pNL4-3 proviral constructs and filtered to remove cellular debris. Virus was pelleted from the filtered supernatants through a sucrose cushion and solubilized in lysis buffer. Viral proteins were immunoprecipitated and analyzed by SDS-PAGE (9%).



form an α-helix with partial amphipathic character (Fig. 1C). Three of these tryptophan residues, the first, third, and fourth in this region, are also completely conserved among sequenced strains of HIV-2 and SIV (Fig. 1B). Two of the tryptophan residues align with the analogous region in the VISNA lentiviral glycoprotein, and a third is present within the membrane-spanning domain of this protein. In addition to the tryptophan residues, the other hydrophobic residues in this region are also very highly conserved among different strains of HIV-1 (Fig. 1A). This region of the HIV-1 glycoprotein also overlaps the C-terminal sequence of a peptide, DP-178, which has been shown to potently inhibit fusion and virus entry (54), as well as the epitope for a neutralizing human monoclonal antibody, 2F5 (Fig. 1A). These factors suggest an important role for the membrane-proximal region in the function of lentiviral glycoproteins.

All of the mutated Env proteins were expressed at levels comparable to those of wild-type Env and, except for AΔK1-K2, were transported and processed normally. This suggests that all of these proteins folded in a manner similar to wild-type Env, since multiple studies suggest that misfolded oligomeric membrane glycoproteins tend not to be transported through the normal secretory pathway but are, rather, retained in the ER and degraded intracellularly (9). In addition, Env proteins with mutations which resulted in reduced levels of fusion were maintained on the cell surface at steady-state levels comparable to those of wild-type Env. Thus, the decreased levels of fusion seen with these mutants do not appear to be due to lower concentrations of Env on the cell surface. Finally, mutated Env proteins exhibiting reduced levels of fusion were capable of binding CD4 as efficiently as wild-type Env, indicating that the effect on fusion was independent of primary receptor binding.

Previous analyses by successive trunction of gp41 have demonstrated that amino acid residues 666-682 of the HIV-1 transmembrane glycoprotein are not sufficient to anchor the protein in the membrane (12, 37). Our results from the deletion of this region (mutant Δ665-682) demonstrate further that this reigon is also not necessary for the stable

anchoring of the envelope glycoprotein complex in the plasma membrane. This is consistent with lysine 683 forming the amino-terminal border of the membrane-spanning domain, rather than lysine 665 as has been previously hypothesized. Our results also demonstrate that residues 666-682 are not necessary for the transport and processing or CD4-binding capability of the envelope glycoprotein.

A previous deletion of 11 residues including the entire 2F5 epitope and overlapping the DP-178 and tryptophan-rich regions was shown to abrogate fusion (38). This deletion overlaps the +FLAG insertion, which also abrogated fusion. Since Δ666-670 did not abrogate fusion, the effect of this deletion appears not to have been due solely to the deletion of the residues within the tryptophan-rich region. The mutant AK2 is similar to a mutation reported by Helseth et al. which substituted an isoleucine for the lysine which forms the amino-terminal border of the membrane-spanning domain (21). However, unlike that mutant, AK2, which substitutes three amino acids including an asparagine for the membrane-bordering lysine, maintained a significant level of fusogenicity. Apparently, the polarity of the asparagine in this position in the AK2 mutant, together with the adjacent serine, preserves the functionality of this region.

The central seven amino acid residues in the tryptophan-rich region, 671-677, appear to be critical for the cell-cell fusion activity of the TM glycoprotein. The reduction of the fusogenicity of the glycoprotein by scrambling these central residues (mutant SC7) suggests that the clustering of hydrophobic residues in an α-helical secondary structure as modeled in Fig. 1C may indeed be important, but not essential, for the function of this region. Residues 666-670 and 678-682 also appear to affect fusion, as deletion of these residues reduced, but did not block, cell-cell fusion. Our results suggest that the specific amino acid sequence of residues 665-667 is not critical for cell-cell fusion, since the AK1 mutant was fusogenic. Therefore, it is unlikely that the epitope for neutralizing monoclonal antibody 2F5 which overlaps these residues is involved in a specific molecular interaction during the cell-cell fusion event. Moreover, since Δ666-670 maintained a reduced level of

fusogenicity, it seems likely that the C-terminal portion of peptide DP-178, which overlaps residues 665-673, is not the critical determinant for the potent inhibition of virus-mediated cell-cell fusion demonstrated by this peptide. This finding is corroborated by a recent report by Neurath et al. that a peptide homologous to DP-178, but lacking these residues, has similar neutralizing acitivity and apparently binds the fusion peptide of the TM glycoprotein (35). Although in an analysis of truncated peptides, this overlapping region appeared to be important to the function of the DP-178 peptide (52). To test this hypothesis, AK1, W(2,3)A, and W666A/W672S were tested for their ability to escape inhibition of cell-cell fusion by DP-178. All of the mutants were inhibited completely by the peptide, as was the wild-type protein (data not shown). These results suggest that the tryptophan-rich may be functionally distinct from the critical region of DP-178.

Although the tryptophan-rich region is predicted to be α-helical, modeling the region as a ß-strand also positions all five tryptophans on the same side of the structure. However, in this case, the conserved hydrophobic residues are interspersed with the non-conserved polar residues. Interestingly, the structure of the fusion peptide of gp41 from peptide studies appears to be a ß-strand until it contacts the lipid bilayer, at which time it transitions to a partly α-helical structure (30). Thus, the tryptophan-rich region, which has similar characteristics to the fusion peptide and could interact with membranes, may also undergo a conformational change involving the conserved tryptophan residues. In support of this hypothesis, the 2F5 epitope which overlaps the N-terminal end of this region can no longer be recognized by antibody following CD4 binding (44), suggesting that this region may indeed undergo a conformational change or become sequestered in the membrane during fusion. Thus, deletions within the tryptophan-rich domain may interfere with this step in virus fusion.

A striking feature of many of the mutations analyzed was the propensity to inhibit virus entry to levels at the limit of detection, while having only a limited effect on syncytium formation. For example, the W672P mutant exhibited less than a 20% reduction

in fusogenicity as compared to wild-type Env, yet reduced the number of virus entry events by almost 90% as compared to wild-type. One possible explanation is that the concentration of Env protein on the surface of the virion, which we have shown is significantly reduced for all of the mutants, is lower than the local concentrations of Env present on the cell surface. A high concentration of Env protein on the cell surface may facilitate fusion, overcoming a block to fusion which occurs in the context of virus, perhaps by enhancing the recruitment of Env oligomers into fusion pores. Studies with influenza HA have demonstrated that variations in the cell surface concentration of fusogenic protein oligomers correspond directly with the efficiency of fusion pore formation (7). In consideration of this point, it is interesting to note that the syncytia induced by COS-1 cells expressing wild-type HIV Env are larger on average than the syncytia induced by Env-expressing 293T cells when both are cultured with HeLa-CD4/LTR-ß-gal cells (data not shown). It could be that this is due to a higher level of Env expression in the COS-1 cells as a result of the effect of COS-1-expressed large T antigen on the SV40-based Env-expression plasmid. W672F and W672P, however, were much more efficient at mediating virus entry relative to the other mutants, even though cell-cell fusion efficiencies were similar. This discrepency could be due to a higher sensitivity of the virus entry assay to subtle differences in fusogenicity.

In an earlier mutational analysis of this region, Cao et al. (3) concluded that the single amino acid substitution W672P resulted in Env proteins which were up to twice as fusogenic as wild-type Env in a syncytium assay. Cell-cell fusion was quantitated by counting the number of syncytia per unit area in a transfected T-cell culture. We were unable to duplicate these results in syncytium assays using adherent cells. Cao et al. also reported that the effect of W672P and other single amino acid substitutions within this region on virus entry was minimal. Virus entry into T-cells was determined using an env trans-complementation system (20). Complemented virus was produced by cotransfecting an *env* expression plasmid with an *env*-defective provirus containing a CAT reporter gene.

Relative levels of entry were determined by measuring the CAT activity in the lysates of T cells following incubation with virus. In a complementation assay such as this, it may be possible to "overload" virions with more Env protein than would normally be incorporated into virus expressed from a complete genome. If such were the case, then the complementation assay might be expected to underestimate the detrimental effects of subtle mutations on virus replication in much the same way as a syncytium assay may underestimate a mutation's effect on membrane fusion. This may explain why the same mutation was much more inhibitory in the context of virus from our analysis than it was in the complementation system from the previous study. Another important point is that the CAT assay is linear within only a very narrow range, while the ß-gal assay has been shown to be linear over at least three logs. Thus, if the CAT assay were to proceed out of the linear range, the detrimental effect of a mutation could be underestimated. Indeed, this is precisely what we have observed with the same complementation assay used by Cao et al. (Data not shown).

It is unclear at present how this region, and the conserved tryptophan residues in particular, are functioning in the membrane fusion mechanism. It is not yet known, for example, whether these mutations have any effect on the ability of the envelope glycoprotein to interact with chemokine receptors during the fusion reaction. However, reports published so far on the interaction of envelope glycoprotein with chemokine receptors suggest that regions of gp120 are sufficient to bind chemokine receptor in a complex with CD4, independent of gp41 (47, 57). Whether domains in gp41 might also interact with chemokine receptors remains to be seen. It is also unclear how even conservative mutations within this tryptophan-rich motif dramatically reduce the efficiency with which glycoprotein is incorporated into virions. Interestingly, however, mutations within the proteolytic cleavage site of gp160 have also been shown to reduce glycoprotein incorporation (10), suggesting that the conformation of the glycoprotein ectodomain can affect molecular interactions during virus assembly. Further studies are required to

elucidate the mechanisms by which mutations within the tryptophan-rich region affect these two steps of the virus lifecycle.

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RETENTION OF THE HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ENVELOPE GLYCOPROTEIN IN THE ENDOPLASMIC RETICULUM DOES NOT REDIRECT VIRUS ASSEMBLY FROM THE PLASMA MEMBRANE

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ABSTRACT

The envelope glycoprotein (Env) of human immunodeficiency virus type 1 (HIV-1) has been shown to redirect the site of virus assembly in polarized epithelial cells. In order to test whether localization of the glycoprotein exclusively to the endoplasmic reticulum (ER) could redirect virus assembly to that organelle in nonpolarized cells, an ER-retrieval signal was engineered into an epitope-tagged variant of Env. The epitope tag, attached to the C-terminus of Env, did not affect the normal maturation and transport of the glycoprotein or the incorporation of Env into virions. The epitope-tagged Env was also capable of mediating syncytium formation and virus entry with similar efficiency as compared to wild-type Env. When the epitope was modified to contain a consensus K(X)KXX ER-retrieval signal, however, the glycoprotein was no longer proteolytically processed into its surface and transmembrane subunits, nor could Env be detected at the cell surface by biotinylation. Endoglycosidase H analysis revealed that the modified Env was not transported to the Golgi apparatus. Immunofluorescent staining patterns were also consistent with the exclusion of Env from the Golgi. As expected, cells expressing the modified Env failed to form syncytia with $CD4^+$, permissive cells. Despite this tight localization of Env to the ER, when the modified Env was expressed in the context of virus, virions continued to be produced efficiently from the plasma membrane of transfected cells. However, these virions contained no detectable glycoprotein and were noninfectious. Electron microscopy revealed virus budding from the plasma membrane of these cells, but no virus was seen assembling at the ER membrane, and no assembled virions were found within the cell. These results suggest that the accumulation of Env in an intracellular compartment is not sufficient to redirect the assembly of HIV Gag in non-polarized cells.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is a C-type retrovirus, assembling its core at the plasma membrane just prior to, or concomitant with, budding from the cell

(for a review see 18). HIV-1 acquires its envelope from a portion of the plasma membrane enriched with viral glycoproteins as it buds. Most retroviral envelope glycoproteins accumulate at the plasma membrane and are incorporated into virions in this manner. However, one exception appears to be the envelope glycoproteins of spumaviruses. This genus of retroviruses was recently found to encode a potential K(X)KXX consensus ER-retrieval signal (reviewed in 26) at the C-terminus of the Env protein (15). This cytoplasmic signal sequence has been identified in several ER-resident membrane proteins (19) and has been shown to bind coatamer, a cytosolic protein component of the coat complex involved in retrograde vesicular cycling of proteins back to the ER (3). While the spumaviruses do appear to accumulate their Env proteins within the ER, it has not yet been determined whether this signal is responsible for Env retrieval or how accumulation of Env in the ER affects the assembly of these viruses.

The method which retroviruses use to enrich their envelopes with Env protein is poorly understood. One possible mechanism may involve active incorporation of Env into the assembling virion via an interaction with Gag protein (18). Mutations within the matrix protein (MA) of HIV-1 have been described which block the incorporation of Env into virus (7, 12, 13, 33), suggesting a necessary interaction between the MA and Env proteins, although this could be explained by a nonspecific steric exclusion of the Env cytoplasmic tail from an accomodating "pocket" in MA. Indeed, Env with a truncated cytoplasmic tail was incorporated into virus containing a mutation in MA (12, 13).

The Env sequence requirements for incorporation appear to differ among different retroviruses, even among retroviruses utilizing the same assembly pathway and within the same retrovirus family (18). The Mason-Pfizer monkey virus requires a full-length Env cytoplasmic tail for normal levels of incorporation into virions (1), while RSV appears to have no requirement for a specific Env cytoplasmic tail, incorporating tailless native Env and foreign glycoproteins containing long cytoplasmic tails (5, 6, 29, 32). SIV and HIV-2 incorporate their Env proteins more efficiently if the cytoplasmic tail is truncated (20, 25).

Conversely, their close relative, HIV-1, requires a full cytoplasmic tail for efficient incorporation of its Env protein in most cell types (10, 12-14, 34).

The earliest evidence for a specific interaction between HIV-1 Env and Gag during virus assembly was the ability of Env to redirect the site of virus budding in polarized epithelial cells (28). HIV-1 Env is preferentially transported to the basolateral membrane of polarized epithelial cells (27). In the absence of Env, HIV-1 Gag assembled and budded from both the apical and basolateral membranes of these cells. But when coexpressed with Env, Gag assembled and budded preferentially from the site of Env accumulation, the basolateral membrane. When truncated forms of Env were analyzed in similar experiments (23), it was found that the ability of Env to redirect the site of virus budding was lost following truncation of its cytoplasmic tail. This indicated that the C-terminus of the Env cytoplasmic tail either interacts with Gag or is important to maintain a specific conformation of the cytoplasmic domain necessary for Env-Gag interaction. More recent evidence for an interaction between Env and Gag will be discussed later in this paper.

In this report, we address the potential for HIV-1 Env containing a K(X)KXX cytoplasmic ER-retrieval signal to redirect the site of virus assembly and budding from the plasma membrane to the ER in nonpolarized cells. An initial Env mutant was generated containing a C-terminal epitope tag to test whether the cytoplasmic tail could accomodate exogenous sequences at its C-terminus and remain functional. The epitope tag was then modified to contain the ER-retrieval signal. The characterization of both of these mutant glycoproteins and their effects on virus assembly are described.

MATERIALS AND METHODS

Cell culture. COS-1, CV-1, and 293T cells were obtained from the American Type Culture Collection. HeLa-CD4/LTR-ß-gal cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH and were originally contributed by Michael Emerman. All cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and penicillin and

streptomycin. HeLa-CD4/LTR-ß-gal cells were additionally maintained in G418 and hygromycin as recommended by the contributor.

Mutagenesis and construction of plasmids. For construction of the FLAG mutant, a nucleotide sequence encoding the eight-amino-acid FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; Kodak-IBI), preceded by a three-amino-acid spacer peptide (Asn-Ser-Gly), was introduced into the *env* gene of the HXB2 strain of HIV-1 immediately preceding the stop codon by using the pALTER mutagenesis system (Promega). The regions complementary to and flanking the mutagenic oligonucleotide were sequenced (Sequenase, USB). A BamHI-XhoI HXB2 env fragment was then subcloned into pSRHS3. This plasmid expresses HXB2 env under control of the SV40 late promotor. The vector also expresses the SV40 large T antigen, contains the SV40 origin of replication, and utilizes the MPMV 3' LTR for transcriptional termination and polyadenylation.

For conversion of the FLAG mutant to the ERRS mutant, a polymerase chain reaction (PCR) product was generated by using pSRHS3 containing the FLAG mutation as the template. The resulting product was then digested with BspEI and XhoI and subcloned into a derivative of pSRHS3 in which a unique BspEI site in the MPMV LTR had been deleted by digestion with BspEI, followed by filling in by the Klenow fragment of *E.coli* DNA polymerase I and religation. The entire coding region originating from the PCR product was sequenced, and two separate clones were selected for phenotypic characterization in parallel. For construction of proviral clones, BamHI-XhoI fragments containing each of the two mutations were subcloned into pNL4.3, a proviral clone of the NL4.3 strain of HIV-1. HXB2 and NL4.3 are highly homologous clade B virus strains.

Glycoprotein expression and radioimmunoprecipitation. 60-mm plates of COS-1 cells at approximately 80% confluency were transfected with 3 µg of pSRHS wild-type or mutant plasmid by using DEAE-dextran. 36-48 h post-transfection, the cells were starved for 20 min in cysteine/methionine-deficient DMEM and then metabolically

radiolabelled for 30 min with 125 μCi of [35S]-cysteine/methionine in 350 μl of cysteine/methionine-deficient DMEM per plate. The cells were then washed twice in DMEM and chased in 1.2 ml complete growth medium for 3 h. Following the chase, the growth medium was collected, and the cells were lysed in lysis buffer for 10 min on ice. Both the cell lysate and the culture medium were microcentrifuged for 5 min to remove cellular debris, and the supernatants were transferred to fresh tubes for immunoprecipitation by incubation with AIDS patient serum for 1 h at 4°C, followed by incubation with fixed *Staphylococcus aureus* (Staph A) for 30 min at room temperature. The immunoprecipitate was pelleted and washed three times in lysis buffer containing 0.1% sodium dodecyl sulfate (SDS) and once in 20 mM Tris-HCl (pH 6.8) prior to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Cell-cell fusion assay. 35-mm plates of COS-1 cells were transfected with 1 μg of pSRHS wild-type or mutant plasmid by using DEAE-dextran. 24 h posttransfection, the cells were trypsinized, mixed with HeLa-CD4/LTR-ß-gal cells at a ratio of approximately 1:10, and replated. 24 h later, the cells were fixed and stained with X-gal as described previously (21). The total number of blue syncytia per plate was determined microscopically using a grid.

Cell surface biotinylation. COS-1 cells transfected and metabolically radiolabeled as described above were washed three times in ice-cold phosphate-buffered saline (PBS) containing 0.1 mM CaCl2 and 1 mM MgCl2 (PBS-C/M) at 36-72 h posttransfection. The cells were then incubated in 0.5 mg of sulfo-NHS-biotin (Pierce) per ml of PBS-C/M for 30 min on ice. To quench the reaction, the cells were washed with DMEM for 10 min on ice and then washed twice with ice-cold PBS-C/M containing 20 mM glycine. The cells were lysed in lysis buffer containing 20 mM glycine and were immunoprecipitated as described above. The immunoprecipitate was boiled for 5 min in PBS containing 2% SDS to denature the antibody, and the Staph A was removed by pelleting. The supernatant was diluted with PBS to a final SDS concentration of 0.05%

and incubated for 1 h with streptavidin-agarose beads (Pierce). The beads were then washed as described above for an immunoprecipitate, and the resulting pellet was resuspended by boiling in protein-loading buffer containing 12% SDS for 5 min prior to SDS-PAGE.

Endoglycosidase H treatment. COS-1 cells transfected and metabolically radiolabeled as described above were lysed immmediately following the pulse or were chased for 1, 2, or 3 h. Lysates were immunoprecipitated as described above, and the resulting washed pellets were boiled for 5 min in 0.04% SDS—2% 2-mercaptoethanol—50 mM sodium citrate (pH 5.3). The Staph A was removed by pelleting at room temperature, and the supernatant was divided into two tubes. An equal volume of 2x endo H buffer (50 mM sodium citrate [pH 5.3], 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 200 μg of soybean trypsin inhibitor per ml, 2 μg of leupeptin per ml, 2 μg of pepstatin A per ml) was added to each tube, and 1 mU of endoglycosidase H (Boehringer Mannheim) was added to one of the tubes. The samples were incubated for 12 h at 37°C. An equal volume of 2x protein loading buffer was then added, and the samples were reboiled for 2 min prior to being loaded onto SDS-PAGE gels.

Immunofluorescent staining. CV-1 cells transfected as described above were plated 24 h posttransfection onto glass coverslips. The cells were fixed and stained for total-cell immunofluorescence 48 h later with an anti-gp120 monoclonal antibody as described previously (30). Cells were photographed at 400x magnification through a fluorescence microscope (Zeiss).

Virus production and entry. For analysis of virus production and virus entry, 293T cells in 100-mm plates were transfected with 10 μg of pNL4.3 wild-type or mutant plasmid by a modified $CaPO_4$-mediated method (2). 48 h post-transfection, the culture medium was filtered through a 0.45-μm filter. To quantitate the relative amount of virus production, 25-μl aliquots were removed for reverse transcriptase (RT) assays in triplicate as described previously (9).

For quantitation of virus entry, after determination of the mean levels of RT activity for each culture, aliquots of filtered medium were normalized for relative RT activity by dilution in complete growth medium. DEAE-dextran was added to a final concentration of 5 μg per ml, and 0.3 ml was added to 8 x 10^5 HeLa-CD4/LTR-ß-gal cells in triplicate wells of a 12-well plate. The virus was allowed to adsorb for 2 h before an additional 2 ml of complete growth medium was added. 48 h post-infection, the cells were stained with X-gal as described previously (21). The number of blue syncytia per 16 mm^2 field was determined for three random, nonoverlapping low-power (100x) microscopic fields in each of the triplicate wells by using a grid. The total number of blue syncytia per well was then estimated by multiplying the mean number of blue syncytia per 16 mm^2 by the total area of the well.

Virus pelleting and EM analysis. For analysis of virus assembly, 293T cells were transfected as described above except that following transfection, the cells were washed twice in DMEM and split into two 100-mm plates.

For analysis of glycoprotein expression and incorporation into virions, one of the plates was metabolically radiolabelled 24 h posttransfection (48 h after the addition of DNA) with 625 μCi of [35S]-cysteine/methionine (Dupont-NEN) in 2.5 ml of cysteine/methionine-deficient DMEM containing 10% complete growth medium for 16 h. The culture medium was then filtered through a 0.45-μm filter, and 0.8 ml was transfered to each of three microfuge tubes for centrifugation at 13,000 x *g* for 1 h. The resulting virus pellets were resuspended in lysis buffer and combined into one tube. The cells were lysed in lysis buffer, and cellular debris was removed by centrifugation for 5 min. Viral proteins were immunoprecipitated from each sample and analyzed by SDS-PAGE as described above.

For analysis of the site of virus assembly and budding, cells from the other plate were suspended in PBS, pelleted, and fixed in 1% glutaraldehyde in preparation for analysis by electron microscopy (EM).

RESULTS

Modification of HIV-1 Env to contain an ER-retrieval signal. Linear peptide signals have been identified which permit errant ER-resident proteins to be retrieved from the Golgi and cycled back to the ER (26). The best characterized of these signals is the KDEL retrieval signal commonly found on resident soluble proteins in the lumen of the ER. More recently, a K(X)KXX consensus sequence found at the terminus of the cytoplasmic tail of ER-resident type I membrane proteins has been identified to function as an ER-retrieval signal (19).

In order to address whether the assembly of HIV-1 can be redirected by the localization of Env to an intracellular compartment, we modified the C-terminus of HIV-1 Env to contain a consensus K(X)KXX ER-retrieval signal (Fig. 1). The C-terminus of HIV-1 Env is hydrophobic and has been proposed to interact with membranes (16). For this reason, we first engineered an epitope tag onto the C-terminus of the cytoplasmic tail of Env to facilitate the recognition of the KKXX signal. We chose the highly charged eight-amino-acid FLAG marker peptide as an epitope because of its high degree of surface accessiblity. We also inserted a three-amino-acid spacer sequence between the Env C-terminus and the FLAG marker peptide to avoid disrupting any membrane interaction or structural conformation crucial to the function of the Env cytoplasmic tail. The sequence of the epitope was then modified to contain the consensus dilysine motif which constitutes the ER-retrieval signal, as well as an additional lysine residue. The additional lysine was added because preliminary data suggested that it facilitates the ER-localization of the HSRV Env protein (24).

HIV-1 Env containing an ER-retrieval signal is not proteolytically processed to yield gp120. The epitope-tagged (FLAG) and ER-retrieval signal-containing (ERRS) Env precursor glycoproteins (gp160s) were expressed and maintained at levels similar to wild-type Env after a 3-h chase period (Fig. 2), indicating that neither Env mutant was degraded significantly. Although the wild-type and FLAG Env

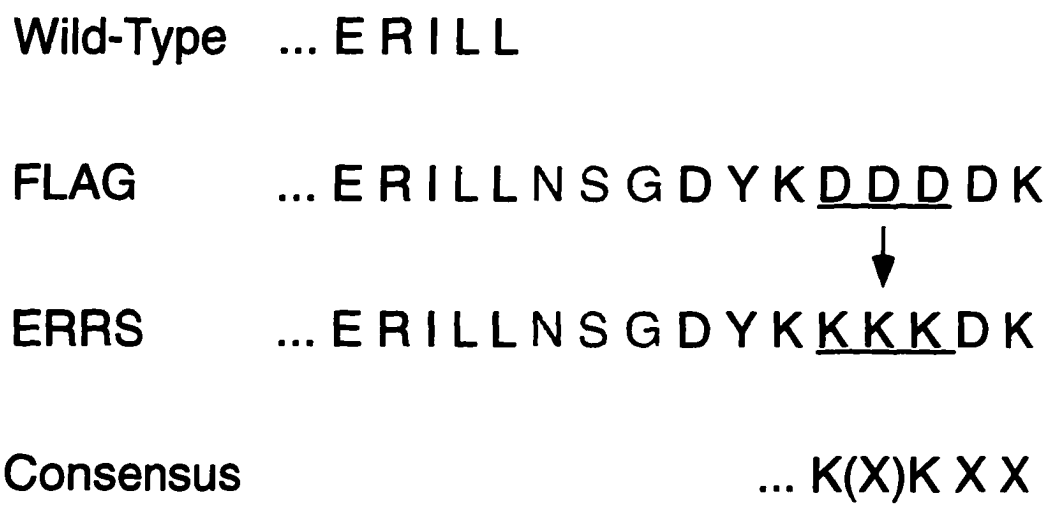
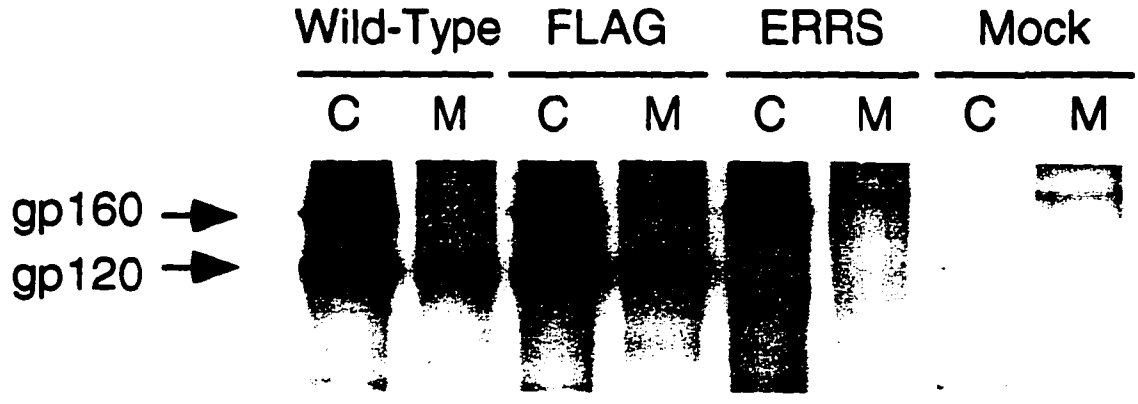


FIG. 1. Conversion of a C-terminal epitope tag to an ER-retrieval signal. The five C-terminal amino acid residues of the HIV-1 envelope glycoprotein cytoplasmic tail are shown at top. The eight-amino-acid FLAG epitope (DYKDDDDK) plus an additional three-amino-acid spacer peptide (NSG) were added to the C-terminus of Env by site-directed mutagenesis. The FLAG epitope sequence was mutated to introduce the consensus ER-retrieval signal plus an additional lysine residue at position -4/-5 which is not a necessary part of the consensus signal motif, shown at bottom.

FIG. 2. Expression and processing of envelope glycoproteins. COS-1 cells expressing glycoproteins were metabolically radiolabeled and chased for 3 h. The cell lysate (C) and culture medium (M) were collected, and HIV proteins were immunoprecipitated for analysis by SDS-PAGE (8%).



proteins were proteolytically processed to yield the surface glycoprotein subunit (gp120) (Fig. 2, lanes Wild-Type [C] and FLAG [C]), no normal gp120 band was detected for the ERRS Env (Fig. 2, lane ERRS [C]). A faint band at approximately 116 kDa, migrating faster than gp120, was produced with the ERRS mutant, which was not present in the mock-transfected cell lysate (Fig. 2, compare lane ERRS [C] with other C lanes). The identity of this band is unknown, but it is suspected to be an Env cleavage product, perhaps gp120 which has had a subset of its carbohydrate sidechains trimmed by mannosidases located in the *cis* compartment of the Golgi apparatus before being cycled back to the ER. Evidence for the identity of this band will be discussed in later sections. Similarly, gp120 was released into the media from cells expressing either wild-type or FLAG Env but not from cells expressing ERRS Env (Fig. 2, M lanes). The 116-kDa band seen in the lysates of cells expressing ERRS Env was also absent from the culture media (Fig. 2, lane ERRS [M]). Finally, no gp160 could be detected in the media of cells expressing any of the three Env constructs, confirming that the C-terminal modifications in Env did not in some way disrupt the stable association of the glycoprotein with the membrane. The lack of production of a normal gp120 product following a 3-h chase suggests that the ER-retrieval signal is functional in ERRS Env, preventing its transport through the Golgi.

ERRS Env does not mediate syncytium formation. Proteolytic cleavage of gp160 is required for the function of Env in mediating membrane fusion following binding to the CD4 receptor. Since no gp120 was detected in cells expressing ERRS Env in the preceding experiment, these cells would not be expected to form syncytia with cells expressing CD4. To test this, COS-1 cells expressing either wild-type or mutant Env were mixed at a ratio of approximately 1:10 with HeLa-CD4/LTR-ß-gal cells and replated. HeLa-CD4/LTR-ß-gal cells express high levels of CD4 and contain a lac Z reporter gene under control of the HIV-1 LTR (21). Since the pSRHS Env expression plasmids also express HIV-1 Tat, if the COS-1 cells express Env which is capable of mediating fusion with the HeLa-CD4/LTR-ß-gal cells, then the Tat produced within the COS-1 cells will

activate expression of the lac Z reporter gene. Upon fixation and staining with X-gal, these cultures would contain clearly visible blue syncytia. The blue staining is most intense within the nuclei, since the ß-galactosidase encoded by these cells has been modified to contain a nuclear localization signal (21). This nuclear staining makes it easy to quantitate the size of syncytia by counting the number of nuclei they contain.

Fig. 3 shows the results of a representative experiment. Both wild-type and FLAG Env produced large numbers of blue syncytia (Fig. 3A). Although the FLAG mutant produced about half as many syncytia as wild-type Env in this experiment, the syncytia produced by both Env proteins were of similar size (Fig. 3B). Since the numbers of syncytia in different cultures can be affected by both relative transfection efficiencies and variations in cell mixing, the average sizes of syncytia produced in different cultures may provide a more accurate measure of the relative fusogenicities of different Env proteins. Based on this criteria, the FLAG mutant appears to be essentially as fusogenic as wild-type Env.

In contrast to the FLAG mutant, however, the ERRS mutant produced only three times as many blue syncytia and single cells as were found in the mock culture as background and only 7% of the number of syncytia produced by the FLAG mutant (Fig. 3A). Perhaps more importantly, the average size of these syncytia was less than that of the background syncytia in the mock culture, indicating that these may not be the result of true Env-mediated cell-cell fusion events (Fig. 3B). Indeed, no syncytia were apparent in unstained cultures when the ERRS construct was transfected directly into HeLa-T4 cells (data not shown), suggesting that the blue syncytia observed here are the background level of the ß-galactosidase assay. Therefore, the ERRS mutant appears to be unable to mediate syncytium formation, presumably due to its inability to be transported to the cell surface and to the lack of normal processing of gp160 to gp120.

ERRS Env is not transported to the cell surface. Although gp120 was not produced from the ERRS Env mutant, suggesting that it is not transported through the

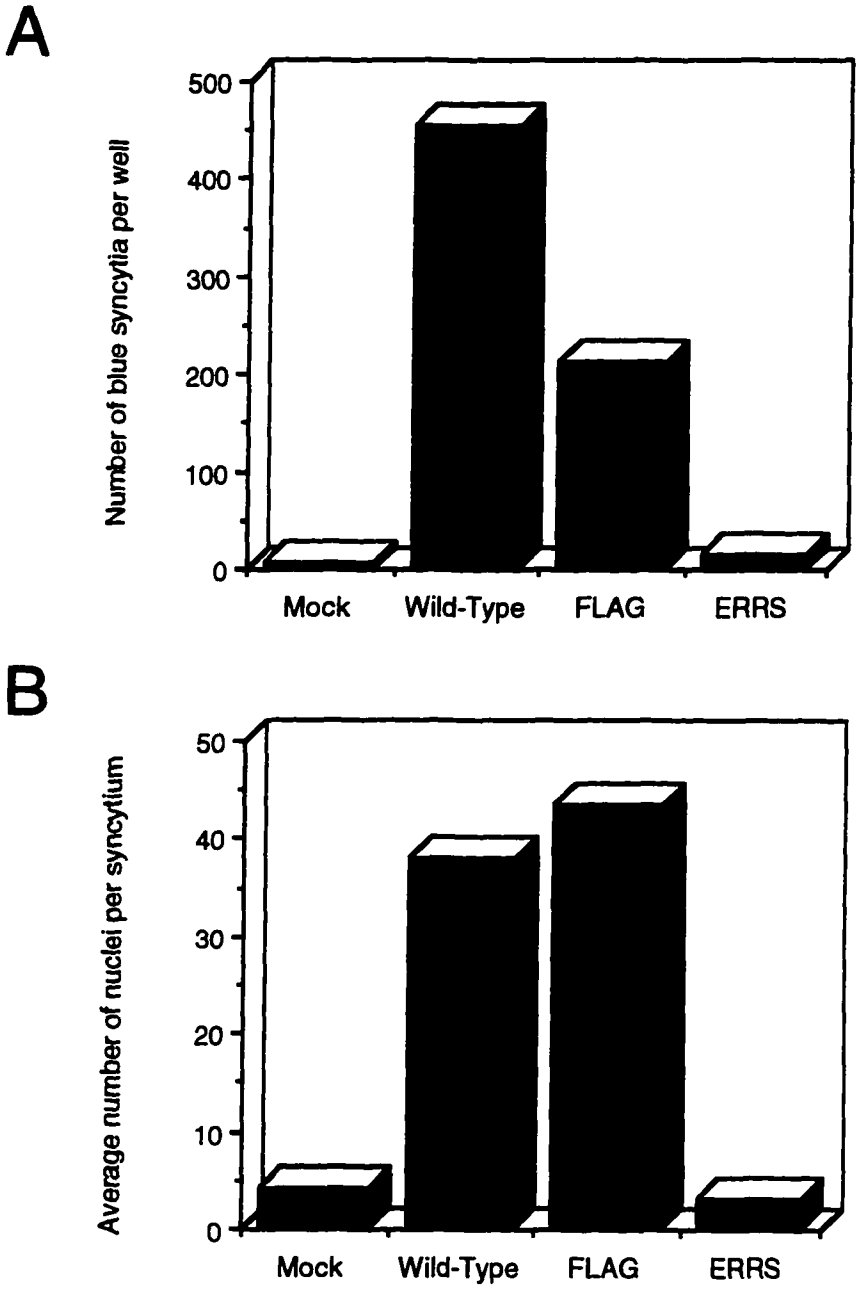


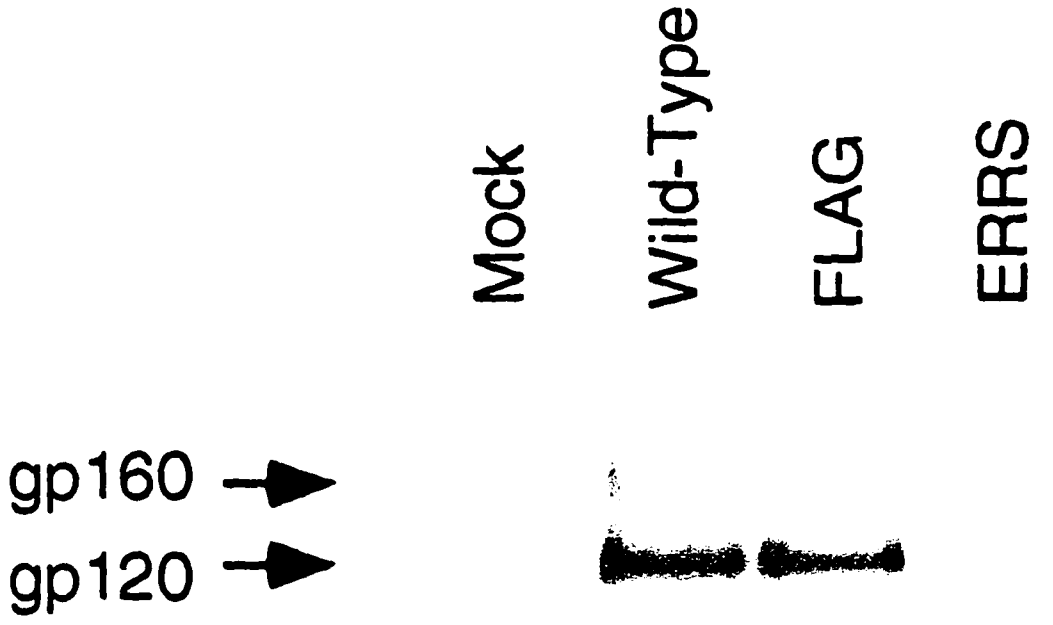
FIG. 3. Envelope glycoprotein-mediated syncytium formation. COS-1 cells expressing glycoprotein were mixed 1:10 with HeLa-CD4/LTR-ß-gal indicator cells and replated. The cells were stained in situ with X-gal, and blue syncytia were quantitated microscopically. (A) The average number of syncytia per 35-mm well was determined from two wells, counting the entire well with the aid of a grid. (B) The average number of nuclei per syncytium was determined by quantitating 25 syncytia in each of two wells and averaging the total.

Golgi, it could be that the mutant is transported through the Golgi but is not processed. In this case, the preceding cell-cell fusion assay would still have failed to indicate the presence of ERRS Env on the cell surface, since it would not have been cleaved and, thus, would have been nonfusogenic. Therefore, to determine whether the mutant glycoprotein was transported to the cell surface, we biotinylated proteins exposed on the cell surface with the membrane-impermeable reagent Sulfo-NHS-biotin. This reagent reacts with primary amines, forming a covalent linkage. After quenching of the biotinylation reaction, radiolabeled Env was purified from cell lysates by immunoprecipitation as usual. The immunoprecipitate was then boiled in 2% SDS to denature antibodies and the Staph A was removed by pelleting. Biotinylated Env proteins were then separated from nonbiotinylated Env by binding to streptavidin-agarose beads.

As can be seen in Fig. 4, equivalent amounts of gp120 are present at the cell surface for both wild-type and FLAG Env. In addition, a small amount of uncleaved gp160 precursor could be detected on the cell surface. This is consistent with our previous findings which demonstrate that, in this expression system, some gp160 can escape proteolytic processing and be transported to the cell surface (30). In contrast, however, no glycoprotein, cleaved or uncleaved, could be detected on the surface of cells expressing the ERRS mutant. Therefore, it appears that the ER-retrieval signal prevents the processing of Env into gp120 by preventing its transport through the Golgi and on to the cell surface.

ERRS Env is localized to the ER. To confirm that the ER-retrieval signal is preventing the transport of Env through the Golgi, we subjected the ERRS glycoprotein mutant to digestion with endoglycosidase H (endo H). This enzyme removes the high mannose-type carbohydrate sidechains which are added to glycoproteins in the ER but cannot remove carbohydrate sidechains which have been modified in the Golgi apparatus (reviewed in reference 17). For this experiment, cells expressing FLAG or ERRS glycoprotein mutants were pulse-labeled and then chased for 1, 2, or 3 h prior to lysis and

FIG. 4. Biotinylation of envelope glycoproteins expressed on the cell surface. Cells expressing radiolabeled glycoprotein were incubated with biotinylation reagent on ice. Following lysis and immunoprecipitation with patient serum to isolate viral proteins, pellets were boiled in SDS to denature antibody and biotinylated proteins were isolated by binding to streptavidin-agarose. The biotinylated viral proteins were then analyzed by SDS-PAGE (8%).



immunoprecipitation. The immunoprecipitates were then divided into two tubes for either digestion with endo H or mock treatment.

As can be seen in Fig. 5, epitope-tagged Env was completely sensitive to endo H digestion following pulse-labeling, resulting in a product of approximately 95 kDa, as would be expected for the fully deglycosylated 867-amino-acid epitope-tagged Env protein (Fig. 5, FLAG, lane Pulse [+]). After a chase period of 1 h or more, a second band which migrated at approximately 80 kDa appeared after endo H treatment, consistent with partially endo H-resistant gp120 resulting from the modification of 13 of the 24 carbohydrate side-chains of gp120 in the Golgi (22).

The band migrating at approximately 116 kDa seen in the lysate from cells expressing ERRS-containing Env in Fig. 1 was not apparent in this experiment. The possible identity of this protein and an explanation for the absence of this band in this experiment will be proposed in the Discussion. Additional bands seen in this experiment from the expression of the ERRS mutant are thought to be artifactual nonspecific protein degradation products resulting from the overnight incubation. No distinct bands in the (+) lanes within the bottom section of the gel could be attributed to endo H-digested forms of these products (data not shown).

If the ERRS-containing Env is indeed not transported to the Golgi, or at least is not allowed to accumulate there, then this lack of Env in the Golgi should be obvious upon immunofluorescent staining of cells expressing this mutant. To demonstrate this, CV-1 cells expressing each of the Env constructs were fixed and stained for indirect immunofluorescence with a monoclonal antibody to the V3-loop of gp120/gp160 and a Texas Red-conjugated secondary antibody.

Fig. 6 shows that in cells transfected with either wild-type or epitope-tagged Env, bright perinuclear fluorescent staining could be seen, consistent with localization of Env in the Golgi apparatus and similar to the fluorescence pattern seen upon staining with

FIG. 5. Endo H treatment of envelope glycoproteins. Cells were radiolabeled and chased for 1 (1H), 2 (2H), or 3 h (3H). Glycoproteins were immunoprecipitated from the resulting cell lysates and split in half for either endo H digestion (+) or mock treatment (-). The FLAG and ERRS mutants were expressed in the top and bottom panels, respectively. $gp160_S$ indicates glycoprotein precursor which was sensitive to endo H digestion. $gp120_R$ and $gp160_R$ indicate processed glycoprotein and glycorprotein precursor, respectively, which was resisitent to endo H digestion.

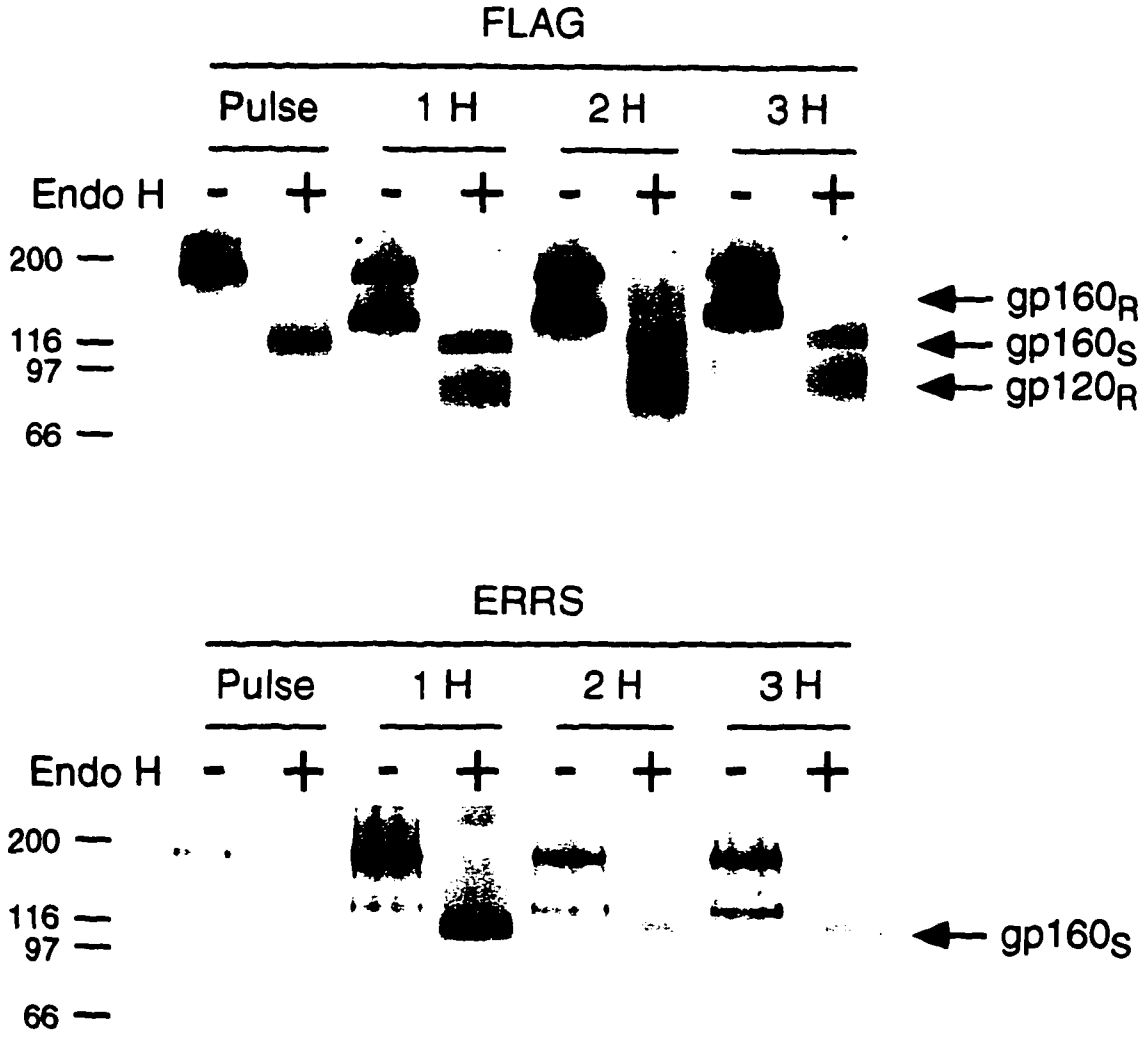
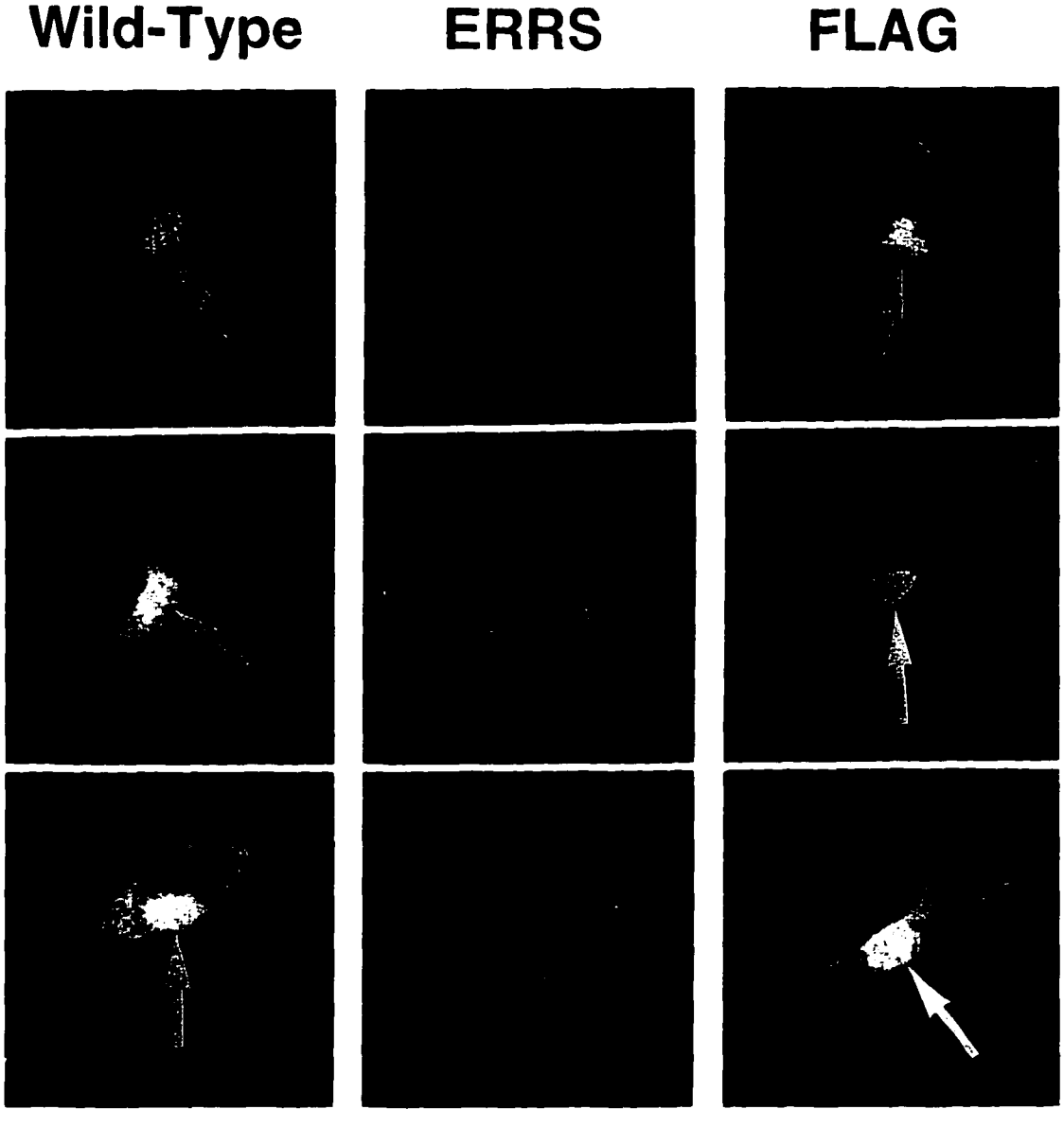


FIG. 6. Internal immunofluorescent staining of envelope glycoproteins expressed in CV-1 cells. CV-1 cells expressing glycoprotein were fixed and stained with a monoclonal antibody to gp120, followed by detection with a Texas red-conjugated secondary antibody. Photographs were taken with a fluorescence microscope.



rhodamine-conjugated wheat germ agglutinin (31). In contrast, cells expressing ERRS Env lacked any discrete region of intense staining, displaying instead only diffuse cytoplasmic staining. This staining pattern is consistent with the localization of the ERRS mutant to the ER and its exclusion from the Golgi apparatus. These results confirm that the ER-retrieval signal in the ERRS mutant is functional.

ERRS Env does not affect extracellular virus production. The preceding sections demonstrate that the ER-retrieval signal incorporated into the C-terminal marker peptide is functional in retrieving Env prior to its transport through the Golgi and that, as a result, Env accumulates in the ER. To test how this intracellular localization of Env affects virus assembly and budding, both the FLAG and ERRS modifications were transferred to the *env* gene of an NL4-3 proviral clone. If the site of virus assembly and budding is dependent on the localization of Env, then virus containing ERRS Env might be expected to assemble at the ER membrane and bud into the ER. Two days after the transfection of 293T cells with the proviral construct, the culture medium was collected and filtered to remove cellular debris. Aliquots of the filtered medium were assayed for reverse transcriptase (RT) activity as a measure of the relative level of virus production. As can be seen in Fig. 7A, each of the proviral constructs produced high levels of RT activity. The FLAG and ERRS proviruses produced only slightly reduced levels of RT activity as compared to wild-type provirus, indicating that these modifications do not significantly affect the production of virus from cells. Therefore, the localization of ERRS Env to the ER does not prevent the efficient budding of virus from the cell.

Virus produced from cells expressing ERRS Env is noninfectious. To determine whether the virus produced from the FLAG and ERRS proviral constructs was infectious and thus contained functional Env, aliquots of the RT-assayed media were used to infect HeLa-CD4/LTR-ß-gal cells. Aliquots were normalized according to their relative RT values and incubated with HeLa-CD4/LTR-ß-gal cells for 2 h. Fresh media was then added, and the cells were fixed and stained with X-gal 2 days later.

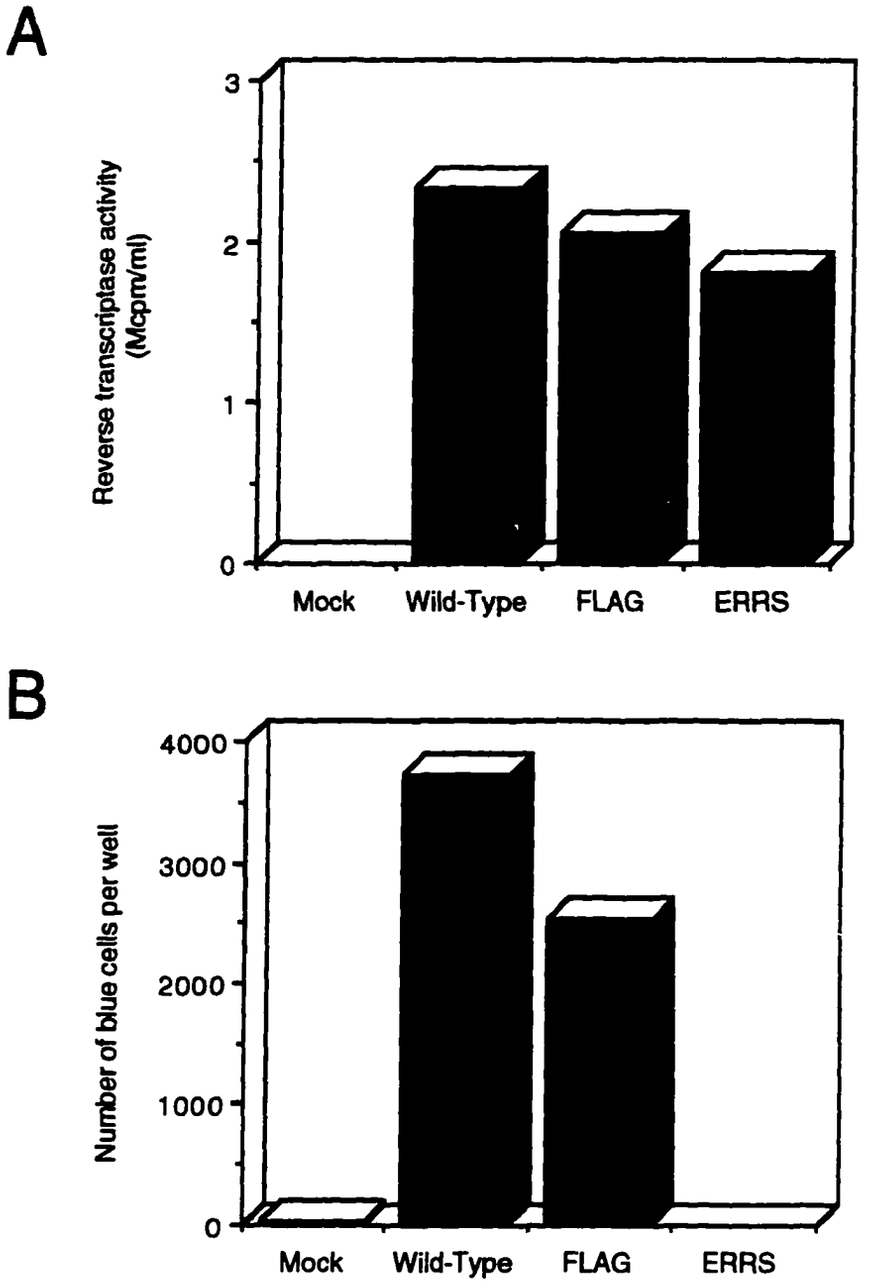


FIG. 7. Virus expression and entry. pNL4-3 proviral constructs were expressed in 293T cells. (A) Culture supernatants were collected and filtered, and relative levels of virus were quantitated by measuring RT activity. (B) The volumes of the supernatants were normalized based on their relative levels of RT activity and used to infect HeLa-CD4/LTR-ß-gal cells. The number of blue cells per well was estimated by counting the number of blue cells in each of three 16-mm2 fields at low power for three separate wells. The average number of blue cells per field was then multiplied by the area of each well.

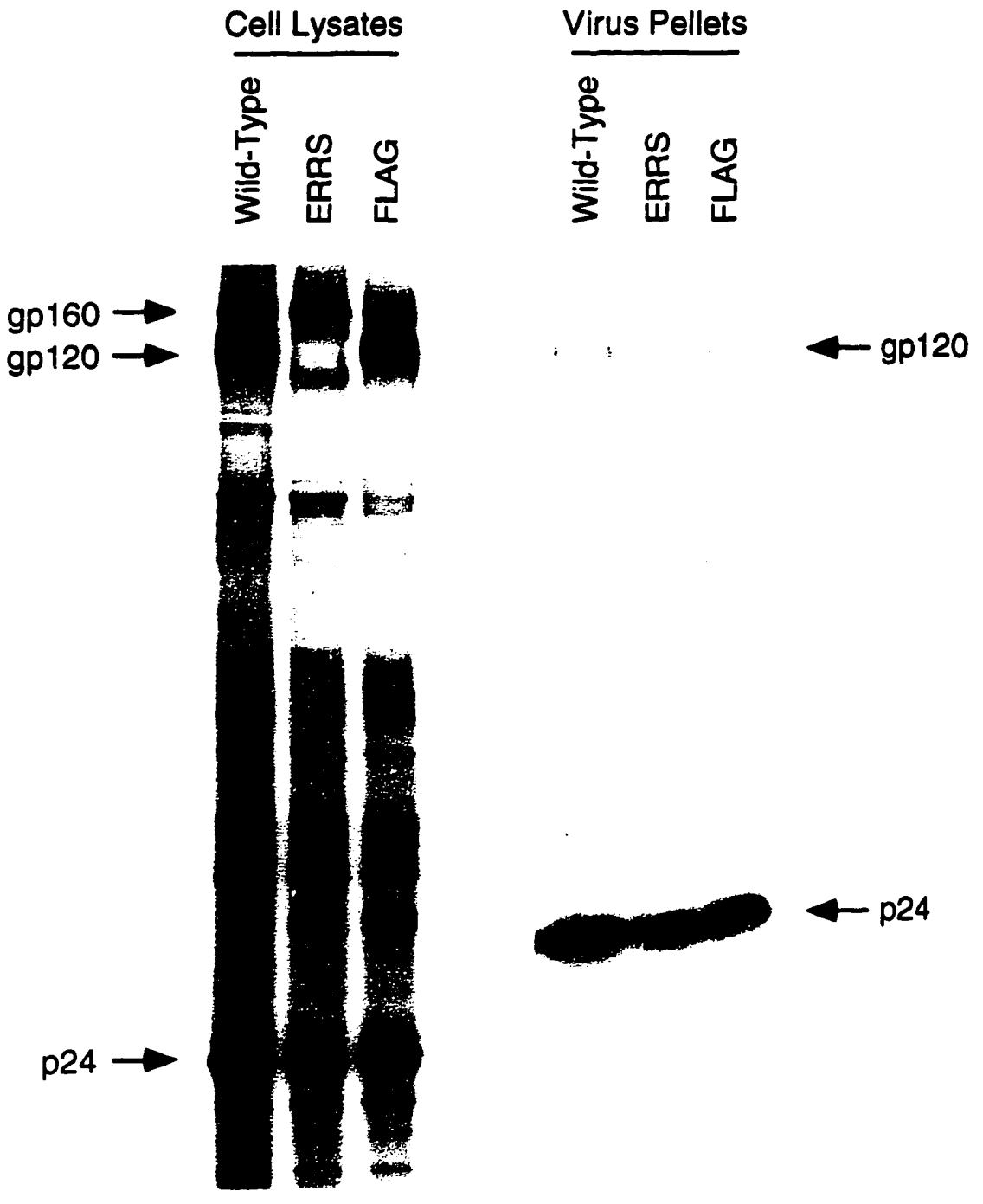
Fig. 7B shows that virus produced from both wild-type and FLAG proviral constructs resulted in large numbers of blue syncytia, indicating that these viruses were highly infectious. In contrast to the FLAG provirus, however, the ERRS construct yielded only background numbers of blue syncytia. Therefore, the virus produced from cells expressing ER-localized Env is completely noninfectious.

ERRS Env is not incorporated into virions. To determine whether the virions produced from the ERRS provirus contained any glycoprotein, virus was pelleted for protein analysis. Twenty-four hours posttransfection with either wild-type or mutant provirus, 293T cells were metabolically radiolabeled overnight. The culture medium was then filtered through a 0.45-μm filter to remove cellular debris and centrifuged to pellet virions. The virus pellet was resuspended in lysis buffer for immunoprecipitation and analysis of proteins by SDS-PAGE.

Fig. 8 demonstrates that glycoprotein was efficiently expressed by all three proviral constructs in the cell lysates. However, once again no gp120 was produced by the ERRS construct. The faint 116-kDa band, as well as a distinct band migrating just ahead of gp160, was also once again detected for this mutant. In the viral pellets, similar amounts of gp120 were detected for both wild-type and FLAG constructs, but no form of glycoprotein was detected in the pellet from the ERRS construct. p24 levels were similar in each of the viral pellets, indicating that the lack of glycoprotein in the ERRS lane was not due to a decreased amount of pelleted virus. These results demonstrate that the localization of Env to the ER results in the efficient production of virions which lack envelope glycoprotein and are thus noninfectious.

Virus produced from cells expressing ERRS Env buds from the plasma membrane. The lack of incorporation of ERRS Env into virions suggested that virus was budding exclusively from the plasma membrane, which was shown in Fig. 4 to be devoid of glycoprotein. Alternatively, virus could be budding into the ER but failing to incorporate Env for some other reason. Yet another possibility was that only a subset of

FIG. 8. Incorporation of glycoprotein into virions. Virus was pelleted from the filtered culture supernatant of radiolabeled 293T cells. The viral pellet was resuspended in lysis buffer and immunoprecipitated in parallel with the cell lysate with patient serum prior to analysis by SDS-PAGE.



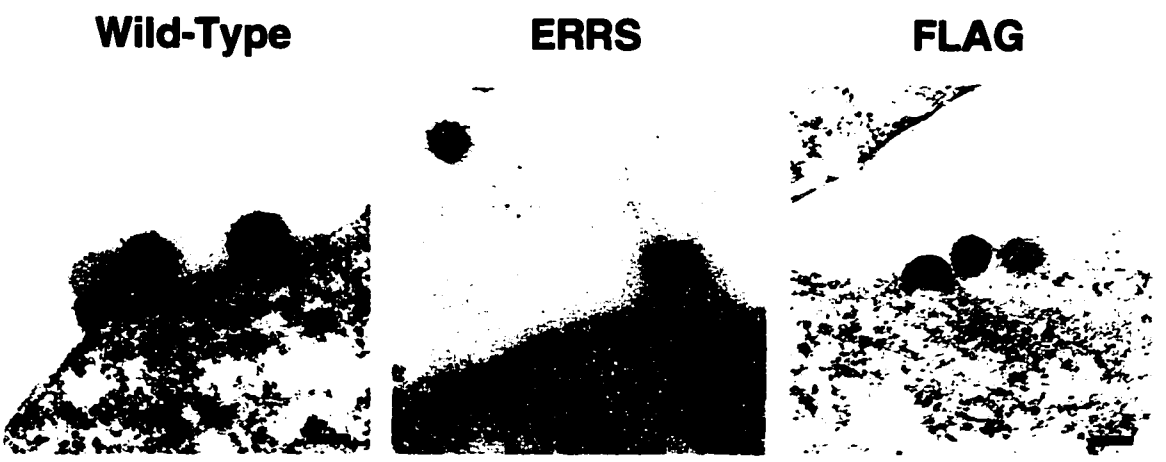
virus was assembling at the ER membrane but was unable to completely bud for some reason. To distinguish between these different possibilities, cross-sections of 293T cells expressing high levels of virus were examined by EM for budding structures. Representative photos are shown in Fig. 9. In each cell culture, regardless of the construct being expressed, virions were found budding from the plasma membrane. No intracellular budding structures could be found in the cells expressing the ERRS construct which were distinguishable from mock-transfected cells. Nor could any budded virus be found in an intracellular compartment. These results confirm that the lack of incorporation of ERRS Env into virions was due to the exclusive budding of virus from the plasma membrane.

DISCUSSION

We have shown that accumulation of the HIV envelope glycoprotein within the ER is insufficient to redirect the assembly of virions at the ER membrane. The introduction of an eight-amino-acid highly charged epitope tag and a three-amino-acid spacer peptide to the C-terminus of Env did not affect the ability of the protein to mediate both cell-cell fusion and virus entry. However, when this tag was modified to contain an ER-retrieval signal, the glycoprotein was no longer transported through the Golgi and thus was not processed normally or expressed on the cell surface. As a result, expression of this Env mutant (ERRS) did not result in cell-cell fusion. Importantly, expression of the ERRS mutant did not affect the normal budding of virions from the plasma membrane, and the virus which was released did not contain glycoprotein and thus was non-infectious. EM analysis of these cells failed to reveal any structures of assembling or budding virus at the ER membrane, and no virions were found within an intracellular compartment. These results, therefore, do not provide any evidence for a functional interaction between Env and Gag proteins at this early step in glycoprotein transport.

While no mature gp120 was produced in cells expressing the ERRS Env construct, a faint band migrating at approximately 116 kDa was apparent. In addition, a similarly faint band was also seen migrating just ahead of gp160. This is consistent with a subset of

FIG. 9. Budding of virus from the plasma membrane. 293T cells expressing pNL4-3 proviral constructs were fixed and sectioned for EM analysis. The bar represents 100 nm.



the glycoprotein reaching the *cis*-Golgi, where the high-mannose sidechains are trimmed, before being retrieved back to the ER, resulting in a faster mobility of the glycoproteins. Since the bands of partially processed gp160 and the 116-kDa underglycosylated surface subunit are of similar intensity, it seems likely that the cellular enzyme which cleaves the glycoprotein precursor is at least partially active in the early compartments of the Golgi. The lack of detection of this band in the endo-H experiment is believed to be due simply to a lower level of expression in that experiment and insufficient resolution by SDS-PAGE. It may also be that the small amount of underglycosylated product was highly susceptible to proteases and was degraded during the incubation, since degradation of endo-H deglycosylated proteins was also evident.

Evidence for the association of HIV-1 Env with Gag has increased in recent years. Deletions within the HIV-1 MA protein have been shown to prevent the incorporation of glycoprotein into virions (7, 33). More recently, individual amino acid substitutions within MA were found to be sufficient for this effect, and the block to glycoprotein incorporation by these mutants was further shown to be overcome by truncating the cytoplasmic tail of Env (12, 13). These results suggested that regions near the center of the cytoplasmic tail were critical for an interaction with Gag. The existence of such an interaction was also strongly supported by a recent report demonstrating that an endocytosis signal within the cytoplasmic tail of Env was down-regulated by coexpression of the Gag precursor protein, pr55 (11). In addition, an interaction between immobilized Env cytoplasmic domain and soluble MA has been demonstrated in vitro.

Nonetheless, the lack of incorporation of glycoprotein into virions, of inhibition of virus budding, and of virus assembly and budding within the ER suggests that localization of Env to the ER is not sufficient to target Gag assembly to this site. How might one explain the discrepancy between our results and the ability of Env to redirect the site of virus budding in polarized epithelial cells? It is unlikely that the lack of retargeting is due to the C-terminal modification of the protein, since the FLAG modification, which is just as

highly charged as the ERRS sequence, had no discernable effect on the functional association of Env and Gag. Therefore, it is likely that the lack of association between Env and Gag is a consequence of its localization to the ER. It is important to note that the composition of the ER membrane is likely to be very different than that of the plasma membrane. It could be that Gag interacts with ER-localized Env, but virions cannot assemble and bud at the ER membrane because of the absence of necessary cellular factors or because certain factors in and around the ER membrane disrupt assembly and budding.

An alternate explanation for our results is that the cellular machinery which is responsible for binding to the ER-retrieval signal and rerouting the protein back to the ER inhibited the interaction of Gag with the Env cytoplasmic tail. The consensus K(X)KXX signal sequence has been shown to bind and drive the polymerization of microtubules in vitro (4). In addition, coatamer protein has also been shown to bind to ER-retrieval signal sequences (3), and it is possible that this binding sterically interferes with Gag association.

Finally, protein sorting in polarized epithelial cells appears to occur in the *trans*-Golgi complex, the site of retroviral cleavage. We and others have shown previously that cleavage-deficient mutants of HIV-1 Env are inefficiently incorporated into virions (8). Thus, it is possible that Gag-Env interactions require conformational changes in the cytoplasmic domain that occur only after transport and proteolytic cleavage of the mature glycosylated gp160.

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DISCUSSION

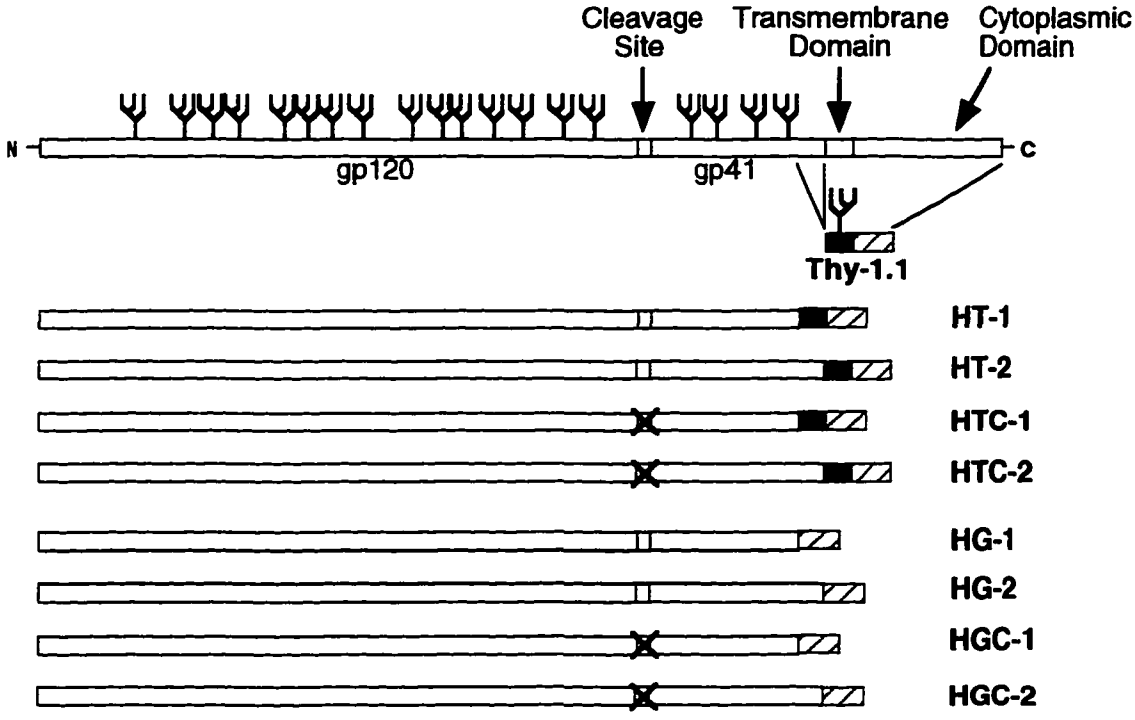
GPI-ANCHORED VIRAL ENVELOPE GLYCOPROTEINS: ROLE OF THE MEMBRANE-SPANNING DOMAIN IN MEMBRANE FUSION

The strategy utilized in the initial paper for the expression of GPI-anchored HIV-1 envelope glycoproteins required the addition of a foreign peptide to the C-terminus of the Env ectodomain. The resulting proteins were nonfunctional, but it was unclear whether the lack of function was due to the fact that they were GPI-anchored or due to interference with the fusion reaction by the heterologous peptide. The protein engineering strategy utilized in the first paper was modified in the second paper to eliminate the intervening non-viral sequence from the ectodomain (see Fig. 1). In this case, it was found that the C-terminus of the HIV-1 Env ectodomain interfered with attachment of the glycolipid membrane anchor by cellular machinery. Truncation of the ectodomain removed the block to GPI attachment, but the accompanying study in the second paper of this dissertation demonstrated that the region deleted in the truncated ectodomain was critical for the function of the full-length glycoprotein. Therefore, the GPI addition strategy was then applied to SIV Env (see Fig. 2). The resulting SIV glycoproteins were efficiently attached to a glycolipid anchor, indicating that the same C-terminal region in SIV Env is not inhibitory to GPI attachment. The GPI-anchored SIV ectodomains were also found to be incapable of mediating membrane fusion. Therefore, lentiviral envelope glycoproteins appear to require a membrane-spanning peptide anchor in order to function.

Similar results with another GPI-anchored HIV-1 glycoprotein have since been reported by another group (196). In addition, glycolipid-anchored forms of RSV Env (75), MuLV Env (156) and flu HA (105) have also been shown to be nonfusogenic. In the case of HA, however, fusion of the outer leaflets of the donor and target cell membranes

FIG. 1. Diagram of HIV-1 envelope glycoproteins containing different GPI addition signals. (A) Diagram of the original four chimeric constructs containing the C-terminal 53-amino acid peptide of Thy-1.1 (top) and the constructs engineered to contain only the minimal GPI-addition signal, lacking the 21 amino acid residues from Thy-1.1 upstream of the GPI-addition site. (B) Schematic representation of the differences in structures of the resulting GPI-anchored ectodomains for each of the two types of constructs.

A



B

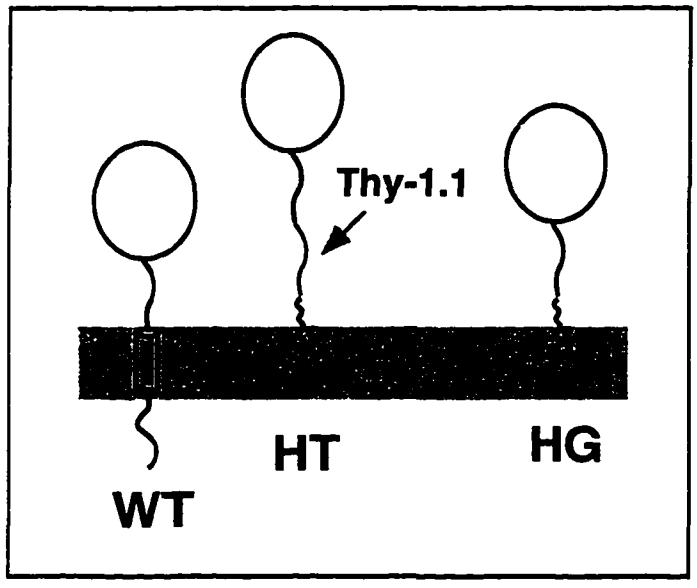
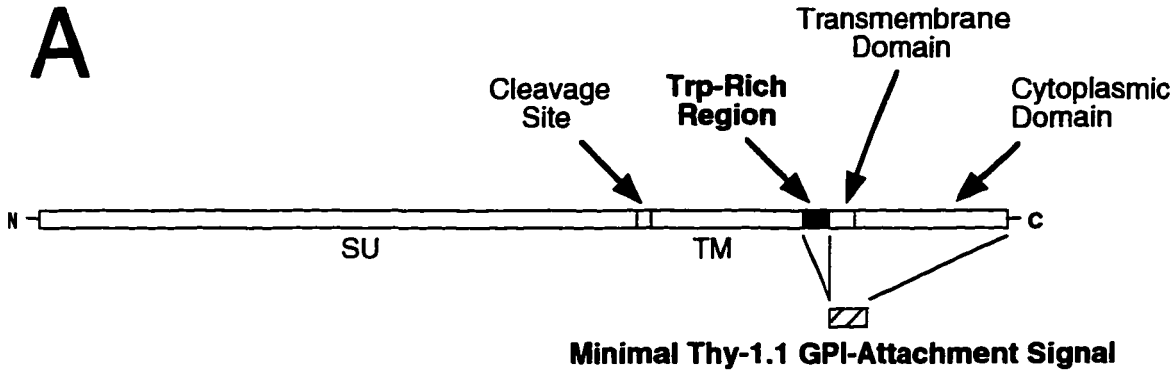
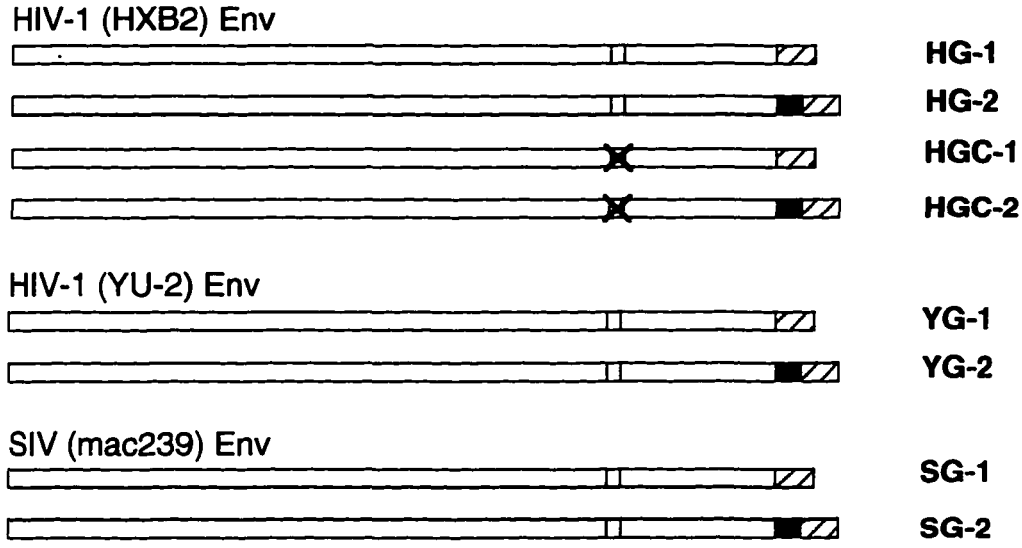
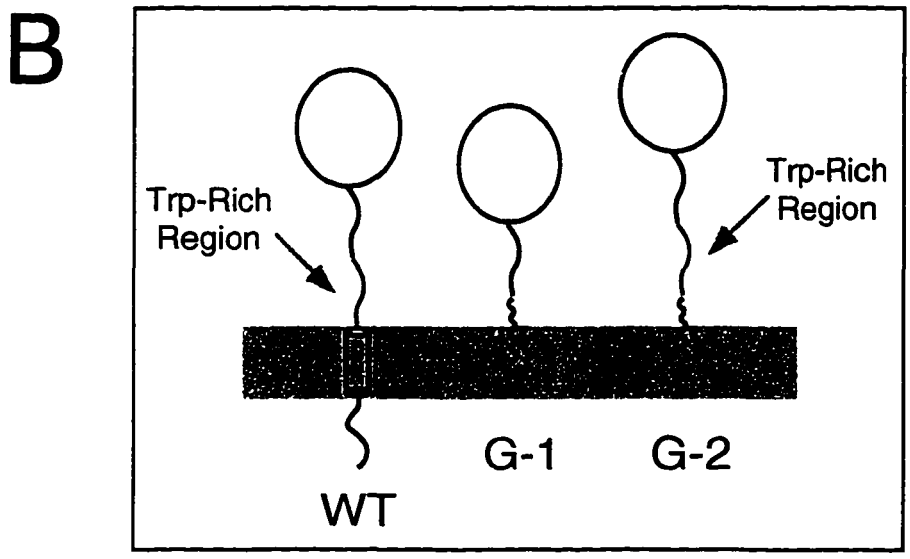


FIG. 2. Diagram of lentiviral envelope glycoproteins containing a minimal GPI addition signal. (A) Diagram of the different lentiviral glycoprotein constructs generated which contain the minimal GPI-addition signal, illustrating the presence or absence of the tryptophan-rich region from each of the constructs. (B) Schematic representation of the expected structural differences between constructs resulting from the placement of the GPI-addition signal at two different positions within the glycoprotein ectodomain.







was detected in the absence of mixing of the aqueous contents of the two cells (105, 133). This phenomenon, termed hemifusion, had been demonstrated previously in synthetic membrane systems but had never been seen previously in biological systems. This suggests that viral envelope proteins must interact with both leaflets of the viral membrane in order to complete the fusion reaction. A model has been proposed in which hemifusion is an intermediate step in all protein-mediated membrane fusion events (105). This model predicts that the resolution of the hemifusion intermediate requires a membrane-spanning peptide domain. Interestingly, truncation of the simian virus 5 fusion glycoprotein has also been shown to result in hemifusion (4). Because truncation of this protein may well have shortened the membrane-spanning domain (the exact border of the membrane-spanning domain has not been defined for this protein), it is likely that the truncation mutants are unable to interact sufficiently with the inner leaflet of the donor membrane. Thus, the shortened transmembrane domain may mimic the GPI-anchored ectodomain in trapping the hemifusion intermediate.

Future studies should be able to determine whether the GPI-anchored constructs described here are capable of mediating hemifusion. The ability to release these Env ectodomains in a soluble form by using phospholipase may prove to be useful for obtaining pure oligomeric complexes for use in structural and functional studies. For example, solubilized glycoprotein ectodomains could be used to determine whether, in the presence of sCD4 and CKR-containing membranes, the fusion peptide (and perhaps other glycoprotein domains) associates with the target membrane. This could be determined by using photoactivatable lipid-soluble tags as was done previously with bromelain-solubilized flu HA (85).

Although it seems reasonable that Env requires a membrane-spanning domain in order to complete the fusion event, it is less clear what the structural and sequence requirements of that domain are. Substitution of the membrane-spanning domain of CD4 for that of HIV Env has been shown to result in a fusogenic protein (192). Similarly, the

membrane-spanning domain of CD22 could also functionally replace that of HIV Env (203). However, in the context of the HIV-1 sequence, mutations within the membrane-spanning domain have been reported to interfere with the fusion. Substitution of a hydrophobic residue for the central arginine of this domain has been reported to abrogate fusion (149). In addition, insertion of three additional amino acids into this domain or the deletion of multiple residues has also been shown to abrogate fusion (89). Even multiple conservative substitutions have been reported to interfere not only with fusion, but with membrane anchoring as well (69). Thus, the role of the membrane-spanning domain of HIV Env in membrane fusion appears to be fairly complex.

How might the structure and/or sequence of the membrane-spanning domain affect the completion of the fusion reaction? Disruption of the viral membrane may occur similar to the presumed disruption of the target cell membrane by the fusion peptide. In this respect, the sequence of the membrane-spanning domain may be critical for the membrane fusion function of Env. For example, the charged residue in the middle of the membrane-spanning domain may play a critical role in membrane disruption. A more likely explanation, however, is that the membrane-spanning domain forms a critical structure at the base of the putative fusion pore complex. Without a complete pore stretching through both leaflets of the viral membrane, the mixing of contents may not occur. In addition, certain structural requirements and sequence specificities of the membrane-spanning domain may influence the formation of higher-order oligomers or the pore, itself.

Interestingly, the GPI-anchored HIV glycoproteins were found to be incorporated into virions very efficiently. Efficient incorporation has also been reported for GPI-anchored MuLV Env (156) and bovine herpesvirus gIII (120). The GPI-anchored cellular protein Thy-1 has also been shown to be incorporated into MuLV virions (17), and GPI-anchored CD55 and CD59 are incorporated into both human T cell leukemia virus type 1 and human cytomegalovirus (175). As will be discussed in the third section of this Discussion, HIV-1 has evolved a mechanism for the efficient recruitment of glycoproteins

into budding virions. This mechanism appears to involve an interaction between the Gag and Env proteins beneath the plasma membrane of the cell. Thus, in the case of the GPI-anchored glycoproteins, which have no cytoplasmic sequences, no such mechanism could be functioning. So how were these proteins incorporated so efficiently? It may be that the increased lateral mobility of the GPI-anchor in the membrane, combined with the absence of an endocytosis signal, allows these proteins to accumulate into a patch of the membrane which has been cleared of other cellular proteins by the underlying Gag proteins. Moreover, it could be that the GPI anchor enriches the concentration of the protein within certain subdomains of the plasma mebrane from which virus buds. A caveat to this efficient incorporation mechanism, of course, is that the incorporated glycoproteins are nonfunctional.

ROLE OF THE TRYPTOPHAN-RICH REGION OF HIV ENV IN FUSION

The work outlined in the third paper centered on mutational analysis of the membrane-proximal region of the gp41 ectodomain to determine its role in fusion. This region is unusually rich in tryptophan residues, containing five which are completely conserved among all strains of HIV within a seventeen-amino-acid stretch of uncharged residues. In addition, the other hydrophobic residues in this region are highly conserved and can be modeled to cluster on one face of an amphipathic α-helix. Deletion of this entire region adjacent to the membrane-spanning domain resulted in a stably membrane-anchored protein which was processed and transported to the cell surface normally and which bound CD4 efficiently. However, this deletion abrogated both Env-mediated cell-cell fusion and virus entry. Substitution of even phenylalanine for individual tryptophan residues reduced the average size and number of Env-induced syncytia by almost 10%, while inhibiting virus entry much more dramatically. This indicated that the tryptophan residues, and not simply the hydrophobic moment, were critical for the function of this domain in fusion. Further substitution of alanines for the first three or all five tryptophans, together, abrogated both cell-cell fusion and virus entry. Moreover, the insertion of a nine-amino-

acid spacer between this tryptophan-rich region and the membrane-spanning domain also abrogated fusion. This was particularly intriguing, since insertion of the same sequence into the analogous position of influenza HA had been shown not to disrupt fusion (105). This suggested that the tryptophan-rich region might interact with the viral envelope during fusion. This hypothesis could be tested in future experiments using fusion-impaired mutants as negative controls for lipid association.

Although the tryptophan-rich region of the HIV-1 envelope glycoprotein is predicted by computer algorithms to form an α-helix, if modeled as a ß-strand, all five of the conserved tryptophan residues lie on the same side of the strand (see Fig. 3). However, unlike in the α-helix model, the tryptophan residues are interspersed with non-conserved polar and neutral residues. Moreover, the other conserved hydrophobic residues lie on both sides of the strand rather than clustering together. Thus, it is tempting to favor the α-helical structure because it suggests a more cohesive functional domain. However, since the glycoprotein does undergo conformational changes, it could be that the tryptophan-rich region adopts two different secondary structures at different stages of fusion. Interestingly, studies of a synthetic HIV-1 fusion peptide suggest that this region forms a ß-sheet up until contacting membranes, at which time the structure rearranges to an α-helix which inserts into the membrane (127).

Although even individual conservative mutations were found to decrease the fusogenicity of the glycoprotein, the tryptophan-rich region proved to be relatively resistant to mutagenic disruption, with most of the mutants mediating some degree of membrane fusion. The pronounced inhibitory effect of the SC7 mutation, however, which was designed to disrupt the predicted structure of this region by scrambling conserved residues, further suggested that the overall structure and sequence arrangement are important in the function of this domain. While the W672P mutant did not disrupt fusion or entry more than other single amino acid substitutions, this does not necessarily preclude an α-helical

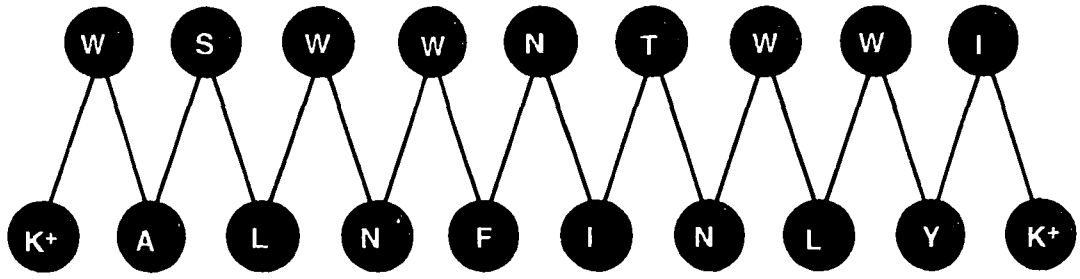


FIG. 3. Potential ß-sheet structure of the tryptophan-rich region of the HIV-1 envelope glycoprotein. All five highly conserved tryptophan residues align on the same side of the structure. Other highly conserved hydrophobic residues, however, fall on opposite sides of the predicted structure.

structure. Many α-helical membrane-interactive peptides, including melittin, contain prolines (and thus have a kink in the helix). Indeed, in the context of membranes, proline residues have been found to stabilize α-helical secondary structure, while disrupting ß-sheet structures (118). It is clear from the striking affect of a conservative single amino acid substitution (W672F) on virus entry that the function of this region in the fusion mechanism is dependent upon the tryptophan residues which lie within it. This result explains the absolute conservation of these residues throughout virus evolution. The conservation among different virus isolates of residues important for efficient membrane fusion suggests that optimal membrane fusion efficiency is selected for in vivo and is critical for virus replication.

2F5, one of only two monoclonal antibodies specific for gp41 which neutralize virus (35, 143, 144), binds an epitope overlapping the amino terminal portion of this tryptophan-rich region. Cao et al. have shown that a single amino acid substitution in the epitope permitted virus to escape neutralization by this antibody (19). 2F5 may block fusion by inhibiting a necessary conformational change in Env which involves the overlapping tryptophan-rich region. In support of this is a study demonstrating that 2F5 binding prevented the exposure of distal epitopes on gp41 and gp120 (146). Alternatively, binding of 2F5 to this region may sterically block the interaction of this region with another peptide domain or with the membrane. Interestingly, the +FLAG mutation, which inserts six amino acids from the FLAG marker peptide into the middle of the 2F5 epitope, also abrogated fusion. It may be that the insertion of the highly charged epitope mimics the binding of 2F5 antibody in inhibiting one of these types of interactions.

In addition to 2F5, a synthetic peptide, DP-178, corresponding to a 36-amino-acid sequence of gp41 which overlaps the tryptophan-rich region has been shown to potently inhibit fusion (200, 202). Studies on truncated versions of this peptide suggested that the portion of the peptide corresponding to the amino terminal half of the tryptophan-rich region was critical to the inhibitory function of the peptide (200). This suggested that the

tryptophan-rich region may be involved in a critical peptide-peptide interaction during fusion for which DP-178 was competing. However, a longer peptide, SJ-2176, which overlaps all of DP-178 except for the C-terminal portion which corresponds to the tryptophan-rich region, has also been shown to inhibit fusion at similar concentrations (101, 145). Thus, it is unclear whether the tryptophan-rich region is interacting with a domain encompassed by the SJ-2176 sequence, whether SJ-2176 binding prevents conformational changes necessary for the interaction of the tryptophan-rich region with other domains, or whether the amino-terminal sequence of the tryptophan-rich region merely adds stability to the shorter versions of the DP-178 peptide which block adjacent interactions. There is evidence that both peptides interact with the fusion peptide (145), as well as evidence that DP-178 interacts with the heptad repeat region (200). In support of the last scenario, amino acid substitutions in the amino-terminal half of the tryptophan-rich region failed to permit escape from the inhition of fusion by DP-178 (163).

Not to be underemphasized are the dramatic differences between the effects these mutations had on cell-cell fusion versus virus-cell fusion. These results, which were obtained using the most sensitive and precise assay systems available for the study of these events, clearly demonstrate that cell-cell fusion assays, alone, can underestimate the impact of certain mutations on virus entry. How do even these subtle mutations result in a dramatically decreased number of virus entry events? Since these mutations did appear to slightly decrease the efficiency with which the envelope glycoprotein can mediate fusion in the cell-cell assay, it may be that the effects of these mutations on fusion were overcome by the multiple interactions and large contact area of cell-cell fusion, but were much harder to compensate for on the surface of the virion. If the probability of a glycoprotein complex initiating (or completing) a membrane fusion reaction (presumably by forming a fusion pore) is reduced by a mutation, then the effect of that mutation on the fusion of two membranes would be expected to be dependent on the number of glycoprotein interactions which can occur between the membranes. Contacts between two adherent cells would be

expected to permit many more glycoprotein interactions with the target membrane than the contact of a virion with the cell. Therefore, the probability of an attached virus particle containing the mutated glycopotein initiating a successful fusion event would be much lower than the probability of a cell expressing the glycoprotein initiating fusion with another cell. Such a scenario may even represent fusion events initiated by wild-type glycoprotein, since every cell expressing glycoprotein in a cell-cell assay forms syncytia, but only approximately one virion in two thousand gains entry into a cell by the virus entry assay utilized in this study. Alternatively, this inefficiency of infection could be due to defects during reverse transcription. Why more studies have not found similar discrepancies between cell-cell fusion and virus entry mediated by glycoprotein mutants is unclear, but very few studies, if any, have studied glycoprotein mutants by quantitating single-round virus entry using uncomplemented virus in parallel with cell-cell fusion.

What role does this region, and the tryptophan residues in particular, play in the membrane fusion mechanism? Tryptophan residues have been suspected to play an important role in a number of biologic processes. Among these is an interaction with cholesterol in biological membranes, similar to that shown with the indole moiety of polyene antibiotics, which has been proposed to facilitate hemolysis (41). Another proposed function is the mediation of inter- and intra-peptide interactions between juxtaposed Trp residues. The aromatic rings of two tryptophan residues can stack on either side of a basic residue such that the pi electrons interact. Thus, the tryptophan residue at position 672 in the HXB2 Env glycoprotein could potentially interact with cholesterol in either the viral envelope or target cell membrane, intra- or inter-chain residues with aromatic ring side chains, or intra- or inter-chain residues with cationic side chains. Such an interaction may be crucial in the membrane fusion event.

Interestingly, a study of the effect of Trp substitutions in the hemolytic bee venom protein melittin found that substitution of a residue on the hydrophilic side of either of the protein's amphipathic helices such that the protein's calculated hydrophobic moment is

decreased actually increased hemolytic activity (13). This configuration facilitated peptide-lipid interaction, whereas substitution of a residue on the hydrophobic sides of the amphipathic helices decreased hemolytic potential, favoring peptide-peptide interaction. This suggests that apart from overall amphipathicity, specific interactions between tryptophan and other residues or with cholesterol are important in hemolysis. From this it is interesting to note that substitution of the tryptophan residue at position 672 with even another highly hydrophobic residue with an aromatic ring side-chain greatly reduced virus entry, supporting in this case as well the importance of a specific Trp-mediated molecular interaction.

Does the tryptophan-rich region associate with membranes? The detrimental effect of the W672F mutation on virus entry suggests that the function of this region is specifically dependent on the conserved tryptophan residues, and that the aromatic sidechains or hydrophobicity of these positions, alone, is not the critical feature. This is unlike the effect of similar mutations in the fusion peptide, in which mutations which increase hydrophobicity enhance cell-cell fusion and virus infectivity (14, 66, 178). It might seem more likely from this result that this region would be involved in a peptide-peptide interaction rather than a peptide-lipid interaction, since peptide-peptide interactions often require specific interactions between residues or precise conformational structures which are dependent upon the peptide sequence. Such an interaction may be with other domain(s) thought to be involved in a conformational rearrangement following receptor binding, such as the heptad repeat or DP-178 region. Conversely, enveloped RNA viruses tend to be quite refractory to conservative mutations within the membrane-interactive fusion peptide or transmembrane domain of their viral fusion proteins.

These generalizations, however, are not absolute. In some cases, conservative substitutions for certain hydrophobic residues in both the fusion peptide and the membrane-spanning domain have been reported to inhibit fusion (42, 69). More specifically, the effect of the relative position of the tryptophan residue within the sequence of the bee

venom peptide as stated above demonstrates directly that peptide-membrane interactions can be specifically dependent upon the positioning of a tryptophan residue. Also, it is important to consider that membrane fusion was significantly inhibited by the W672F mutation only in the context of virus entry and not in a syncytium assay. Thus, it seems more likely that the function of the tryptophan residues in this region is less specific than that which might be expected to involve in a peptide-peptide interaction, since the requirement for individual residues is not absolute. Indeed, it is not until at least three of the tryptophan residues have been changed to alanine that cell-cell fusion is lost. Moreover, even such gross mutations as substitution of alanines for five of the trytophan residues, scrambling of the central seven residues of this sequence, and deletion of five residues altogether fail to completely abrogate fusion. This is in stark contrast to mutagenic analysis of regions which have been proposed to be involved in peptide-peptide interactions during the fusion event in which even single amino acid substitutions abrogate fusion (19, 51). Therefore, although even conservative single-amino acid substitutions dramatically inhibit virus entry, the functionality of this region in the membrane fusion mechanism is relatively resilient to mutation. Thus, it makes sense to think that this region interacts with membranes during the fusion reaction and that the tryptophan residues are specifically important for the enhancement of this interaction. This enhancement may be critical for efficient virus entry, and thus, these residues would have been highly conserved. Interestingly, this region behaved in a similar fashion to the analogous region of the Newcastle disease virus fusion protein, which has also been shown to be relatively resistent to single amino acid substitutions but sensitive to multiple substitutions. This region contains a leucine-zipper-like motif and was hypothesized to interact with membranes during fusion because of its proximity to the membrane (157).

In support of the interaction of this region with lipid is the abrogation of fusion by the insertion of the nine-amino-acid peptide from human DAF. The same peptide inserted into the analogous position of influenza HA has been shown not to inhibit fusion by that

molecule (105). This result suggests that the tryptophan-rich region must be immediately adjacent to the membrane-spanning domain and, therefore, may interact with the membrane in which the protein is anchored at some point during fusion. It is important to note, however, that this region could interact both with other peptide domains as well as with lipid at either the same or different stages of the fusion reaction. One such possiblity is that the region provides a hydrophobic "pocket" which accomodates the fusion peptide following processing of the precursor protein. Upon the triggering of conformational changes by the receptor, the fusion peptide may then be permitted to insert into the target membrane, whereas the tryptophan-rich region may then interact with lipid in such a way as to facilitate the disruption of the donor membrane. It is also interesting that this region interfered with GPI addition in HIV-1 ectodomains, suggesting that this region may be buried within the folded protein prior to initiation of fusion, much like the fusion peptide.

Interestingly, Sattentau et al. have shown that a monoclonal antibody to this region which binds the native protein is unable to bind its epitope following incubation with sCD4 (169). This indicates that this region either undergoes a conformational change or becomes masked following the binding of CD4 and the accompanying conformational change in Env. Masking could simply be due to steric hindrance from the sCD4 molecule, though mutations in this region do not affect CD4 binding. Masking could also be due to an interaction of another peptide domain with this region. Alternatively, the epitope could become sequestered in the membrane as a result of a conformational change in gp41.

The tryptophan-rich region has recently been suggested to be an immunodominant domain of the TM glycoprotein (18). In that study, a C-terminal epitope was delineated from the 2F5 neutralization epitope previously described. This suggests that this domain may be accessible to antibody and, thus, the aqueous environment. However, it is not clear which residues are critical for the antibody contacts. Therefore, it is possible that the residues exposed to antibody and the aqueous environment are not the residues which are involved in the membrane fusion mechanism. This could explain why these antibodies

specific for residues C-terminal to the 2F5 epitope are nonneutralizing — they may not interact with the critical residues for the function of the domain.

It is unlikely that this region interacts with chemokine receptors (CKRs) during virus entry, since gp120 has been shown to bind CKRs independently of gp41, and tropism determinants within gp120 have been shown to confer the ability to utilize specific subsets of CKRs in fusion. However, it remains possible that gp41, as well as gp120, interacts with CKR through a conserved domain which is functional in conjunction with a broad range of CKRs. The fusion mechanism may require conformational changes in gp41 induced by CD4 and/or, perhaps, CKR which involve the tryptophan-rich region. Studies of CD4-induced conformational changes by epitope exposure could be performed on the fusion-impaired mutants in future studies to determine if they are defective in this step of the fusion process.

It will also be interesting in future studies to determine whether this region is similarly important in the entry of other lentiviruses. For example, is the W(1,3,4)A mutation, which failed to abrogate fusion of the HIV-1 glycoprotein, analogous to the lethal W(1-5)A in the context of the SIV glycoprotein, since it removes all three of the tryptophan residues in that protein? Also, since insertion of the bee venom peptide melittin into the C-terminal membrane proximal domain of the MuLV glycoprotein has been found to functionally replace the region in membrane fusion (3), it will be interesting to determine whether the same peptide, which is approximately the same length as the tryptophan-rich region, can functionally replace the N-terminal membrane-proximal region of the HIV-1 glycoprotein. Finally, since potent peptide inhibitors have been found which correspond to other critical regions of the glycoprotein, it will be interesting to determine whether specific peptide inhibitors can be designed to inhibit the function of the tryptophan-rich region based on the information gained from this study.

ENV-GAG INTERACTIONS IN VIRUS ASSEMBLY

In the fourth paper of this dissertation, an endoplasmic reticulum (ER)-retrieval signal was engineered onto the C-terminus of the HIV-1 Env cytoplasmic tail in order to determine if such a protein can redirect the site of virus assembly from the plasma membrane to this organelle. This work has demonstrated that the signal is fully functional, resulting in the lack of transport of the glycoprotein to the Golgi. Importantly, a control construct containing a similar but nonfunctional signal was efficiently incorporated into virions and mediated virus entry. However, intracellular accumulation of the ER-retained glycoprotein failed to redirect virus assembly and budding to the ER membrane. Thus, the cellular machinery which interacts with the ER-retrieval signal may compete with viral structural proteins for binding to the Env cytoplasmic tail.

Recently, it was shown that the membrane-proximal region of the cytoplasmic tail of HIV-1 Env contains an active endocytosis signal (57). Moreover, it was demonstrated that coexpression of Env with the major Gag precursor protein, Pr55, blocked the endocytosis of Env. This suggests that the Gag protein blocks the recognition of the endocytosis signal by the cellular endocytosis machinery by associating with the cytoplasmic tail of Env. The endocytosis signal in Env may have been selected for because it aided in evading immune recognition of the infected cell. Once enough Gag had been produced to override the endocytosis signal, virus budding would have taken precedence over escaping immune surveillance. In vivo studies of virus mutants with defective endocytosis signals may elucidate such a role in pathogenesis in the future.

The aforementioned study suggested that there was a direct association between Gag and the cytoplasmic tail of Env and that this association was tight enough to compete away the cellular endocytosis machinery. Mutations within the cytoplasmic tail of Env have been shown to reduce the efficiency with which glycoprotein is incorporated into virions (64, 213), suggesting that the tail region does contain the structural information required for incorporation. Other indirect evidence for a Gag-Env association comes from

two reports demonstrating that Env can redirect the site of virus budding in polarized epithelial cells (151) and that truncation of the Env cytoplasmic tail abrogates this effect (121). Still more evidence comes from reports demonstrating that mutations within the MA protein of Gag can block the incorporation of Env into virions (64, 65, 212). Thus, the sum of this evidence suggests strongly that HIV Env is selectively incorporated into virus by an interaction with Gag.

Such an Env-Gag interaction could be either direct or mediated by a cofactor. A direct interaction could be tight, strengthened by noncovalent bonds or an "induced fit" involving a conformational change. Or the interaction could be loose, if, for example, one protein forms a "pocket" into which the other protein happens to fit. Moreover, the incorporation could be active or passive. Evolution of an active mechanism for Env incorporation would suggest that incorporation would be inefficient if the mechanism were disrupted. Interestingly, however, the first paper of this dissertation demonstrates that glycolipid-anchored Env is incorporated very efficiently into virions. These constructs lack a membrane-spanning domain and cytoplasmic tail and, thus, could not interact with Gag. Another interesting finding is that RSV is capable of efficiently incorporating not only truncated RSV Env (153), but also the full-length Env proteins from HIV and influenza (49, 50) as well as CD4 (211), suggesting that the incorporation mechanism utilized by that virus does not require a specific interaction. MPMV Env, on the other hand, requires a full-length tail for incorporation (15). Perhaps most interesting, though, is the fact that SIV and HIV-2 incorporate their Env proteins more efficiently when they are truncated (102, 141), in direct contrast to HIV-1. Clearly, all retroviruses do not utilize the same mechanism for Env incorporation.

Since SIV Env also contains an endocytosis signal, the discrepancy between the effects of HIV and SIV Env truncation on incorporation could be due to differences in the recognition of this signal by the endocytosis machinery or to the ability for Gag to compete for this signal in the context of the mutated cytoplasmic tails or both. For example,

truncation of SIV Env may alter the conformation of the cytoplasmic tail so that the endocytosis signal is recognized less efficiently, whereas truncation of HIV Env may not affect the recognition of the signal. In addition, Gag may interact with region(s) of the SIV Env cytoplasmic tail which are closer to the amino-terminal membrane-spanning domain than the analogous regions in HIV Env. Thus, Gag may be able to bind and compete with the endocytosis machinery even better in the truncated SIV Env, whereas critical Gag binding regions in HIV Env may be lost upon truncation.

If we assume that there is some sort of interaction between Gag and Env during HIV assembly, then how are cellular proteins excluded from the viral envelope during budding? One theory is that the assembly of Gag proteins underneath the plasma membrane excludes other membrane proteins that have associations with the cytoskeleton or other cytoplasmic components from a "patch" of membrane that is to become the viral envelope (see Fig. 4) (96). This model could be extended further if the MA protein were to have a restrictive pocket into which the cytoplasmic tail of HIV had evolved to fit. In this case, mobile cellular membrane proteins that lack a cytoskeletal association but do not fit into this pocket would also be excluded. In addition, proteins with multiple membrane-spanning domains and decreased lateral mobility in the membrane may be less likely to "flow" into the extruding membrane. Finally, viral glycoproteins may associate preferentially with a specific subset of lipid molecules. Thus, as the Env proteins congregated in association with the Gag proteins underlying the membrane, cellular proteins which associate preferentially with different lipids would be excluded. Whatever the mechanism, the exclusion of cellular proteins from HIV virions does not appear to be absolute. There have been several reports of incorporation of cellular proteins into HIV virions, including CD55 and CD59 (175), ICAM-1 (63), and chimeric HIV glycoproteins containing the transmembrane domain of CD22 (203). Two of these proteins, CD55 and CD59, are GPI-anchored. As with the GPI-anchored HIV Env proteins described in the

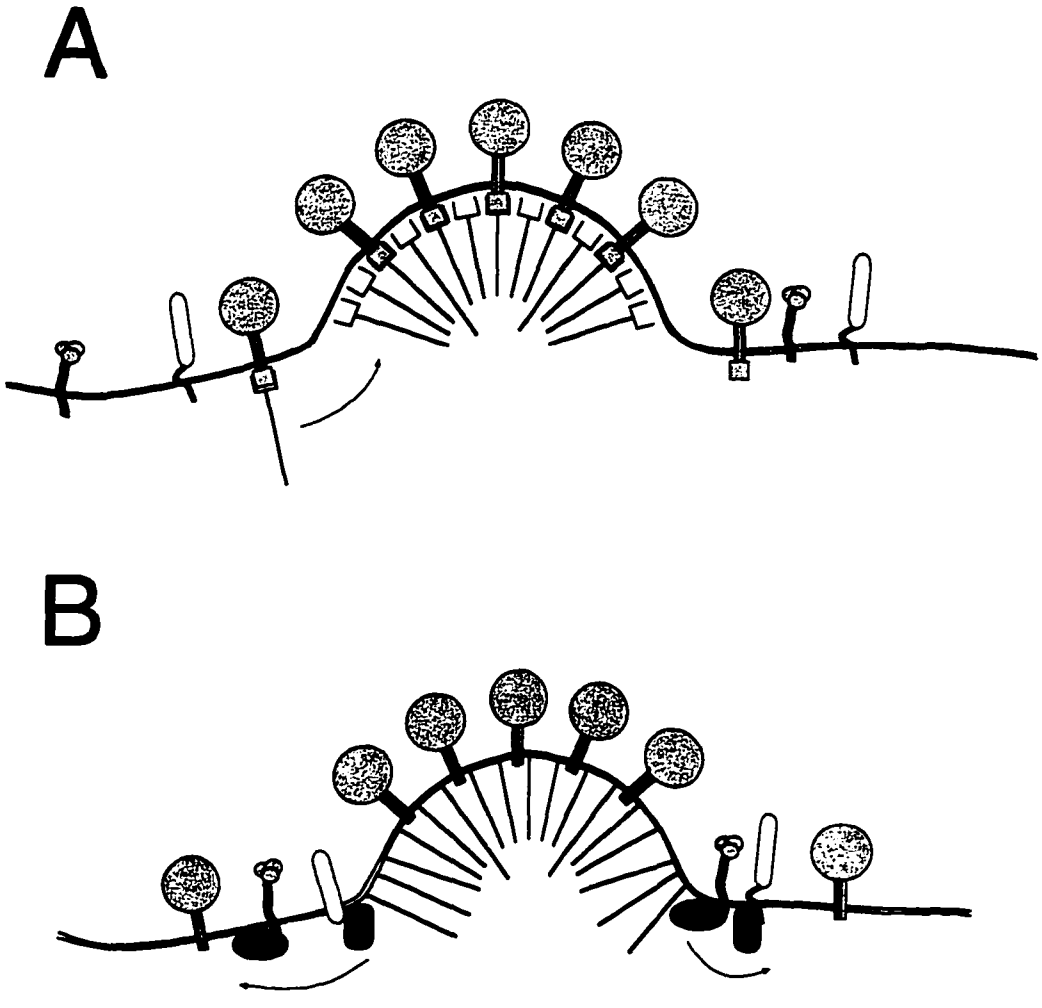


FIG. 4. Potential mechanisms for the incorporation of retroviral envelope glycoproteins into virions. (A) Glycoproteins can be "actively" incorporated into budding virions via a specific interaction with the underlying Gag precursor protein. (B) Envelope glycoprotein may be incorporated "passively" as a result of the exclusion of cellular proteins which interact with cytoplasmic factors or which contain large cytoplasmic domains from a portion of the plasma membrane lined with Gag proteins. Increased lateral mobility or preferred lipid interactions may also facilitate the enrichment of this portion of the plasma membrane with viral glycoproteins. Both of the mechanisms shown here appear to play a role in the incorporation of HIV-1 envelope glycoprotein.

first paper, these proteins may be efficiently incorporated due to their lack of inhibitory transmembrane regions and their high lateral mobility conferred by the GPI anchor.

In conclusion, the papers which constitute the body of this dissetation demonstrate that the membrane-spanning domain and the outer membrane-proximal region of gp41 are critical in the function of the envelope glycoprotein complex during membrane fusion and virus entry. The membrane-spanning domain is likely required to resolve a hemifusion intermediate during fusion, leading to mixing of the soluble contents of the two membrane compartments and completion of the fusion reaction. The tryptophan-rich, membrane-proximal region is important in both fusion and incorporation of Env into the budding virion during assembly. This region is relatively resistant to mutations in its function during fusion, although more severe mutations are capable of completely abrogating fusion. Conversely, the tryptophan motif is highly sensitive to mutations in its capacity to facilitate incorporation of glycoprotein into virions, yet even the deletion of this entire region fails to completely block the incorporation of low levels of glycoprotein. The effect of mutations on the function of this region in both of these steps in the viral lifecycle could be due to either one common or two separate mechansims. The mutations could cause a subtle alteration in the conformation of Env, they could block a necessary peptide-peptide interaction, or they could interfere with an interaction of this region with membrane lipid. Finally, the interaction of Gag structural proteins with Env appears to be dependent on the environment of the intracellular site. While this interaction clearly is efficient and specific at or near the plasma membrane, interactions at the ER membrane, if they occur, are insufficient to mediate virus assembly and budding. These findings add to our understanding of both the structural and functional requirements of the HIV-1 envelope glycoprotein and provide the foundation for further investigations into these matters.

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Major Subject Microbiology

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