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Only Five of 10 Strictly Conserved Disulfide Bonds Are Essential for Folding and Eight for Function of the HIV-1 Envelope Glycoprotein

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Protein folding in the endoplasmic reticulum goes hand in hand with disulfide bond formation, and disulfide bonds are considered key structural elements for a protein's folding and function. We used the HIV-1 Envelope glycoprotein to examine in detail the importance of its 10 completely conserved disulfide bonds. We systematically mutated the cysteines in its ectodomain, assayed the mutants for oxidative folding, transport, and incorporation into the virus, and tested fitness of mutant viruses. We found that the protein was remarkably tolerant toward manipulation of its disulfide-bonded structure. Five of 10 disulfide bonds were dispensable for folding. Two of these were even expendable for viral replication in cell culture, indicating that the relevance of these disulfide bonds becomes manifest only during natural infection. Our findings refine old paradigms on the importance of disulfide bonds for proteins.

INTRODUCTION

A fundamental question in biology is how proteins fold to attain their three-dimensional structure and how structure relates to function. Proteins that traverse the secretory pathway obtain disulfide bonds while they fold in the endoplasmic reticulum (ER; Ellgaard and Helenius, 2003; van Anken and Braakman, 2005). Disulfide bonds are considered essential for folding and/or for structure and function of mature proteins, which explains why the disulfide-bonded structure of proteins usually is conserved across species and within protein families. An intriguing example is the Envelope glycoprotein (Env) of human immunodeficiency virus-1 (HIV-1): it has completely conserved disulfide bonds in the context of a hypervariable sequence. This and the fact that Env has easily testable and well-defined functions for the virus made Env an excellent model for studies on the role of individual, conserved disulfide bonds for a protein's folding and function.

Env is the sole viral protein present on the surface of HIV-1 virions (Allan *et al.*, 1985). It is synthesized as a 160-kDa precursor type I membrane protein (gp160). The protein folds and trimerizes in the ER of the host cell, where it obtains 10 disulfide bonds and ~30 N-linked glycans (Leonard *et al.*, 1990). In the Golgi complex, gp160 is cleaved by a cellular furin(-like) protease into its soluble subunit gp120 and transmembrane subunit gp41 (Stein and Engleman, 1990; Moulard and Decroly, 2000). These remain noncovalently associated on the surface of infected cells and virions. Together, the two Env subunits mediate viral entry: gp120 is responsible for binding to its receptor (CD4) and coreceptor (CCR5 or CXCR4) on the host cell, and gp41 is needed for subsequent fusion of the viral and cellular membranes (Wyatt and Sodroski, 1998; Doms and Moore, 2000; Eckert and Kim, 2001).

Sequence variability of Env among different HIV strains is notorious and in part explains why the humoral immune response against HIV-1 is inadequate in patients. Amid the countless changes in surrounding residues, the cysteine residues are strictly conserved among all isolates of HIV-1 (Louwagie *et al.*, 1995; Los Alamos HIV sequence database: http://hiv-web.lanl.gov). This suggests that the antigenic shield of Env may vary, but that the disulfide-bonded structure of Env and hence the basic architecture of the molecule is constant.

Disulfide bonds may be important for the folding of Env in the ER. Lack of particular disulfide bonds often renders newly synthesized proteins in the ER maturation incompetent: they either aggregate and fail to fold or they partially oxidize into species similar to folding intermediates of the wild-type protein. Alternatively, disulfide bonds contribute

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Abbreviations used: endoH, endoglycosidase H; Env, envelope glycoprotein; ER, endoplasmic reticulum; HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cell.

to the structural integrity of the mature protein. In the case of Env, disulfide bonds seem to be important also for the conformational changes during receptor binding and subsequent fusion of the viral and host cell membranes (Barbouche *et al.*, 2003; Matthias and Hogg, 2003; Markovic *et al.*, 2004).

Cysteine mutants of ER client proteins are excellent tools to provide insight into the relevance of individual disulfide bonds, but so far no systematic cysteine mutation studies of highly disulfide-linked ER clients have been published. Some Env cysteine mutants have been analyzed in different strains (Hemming *et al.*, 1989; Tschachler *et al.*, 1990; Bolmstedt *et al.*, 1991; Freed and Risser, 1991; Syu *et al.*, 1991; Dedera *et al.*, 1992; Lekutis *et al.*, 1992), but together they do not give a consistent notion of the relative importance of the 10 disulfide bonds of Env for folding and function.

To systematically assess the role of each individual disulfide bond in Env folding and function, we analyzed a complete series of mutants, where all cysteines were replaced by alanines, both individually and pair-wise. In spite of the strict conservation of all 10 disulfide bonds, only five turned out to be essential for folding of Env in the ER, whereas two disulfide bonds were expendable not only for folding but even for function of Env.

MATERIALS AND METHODS

Site-directed Mutagenesis and Molecular Cloning

Both single mutants and double mutants of cysteine pairs were generated to eliminate corresponding disulfide bonds in Env by site-directed mutagenesis, either by the pALTER system (Promega, Madison, WI), or by the Quickchange system (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions, using mutagenic oligos where the TGT and TGC cysteine-encoding codons were changed into GCT, respectively, into GCC alanine encoding codons with flanking sequences of 12–14 nucleotides on either side. An extra round of site-directed mutagenesis of one of the corresponding single mutants generated double mutants. Mutations were confirmed by dideoxynucleotide sequencing and mutant Env open reading frames (ORFs) were cloned into pcDNA3 (Invitrogen), yielding pcDNA3-gp160 constructs. A stop codon was introduced at the cleavage site between the gp120 and gp41 sequences by PCR, yielding corresponding pcDNA3-gp120 constructs. For functional assays, mutant Env ORFs were cloned into a full-length molecular clone pLAI (Peden *et al.*, 1991) of the HIV-1_{LAI} isolate. Although we studied Env of the LAI isolate, we followed the canonical HXB2 residue numbering, which relates to the LAI numbering as follows: because of an insertion of five residues in the V1 loop of LAI Env, all cysteine residues beyond this loop have a number 5 residues lower in HXB2 than in LAI: until Cys131, numbering is identical, but Cys162 in LAI becomes 157 in HXB2, etc.

Cells

HeLa cells were cultured in MEM (Life Technologies, Rockville, MD) supplemented with 10% FCS (Life Technologies), penicillin and streptomycin (100 U/ml), 2 mM glutamax (Life Technologies), and nonessential amino acids (Life Technologies). C33A cervix carcinoma cells were maintained in DMEM (Invitrogen), supplemented with 10% FCS, penicillin, and streptomycin. SupT1 T-cells were cultured in RPMI medium supplemented with 10% FCS, penicillin, and streptomycin. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh buffy coats (Central Laboratory Blood Bank, Amsterdam) by standard Ficoll-Hypaque density centrifugation. PBMCs were frozen in multiple vials at a high concentration and, when required, were thawed and activated with 5 μg/ml phytohemagglutinin (Sigma, St. Louis, MO) and cultured in RPMI medium (Life Technologies) containing 10% FCS, penicillin and streptomycin (100 U/ml) with recombinant interleukin-2 (rIL-2; 100 U/ml). On day 4 of culture, PBMCs underwent $CD4^+$ enrichment by incubating them with CD8 immunomagnetic beads (Dynal, Lake Success, NY) and separating out the $CD8^+$ lymphocytes.

Folding Assays

Subconfluent HeLa cells were infected with recombinant vaccinia virus expressing T7 polymerase (Fuerst *et al.*, 1986) to drive expression of Env mutants under control of the T7 promoter. Thirty minutes after infection, cells were transfected with a mixture of 4 μg of mutant or wild-type pcDNA3-gp120 or pcDNA3-gp160, and 10 μl lipofectin (Invitrogen), according to the manufacturer's instructions. Pulse-chase experiments were performed essentially as described (Braakman *et al.*, 1991; Land *et al.*, 2003). Five hours after infection, cells were depleted of cysteine and methionine for 15–30 min before they were pulse-labeled with 50 μCi of Redivue promix L-[^{35}S] in vitro labeling mix (Amersham Biosciences, Piscataway, NJ). Cells were chased for various intervals in medium containing an excess of unlabeled cysteine and methionine. Chase samples were stopped by aspirating the medium and adding ice-cold HBSS (Invitrogen-BRL) containing 20 mM iodoacetamide (IAM) to block free sulfhydryl groups. Cells were lysed in ice-cold MNT (20 mM MES, 100 mM NaCl, 30 mM Tris-HCl, pH 7.5) containing 0.5% (vol/vol) Triton X-100, 20 mM IAM, and protease inhibitor cocktail (10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin, 1 mM PMSF, and 1 mM EDTA). Cell lysates were spun for 10 min at 15,000 $\times$ *g* to pellet nuclei and postnuclear lysates were immunoprecipitated with a polyclonal antibody that recognizes all forms of HIV-1 Env. In addition, secreted or shed gp120 molecules were immunoprecipitated from the culture media at later chase times. Washed immunoprecipitates were resuspended in 0.2% SDS in 100 mM sodium acetate, pH 5.4, and incubated at 95°C for 5 min. An equal volume of 100 mM sodium acetate, pH 5.4, was added, which contained 2% Triton X-100 in MNT, protease inhibitor cocktail, and 0.0025 U endoglycosidase H (endoH; Roche, Rotkreuz, Switzerland). Samples were incubated for 2 h at 37°C. After incubation, SDS-PAGE sample buffer was added, and samples were incubated at 95°C for 5 min. Samples were analyzed by reducing or nonreducing 7.5% SDS-PAGE. Gels were dried and signals were detected on Biomax MR films (Eastman Kodak, Rochester, NY).

Viruses and Infections

Virus stocks were produced as follows: C33A cells were transfected with 10 μg wild-type or mutant pLAI constructs by calcium phosphate precipitation (Das *et al.*, 1999). Three days after transfection, virus-containing culture supernatants were harvested, filtered, and stored at −80°C. Virus concentrations were quantitated by capsid CA-p24 ELISA (Jeeninga *et al.*, 2000). These values were used to normalize the amount of virus for infection experiments. SupT1 cells (50×10^3) were infected with the equivalent of 500 pg CA-p24 of C33A-produced HIV-1_{LAI} per well in a 96-well plate, and virus spread was measured for 14 d using CA-p24 ELISA. The 50% tissue culture infectious dose ($TCID_{50}$) in SupT1 cells was determined after 14 d by endpoint dilution.

Ultracentrifugation of Virions

C33A cells were transfected with 40 μg pLAI per T75 flask. Medium was refreshed at day 1 after transfection. At 3 d after transfection, culture media were harvested, centrifuged, and passed through a 0.45-μm filter to remove residual cells and debris. Virus particles were pelleted from filtered culture media by ultracentrifugation (100,000 $\times$ *g* for 45 min at 4°C) and resuspended in 0.5 ml lysis buffer (50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 100 mM NaCl, 1% SDS).

Quantitation of gp120 by ELISA

Concentrations of gp120 in cell, virion, and supernatant fractions were measured as described before (Moore and Ho, 1993; Sanders *et al.*, 2002), with some modifications. ELISA plates were coated overnight with sheep antibody D7324 (10 μg/ml; Aalto Bio Reagents, Ratharnham, Dublin, Ireland), directed to the gp120 C5 region, in 0.1 M $NaHCO_3$. After blocking by 2% milk in Tris-buffered saline (TBS) for 30 min, gp120 was captured by incubation for 2h at room temperature. Recombinant HIV-1_{LAI} gp120 (Progenics Pharmaceuticals, Tarrytown, NY) was used as a reference. Unbound gp120 was washed away with TBS and purified $HIV\text{-}1^+$ serum Ig (HIVIg) was added for 1.5 h in 2% milk, 20% sheep serum (SS), 0.5% Tween. HIVIg binding was detected with alkaline phosphatase–conjugated goat anti-human Fc (1:10,000, Jackson ImmunoResearch, West Grove, PA) in 2% milk, 20% SS, and 0.5% Tween. Detection of alkaline phosphatase activity was performed using AMPAK reagents (DAKO, Carpinteria, CA). The measured gp120 contents in virus were normalized for CA-p24.

HIV-1 Neutralization Assay Using Primary $CD4^+$ Lymphocytes

Viruses were tested for their relative inhibition sensitivity against increasing concentrations of either sCD4 (BD ImmunoDiagnostics, Orangeburg, NY) or the CXCR4 binding compound AMD3100 (a generous gift from Dr. D. Schols, Katholieke Universiteit, Leuven, Belgium). $TCID_{50}$ values were determined on purified $CD4^+$ lymphocytes isolated from an individual who did not carry the Δ32CCR5 allele ($CCR5^{+/+}$) screened for by standard PCR. $CD4^+$-enriched lymphocytes were plated at 2×10^5 cells/well in 96-well plates with fivefold serial dilutions of the virus. Cells were fed on day 7 with fresh media and scored on day 14 for CA-p24 levels, with the number of positive wells being used to identify the $TCID_{50}$ value for each virus. Each virus (100 $TCID_{50}$ in 50 μl of the culture RPMI medium) was mixed with an equal volume of serially diluted compound for 1 h at 37°C in flat-bottomed 96-well plates, and all neutralization reactions were performed in triplicate. After 1 h of incubation 2.0×10^5 positively selected $CD4^+$ lymphocytes were added to each well in 0.1 ml of culture media containing rIL-2 (100 U/ml). For each neutralization experiment a positive control, virus incubated with cells in the absence of inhibitory compound, and a negative control, virus in the absence of cells, was

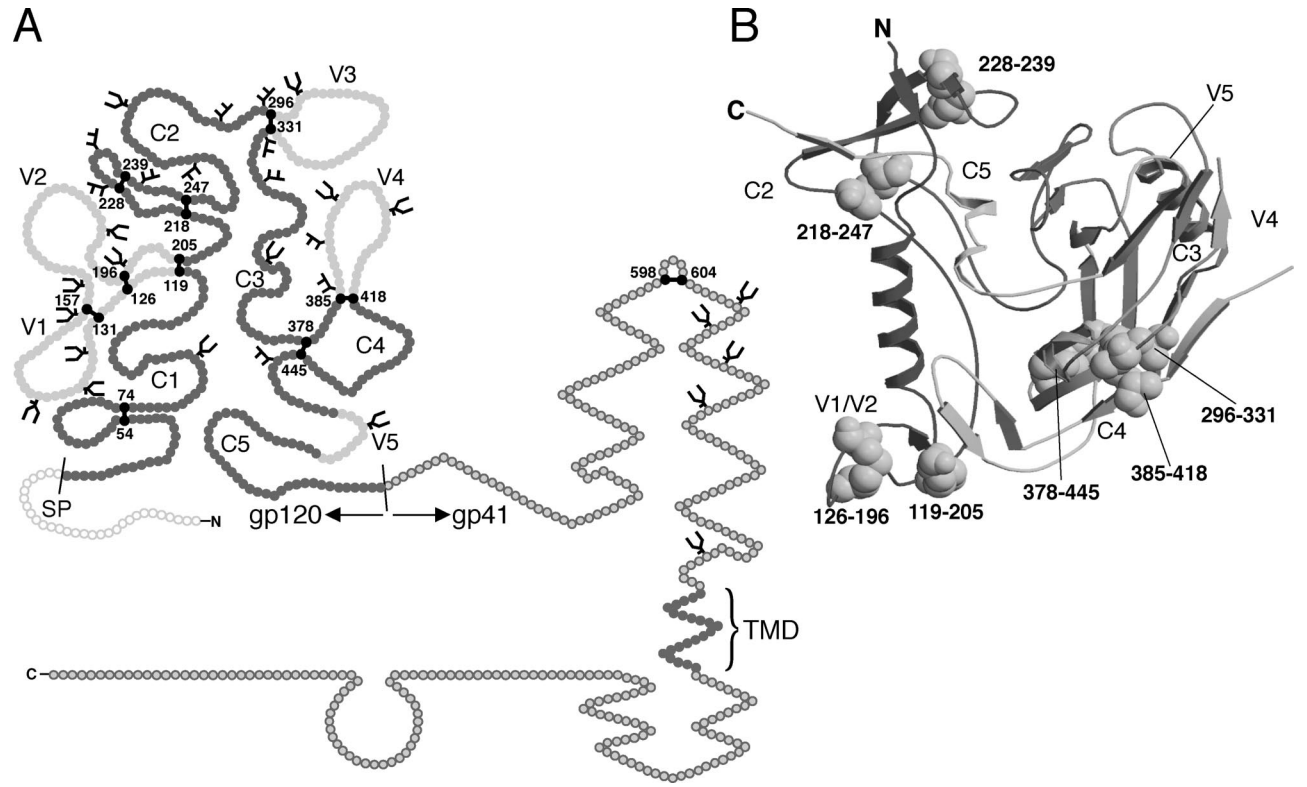

Figure 1. Schematic representation of the gp160 precursor of HIV Envelope. (A) Residues are displayed as spheres. The soluble subunit gp120 with constant (C) – in dark gray – and variable (V) – in light gray – domains and the signal peptide (SP) – in white, and the transmembrane subunit gp41 with its transmembrane domain (TMD) – in darker gray – are indicated. Disulfide bonds (with residue numbers of cysteines) and attached glycans of both the complex (Ψ) and oligomannose/hybrid type (Ƴ) as well as the N-terminus (N) and C-terminus (C) are indicated. (B) Ribbon representation of the crystal structure of gp120 (Kwong *et al.*, 1998). Atoms of cysteine pairs are shown as space filling spheres. Note that the C1 region including the 54–74 disulfide bond is absent. Only the stem of the V1/V2 loops is present, excluding the 131–157 disulfide bond, and the V3 and V4 loops are absent from the core crystal structure of gp120.

included. The negative control CA-p24 concentration was subtracted from all test results. Supernatant was taken on days 7, 10, and 14 and assayed for CA-p24 with fresh media added to the cells. Neutralization responses were determined for each virus at the day CA-p24 levels peaked. We calculated the CA-p24 production in the presence of inhibitory compound as a percentage of that in the cultures with virus only.

RESULTS

Disulfide Bonds and Cysteine Mutants of Env

We set out to assess the role in folding and function of individual disulfide bonds in the Envelope glycoprotein of the HIV-1_{LAI} isolate. The gp120 part of Env contains nine disulfide bonds, whereas gp41 harbors a single disulfide bond (Figure 1A). The gp120 soluble subunit has a core that is composed of conserved regions C1 to C5. The variable regions V1 to V5 are present on protein loops that protrude from this core. The gp120 core largely overlaps with the available crystal structures of gp120 (Kwong *et al.*, 1998; Chen *et al.*, 2005; Figure 1B). Six disulfide bonds locate to the gp120 core. The C1 region contains a single disulfide bond, 54–74, whereas the C2 region contains two disulfide bonds: 218–247 and 228–239. The 378–445 disulfide bond covalently links the C3 and C4 regions. Two bonds are found at the base of variable loops: 296–331 (V3) and 385–418 (V4). The two most prominent loops, which encompass the V1 and V2 regions, have three disulfide bonds at their stem(s): 119–205, 126–196, and 131–157. The disulfide bond in gp41 closes off a very small loop: 598–604. We systematically replaced all cysteines in the ectodomain of Env by alanines and refer to mutants as follows: C54A and C74A are the single mutants, and C54/74A is the double mutant of disulfide bond 54–74.

Biosynthesis and Maturation of Env

To monitor maturation of Env and to compare wild-type Env with all cysteine mutants, we used a pulse-chase assay we previously developed (Land *et al.*, 2003). In HeLa cells, we expressed Env from plasmids under control of the T7 promoter, using a recombinant Vaccinia virus vector system (Fuerst *et al.*, 1986). We radioactively pulse-labeled cells and chased them in the presence of excess unlabeled amino acids. Immediately after the pulse or after various chase periods, cells were cooled on ice, incubated with an alkylating agent to block free sulfhydryl groups, and lysed. We immunoprecipitated Env from the lysates and deglycosylated samples with endoH. At the end of longer chase periods, we also immunoprecipitated Env from culture media. We then analyzed folding kinetics of Env by reducing and nonreducing SDS-PAGE. We not only expressed the gp160 Env precursor but also the soluble subunit gp120. The pulse-chase provides three assays for maturation of Env, two of which are based on differences in mobility of Env conformers in SDS-PAGE (Figure 2).

As a first assay for Env maturation, we analyzed the mobility of alkylated Env using nonreducing SDS-PAGE to detect disulfide bond formation (Land *et al.*, 2003). Immedi-

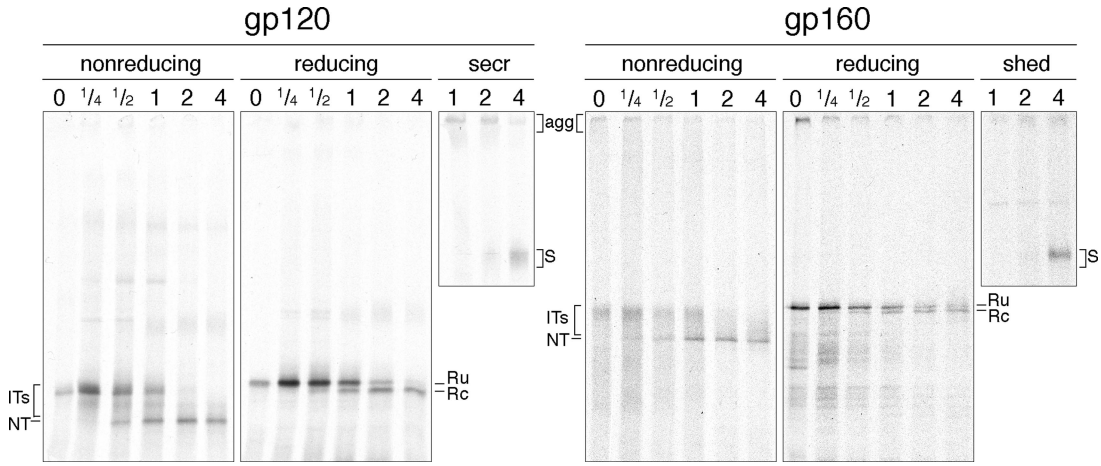

Figure 2. Folding of wild-type gp160 and gp120. Wild-type Env, as precursor gp160 or as the soluble subunit gp120 alone, was expressed in HeLa cells using the Vaccinia T7 heterologous expression system. Cells were pulse-labeled for 2 min (gp120) or 5 min (gp160) and chased for various times (h) as indicated. Cells were lysed and Env proteins were immunoprecipitated from lysates and from the chase medium. Immunoprecipitates from lysates were fully deglycosylated with endoH (see *Materials and Methods*) and analyzed by nonreducing (NR) or reducing (R) 7.5% SDS-PAGE. Immunoprecipitates from the chase media of secreted or shed (S) gp120 were analyzed directly on reducing 7.5% SDS-PAGE. Aggregates (agg), folding intermediates (ITs), the native form (NT), and the reduced protein from which the signal peptide was cleaved off (Rc) or not (Ru), as well as the shed or secreted gp120 (S) are indicated.

ately after the pulse, wild-type gp160 and gp120 appeared as a fuzzy band in nonreducing gels (Figure 2, 0 h, nonreducing) with mobility close to that of the corresponding reduced protein. The reduced samples appeared as a discrete band however (Figure 2, 0 h, reducing), implying that some disulfide bonds had formed already before the end of the pulse. With time, "smears" of faster running protein appeared in the nonreduced samples, corresponding to various species of partially oxidized folding intermediates (ITs). After 4 h of chase, most of the wild-type gp160 and gp120 proteins appeared as a sharp band in nonreducing gels (Figure 2, 4 h, nonreducing), indicating that they reached the fully oxidized native state (NT).

Proteins that fold in the ER are targeted to enter this compartment by a signal peptide (Walter and Johnson, 1994), which in soluble or type I proteins is cleaved off already during synthesis. A striking exception however is the signal peptide of HIV-1 Env, which is removed long after synthesis has been completed (Li *et al.*, 1994). The signal peptide of Env can be removed only after the protein has gone through some initial folding steps, making this cleavage another indicator of Env maturation (Land *et al.*, 2003). By analyzing the chase samples via reducing SDS-PAGE, we hence used signal peptide cleavage as a second assay for Env maturation. Immediately after the pulse only a single band was visible (Figure 2, 0 h, reducing), which corresponds to the reduced protein with its signal peptide still attached (Ru = reduced uncleaved). With time, a second band appeared below Ru, which corresponds to the reduced protein from which the signal peptide had been removed (Rc = reduced cleaved). After 4 h of chase, the signal peptide had been removed from the majority of wild-type gp160 and gp120 molecules. Except for some faint band at the top of the gel, which likely represents aggregated Env, we detected no other bands or smears in the reducing gel. This implies that intracellular pools of Env were fully deglycosylated by endoH, as we previously reported (Land *et al.*, 2003). Env did undergo heterogeneous glycan modifications in the Golgi as is evident from the "smeary" character of the shed and secreted gp120.

This gp120 harvested from culture media was analyzed by reducing SDS-PAGE as a third assay for Env maturation. Correctly folded Env exits the ER and travels along the secretory pathway. Env expressed as soluble subunit gp120 is secreted, whereas gp160 undergoes proteolytic cleavage into gp41 and gp120 subunits. Because the gp120 subunits are no longer covalently attached to the remainder of the Env spikes, they quantitatively shed into the culture medium. As anticipated, substantial amounts of both shed and secreted (endoH resistant) wild-type gp120 were present in the culture medium after 4 h of chase (Figure 2, 4 h, secr/shed). The pool of endoH-resistant intracellular Env is too small to detect because the average time it takes Env to travel via the Golgi to the cell surface and be cleaved and shed or secreted is very short compared with the time the protein resides in the ER.

Altogether, we concluded that wild-type Env matured in the vaccinia virus T7 heterologous expression system similar as in SupT1 cells infected with HIV-1 (Das *et al.*, 1999) and in HeLa or CHO cells infected with gp120 or gp160 expressing recombinant vaccinia viruses (Kieny *et al.*, 1986; Land *et al.*, 2003). As shown before (Land *et al.*, 2003), oxidative folding of the gp120 subunit was similar to that of the gp160 precursor, except that gp120 folded slightly faster and that differences in mobility in SDS-PAGE of Env species are more evident for gp120 because of its smaller size. Maturation of Env is slow, but after 4 h the majority of wild-type Env reaches the native state (Earl *et al.*, 1991; Otteken *et al.*, 1996; Land *et al.*, 2003). We therefore compared maturation of Env mutants after 4 h of chase (Figure 3A) and quantified the results.

Five Disulfide Bonds Are Dispensable for Folding of Env

The 0-h chase samples of all mutants migrated similarly to that of wild type in both reducing and nonreducing gels, indicating that the starting point of folding was indistinguishable for mutants and wild-type proteins (Figure 3A,

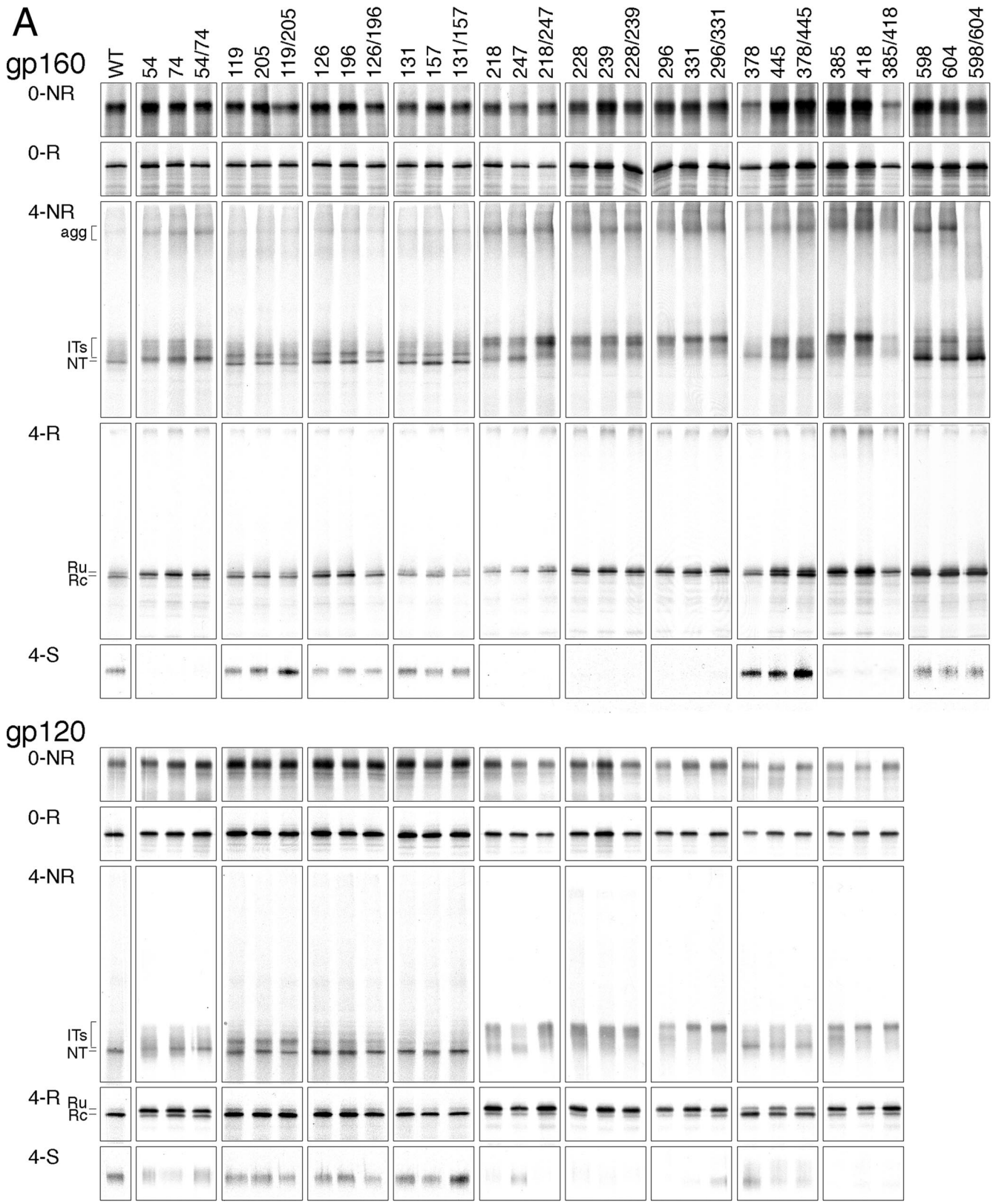

Figure 3. Folding "endpoint" analysis of Env cysteine mutants. (A) Wild-type or cysteine mutants of gp160 or gp120 were expressed in HeLa cells using the Vaccinia T7 heterologous expression system. Cells were pulse-labeled for 10 min and chased for 0 or 4 h. Cells were lysed and Env proteins were immunoprecipitated from lysates and chase medium. Samples were analyzed as in Figure 2. (B) Data were quantified with ImageQuant (Molecular Dynamics, Sunnyvale, CA), and percentage aggregation, NT formation, Rc formation, and shedding/secretion are depicted in histograms.

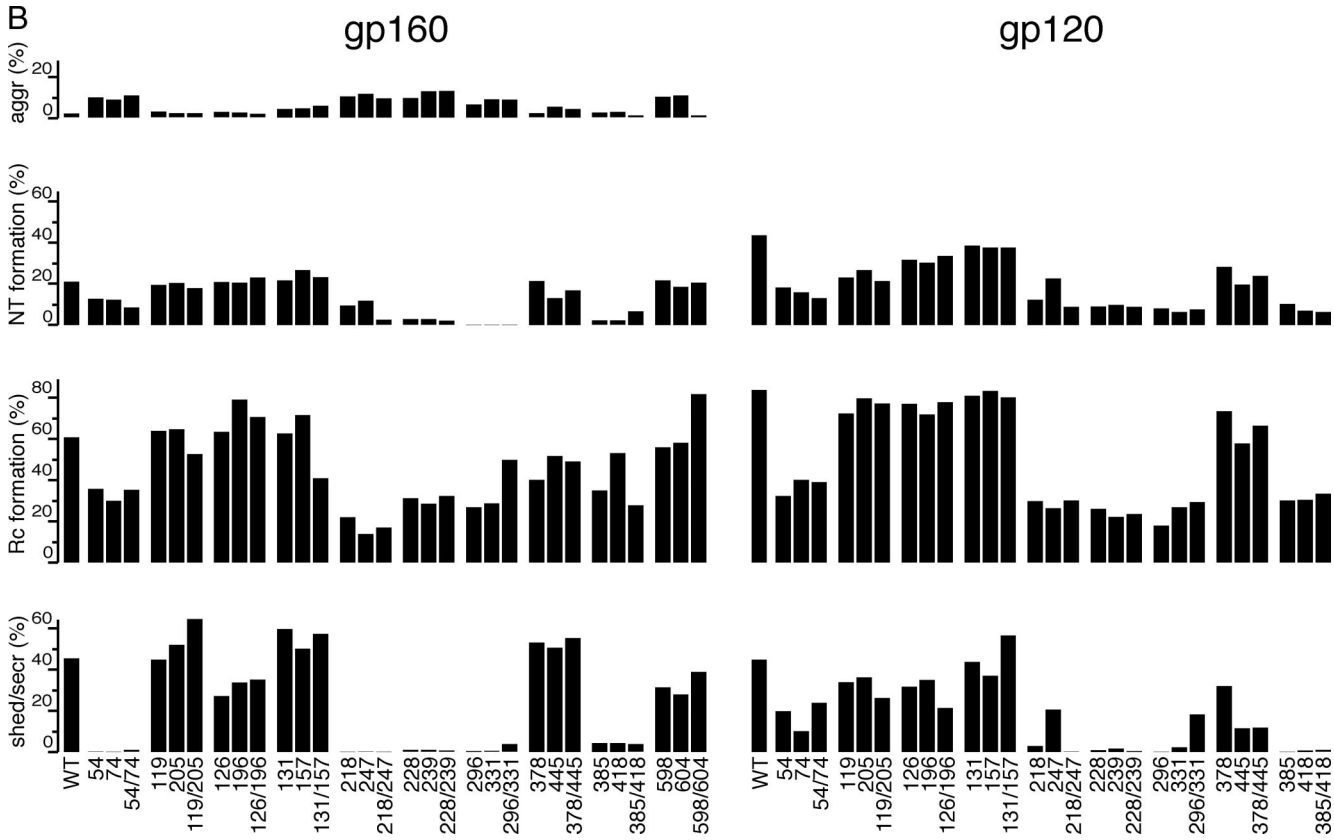

Figure 3. (cont).

0-NR/0-R). After 4 h of chase, however, the Env mutants displayed many differences in maturation (Figure 3A, 4-NR/4-R/4-S).

Based on the three assays, mutants of five disulfide bonds were maturation competent: mutants of the three disulfide bonds in the V1/V2 loops (119–205, 126–196, and 131–157), of the disulfide bond that bridges C3 and C4 (378–445) and of the single disulfide bond in gp41 (598–604). These mutants reached a native state (Figure 3A, 4-NR), and signal peptide removal was substantial (Figure 3A, 4-R). Accordingly, they could exit the ER as was evident from secretion or shedding of gp120 (Figure 3A, 4-S).

The folding patterns of maturation competent mutants were not necessarily identical to those of wild type. In case of the 378–445 mutants, fewer molecules than wild type had reached the native state and had lost their signal peptide (Figure 3A, 4-NR/4-R). Shedding from the 598–604 mutants was poorer than from wild type (Figure 3A, gp160, 4-S), and mutants of the V1/V2 loops accumulated a folding intermediate that was almost undetectable for wild type (Figure 3A, 4-NR). Results were similar for gp160 and gp120. We therefore concluded that five disulfide bonds (119–205, 126–196, 131–157, 378–445, and 598–604) were not strictly essential for Env to reach a folded state similar to wild type, but rather improved folding yields and rates.

Mutants of the remaining five disulfide bonds displayed severe folding defects: of the single disulfide bond in the C1 region (54–74), of the two disulfide bonds in the C2 region (218–247 and 228–239), and of the single disulfide bonds at the stem of the V3 loop (296–331) and the V4 loop (385–418). Compared with wild type, signal peptide cleavage of these mutants was poor (Figure 3A, 4-R). Disulfide bond formation varied: the persistence of the reduced-like band that was already present at the end of the pulse, in mutants of the 296–331 and 385–418 disulfide bonds, indicated that few if any disulfide bonds were formed in the majority of these Env species (Figure 3A, 4-NR). In contrast, mutants of the 54–74 disulfide bond and, to a lesser extent, of the 228–239 and 218–247 disulfide bonds did reach a native-like band (Figure 3A, 4-NR). These mutants nevertheless failed to shed gp120 (Figure 3A, gp160, 4-S). Conversely, mutants of the 385–418 disulfide bond shed marginal amounts of gp120 into the chase medium (Figure 3A, gp160, S; Figure 3B, gp160, shedding).

None of the gp120 mutants formed aggregates, whether folding deficient or not (Figure 3A, gp120, 4-NR). In case of the gp160 mutants, high-molecular-weight species were present after 4 h of chase, which represent aggregates of trimeric and larger size (Figure 3A, gp160, 4-NR/R). Aggregation was modest (never exceeding 13% of the signal; Figure 3B), but was more apparent for mutants that were maturation incompetent (54–74, 218–247, 228–239, 296–331, and 385–418) or displayed slower maturation (378–445). The single mutants C598A and C604A produced more of these high-molecular-weight species than the corresponding double mutant because of their odd number of cysteines, which leaves one free for intermolecular bonding.

Aggregates were fully reduced in the reducing gels (Figure 3A, gp160, 4-R), revealing that Env from lysates was fully endoH sensitive after 4 h. On the one hand this implies that the travel of the folding competent mutants through the Golgi and subsequent surface expression and shedding were similarly as fast as for wild type, because endoH resistance develops only as a result of glycan modifications in the

Golgi. On the other hand, the lack of shedding (Figure 3A, gp160, 4-S) and of endoH resistance of the mutants with folding problems (Figure 3A, gp160, 4-R) implied that they where strictly retained in the ER, as we anticipated. Still, for some of these mutants gp120 was released from cells more by secretion than by shedding from gp41, in particular for the gp120s from the 54–74 mutants, for C247A, and for C296/331A (Figure 3A, gp120, 4-S). Retention in the ER hence was less stringent for gp120 than for gp160, possibly because gp120 is a monomeric soluble protein whereas gp160 must trimerize and is a membrane protein (Singh *et al.*, 1990). We concluded that elimination of any of the 54–74, 218–247, 228–239, 296–331, or 385–418 disulfide bonds led to severe folding deficiencies in the ER. We categorized them as class 1 disulfide bonds, as opposed to class 2 disulfide bonds, which were expendable for productive folding.

Folding patterns were almost identical for gp120 and gp160, but the resolution was better for gp120, because of its smaller size (Figures 2 and 3A). We therefore used gp120 constructs for all mutants except for the 598–604 mutants (because these mutations reside in the gp41 domain), to analyze the effects of disulfide deletions on folding kinetics of Env in a time-course pulse-chase assay from 0 to 4 h (Figure 4). For wild-type gp120, the half-time of folding (of reaching the native state) and of signal peptide cleavage was >30 min (Figure 4, gp120 wt), consistent with our previous results (Land *et al.*, 2003). Gp160 was somewhat slower with a half-time of folding and signal peptide cleavage of >60 min. Note that the gain in signal between the pulse and the first chase sample reflects elongation and completion of ribosome-bound nascent chains during the first 15 min of chase.

Three class 2 mutants displayed folding kinetics that were almost identical to the wild-type protein (Figure 4, 126–196, 131–157, and 598–604). The other two class 2 mutants folded with a similar pattern as wild type but at a somewhat (119–205) or considerably (378–445) lower pace. The class 1 mutants (Figure 4, 54–74, 218–247, 228–239, 296–331, and 385–418, nonreducing) displayed various patterns of disulfide bond formation. For some mutants the majority of Env species reached a native-like band, as was most evident for C239A and the mutants of disulfide bond 54–74. In contrast, from other folding deficient mutants only very few molecules underwent disulfide bond formation, as was clearest for C418A and C385/418A. The remainder of folding deficient mutants gave phenotypes between these two extremes with nonreduced forms smearing from reduced to native positions.

The extent to which folding intermediates of a mutant obtained disulfide bonds, correlated in most cases with their recognition by signal peptidase: the more oxidized, the better signal peptide cleavage (Figure 4, nonreducing vs. reducing). One exception was C385/418A: Env obtained few if any disulfide bonds, whereas after 4 h of chase the signal peptide was removed from a substantial portion of the mutant molecules. Before the signal peptide can be cleaved, Env must undergo some disulfide bond formation (Land *et al.*, 2003). The pool of Env molecules that had their signal peptides removed in the case of the 385–418 mutants therefore is likely to correspond with the pool that displays some oxidation. Another exception was C54/74A, which showed the opposite: it reached an oxidized "endpoint" that migrated in nonreducing gels only slightly slower than native Env, yet signal peptide cleavage was minimal even at later chase times, suggesting that formation of the 54–74 disulfide bond in particular is important for subsequent signal peptide removal.

In general, ER client proteins that fail to fold correctly eventually are disposed of, for instance by a process that is referred to as ER-associated degradation (ERAD; Meusser *et al.*, 2005). Signals decreased only slightly with time for both wild type and most mutants irrespective of their class (Figure 4), which suggested that even the most defective mutants were poor substrates for degradation. The mutants that were secreted, like those of the 54–74 disulfide bond, of course were expected to be lost from the cell lysates (Figure 3A, gp120, 54–74, 4-S).

Two Disulfide Bonds of Env Are Expendable for Function in the Virus

Because all disulfide bonds of Env are strictly conserved, we expected that the disulfide bonds that were expendable for folding would be essential for viral functions of Env instead. To examine the importance of particular disulfide bonds for viral fitness, we analyzed the replication of HIV-1 bearing wild-type or mutant Env proteins in cell cultures. Virus stocks were generated in C33A cells and SupT1 cells were infected with equivalent amounts of virus, as determined by CA-p24 content. Virus spread was monitored for 14 d by CA-p24 ELISA (see Figure 5A). In an independent assay we established whether the double cysteine mutants could mediate viral infectivity: the 50% tissue culture infectious dose ($TCID_{50}$) in SupT1 cells was calculated by endpoint dilution (see Figure 5B).

As anticipated for class 1 mutants, viruses with mutations in the 54–74, 218–247, 228–239, or 296–331 disulfide bonds did not replicate (Figure 4A) nor display infectivity (Figure 5B). The C385/418A mutant virus however conveyed some infectivity (Figure 5B), and its replication was very poor, although slightly better than its corresponding single mutants or any of the other class 1 mutants (Figure 5A). The residual viral function of this mutant was surprising, considering its severe folding difficulties. Still, in contrast to the other class 1 mutants, the 385–418 disulfide mutants displayed shedding of gp120 after 4 h of chase, albeit at a marginal level (Figure 3, gp160, 4-S). Altogether, a minority of Env molecules matured, and few but functional Env spikes were incorporated into these mutant viruses. We therefore subdivided the class 1 disulfide bonds into class 1b, comprising only 385–418, whose elimination resulted in severe folding deficiencies but still allowed residual viral function in some molecules, and class 1a, containing the disulfide bonds that were absolutely required for both folding and function (54–74, 218–247, 228–239, and 296–331).

Mutants of the 126–196, 131–157, or 598–604 disulfide bonds were folding competent, but the corresponding mutant viruses did not replicate (Figure 5A). These disulfide bonds of Env indeed were essential for function rather than for folding. Contrary to our expectations, the remaining two disulfide bonds were required neither for folding nor for function: virus lacking the 119–205 or the 378–445 disulfide bonds in Env sustained viral infectivity (Figure 5B) and replicated efficiently in SupT1 T-cells, as did the C378A mutant (Figure 5A). Still, the corresponding single mutants of 119–205, C119A and C205A, did not replicate, whereas C445A replicated markedly poorer than C378/445A, suggesting that an odd number of cysteines in gp120 is disadvantageous for virus replication. Also in primary T-cells, natural host for HIV, the C119/205A and C378/445A double mutants replicated efficiently (data not shown). We therefore subdivided class 2 disulfide bonds into class 2a: expendable for folding, but essential for the virus (126–196, 131–157, and 598–604) and class 2b: expendable for both folding and function (119–205 and 378–445).

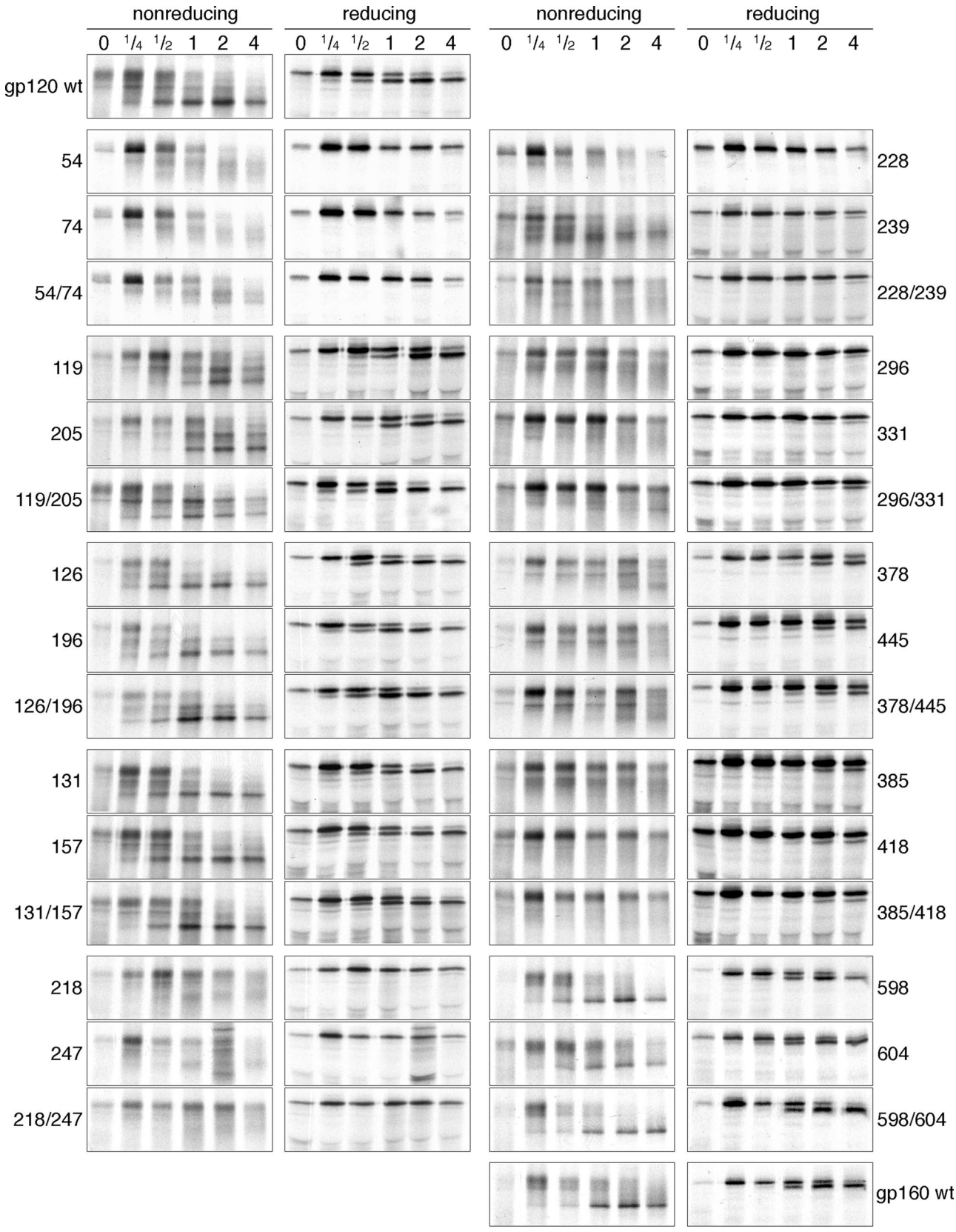

Figure 4. Folding kinetics of Env cysteine mutants. Wild-type or cysteine mutants of gp120 as well as 598–604 disulfide mutants of gp160 were expressed in HeLa cells using the Vaccinia T7 heterologous expression system. Cells were pulse-labeled for 2 min (gp120) or 5 min (gp160) and chased for various times (h) as indicated. Cells were lysed and Env proteins were immunoprecipitated from lysates, deglycosylated with endoH, and analyzed by nonreducing (NR) or reducing (R) 7.5% SDS-PAGE.

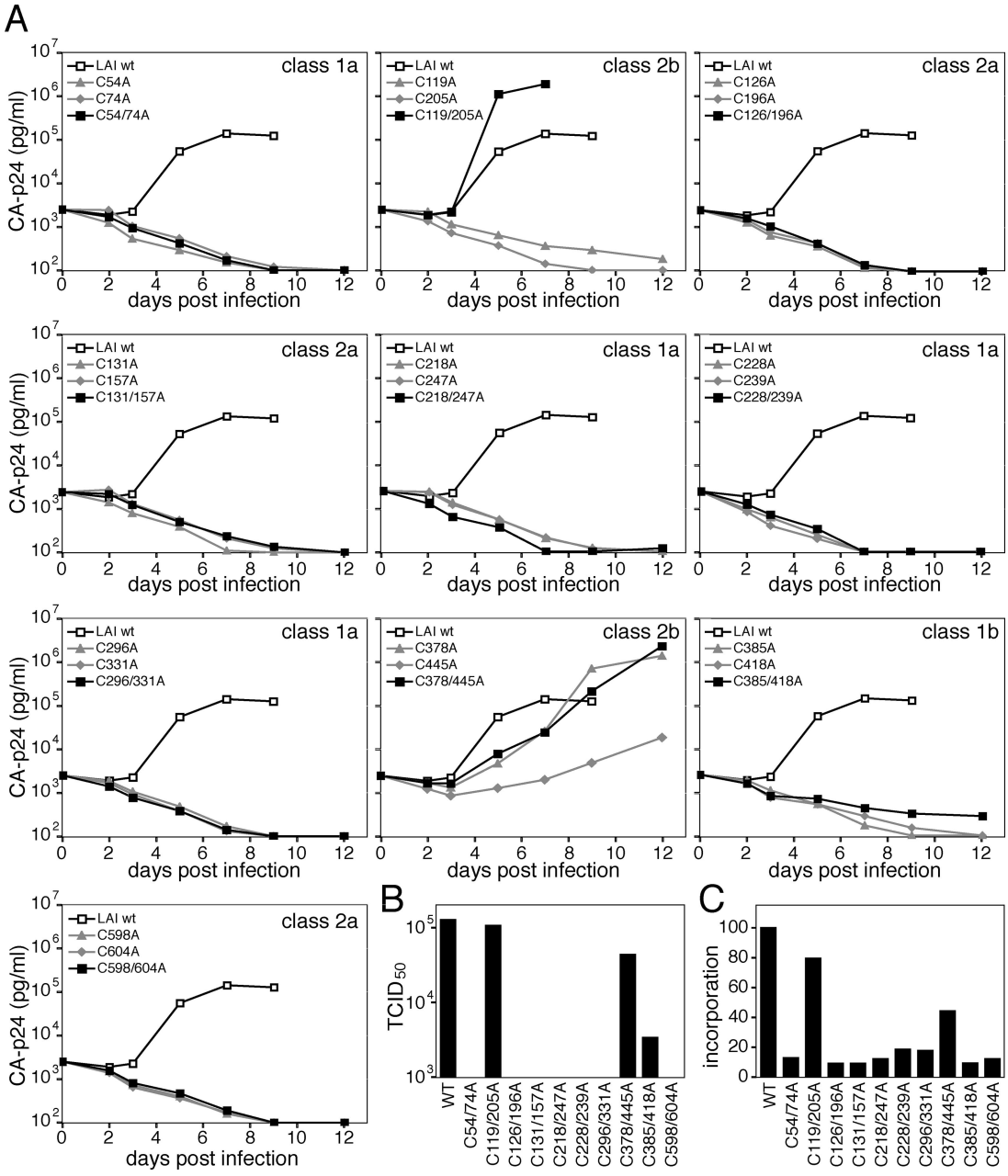

Figure 5. Effects of Env cysteine deletions on viral replication, infectivity, and spike incorporation into the virus. (A) SupT1 cells (50×10^3) were infected with 500 pg CA-p24 equivalents of HIV-1_{LAI}, yielded from supernatants of C33A cells that were transfected with genomic pLAI bearing the wild-type or mutant Env gene. Virus spread was measured for 14 d using CA-p24 ELISA. (B) The infectivities ($TCID_{50}$) in SupT1 cells of virus stocks as in A were measured by endpoint dilution. (C) Virus particles were harvested by ultracentrifugation, and gp120 and CA-p24 contents of virus particles were measured by ELISA. The gp120 amounts were standardized for CA-p24 input and the gp120 contents of mutants are given as percentages of wild type.

Lack of infectivity and replication of class 2a mutant viruses could result from dysfunctional Env on virions or from their absence in viral particles. We therefore measured incorporation of Env in virus particles by gp120 ELISA on purified virus fractions and found that the molar Gag-to-Env ratio in the wild-type virus sample was ~100:1 (data not shown). This corresponds to an average of 4–8 trimeric Env spikes per virus particle, which agrees with previously published results (O'Brien *et al.*, 1994; Chertova *et al.*, 2002). As expected, considerable amounts of C119/205A Env spikes were incorporated onto virions (~80% of wild-type levels; Figure 5C). Incorporation of C378/445A spikes was lower (~45% of wild-type levels; Figure 5C), which correlated with the reduced folding rate (Figures 3 and 4), delayed replication (Figure 5A), and slightly reduced viral infectivity (Figure 5B) compared with wild type. We did not anticipate to detect any incorporation of those mutants that could not fold and exit the ER. The residual incorporation we found for the class 1a mutants (Figure 5C) therefore likely represents background. Incorporation of the class 2a mutants that did fold but failed to replicate, C126/196A, C131/157A, and C598/604A, was in that same range. We concluded that the

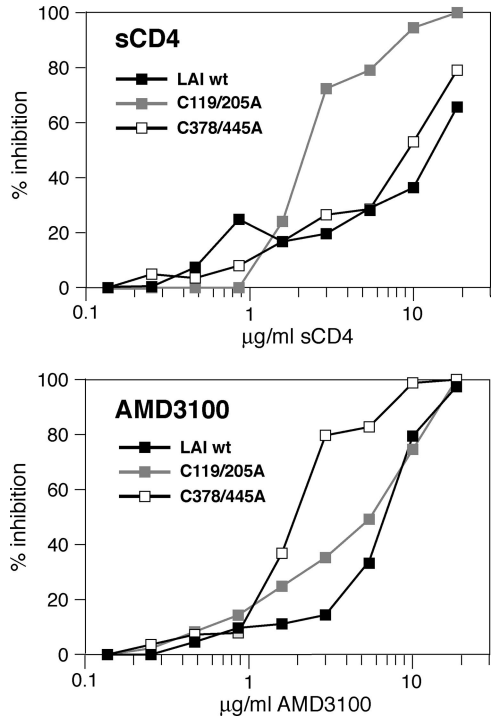

Figure 6. Effect of inhibition of (co-)receptor binding on replicating mutant viruses. $CD4^+$ lymphocytes (2.0×10^5/well in 96-well plates) were inoculated with equal amounts (100 $TCID_{50}$) of the parental HIV-1_{LAI} or mutant virus containing Env lacking either the 119–205 or the 378–445 disulfide bond. CD4 receptor binding was inhibited by limiting dilutions of sCD4 (top) and CXCR4 coreceptor binding with AMD3100 (bottom). As a measure for inhibition, the percentage reduction in CA-p24 production at various concentrations of either compound as compared with the CA-p24 production in the absence of compound was calculated. The experiment was repeated several times, and results were reproducible.

lack of viral replication of the class 2a mutants was due to absence or low abundance of functional Env spikes on the mutant virions.

Functional Env Mutants Are Similar But Not Identical to Wild Type

To ascertain whether the class 2b mutant viruses had different Env properties than the parental strain, we tested usage of the receptor (CD4) and coreceptor (CXCR4 for the LAI isolate) for C119/205A and C378/445A. $CD4^+$-enriched lymphocytes were inoculated with equivalent amounts of mutant and wild-type virus, but viral entry, and hence propagation, was inhibited with limiting dilutions of either soluble CD4 (sCD4) or the CXCR4-binding compound AMD3100. Compared with the LAI parental strain and the C378/445A virus, less sCD4 was required to inhibit viral replication of the C119/205A mutant virus (Figure 6, top), which indicates that receptor usage was different for this mutant. The C378/445A mutant on the other hand displayed alterations in CXCR4 coreceptor usage, with lower concentrations of AMD3100 required to inhibit virus replication than for either the wild-type or C119/205A viruses (Figure 6, bottom).

The changes in specific molecular usage of receptor (C119/205A) or coreceptor (C378/445) could be attributed to, for instance, increased binding affinity or increased ability to undergo the conformational changes that accompany (co-)receptor binding. In any case, these differences indicated that the lack of either disulfide bond changed the architecture or flexibility of the Env spikes, be it that these changes did not prevent the virus from propagating in tissue culture cells.

DISCUSSION

Elimination of disulfide bonds from proteins often leads to misfolding, aggregation, and loss of function. In contrast, we found that Env was remarkably tolerant toward manipulation of its disulfide bonds even though they are strictly conserved among all HIV sequences. Two of the 10 disulfide bonds were completely expendable for function (receptor binding and/or fusion). Three other disulfide bonds were essential for the virus, but dispensable for oxidative folding of Env in the ER and subsequent exit from the compartment.

Classification of the 10 Disulfide Bonds of Env

Because all disulfide bonds in Env are strictly conserved, we anticipated that most if not all would be crucial for Env folding, let alone for its function. In the case of influenza virus hemagglutinin (HA), for instance, all disulfide bonds are necessary for the protein to reach the native state, be it that one displayed a conditional phenotype (Braakman *et al.*, unpublished data). To our surprise, we found that disruption of several disulfide bonds was compatible with Env folding and/or function. We categorized the 10 disulfide bonds of Env, based on the consequences of their elimination for folding and function: class 1 comprises disulfide bonds whose elimination led to severe folding deficiencies: 54–74, 218–247, 228–239, and, as reported earlier, 296–331 (Tschachler *et al.*, 1990; Freed and Risser, 1991; Travis *et al.*, 1992) and 385–418 (Hemming *et al.*, 1989; Tschachler *et al.*, 1990; Bolmstedt *et al.*, 1991; Lekutis *et al.*, 1992). Because deletion of the latter disulfide bond was compatible with some residual viral function in spite of folding failure, we further distinguished class 1 into class 1b, which only contained 385–418, with residual viral function despite folding deficiency, and class 1a, which contained the other disulfide bonds in class 1. Likewise, we divided the disulfide bonds whose elimination still allowed proper folding into class 2a, containing the disulfide bonds that were essential for viral function despite their dispensability for folding, 126–196, 131–157, and 598–604, and class 2b, containing the disulfide bonds that were expendable for both folding and function, 119–205 and 378–445 (Figure 7).

The Folding Pathway of Env

All Env mutants underwent oxidative folding, meaning that at 4 h of chase they either reached a fully oxidized state or they displayed a smear of partially oxidized species. Most single cysteine mutants displayed folding kinetics similar to that of the corresponding double mutants, which signifies that orphan cysteines did not promiscuously team up with nonnative cysteine partners. This suggests that nonnative disulfide bonds are highly transient in nature and are easily broken again. In support of such a scenario, we have witnessed waves of oxidation and reduction in gp120 in the presence of low concentrations of reductant (Land *et al.*, 2003). The smears therefore may represent heterogeneous but "genuine" folding intermediates. Altogether, we cannot pinpoint a single obligatory "initiation" disulfide bond that must form before any of the others can. It was therefore impossible to delineate a straightforward folding pathway whereby formation of one disulfide bond was essential for formation of the next. Instead, we found evidence that different modules of Env fold independently of each other.

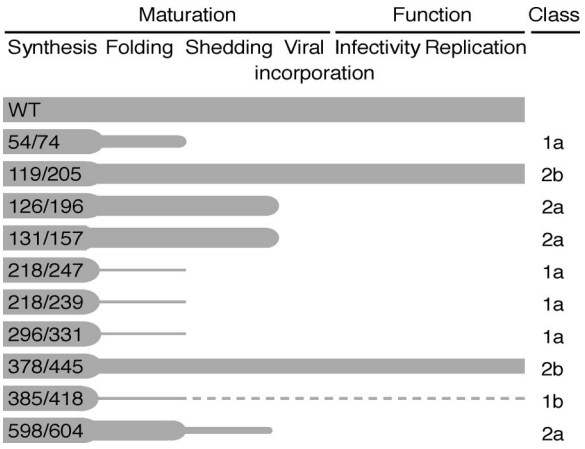

Figure 7. Classification of disulfide bonds of Env. HIV-1 Envelope disulfide mutants were clustered in four classes based on the "bottlenecks" in their maturation and/or function in comparison to wild-type. Bottlenecks are depicted on an arbitrary scale. Class 1a mutants are incompetent for folding and function; class 1b mutants display residual viral function (depicted by a dashed line) despite severe folding problems; class 2a mutants are folding competent, but function incompetent. Finally, class 2b mutants are folding and function competent. For clarity, only double mutants are shown in this cartoon.

For one, gp120 folding appeared largely independent of gp41, because mutations in 598–604 did not affect oxidative folding of the gp120 moiety in gp160 and because folding of gp120 alone was almost identical to gp160. Likewise, folding of the V1/V2 region seemed to be independent. All mutants of the V1/V2 region reached a native state with rates similar to wild type, except that a distinct folding intermediate was more prominently present. This suggests that the mutations did change the kinetics of folding or the folding path, but did not interfere with folding of Env as a whole. This may come expected, because Env can function even in the absence of the complete V1/V2 loops (Wyatt *et al.*, 1995; Stamatatos *et al.*, 1998).

Quality Control of Env

Current models of ER quality control surveillance (Ellgaard and Helenius, 2003) predict that only correctly folded proteins can exit the ER and that ER client proteins that fail to fold aggregate (Kopito and Sitia, 2000) and/or are cleared from the ER lumen via ERAD, by retrotranslocation into the cytosol and subsequent proteasomal degradation (Meusser *et al.*, 2005). We found that the Env mutants did not always obey these "rules."

None of the mutants appeared prone to substantial aggregation. The gp160 mutants did display some aggregation, most prominently in the case of poorly folding mutants, but aggregates were not detected in the corresponding gp120 mutants. The class 1 mutant molecules, at the same time, seemed poor ERAD substrates, because their decrease was similar to that of the class 2 mutants. This could mean that the ER quality control mechanisms either failed to "brand" them as misfolded or that they are recognized as misfolded, but reside in large Env-chaperone complexes, too complex or bulky to be retrotranslocated.

ER quality control of the class 2a mutants failed from another perspective. The "approval" to exit from the ER hopefully would warrant a protein's functionality, but the class 2a mutants were of no use to the virus despite their folding success. In agreement, defective cell entry of C131G and C196V single mutants (Tschachler *et al.*, 1990) and reduced gp160 precursor cleavage and replication deficiency of 598–604 mutants (Syu *et al.*, 1991; Dedera *et al.*, 1992) had been reported before. We found that these mutants were not incorporated into virions. This implies that for the class 2a mutants quality control is exerted at the level of virion assembly/budding and not at the level of exit from the ER, because shedding, and hence cell surface appearance were similar to wild type.

Dispensability of Disulfide Bonds for Function of Env

The fate of the class 1b mutant is perhaps the most puzzling: how can we reconcile misfolding with marginal viral function of Env? The minimal shedding of the 385–418 mutants suggests that a minority of these mutant Env molecules did manage to fold "correctly," exit the ER, appear on the cell surface and therefore did get incorporated into virus particles as functional spikes, which would account for the residual infectivity and replication. Altogether, the folding defects of the class 1b mutants appeared severe but not insurmountable. Accordingly, we found that in vitro evolution of the C385/418A mutant easily gave rise to Env revertants with increased replication capacity (Sanders *et al.*, 2008a; Sanders *et al.*, 2008b).

The dispensability of the class 2b disulfide bonds (119–205 and 378–445) raises the question why they have been conserved. Loss of disulfide bonds in the course of evolution would involve Env variants with odd cysteines, because simultaneous pairwise elimination of cysteines is unlikely to occur. We found that the single cysteine mutants of the 119–205 disulfide bond replicated with lower efficiency than the corresponding double mutants. Perhaps a single cysteine intermediate is too large an obstacle on the evolutionary path toward Env variants lacking this disulfide bond, which would rule out its occurrence. This argument does not hold for the 378–445 disulfide bond, however, because the C378A single mutant replicated with efficiency similar to the double mutant.

Alternatively, the importance of these two disulfide bonds becomes manifest only during natural infection, when Env and the virus particles at large are challenged by the host's immune system. The differences in (co-)receptor usage suggest that the architecture or flexibility of these mutant Env molecules was slightly altered compared with wild type. The class 2b disulfide bonds may thus contribute to the integrity of the outer antigenic shield that disguises the immunosusceptible core of Env.

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