Folding of HIV-1 Envelope glycoprotein involves extensive isomerization of disulfide bonds and conformation-dependent leader peptide cleavage

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ABSTRACT **Human immunodeficiency virus binds and enters cells via the Envelope glycoprotein gp160 at its surface. In infected cells, gp160 is found not only on the plasma membrane but also in the endoplasmic reticulum (ER). Our aim was to establish rate-determining steps in the maturation process of gp160, using a radioactive pulse-chase approach. We found that gp160 has an intricate folding process: disulfide bonds start to form during synthesis but undergo extensive isomerization until the correct native conformation is reached. Removal of the leader peptide critically depends on formation of at least some disulfide bonds in subunit gp120 during folding. Envelope folds extremely slowly and therefore resides in the ER longer than other proteins, but the yield of properly folded molecules is high and degradation is undetectable. The large quantity of gp160 in the ER hence is a result of its slow transit through this compartment. We show here that newly synthesized HIV-1 Envelope glycoprotein apparently follows a slow but high-yield folding path in which co- and post-translational formation of disulfide bonds in gp120, disulfide isomerization and conformation dependent removal of the leader sequence are determining and intertwined events.—Land, A., Zonneveld, D., Braakman, I. Folding of HIV-1 Envelope glycoprotein involves extensive isomerization of disulfide bonds and conformation-dependent leader peptide cleavage. *FASEB J.* 17, 1058-1067 (2003)**

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This paper reports our analysis of the folding process of human immunodeficiency virus type 1 (HIV-1) Envelope (Env) glycoprotein gp160 and its isolated soluble subunit gp120. Infection with HIV-1 is the cause of AIDS. Envelope plays a critical role in infection, because it mediates HIV binding to the target cell surface and subsequent fusion of the cellular and viral membranes (1, 2). Gp160 is a type I integral membrane protein that possesses ~ 30 potential N-linked glycosylation sites (~25 of which in gp120) dependent on virus strain and 10 disulfide bonds (9 in gp120) (3, 4). It is cotranslationally translocated into the ER, where it folds (5, 6) and trimerizes (7–9). The ER lumen is highly specialized for this task (10–13): during folding, gp160 is assisted by folding factors in the compartment (5, 14). HIV-1 Envelope associates with BiP (5), calnexin, and calreticulin (14–16), and its leader peptide is removed late after synthesis (17, 18). Gp160 is then transported to the Golgi complex where it is cleaved into two noncovalently associated subunits: the soluble subunit gp120 and the transmembrane domain-containing subunit gp41 (19, 20).

Secretion of gp120 is improved when the original signal sequence is replaced by other ER leader peptides, which are removed faster (18), suggesting that the rate of release from the ER is determined at least to some extent by the rate of leader peptide removal. That folding of proteins is affected by proper timing and the proper site of cleavage of their signal sequence has been established in studies using cleavage mutants of various proteins (21–23).

Conformational maturation of secretory and membrane proteins is determined not only by their amino acid sequence but also by co- and post-translational covalent modifications. Correct disulfide bonds need to be formed and N-linked glycans need to be attached. Without these modifications, proteins aggregate and remain in the ER (10, 11). In HIV-1-infected cells as well as in cells transfected with a plasmid coding for gp160, the majority of gp160 was found in the ER, as seen by Western blot and indirect immunofluorescence. This ER localization was generally explained as the result of misfolding and degradation of a large fraction of newly synthesized gp160 (24–26). We now show, however, that steady-state ER localization is a consequence of extremely slow maturation kinetics of this protein. We found that extensive isomerization of disulfide bonds occurs and next to the already reported effect of the remarkably late cleavage of the leader peptide on folding, the folding state of gp120 (alone or as part of gp160) in turn determines the timing of leader peptide cleavage.

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MATERIALS AND METHODS

Antibodies and other reagents

Polyclonal rabbit antiserum 16 (Ab16) was generated against purified HIV-1 LAI gp160 and gp120 expressed in Vaccinia-infected mammalian cells. Polyclonal antibody 40336 was generated against purified HIV-1 IIIB gp160 expressed in Baculovirus-infected insect cells. Both antibodies were generously provided by Dr. H. G. Huisman, CLB, Amsterdam. Endoglycosidase H (Endo H) (27) and endoglycosidase F (Endo F) (28, 29) were purchased from Boehringer Mannheim GmbH (Mannheim, Germany).

Cell lines and expression systems

HeLa cells were grown in minimal essential medium with 10% FCS. All media and additives were obtained from Gibco-BRL (Gaithersburg, MD). To express wild-type gp160 and its soluble subunit gp120 from the HIV-1 LAI strain, we used a recombinant Vaccinia virus expressing these proteins, kindly provided by Dr. M. P. Kieny (30). Purified virus was used to allow infection of all cells for high expression levels. Cells were used ~5 h postinfection, as indicated in the legends.

Metabolic labeling of adherent cells

Pulse-chase experiments were done essentially as described before (31) with some modifications. Subconfluent cells expressing gp160 or gp120 were washed once with Hanks' balanced salt solution (HBSS; Gibco-BRL) and preincubated in starvation medium lacking methionine and cysteine for 15 min at 37°C. The cells were pulse-labeled with 50 μCi of Redivu™ pro-mix™ L-[^{35}S] in vitro cell labeling mix (Amersham Pharmacia Biotech AB, Buckinghamshire, UK) as indicated, then chased for various times. The chase was started by adding complete medium containing excess unlabeled cysteine and methionine. Cycloheximide was included in the chase to stop elongation of nascent chains.

The chase was stopped by transferring the cells to ice and washing them with ice-cold HBSS containing 20 mM iodoacetic acid, iodoacetamide, or N-ethylmaleimide to alkylate-free sulfhydryl groups. The cells were lysed in 0.5% Triton X-100 in MNT (20 mM MES, 100 mM NaCl, 30 mM Tris-HCl pH 7.4) containing 20 mM iodoacetic acid, iodoacetamide or N-ethylmaleimide and protease inhibitors (chymostatin, leupeptin, antipain, pepstatin, PMSF, and EDTA). Lysates were either used directly for immunoprecipitation or snap-frozen in liquid nitrogen and stored at −80°C until further analysis.

Immunoprecipitation

Protein A Sepharose 4B fast-flow beads (Amersham Pharmacia Biotech AB) were incubated for 30 min at 4°C with a polyclonal antiserum (1–8 μL). Lysates (100–400 μL were then added and incubated with the beads for 18 h at 4°C. The immunoprecipitates were washed twice with a buffer that contained 0.05% Triton X-100, 0.1% or 0.05% SDS, and 300 mM NaCl in 10 mM Tris-HCl, pH 8.6, for 5 min at room temperature. The washed pellets were resuspended in 10 μL 10 mM Tris-HCl, pH 6.8, after which nonreducing sample buffer was added to a final concentration of 200 mM Tris-HCl pH 6.8, 3% SDS, 10% glycerol, 0.01% bromophenol blue, and 1 mM EDTA. After heating for 5 min at 95°C, samples were subjected to nonreducing and reducing SDS-PAGE (31).

Folding assay, endoglycosidase H/F digestion

To identify folding intermediates, glycans were removed by digestion with Endo H or Endo F. The washed immunoprecipitates were resuspended in 0.2% SDS in 100 mM sodium acetate pH 5.5 and heated to 95°C for 5 min. An equal volume of 100 mM sodium acetate pH 5.5 and Triton X-100 to a final concentration of 2% was added, followed by protease inhibitors and the deglycosylation enzyme. The samples were incubated for 2 h at 37°C with 0.0025 U Endo H or 0.5 U Endo F. A quarter volume of fivefold concentrated sample buffer was added and the sample was mixed and heated to 95°C for 5 min. Samples were analyzed by nonreducing and reducing (40 mM DTT) SDS-PAGE.

SDS-PAGE, fluorography, and PhosphorImaging

Immunoprecipitated samples containing radiolabeled gp160 or gp120 were loaded onto a 7.5% SDS-polyacrylamide slab gel for electrophoresis according to Laemmli et al. (32). For 2D gels, samples were loaded onto a tube gel and electrophoresed. The tube gel then was extracted from the capillary, boiled for 10 min in reducing sample buffer, and placed on a regular 7.5% SDS polyacrylamide slab gel for the second dimension. Reduced sample was loaded in a separate lane close to the edge of the slab gel. Gels were stained with Coomassie Brilliant Blue R 250, neutralized, and impregnated with salicylic acid for fluorography. Gels were exposed on Kodak XAR-5 film, Kodak Biomax MR film, or on storage phosphor screens (Kodak) and analyzed on the STORM (Amersham) or Bio-Rad Personal Molecular Imager FX using Image Quant software (Amersham). The apparently lower intensity of many nonreduced samples vs. their reduced equivalents was caused by the smearing of protein signal over a large surface area in the gel. We quantified and determined that total signal of nonreduced smear in a lane was the same as total signal of the corresponding reduced band(s).

RESULTS

Slow, similar folding of gp160 and gp120

To characterize the earliest events in the biosynthesis of HIV-1 gp160 and gp120, we used a recombinant Vaccinia virus (33) in which the coding sequence for either protein was inserted behind the E7.5K Vaccinia promoter (30). Subconfluent mammalian cells in culture were infected with the recombinant virus carrying either the gp160 cDNA (VV160) or the gp120 cDNA (VV120). Five hours postinfection, cells were pulse-labeled for 2 (gp120) or 4 min (gp160) with ^{35}S-labeled methionine and cysteine, after which labeled protein was chased for various times up to 4 h. Triton X-100 lysates of the cells were immunoprecipitated with an antiserum that recognizes all forms of gp160 and gp120.

To study disulfide bond formation in newly synthesized gp160 and gp120, immunoprecipitated samples were deglycosylated by digestion with Endo H before analysis by nonreducing (NR) SDS-PAGE. During the folding process the number of disulfide bonds increases, the protein will become more compact, and this will increase its electrophoretic mobility (31).

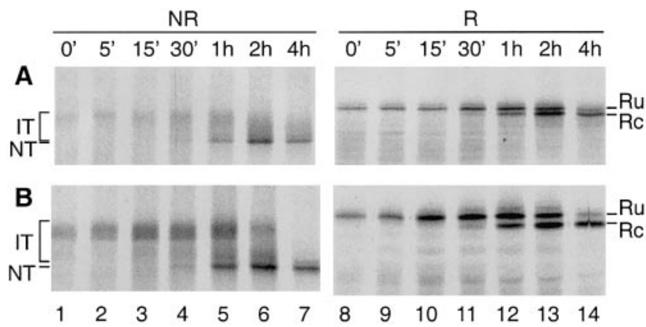

Figure 1. Oxidation and signal peptide cleavage of gp160 and gp120. 5 h after infection with recombinant Vaccinia virus expressing gp160 (*A*) or gp120 (*B*), HeLa cells were pulse-labeled with ^{35}S amino acids for 4 (*A*) or 2 min (*B*) and chased for the indicated times. Lysates of the cell pellets were immunoprecipitated with Ab16 and, after Endo H treatment, were subjected to nonreducing (NR; lanes 1–7) and reducing (R; lanes 8–14) 7.5% SDS-PAGE. IT, folding intermediates; NT, native form from which the signal peptide is removed; Ru, reduced uncleaved (signal peptide still attached); Rc, reduced and cleaved.

Immediately after the pulse, gp160 ran as a smear representing early folding intermediates (IT) with incomplete sets of disulfide bonds (**Fig. 1***A*, lane 1). These gradually disappeared during the chase (lanes 4–7). After 30 min one sharp band appeared (lane 4), which increased in intensity over time (lanes 4–7). After a 4 h chase, the majority of gp160 now ran as this lower band (lane 7), which represented native (NT), completely folded gp160 containing all 10 disulfide bonds.

Gp120 displayed a similar oxidation pattern (Fig. 1*B*). Immediately after the pulse, a smear of IT was detected (Fig. 1*B*, lane 1). NT appeared at around the same time for gp120 and gp160: ~30 min after synthesis. Most of the gp120 folding intermediates had been converted into the native conformation after a 4 h chase (Fig. 1*B*, lane 7). This is different for gp160, where a reasonably large portion of molecules was not yet completely folded by that time (Fig. 1*A*, lane 7). The slowest molecules of gp160 apparently matured slower than the slowest gp120 molecules, either because of gp41 in gp160 or because of gp160's membrane attachment. Both proteins were similar in their display of substantial heterogeneity in maturation rates; the average gp120 or gp160 molecule took hours to mature.

Neither gp160 nor gp120 was ever present as reduced full-length protein, implying that the first disulfide bonds must form during synthesis. The folding intermediates appeared as fuzzy, broad smears instead of discrete bands on the gel, perhaps because gp160 and gp120 contain large numbers of small loop-forming disulfide bonds.

The corresponding deglycosylated reduced samples are shown in Fig. 1*A*, *B*, lanes 8–14. During the first 15 min after synthesis, the folding intermediates collapsed upon reduction into one sharp band of ~100 kDa for gp160 (Fig. 1*A*, lanes 8–10) and ~70 kDa for gp120 (Fig. 1*B*, lanes 8–10). After 30 min of chase (Fig. 1, lanes 11), cleavage of the 31 amino acid long leader peptide starts (16, 17) and cleaved gp160 and gp120 become detectable (Rc, lanes 11–14). This was confirmed by the lack of recognition of both cleaved proteins by an antibody directed against the gp120 leader sequence (16) (data not shown). A significant fraction of gp160 and gp120 still contained their signal peptides after 4 h of chase, again indicative of the enormous heterogeneity in maturation rates.

The decrease in signal after 4 h of chase was caused by secretion of gp120 and by cleavage of gp160 into gp120 and gp41 in the Golgi complex and subsequent release of gp120 into the culture medium (not shown) (19, 34). Proteasome inhibitors did not affect expression levels or folding patterns, implying that no significant degradation of gp160 or gp120 occurred in our system. Indirect immunofluorescence of HeLa cells expressing gp160 after recombinant Vaccinia virus infection established that gp160 reached the cell surface (**Fig. 2**). In cells fixed and permeabilized with methanol, we detected gp160 in an internal ER-like staining

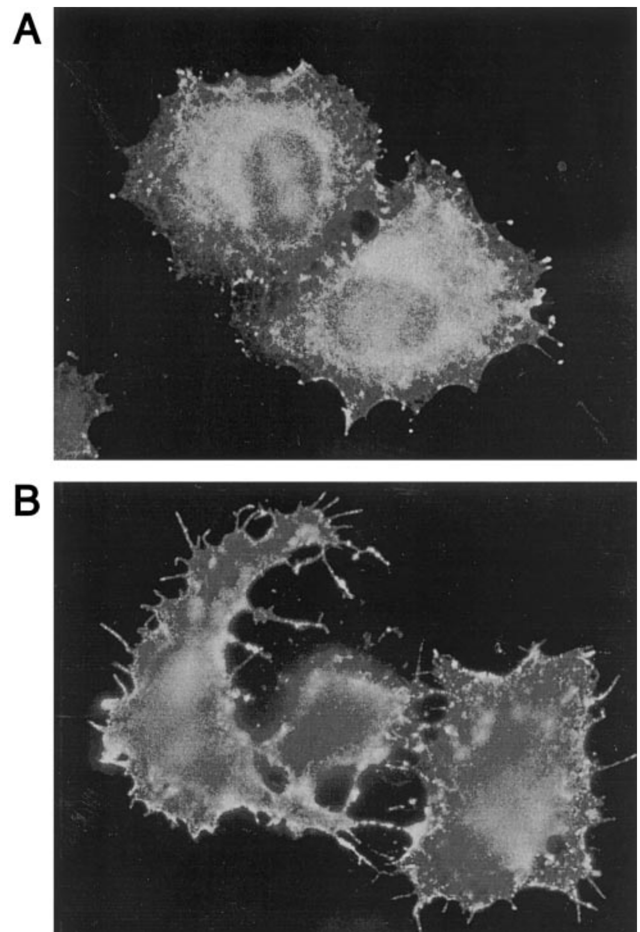

Figure 2. Localization of gp160 to the plasma membrane. Indirect immunofluorescence was performed on HeLa cells expressing gp160. HeLa cell were infected with recombinant Vaccinia virus expressing gp160 for 12 h with an m.o.i. of 0.1 pfu/cell. Cells were fixed and permeabilized with methanol (*A*) or fixed with paraformaldehyde (*B*), probed with 40336, a polyclonal rabbit antiserum against gp160, and subsequently with Cy3-conjugated secondary antibody.

pattern and on the cell surface (Fig. 2*A*). In cells fixed with paraformaldehyde and left intact, only the bright surface staining was visible (Fig. 2*B*), indicative of a large quantity of gp160 on the plasma membrane.

Two-dimensional SDS-PAGE reveals timing of leader peptide removal

Because at least some disulfide bonds had already formed before the signal sequence was removed (Fig. 1*A, B*, lanes 1), we tested whether and to what extent gp120 cleavage and oxidation state correlated. We therefore performed 2-dimensional (2D) SDS-PAGE, NR in the first dimension (horizontal in Fig. 3) and reducing (R) in the second dimension (vertical in **Fig. 3)** (35). All proteins lacking disulfide bonds will migrate on the diagonal (Fig. 3, gray line) because of their identical mobilities in both dimensions. Molecules that contain intrachain disulfide bonds will be more compact and will run above the diagonal in Fig. 3; proteins that form interchain disulfide-linked complexes will run below the diagonal.

After a 10 min pulse, gp120 ran as a horizontal stripe of folding intermediates with different sets of disulfide bonds but with the leader peptide still attached (Fig. 3*A*, arrow; cf. with reference lane and with Fig. 1*B*, lanes 2 and 9). After a chase of 30 min, a second stripe appeared with a slightly higher electrophoretic mobility, representing folding intermediates from which the signal sequence had been removed (Fig. 3*B*, arrow). Its mobility further away from the diagonal indicates that gp120 molecules in the lower stripe had formed more disulfide bonds than gp120 molecules migrating in the first stripe. This implied that the protein had acquired quite a few disulfide bonds before the leader peptide was removed. The increased intensity at the end of the stripe in Fig. 3*C* (arrow) represents native gp120 lacking its leader sequence, in which all disulfide bonds have been formed.

In contrast to leader peptides of other proteins, the Env leader is removed only after some folding has occurred, suggesting a relationship between the two. In Fig. 3*B*, we clearly show that removal of the leader sequence was not restricted to one particular compactness of gp120, but could occur in gp120 forms with different disulfide composition. To determine whether the gp120 folding rate affected the timing of leader cleavage, we slowed down folding by lowering the temperature during the chase period. Although folding was temperature dependent within the range of 37–20°C tested, the folding pathway as represented by the smear of folding intermediates was not affected (not shown). Removal of the leader peptide was delayed to the same extent as the folding process, confirming a relationship between the two.

Manipulating disulfide bond formation affects leader peptide removal

To hamper oxidative folding of gp120 and examine the effect on signal peptide removal, we used the mild reducing agent β-mercapto-ethanol. HeLa cells expressing gp120 were chased in the presence of 15 mM β-mercapto-ethanol. Immediately after the pulse, the folding intermediates appeared as a smear ranging all the way from reduced to around native gp120 (**Fig. 4***A*, lane 1). During the chase, their mobility not only increased, as expected, but exhibited transient decreases, especially between 30 and 60 min of chase (Fig. 4*A*, lanes 4–5) and again between 90 and 120 min of chase (Fig. 4*A*, lanes 6–7). From 2 h onward, the smear of folding intermediates eventually became more compact (Fig. 4*A*, lane 7) and the first leader peptides were removed, as can be seen in the corresponding reducing gel (Rc; Fig. 4*B*, lane 7). Gp120, however, did not yet reach an obvious native form (Fig. 4*A*, lanes 7–10). Similar to the results at lower temperature, we concluded that leader peptide removal indeed was severely delayed when disulfide bond formation was hindered (Fig. 4*B*, lanes 7–10).

In the reducing gel (Fig. 4*B*) all disulfides were reduced. Nevertheless, we noticed that the "wave" in the nonreducing gel was reflected weakly in the reducing gel (Fig. 4*B*, lanes 1–10). As in Fig. 4*A*, the single, reduced band representing gp120 with the signal sequence still attached (Ru) also decreased mobility between 30 to 60 and 90 to 120 min of chase (Fig. 4*B*, lanes 4–7). Since samples were completely reduced and deglycosylated before analysis by SDS-PAGE, the effect could only have been caused by our sample preparation. Postlysis oxidation of gp120 is prevented by alkylation with iodoacetic acid. Every molecule of iodo-

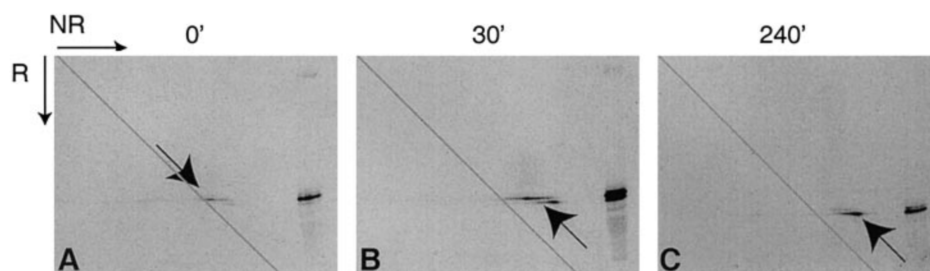

Figure 3. Relation between disulfide bond formation and cleavage of the signal peptide (2D electrophoresis). As in Fig. 1*B*, HeLa cells expressing gp120 were pulse-labeled for 10 min and chased for 0, 30, or 240 min. Lysates were immunoprecipitated with Ab16 and, after Endo H treatment, analyzed in the first dimension under nonreducing conditions (NR; horizontal) and in the second dimension under reducing conditions (R; vertical). Arrow, folding intermediates with the signal peptide still attached (*A*) or removed (*B*); arrow in panel *C*, native gp120 without its signal peptide. A lane to the right of the first-dimension tube gel contained the corresponding reduced sample.

acetic acid bound will cause a decrease in the electrophoretic mobility of gp120 because it adds mass and negative charge. To investigate this, completely reduced samples of a similar experiment as in Fig. 4 were treated with iodoacetic acid before analysis by reducing SDS-PAGE. All sulfhydryl groups now were reduced and alkylated; as a consequence, gp120 indeed had the same mobility at all chase times (data not shown). The mobility shift of the reduced samples in Fig. 4*B* therefore represented the variable number of iodoacetic acid molecules bound to gp120, attesting to the variable number of free sulfhydryl groups in the protein. We concluded that at least some disulfide bonds in gp120 were repeatedly broken and formed, implying extensive isomerization during folding. This phenomenon is likely to contribute significantly to the lack of discrete bands but, instead, a fuzzy appearance of the folding intermediates.

Whereas Fig. 1 shows the prevalent folding assay for gp120, we sometimes did observe a "mobility wave" of early folding intermediates in unmanipulated cells. An example of such an experiment is shown in Fig. 4*C, D*, in which the mobility decrease occurred from 15 to 30 min. Again, the wave in the nonreducing gel (Fig. 4*C*) was reflected in the reduced alkylated samples (Fig. 4*D*). With or without reducing agent, this "wave" was not always detectable under the same conditions or at the same chase times. There was no correlation whatsoever between the decrease of gp120 mobility and additions of fresh reducing agent during some chases, or with the use of a particular alkylating agent (N-ethylmaleimide, iodoacetamide, and iodoacetic acid). Since folding and disulfide bond formation depend strongly on the redox conditions in the cell and redox milieu fluctuates with cell cycle, cell confluency, and exact medium composition, more synchronized folding perhaps could only be obtained with cells cultured in a chemostat.

Leader peptide removal is prevented when disulfide bonds are absent

In all experiments performed, using two additional Envelopes from different HIV-1 strains (IIIB and Ba-l) and using various cell lines, expression systems, and conditions, we always found a correlation between the rates of folding and leader peptide removal. To determine whether signal sequence cleavage could be prevented altogether by holding up formation of disulfide bonds, we kept gp120 reduced by adding 10 mM DTT to the pulse-labeling and chase media. Leader peptidase activity is not affected by the presence of DTT (36, 37). Disulfide bonds indeed were not formed in gp120 (**Fig. 5***A*, lanes 1–5) and, as a consequence, removal of the signal peptide did not occur anymore (Fig. 5*A*, lanes 6–10). Instead, part of gp120 misfolded and disappeared from the gel either by degradation or by aggregation into complexes too large to enter the stacking gel. These data, which were identical for gp160 (not shown), indicate that removal of the leader peptide can be prevented by blocking disulfide bond formation in gp120; cleavage depended on the presence of at least one disulfide bond in gp160 and gp120.

Folding and leader cleavage in gp120 can be postponed by a reductive incubation

Since reduced full-length gp120 and gp160 were never detected in the ER, some disulfide bonds must always form cotranslationally (Fig. 1 and Fig. 4*C*). By preventing disulfide bond formation during the pulse (Fig. 5*A*), we may have caused misfolding of gp120, as suggested by its disappearance during the subsequent reducing chase (Fig. 5*A*, lanes 2–5 and 7–10). To examine whether cotranslational formation of disulfide bonds was required for proper folding and to determine whether leader peptide cleavage was still possible

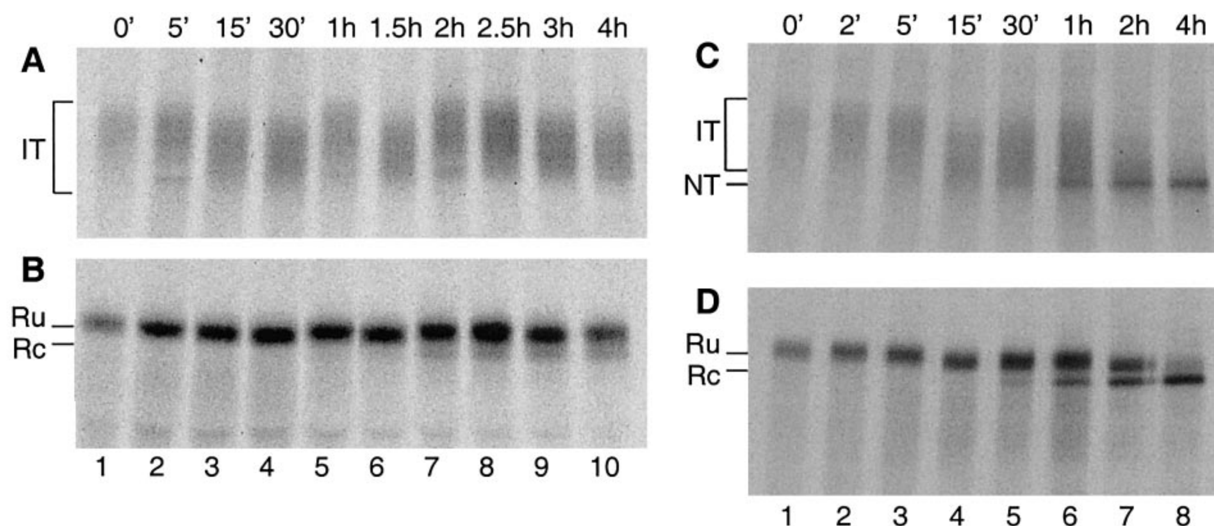

Figure 4. Folding "waves" with or without β-mercapto-ethanol. HeLa cells expressing gp120 were pulse-labeled for 2 min. Cells were chased in the presence (*A, B*) or absence (*C, D*) of 15 mM β-mercapto-ethanol (final concentration). Detergent lysates were immunoprecipitated with 40336 and analyzed as in Fig. 1. *A, C)* Nonreduced samples; *B, D)* reduced samples.

after a reducing incubation, we allowed oxidation of newly synthesized reduced gp120 (Fig. 5, lanes 1) by removing DTT from the chase. Reduced gp120 now disappeared and the folding intermediates appeared (Fig. 5*B*, lane 2). After 30 min of chase, the cleaved form of gp120 started to accumulate (Fig. 5*B*, lanes 2 and 7), and native, oxidized, cleaved gp120 increased with time (Fig. 5*B*, lanes 2–5 and 7–10). The results were the same for gp160 (not shown), with similar rates of folding and signal peptide removal as in control conditions. Although in principle gp120 and gp160 could form their disulfide bonds post-translationally, not all molecules could be rescued from the reducing insult. A fraction of each protein misfolded and accumulated into aggregates too large to enter the stacking gel. This led to a decrease in signal during the chase (Fig. 5*B*, lanes 2–5 and 6–10). Again, leader peptide cleavage occurred only after some disulfide bonds had been formed. The cleavage event thus can be turned on and off at will by allowing or preventing disulfide bond formation in gp120 during folding.

Effect of glycosylation on folding and leader peptide removal of gp120

Heavily glycosylated proteins require their glycans for proper folding. Gp120 and gp160 are no exception: unglycosylated gp120 does not fold into a biologically active conformation (38). Even the deletion of only three conserved glycosylation sites within the gp41 domain (Asn-621, –630, and –642) prevented gp160 from leaving the ER (39). To test whether unglycosylated gp120 and gp160 would be substrates for the leader peptidase, we treated cells with the glycosylation inhibitor tunicamycin (40). Gp120 and gp160 behaved the same; **Fig. 6** shows that gp120 disappeared from its usual position in the gel (lanes 1–3) upon treatment with tunicamycin (lanes 4–6). It ran at ~70 kDa, the expected position without glycans. It ran below Endo F-treated gp120 control samples (lane 7), because this enzyme leaves behind a single GlcNac residue of every glycan chain it digests, which in gp120 accumulates to ~25 monosaccharides, accounting for the ~4 kDa difference between lanes 4 and 7. Whereas in control cells, gp120 folds (lanes 10–12) and loses its leader sequence (lanes 7–9), electrophoretic mobility of gp120 did not change in tunicamycin-treated cells. Without prior reduction, gp120 was present quantitatively in disulfide-linked aggregates at the end of the pulse (lane 13), which were accessible to reduction by DTT irrespective of previous incubations with endo F at 37°C (lanes 4–6). The aggregates did not change during the chase. Clearly, gp120 was misfolded when synthesized in the absence of glycosylation. Again, the leader peptide was not removed, confirming that proper conformation was required for leader peptide cleavage.

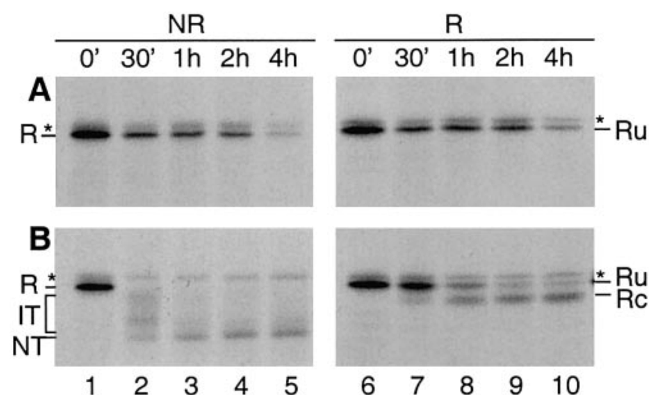

Figure 5. Conformation dependent cleavage of the signal sequence and complete post-translational folding. HeLa cells expressing gp120 were preincubated for 5 min in the presence of 10 mM DTT (final concentration) in medium lacking cysteine and methionine. Cells were then pulse-labeled for 10 min in the presence of 10 mM DTT and chased for the indicated times in the presence (*A*) or absence (*B*) of 10 mM DTT . Lysates were immunoprecipitated with 40336 and, after treatment with Endo F, subjected to nonreducing (NR; lanes 1–5) and reducing (R; lanes 6–10) 7.5% SDS-PAGE. *Vaccinia virus background band.

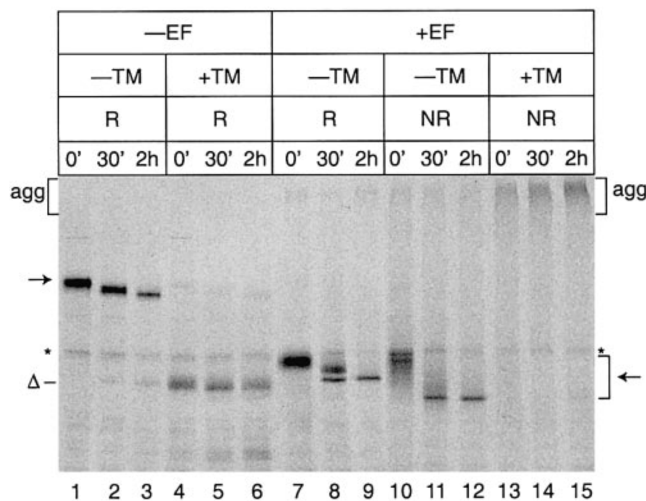

Figure 6. Effect of tunicamycin on oxidation and signal peptide cleavage of gp120. HeLa cells were infected as in Fig. 1 and incubated in medium containing 5 μg tunicamycin (+TM) or DMSO (–TM) from 30 min before the pulse until the end of the chase. 5 h postinfection, the cells were pulse labeled for 10 min. Immunoprecipitates were divided in two; one-half was treated with endo F before nonreducing (NR; lanes 10–15) and reducing (R; lanes 1–9) 7.5% SDS-PAGE analysis as in Fig. 1. Arrows indicate gp120 from cells not treated with TM, either mock (lanes 1–3) or endo F treated (lanes 7–12); Δ, nonglycosylated reduced gp120 (lanes 4–6); agg, aggregates of misfolded protein. *Vaccinia virus background band.

DISCUSSION

Here we show that during folding of HIV-1 Envelope, a close relationship exists between disulfide bond formation and leader peptide cleavage. At least some intrachain disulfide bonds need to form before cleavage of

the signal peptide can occur. Two-dimensional SDS-PAGE analysis revealed that cleavage is not limited to one particular compact form of the protein, i.e., one Envelope form that has a defined set of disulfide bonds. Instead, a minimal compactness caused by a number of disulfide bonds was sufficient for cleavage, but more compact forms could be substrate for the leader peptidase as well. The disulfide bonds that form in Envelope during folding undergo extensive isomerization, suggesting transient nonnative cysteine pairing in its folding process. Although gp120 and gp160 fold extremely slowly in a series of intricate steps, this results in a high yield of correctly folded molecules without any apparent loss of protein through degradation.

Two rate-determining processes in gp120 govern Env folding: disulfide isomerization and conformation-dependent signal peptide removal. This was identical for gp120 and gp160, showing that gp120 is the dominant subunit during Env folding. We found that gp120 needed to acquire a certain conformation to allow signal peptide cleavage, despite the well-known fact that signal peptidases act early and conformation independently on other proteins. Most likely the cleavage site is shielded during and shortly after gp120 synthesis. Manipulation of the rate and efficiency of gp120 and gp160 folding, using β-mercapto-ethanol or a change in temperature, therefore resulted in a similar change in the cleavage event. When proper folding was impossible because of prolonged treatment with DTT or when gp120 or gp160 was synthesized in the presence of tunicamycin to prevent N-linked glycosylation, cleavage was prevented altogether. Since glycans are numerous on gp120 and gp160 (41), it was no surprise that without them both proteins misfolded (42, 43; present paper). The state of folding and oxidation of gp120 and gp160 apparently determine whether and when their leader peptides are removed.

Where we show that removal of the signal peptide depended on the folded state of gp120 and gp160, Kang and co-workers showed that the inverse was true as well: late cleavage of the signal peptide retarded folding and secretion of the isolated gp120 subunit (16, 18, 44). This mutual dependence of folding and cleavage may help regulate the proper outcome of the folding process. A replacement of the Env signal peptide or a decrease in positive charges in the Env signal peptide both increase the rate of gp120 secretion and release from calnexin (16), indicating that the low rate of signal removal contributed to the low rate of folding. A lower folding rate usually leads to an increase in yield of correctly folded molecules, which may be of benefit to HIV. Why would this rapidly evolving virus otherwise maintain such a slow release? An alternative explanation could be found in a putative function of the leader peptide itself. The cleaved peptide is released into the cytosol, where it binds calmodulin and activates the NF-κB cascade (45). Perhaps gp160 is a slow-release depot of this signal, ensuring presence of the signal peptide over a time course of many hours.

Recently, Ploegh and co-workers demonstrated delayed cleavage of the leader peptide from human cytomegalovirus US11, which depended on the presence of the distant US11 transmembrane domain (46). A relation to a transmembrane domain does not apply to HIV Env, since gp160 and gp120 leader peptide cleavage was equally slow.

The conformational state of gp120 that allows signal sequence removal is difficult to define, since extensive heterogeneity existed in the folding intermediates that became substrate for the signal peptidase. Perhaps conformation and stability brought by one particular (set of) disulfide bonds in only a part of gp120 structure is required. Further studies using cysteine deletion mutants may shed light on this question. The issue is complicated by our observation that massive isomerization of disulfide bonds accompanied folding of gp120 and gp160. We showed that during gp120 folding, not all cysteines were locked constantly in a disulfide bond. They existed transiently as free sulfhydryl groups, susceptible to alkylation by iodoacetic acid. These transient disulfides are very likely to be non-native, as they are present in folding intermediates that are destined to change conformation again.

The likelihood of non-native disulfide bond formation is easy to understand. When a cysteine in a ribosome-associated nascent polypeptide chain is translocated into the ER lumen it needs to await its partner cysteine, which may not be the next cysteine in the chain. Because of the relatively oxidizing environment of the ER lumen, aberrant disulfide bonds can easily form when any two cysteines get close together before the protein is completely folded. This is a likely event for gp120, since five of its disulfide bonds connect cysteines that are separated from each other by at least one cysteine belonging to another bond.

A major question that remains for many proteins is whether non-native disulfide bonds exist on the proper folding route or whether they lead to misfolding. We show here that disulfide isomerization does not coincide with or lead to misfolding for HIV-1 Env. We did not detect any degradation nor did the folding intermediates form aggregates during the chases. The role of non-native disulfide bonds during the folding process has often been debated. In vivo data are scarce, and in vitro data on this topic may not be representative for intracellular events. For bovine pancreatic trypsin inhibitor, a model protein with one of the best-characterized folding pathways, non-native disulfide bonds were found as obligatory transient intermediates in vitro (47, 48). These bonds, however, are not needed as intermediates when disulfide isomerases are present (49).

Because of the large number and abundance of disulfide isomerases—in particular, protein disulfide isomerase (PDI)—non-native disulfide bonds and their shuffling are likely to be plentiful in the ER. Pilot experiments (A. Land, Marije Liscaljet, and I. Braakman, unpublished results) sometimes showed an association between gp120 and PDI. The weakness of the interaction may represent the transient association and

the dynamics of the folding process, or may be a true reflection of the in vivo situation, where other disulfide isomerases may play a role and where other chaperones such as calnexin and calreticulin could hinder the association of PDI with gp120. Surface glycoproteins E1 and p62 of Semliki forest virus were shown to form mixed disulfides with PDI and ERp57, which are directly involved in disulfide oxidation and isomerization in these substrate proteins (50). Binding of ERp57 and PDI appears to be substrate specific, with ERp57 being associated with calnexin and calreticulin (51). So far we have not been able to detect a direct interaction between ERp57 and gp120 or gp160.

Whether non-native or native, we established that gp120 and gp160 needed disulfide bonds to become folded. Disulfide bond formation in gp120 already starts on the nascent chain and proceeds post-translationally. The importance of cotranslational oxidation became visible when disulfide bond formation was delayed until after synthesis. A significant number of molecules now misfolded and aggregated into disulfide-cross-linked aggregates. Cotranslational disulfide bond formation increased the ratio of intrachain over interchain disulfide bonds in gp120, the latter apparently being more difficult to resolve. In general, the main function of disulfide bonds is to stabilize protein conformation during and/or after folding (52). HIV-1 Env apparently needs its first set of (most likely non-native) disulfide bonds to optimize the outcome of folding, perhaps by preventing nonproductive interchain interactions.

The complex events that occur during Env folding, disulfide isomerization and folding-related cleavage of the signal peptide, may be the main reason for its extremely low folding rate. Despite their long residence time in the ER, we show that gp160 and gp120 remain on productive folding pathways and are not subject to degradation. This warrants the conclusion that HIV-1 Env is a protein that folds efficiently, with high yield, and for which its steady-state ER localization is a logical result of its low folding rate.

In many respects gp160 and gp120 are similar to other proteins for which folding in the ER was studied. Disulfide bonds are already formed cotranslationally and release from calnexin and calreticulin at the time of completion of monomer folding is observed for many other proteins, like influenza virus hemagglutinin (53), VSV G-protein (54), and tPA (55). On the other hand, gp160 and gp120 were already released before the monomer was completely folded, suggesting transfer to another chaperone (5). Perhaps more peculiar was the relative lack of influence by the cellular environment on folding. Whereas the efficiently folding influenza hemagglutinin molecule displayed widely varying folding rates in different cell lines (31), gp160 and gp120 seemed rather cell type independent. Although the presented data concern gp120 and gp160 overexpressed using a recombinant Vaccinia virus system, we did not see a difference with other means of expression. A comparison with our previous studies on folding of gp160 in the HIV-1-infected (CD4-positive) SupT1 cell line showed that neither the presence of CD4, the expression of other HIV-1 proteins, nor infection with recombinant Vaccinia virus made a notable difference (56). Because folding is slow and difficult for Env, it may be determined more by the protein itself than by the cell. The events in the ER were rate limiting for both gp160 and gp120, since the only significant quantity of post-ER localized gp160 or gp120 was detected on the cell surface and in the medium.

Summarizing our results, we conclude that HIV-1 Envelope is a slow but efficiently folding molecule. Its rate of folding is so low and heterogeneous that at any time after HIV infection, a significant fraction of gp160 resides in the ER. The timing of cleavage of the leader peptide that directed gp160/gp120 to the ER is determined by the conformation of the protein. Formation of at least some intrachain disulfide bonds in gp120 is required for leader peptide cleavage to proceed. Since leader peptidases are conformation insensitive, the structure of this viral protein must dictate the delay in processing by shielding its cleavage site. Cotranslational formation and extensive isomerization of disulfide bonds during folding both contribute to a highly productive folding process. FJ

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