Characterization of the Biosynthesis of Human Immunodeficiency Virus Type 1 Env from Infected T-cells and the Effects of Glucose Trimming of Env on Virion Infectivity*

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HIV (human immunodeficiency virus)-1 Env is displayed on the surface of infected cells and subsequently incorporated into virions, which is necessary for the initiation of a viral infection by recognition of the CD4 and the chemokine receptors (such as CCR5 or CXCR4) on the surface of new target cells. As a type 1 integral membrane glycoprotein, Env is cotranslationally translocated into the endoplasmic reticulum. In this report, we characterized the synthesis of Env, which did not occur at a constant rate but by translational/translocational pausing that has not previously been shown with a viral encoded glycoprotein. Overall translation was not impeded by the presence of the reducing agent dithiothreitol in vivo, although this did influence the cleavage of the precursor gp160 into its mature form, gp120. Env interacts transiently with resident components of the endoplasmic reticulum such as calnexin, which had maximal association at a 10-min post-translational addition. Addition of the glucosidase inhibitor, castanospermine, failed to significantly influence the association of Env with calnexin, consistent with the notion that calnexin recognizes components other than α-terminal glucose. Moreover, castanospermine treatment failed to affect the infectivity of virions. Taken together, this report demonstrates the existence of translational/translocational pausing for a viral glycoprotein and suggests that trimming of glucose from HIV-1 Env is not essential for the initiation of virus infection.

HIV-1, as with all retroviruses, encodes gag, pol, and env genes. Protein expression of Gag and Pol is directed from full-length viral genomic RNA, which is also packaged as a dimer into virions. Gag protein expression is sufficient to drive the budding and assembly of virions from the plasma membrane (1), while the Pol proteins act as the major enzymatic components of the retrovirus life cycle. Env (gp160) traffics through the secretory pathway as a type I integral membrane protein and is cleaved into its gp120 and gp41 forms by a cellular protease that resides in the medial to trans-Golgi network (2–5). During the budding process, virions acquire a portion of the host cell membrane containing the Env molecule, which is composed of two subunits, the surface domain or gp120 and the transmembrane protein or gp41. After gp120 successfully binds CD4 and its respective coreceptor, it is thought that a conformational switch occurs to expose the fusion peptide of gp41. This then leads to the fusion of the viral membrane and the new target cell to initiate a new round of infection.

The translation of Env (gp160) begins as a coupled event with ER translocation. The N terminus of Env encodes a signal sequence, which as it emerges from a translating ribosome is recognized by the signal recognition particle complex. The binding of the signal recognition particle to the leader sequence recruits the emerging nascent polypeptide to the Sec61p/translocon of the ER (6). The ribosome then forms a tight seal over the cytosolic side of the ER membrane at the translocon pore as the protein is translated. Translocation then proceeds with the proposed assistance of ER luminal components such as BiP (7–10). It has been demonstrated that certain proteins do not undergo a translationally coupled translocation at a steady rate, but rather that amino acid coding regions of certain proteins dictate translocational pausing (11). These pause-transfer sequences mediate the stopping and restarting of translocation during which the protein-conducting channel becomes partially opened, exposing the nascent chain polypeptide to the cytosol (12). These studies have raised the notion that in addition to the ribosome and the luminal components of the ER, the nascent chain may regulate translocation.

During protein translocation into the ER, the signal peptide is cleaved by the signal peptidase, and the growing nascent polypeptide chain is modified by the addition of N-linked oligosaccharides within the lumen of the ER. Alteration in the glycosylation of Env has been shown to impact the folding of Env (13–15). In addition to the role of the ER luminal chaperones in translocation of nascent polypeptides, BiP, protein-disulfide isomerase, and 94-kDa glucose-regulated protein act to prevent the misfolding of these proteins (16, 17). The lectin-like molecular chaperones, calnexin and calreticulin, have been shown to promote the folding and assembly of glycoproteins within the ER (18–20). Data have emerged suggesting that the association of the oxidoreductase, ERp57, in a complex with either calnexin or calreticulin regulates oxidation and isomerization of newly synthesized glycoproteins (21, 22). It has been proposed that calnexin acts on emerging glycoproteins through their association with their sugar residues (23); however, this has been disputed in a study in which calnexin was shown to prevent the aggregation of nonglycosylated proteins in vitro.

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1 The abbreviations used are: HIV-1, human immunodeficiency virus type 1; Env, envelope; ER, endoplasmic reticulum; BiP, immunoglobulin heavy chain binding protein; CST, castanospermine; DNJ, deoxynojirimycin; NB-DNJ, N-butyldeoxynojirimycin; PNGase, N-glycosidase F; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CN, calnexin.

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HIV-1 Env Biosynthesis and Maturation

(24). Several reports have shown that the treatment of cells with drugs that inhibit α-glucosidase, such as castanospermine (CST) and deoxynojirimycin (DNJ), interfere with the ability of calnexin to associate with glycoproteins in the ER (25). This results in the inability of these molecules to fold properly leading to their retention within the ER (26). Following the misfolding of proteins within the ER, they undergo a degradative process, probably mediated by the cytosolic 26 S proteasome (27). The treatment of HIV-1-infected cells with α-glucosidase inhibitors was shown to interfere with sncytia formation (28, 29). However, gp120, expressed in the presence of DNJ (13), and NB-DNJ demonstrated no difference in their abilities to bind to CD4 or to bind to T-cells (30). These studies brought us to re-examine the effects that α-glucosidase inhibitors have on HIV-1 infection.

In this report, we examined the biosynthesis of gp160 Env as it is translated and translocated into the ER. It was revealed that Env undergoes several translational/translocational pauses, which are ATP-dependent but not dependent on disulfide bond formation. Additionally, Env associates with calnexin in HIV-1-infected T-cells independent of the glucose trimming pathway. Moreover, the treatment of HIV-1-infected T-cells with CST fails to interfere with its secretory pathway trafficking or infectivity on susceptible target cells.

EXPERIMENTAL PROCEDURES

Cells, DNA Transfection, and Infection—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and antibiotics and passed upon confluence. H9 cell lines were grown in RPMI 1640 with 10% fetal bovine serum and antibiotics and maintained at a density of <1 × 10⁶ cells/ml. COS-7 cells were transfected by the DEAE-dextran method. Briefly, COS-7 cells were trypanosed and seeded at 50% confluence 24 h prior to transfection. Cells (5 × 10⁸) were then trypsinized, pelleted, and resuspended in 1 ml of TD buffer (25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5 mM KCl, and 0.7 mM K₃HPO₄) containing 500 µg of DEAE-dextran and 5 µg of HXB2 or HXB2NEO (31). Transfection was carried out at 37 °C for 30 min, and the cells were then washed in 5 ml of complete medium and reseeded in T-75 flasks. CST was added directly to the tissue culture supernatants containing viral particles were harvested 30 min, filtered through a 0.2-μm filter, and assayed for viral infectivity. Virions were pelleted at 100,000 × g for 90 min.

Pulse-Chase Labeling and Immunoprecipitation—H9 cells chronically infected with HXB2NEO were washed twice with PBS and incubated in cysteine-free RPMI 1640 supplemented with 2% fetal bovine serum. When performing rapid pulse-chase, pulse labeling was performed for 2 min in cysteine-free RPMI 1640 supplemented with 10 μCi/ml [³⁵S]cysteine (PerkinElmer Life Sciences), otherwise longer pulse labelings were performed in the presence of 200 μCi/ml [³⁵S]cysteine. The pulse was ended by adding prewarmed RPMI 1640 supplemented with 10% fetal bovine serum with 5 mM cysteine. After various chase time periods, the cells were transferred to ice and washed twice in ice-cold PBS (32). Alternatively, following the 2-min pulse labeling either cycloheximide (CHX, 0.5 mM) or dithiothreitol (DTT, 5 mM) was added to the chase media, or ATP was depleted from cells by the addition of prewarmed glucose-free media with 20 mM 2-deoxy-D-glucose and 10 mM sodium azide (33). Additionally, H9 cells were incubated with 200 μCi/l CST (Sigma) for 45 min prior to pulse labeling, and the drug was remained present throughout the pulse-chase period. For immunoprecipitation of Env, cells were lysed in a buffer containing 1% Triton, 10 mM Tris (pH 7.6), 300 mM NaCl, and protease inhibitors. For communoprecipitation of Env and calnexin, cells were lysed in a buffer containing 2% CHAPS, 50 mM Hepes (pH 7.6), 200 mM NaCl, and protease inhibitors. Nuclei were pelleted at 5 min at 1,500 × g, and postnuclear supernatant was incubated with either sheep anti-gp120 antiserum (AIDS Research Reagents Program, catalog number 288) or rabbit anti-calnexin antiserum (Stressgene) and protein A-Sepharose beads (Sigma) for 3 h. Immunoprecipitated samples were washed 3 times in their respective lysis buffers, separated by 7.5% SDS-PAGE, and analyzed by autoradiography.

Immunoblotting—Virion-associated viral proteins were prepared from cell culture supernatants by centrifugation at 3,000 × g for 30 min in a Sorvall RT 6000B centrifuge followed by filtration of the supernatants through a 0.2-μm membrane. Virus-particle-containing supernatants were concentrated by centrifugation through a 30% sucrose cushion at 100,000 × g for 90 min in a Sorvall Ultra 80. Viral pellets were resuspended in PBS. Cells were lysed in viral pellets by direct lysis of cells (1 × 10⁶) in 1 × lading dye (0.08 mM Tris, pH 6.8, 2.0% SDS, 10% glycerol, 0.1 M diethiothreitol, and 0.2% bromphenol blue), samples were boiled for 10 min, and proteins were separated by SDS-PAGE. Membranes were probed with HIV-1-positive human serum (1:200) or sheep anti-serum against gp120 (1:500). Blots were developed using an alkaline phosphatase reaction.

PNGase F Treatment—Cell-associated viral proteins were lysed in the presence of 1% Triton, 10 mM Tris (pH 7.6), 300 mM NaCl, 50 mM β-mercaptoethanol, and protease inhibitors. Postnuclear supernatant were then boiled for 5 min and treated with PNGase F (Genzyme) for 18 h at 37 °C in the presence of 1% Nonident P-40. Samples were boiled for 10 min in SDS-PAGE loading dye and subjected to immunoblotting.

MAGI Assay—Viral supernatants from COS-7 transfected cells were pelleted through 30% sucrose as described above and resuspended in PBS. Virions were normalized by reverse transcriptase activity as described previously (31), and 10,000 cpm equivalent of virus was used to initiate an infection. Infections were performed in triplicate using HeLa CD4-LTR-β-gal cells as targets. Assays were performed as described previously (34).

RESULTS

The HIV-1 Env protein is synthesized as a 160-kDa glycoprotein (gp160). The cleavage of gp160 to gp120 and gp41 indicates the trafficking of Env through the Golgi stacks. The mature gp120 molecule arises as a cleavage product of gp160 initially at ~80 min post-translation as demonstrated in a pulse-chase analysis of H9 infected cells (Fig. 1A). Additionally, a band of faster mobility than gp160, which reacted with the anti-Env antibody, was observed in the 0 min chase lane (as indicated with an asterisk; Fig. 1A). Because this band was chased out at later time points, we reasoned that it might be a translational/translocational intermediate of gp160. In steady-state labeling of H9 infected cell lines, the presence of the Env precursor (gp160) and the mature cleaved form of gp120 was evident (Fig. 1B). Upon treatment of gp160 and gp120 with PNGase F to remove the N-linked sugars, these molecules migrated more rapidly, consistent with molecular mass of ~100 and 60 kDa, respectively. A light band between gp120 and gp160 was detected (Fig. 1B, left panel). This protein band is likely to be a translationally arrested product because its size dropped to ~90 kDa after treatment with PNGase F (Fig. 1B, right panel).

We next examined the biosynthesis of gp160 during the time of protein translation. HIV-1-infected cells were pulse-labeled for 2 min and chased for up to 10 min to examine the translational intermediate products. Surprisingly, distinct bands, which were immunoprecipitated with an antiserum against gp160 but of lower molecular weight, were observed (Fig. 2A). These molecules were chased out during the time period examined. To examine whether these molecules were translational/translocational intermediates, CHX was added during the pulse period to stall translation, which resulted, in the sustained presence of these lower molecular weight Env (gp160) intermediates (Fig. 2B). We additionally analyzed Env-related polypeptides, which were either treated with CHX or left untreated during their biosynthesis, and ran them side-by-side on SDS-polyacrylamide gels (Fig. 2C). With the addition of deoxy-d-glucose and sodium azide to the tissue culture system to deplete ATP (33), we observed a similar pausing of the Env protein during translation (Fig. 2D). Disruption of disulfide bond formation by the addition of DTT to the chase media of infected cells (35) failed to significantly impede translation of Env (Fig. 2E). Because translation and ER translocation are
thought to be coupled in mammalian cells, it remained difficult to make a distinction between these two steps. These data demonstrate that the synthesis of the Env glycoprotein does not proceed at a steady rate but rather is marked by periods of translational/translocational pausing.

We next wanted to test the effect of DTT addition on the stability and cleavage of Env in infected cells. In a pulse-chase analysis, the addition of DTT to the chase media interfered with the cleavage of gp160 into its gp120 form (Fig. 3B). When steady-state labeling experiments were performed in the presence of DTT, not only were the higher molecular weight Env-related complexes disrupted (as indicated by arrowheads in Fig. 3C), but the overall abundance of gp160 was diminished. These data are consistent with the notion that misfolding of Env causes it to be subjected to the quality control mechanisms within the ER, leading to its ER retention and removal by decay pathways.

One of the mechanisms by which functional maturation of glycoproteins proceeds is through their association with resident ER chaperones. One such molecule, calnexin, has been shown to retain misfolded molecules within the ER in part via recognition of the α-terminal glucose of N-linked glycoproteins (25). Again, using pulse-chase analysis with antisera against Env (Fig. 4A) and calnexin (Fig. 4B), an association between

Fig. 1. Pulse-chase analysis of HIV-1 Env and analysis of N-linked glycosylation. A, H9 infected cells were pulse-labeled with [35S]cysteine for 10 min and chased for up to 240 min. Cell lysates were analyzed by immunoprecipitation with an antiserum to HIV-1 Env at the time intervals indicated. Lanes containing uninfected cell lysates are indicated as mock. Translation/translocation intermediate is indicated by an asterisk. B, H9 infected cells were steady-state-labeled with [35S]cysteine for 2 h. Cell lysates were additionally subjected to treatment with PNGase F followed by immunoprecipitation with an antiserum to HIV-1 Env.

Fig. 2. Analysis of translation/translocation pausing in the biosynthesis of HIV-1 Env. H9 infected cells were pulse-labeled with [35S]cysteine for 2 min, then incubated with either CHX or DTT or depleted of ATP by the addition of 2-deoxy-D-glucose and sodium azide, and chased for up to 10 min. Cell lysates were analyzed by immunoprecipitation with an antiserum to HIV-1 Env at the time intervals indicated. Following the 2-min pulse labeling H9 infected cells were either untreated (A), treated with 0.5 mM CHX (B and C), depleted of ATP by the addition of prewarmed glucose-free media supplemented with 20 mM 2-deoxy-D-glucose and 10 mM sodium azide (D), or treated with 5 mM DTT (E). C, uninfected and infected H9 cell lysates, which were either treated with 0.5 mM CHX or remained untreated during a 10-min chase period, were run side-by-side and analyzed by SDS-PAGE. Translation/translocation intermediates are indicated by asterisks.
gp160 and calnexin was shown to have a $t_{1/2}$ of 10 min after the initiation of Env synthesis (Fig. 4B). To examine what role the α-terminal glucose had on the association of Env with calnexin, cells were treated with an α-glucosidase inhibitor, CST, prior to radiolabeling. Mock-infected and HXB2-infected cells were pulse-labeled and chased for 60 min followed by immunoprecipitation with antiserum to Env and CN. Here we observed no significant disruption of the association of gp160 and calnexin with CST treatment (Fig. 5A). Treatment of infected cells with CST caused Env to have a slower mobility by SDS-PAGE (indicated by arrowheads). As a control, infected cells that were labeled with [35S]cysteine for 10 min in the presence of DTT caused the association between gp160 and CN to be significantly disrupted (Fig. 5B, lanes 6 and 8). In contrast, the treatment of HIV-1-infected cells with CST did not appear to impact the interaction between Env and CN (Fig. 5B, lanes 5 and 7). Besides the effects that CST treatment has on the glucose trimming of Env, we addressed whether it had any additional impact on the formation and infectivity of virions. When CST was added in increasing concentrations, a mobility shift in Env was evident for the cell-associated molecules (Fig. 6A, indicated by arrows), however no significant differences were observed for the Gag-related proteins or for reverse transcriptase within the cell (Fig. 6B). Env was present in similar quantities within cell-free virions irrespective of whether viral producer cells were grown in CST (Fig. 6C), indicating that CST has little influence on the trafficking or retention of Env through the secretory pathway. In addition, virion-associated Gag, Integrase, reverse transcriptase, and gp41 were not significantly altered by CST treatment (Fig. 6D) nor was the infectivity of CST-treated virions impeded in a MAGI assay (Fig. 6E).

**DISCUSSION**

Evidence has emerged suggesting that the nascent chain polypeptide may be exposed at times to the cytosolic side of the ER membrane during cotranslational translocation (36). In fact, nascent polypeptides containing specific amino acid sequences, termed pause-transfer sequences, have been demonstrated to induce temporary pauses in translocation that coincide with nascent chain accessibility to the cytosolic side of the ER membrane (11, 12). These observations for translational/translocational pausing from an *in vitro* system have also been supported by evidence in tissue culture cells with apolipoprotein B (37). In this report we examined the biosynthesis of the Env protein in HIV-1-infected T-cells during the time of translation/translocation into the ER. We find several pause-transfer points as part of normal ER translocation, experiments which have not been reported for a viral protein previously (Fig. 2). This temporary pausing and restarting of cotranslational translocation was, as expected, dependent on the ribosomal passage over the *env* mRNA because CHX treatment impeded further translation/translocation. Additionally, restarting of the paused intermediates was dependent on ATP, which might suggest the involvement of the ER translocation components rather than strictly the translational machinery. It has previously been noted that ER luminal proteins, which use ATP as an energy source, may regulate the translocation of nascent chain proteins into the ER (7–9). However, demonstration of cytosolic Hsp70 binding to nascent chains of apolipoprotein B in an ATP-dependent manner suggests the additional involvement of non-ER luminal components in cotranslational translocation (38). Although the existence of pause-transfer sites may be dependent on the amino acid sequences within the nascent chain polypeptide itself (11), the restarting of these pausing events may require additional associating molecules. The varied rate of translation/translocation of HIV-1 Env highlights an additional level of regulation. By pausing during translation/translocation of the nascent chain polypeptide, the addition of sugars and chaperone-assisted folding may occur in a regional manner to ensure the production of functional Env. Our data suggest that the restarting of Env pause sites during its biosynthesis appears to be dependent on translation as well as ATP and not the formation of disulfide bonds.

The ER houses several molecule chaperones such as BiP, protein-disulfide isomerase, 94-kDa glucose-regulated protein, calnexin, calreticulin, and ERp57, which serve to prevent the misfolding of newly synthesized glycoproteins (25). To this end, misfolded proteins tend to be retrieved from further transport through the secretory pathway until properly folded. This un-
Understanding has raised the notion that the ER acts as a cellular quality control compartment (25). Retention of misfolded molecules within the ER usually leads to their degradation (26), a process dependent on the cytosolic 26 S proteasome (27). ER misfolding pathways may be studied by the introduction of mutations into glycoproteins and by the addition of chemicals, such as DTT, which disrupt the disulfide bonding of these molecules (35). The use of DTT to treat HIV-1-infected T-cells had little effect on the restarting of Env translational/translational pause intermediates (Fig. 2E), however it did result in a failure of gp160 to reach the Golgi compartment as evidenced by the absence of the cleaved gp120 form of Env (Fig. 3). DTT addition in vivo consequently leads to a marked reduction in the overall levels of Env (Fig. 3, lane 2), which was independent of its translation/translocation (Fig. 3B). ER resident chaperones are thought to associate with newly synthesized glycoproteins to regulate their folding and retain misfolded molecules within the ER. Because the addition of DTT disrupted the association between Env and calnexin (Fig. 5B), it suggests that other chaperones may dictate the retention of
misfolded Env within the ER. For example, BiP has been shown to bind newly synthesized glycoproteins in a sequential manner prior to calnexin (39).

It has been shown that treatment of cells with CST, an a-glucosidase inhibitor, can disrupt the association of calnexin with various viral glycoproteins because calnexin is thought to recognize the trimmed monoglucosylated form of these glycoproteins (22, 25, 39, 40). However, the association of HIV-1 Env with calnexin after treatment with CST appeared to be unaltered (Fig. 5). This is consistent with a previous report that demonstrated that CST treatment failed to abolish the association of gp160 with calnexin (41). These studies suggest that calnexin does not recognize HIV-1 Env only through its interaction with the monoglucosylated core glycans. In a recent report, it was demonstrated that calnexin recognized the polypeptide portion of unfolded molecules in vitro, suggesting that it may not be acting solely as a lectin-associating chaperone (24). Although association of calnexin and Env in infected T-cells may not be dependent on the glucosylation state of Env, we cannot rule out the possibility of subtle interaction differences between CST-treated and untreated samples. Additionally it could be argued that Env association with calnexin may be through a trimeric complex with ERp57 and that this thiol-dependent reductase plays a more critical role in this particular association (21, 22). It remains possible that calnexin binds the unfolded state of Env and plays a role in preventing the aggregation of misfolded molecules. This remains intriguing considering the association of translation/translocation intermediates of gp160 with calnexin in the earliest chase times (Fig. 4B, 2 and 5 min). Although the inhibition of terminal glucose trimming demonstrated limited disruption in calnexin association with Env, their inaction may still be critical for the formation of functionally active Env.

Previous studies have examined the effects of CST on HIV-1-infected cells and have shown a reduction in the formation of syncytia and virus infectivity (28, 29). These reports have either assayed the effect of CST on viral replication in multiple rounds or have used cell culture supernatants containing CST as a source of virus input. Because CST does not specifically target viral glycoproteins, it remained plausible that its use may confer sublethal cell toxicity that could alter other molecules of cellular origin effecting virus cell-to-cell spread. In the present study, we observed little to no effect in the inhibition of infectivity of cell-free virions produced in the presence of CST (Fig. 6E). The differences in the results may be explained by the fact that we tested cell-free virions in a one-round infectivity assay rather than growing virally infected cells in the presence of a-glucosidase inhibitors over the course of several days. Additionally, we removed all CST from the cell culture supernatants by pelleting virus before infecting target cells, thus minimizing the effects CST may have on these cells. In fact, we demonstrate that CST caused a retardation in the mobility of both gp120 and gp160, characteristic of the absence of glucose trimming of these molecules (Fig. 6). Additionally, the treatment of HIV-1-infected cells with NB-DNJ has no significant impact on the virion incorporation of gp120 and gp41, suggesting that trafficking of Env through the secretory pathway was unimpeded. These results are consistent with reports using DNJ and NB-DNJ to inhibit glucosidase I and II in the production of Env, which demonstrated no effect on CD4 binding (13, 30). However, treatment of HIV-1-infected cells with NB-DNJ did display a post-CD4 binding entry defect (30), which was not observed with CST treatment (Fig. 6E). These differences may be explained because NB-DNJ inhibits ER glucosidases gener-
ally (42), potentially conferring a more severe phenotype. Our data suggest that glucose trimming is not essential for the initiation of cell-free virus infection nor does it significantly influence the association of HIV-1 Env with calnexin.

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