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Folding, Assembly, and Intracellular Trafficking of the Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Analyzed with Monoclonal Antibodies Recognizing Maturational Intermediates

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Monoclonal antibodies (MAbs) that bind linear or conformational epitopes on monomeric or oligomeric human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins were screened for their recognition of maturational intermediates. On the basis of reactivities with gp160 at different times after pulse-labeling, the MAbs were sorted into groups that exhibited binding which was immediate and constant, immediate but transient, delayed, late, or very late. This grouping was consistent with the selectivity of the MAbs for structural features of gp160. Thus, a MAb to the V3 loop reacted with envelope proteins at all times, in accord with the relative conformational independence and accessibility of the epitope. Several MAbs that preferentially react with monomeric gp160 exhibited diminished binding after the pulse. A 10-min lag occurred before gp160 reacted with conformational MAbs that inhibited CD4 binding. The availability of epitopes for other conformational MAbs, including some that react equally with monomeric and oligomeric gp160 and some that react better with oligomeric forms, was half-maximal in 30 min and closely followed the kinetics of gp160 oligomerization. Remarkably, there was a 1- to 2-h delay before gp160 reacted with stringent oligomer-specific MAbs. After 4 h, approximately 20% of the gp160 was recognized by these MAbs. Epitopes recognized by monomer-specific or CD4-blocking MAbs but not by oligomer-dependent MAbs were present on gp160 molecules associated with the molecular chaperone BiP/GRP78. MAbs with a preference for monomers reacted with recombinant or HIV-1 envelope proteins in the endoplasmic reticulum, whereas the oligomer-specific MAbs recognized them in the Golgi complex. Additional information regarding gp160 maturation and intracellular trafficking was obtained by using brefeldin A, dithiothreitol, and a low temperature.

The envelope glycoprotein (Env) of human immunodeficiency virus type 1 (HIV-1) is an oligomer composed of extracellular (gp120) and transmembrane (gp41) proteins (22, 27). The gp120 subunits are responsible for the adsorption of virions to CD4 receptors on the host cells, whereas the gp41 subunits anchor the complex in the viral membrane and mediate cell fusion (9, 26). Since Env is required for infectivity and is the target of neutralizing antibodies, the structure, assembly, and function of Env are subjects of intense investigations. The Env precursor gp160 is a type I integral membrane protein that becomes heavily glycosylated in the lumen of the endoplasmic reticulum (ER), where formation of disulfide bridges and binding to BiP/GRP78 also occur (19, 40, 47). Folding of gp160 to enable CD4 binding, dissociation from BiP/GRP78, and oligomerization occur within about 30 min after synthesis, either in the ER or during transit to the Golgi complex. Subsequently, Env is proteolytically cleaved in the Golgi compartment to yield gp120 and gp41 and transported to the plasma membrane.

Monoclonal antibodies (MAbs) have been used to resolve the steps in intracellular trafficking, folding, assembly, and processing of several viral glycoproteins, most notably the influenza virus hemagglutinin (46, 48). Previously, a panel of 138 MAbs that were raised against soluble monomeric, dimeric, or tetrameric forms of a recombinant HIV-1 Env protein (gp140) and that lack transmembrane and cytoplasmic domains was described (5, 15). The MAbs were screened by immunoprecipitation assays, and they recognized mostly conformation-dependent epitopes in gp120 or gp41, although some also reacted with denatured protein. Interestingly, some MAbs bound preferentially to oligomeric or monomeric gp160, whereas others exhibited no evident preference for quaternary structure (5). MAbs that reacted equally well with monomeric and oligomeric gp160 were termed oligomer independent, whereas those that reacted more strongly with monomers or oligomers were defined as monomer or oligomer sensitive, respectively. An additional class of MAbs bound only to oligomeric Env molecules and was called oligomer specific. Now, we have analyzed the ability of representative MAbs to bind gp160 at successive times after pulse-labeling and compared the cellular distributions of maturational forms of gp160. In addition, we determined the stage at which gp160 is associated with the molecular chaperone BiP/GRP78 and acquires the ability to bind the receptor CD4. Finally, we examined the effects of inhibitors such as dithiothreitol (DTT), brefeldin A (BFA), and low temperature on gp160 folding, oligomerization, and intracellular trafficking.

MATERIALS AND METHODS

Recombinant viruses. A clone of the BH8 isolate of HIV-1 (36) was the source of the *env* gene inserted into the previously described vaccinia viruses vPE12 and vPE16 (14, 16). The *env* gene in vPE12 contains a deletion at the junction between the gp120 and gp41 coding regions, and consequently a noncleavable form of gp160 is made. For CD4 gene expression, the recombinant vaccinia virus vCB-7 (4) encoding full-length CD4 was used.

MAbs. MAbs were produced by murine B-cell hybridomas in serum-free tissue

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culture medium (5, 15). In some cases, the MAbs were purified on protein G-Sepharose (Pharmacia).

Vaccinia virus infection, pulse-labeling, and immunoprecipitation. BS-C-1 cells were infected with 10 PFU of recombinant virus per cell. At 16 h after infection, cells were placed in methionine-free medium for 1 h, after which they were labeled for 15 min with 100 μCi of [^{35}S]methionine (1 mCi/mmol; Amersham) per ml. One sample was harvested and lysed immediately after the pulse, while others were obtained after the radioactive medium was replaced with normal medium supplemented with a 20-fold excess of cold methionine. Cells were lysed in 100 mM Tris-HCl (pH 8.0)–100 mM NaCl–0.5% Nonidet P-40 on ice for 10 min and centrifuged at 4°C for 10 min. The supernatant fractions were incubated with MAbs and protein A-Sepharose beads as previously described (15). For immunoprecipitations, gp160-directed rabbit antiserum 2144 (a gift of C. Broder) served as a positive control.

Immunofluorescence assay. HeLa cells grown on glass coverslips (Clay Adams) were infected with 10 PFU of recombinant vaccinia virus per cell and cultivated for 16 h. Alternatively, Jurkat cells were infected with 2,000 50% tissue culture infective doses of HIV-1_{NL4-3} (1), cultivated for 1 week, and attached to concanavalin A-coated coverslips. The coverslips had been coated by being incubated in 5 mg of concanavalin A (Sigma) per ml–30 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (Pierce) per ml–0.1 M sodium acetate (pH 5.0) for 1 h at room temperature and washed in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–140 mM NaCl–0.5 mM KCl–3 mM dextrose (pH 7.1). Subsequent steps were performed at room temperature unless specified otherwise. The cells were fixed with 3% paraformaldehyde for 20 min, permeabilized with 0.05% saponin (Calbiochem), and incubated for 60 min with MAbs appropriately diluted in phosphate-buffered saline containing 0.5% bovine serum albumin. Subsequently, an incubation with a 1:500 dilution of rhodamine-conjugated anti-mouse immunoglobulin G (Dakopatts) was performed for 30 min. For colocalization studies, the ER was stained with a 1:100 dilution of antibody directed to the ER-residential protein disulfide isomerase (Stressgen) and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody. For Golgi complex colocalization, the infected cells were first incubated at 0°C for 30 min in a 1:50 dilution of unlabeled lectin *Ricinus communis* agglutinin I (RCA I) (Vector Laboratories) to minimize subsequent surface staining. After fixation, cell surface blocking with RCA I was repeated at room temperature. Following permeabilization, the cells were incubated with FITC-conjugated RCA I (EY-Laboratories) for 30 min in a 1:50 dilution. Then HIV-1 MAb T6 was added to the cells, which were incubated for 60 min, and subsequently detected by rhodamine-conjugated anti-mouse immunglobulin G. Fluorescence was viewed with a Zeiss Axiophot microscope.

Association of gp160 with BiP/GRP78. Immunoprecipitations with anti-BiP/GRP78 were performed as previously described (19). In brief, cells were infected and labeled as described above except that 800 μCi of [^{35}S]methionine per ml was used. Cells were lysed with 1% Triton X-100 in 150 mM NaCl–10 mM glucose–10 mM iodoacetamide–50 mM Tris-HCl (pH 7.4)–5 IU of hexokinase per ml. The extract was incubated for 1 h with a BiP/GRP78-directed MAb, after which protein A-coated Sepharose was added and the mixture was incubated for 30 min. Then the complexes were washed three times with 10 mM Tris-HCl (pH 7.4)–150 mM NaCl–0.1% Triton X-100 and once with 25 mM Tris-HCl (pH 7.4)–140 mM NaCl–1 mM $MgCl_2$–10 mg of ovalbumin per ml. The gp160 bound to BiP/GRP78 was released to the supernatant by incubating the protein A-Sepharose beads with 2 mM ATP for 20 min at 37°C (32). The released gp160 was then captured by gp160-directed MAbs and protein A-Sepharose.

BFA treatment. The fungal metabolite BFA (Boehringer Mannheim) was added to the medium (final concentration of 5 μg/ml) beginning 1 h before pulse-labeling. For immunofluorescence studies, BFA was added 4 h before fixation of the cells.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were prepared in Laemmli buffer with 1% SDS and 1% 2-mercaptoethanol. Proteins were then separated on 10% polyacrylamide gels. After electrophoresis, the gels were analyzed with a PhosphorImager (Molecular Dynamics).

RESULTS

MAbs recognize specific maturational intermediates of gp160. Representative MAbs were examined for their ability to discriminate between biosynthetic intermediates of HIV-1 gp160. For most experiments, a recombinant vaccinia virus that expresses a noncleavable gp160 was used because such oligomers exhibit increased stability (16). The MAbs were sorted into groups that bound gp160 at successive times after metabolic pulse-labeling (Fig. 1). Within a group, the MAbs had similar temporal patterns of reactivity; therefore, the kinetics of gp160 binding to one representative MAb of each group is shown in Fig. 2A. The MAbs were sorted according to the following criteria. (i) Immediate and constant reactivity. The example, MAb D47, is directed to a linear epitope on the V3 loop of the gp120 subunit. Binding of this MAb to gp160 was relatively constant over the chase period. (ii) Immediate but transient reactivity. Examples of MAbs in this group are the monomer-sensitive MAbs D18, T18, T24, and M15. They recognized linear epitopes on gp120 that are accessible immediately after synthesis. Maximum binding of gp160 occurred immediately after the pulse, and binding decreased with a half-life ($t_{1/2}$) of 30 min. (iii) Delayed reactivity. Examples are MAbs D20, D27, T20, and T22, which can bind gp120 and interfere to various degrees with CD4 binding. The epitopes recognized by these MAbs formed with a $t_{1/2}$ of about 10 min. Although these epitopes may be spatially related, they are not identical, since the MAbs have different patterns of cross-reactivity with Env proteins from other HIV-1 isolates (20). (iv) Late reactivity. These conformational MAbs do not recognize gp120 and are divided into two subgroups consisting of oligomer-independent MAbs D1, D16, and T37, which recognize conformational epitopes on oligomeric and monomeric gp160 equally, and oligomer-sensitive MAbs D4, D10, D11, D12, and D41, which bind oligomers preferentially (5). The epitopes reactive with these MAbs formed with a $t_{1/2}$ of 30 min after gp160 synthesis. (vi) Very late reactivity. This group contains the oligomer-specific MAbs T4 and T6 (5), whose antigenic determinants appeared between 1 and 2 h after gp160 synthesis.

In pulse-chase experiments with oligomer-sensitive MAbs, faint bands were visible after precipitation of early-chase extracts (Fig. 1B). This result could be explained by rapid folding of a small proportion of gp160 so that heterogeneous species are present at any time or by varied affinities of the MAbs for different conformational forms of gp160. To distinguish between these possibilities, we subjected the supernatant obtained after a first round of immunoprecipitation of an early-chase extract with oligomer-sensitive MAbs to a second round of immunoprecipitation with more of the same MAb. We observed no additional precipitation of gp160 (data not shown), indicating that small amounts of rapidly folded gp160 molecules were removed during the first round of immunoprecipitation with the oligomer-sensitive MAb and that the remaining gp160 molecules could not be recognized by such a MAb with low affinity. We also detected faint bands after immunoprecipitation of late-chase extracts by monomer-sensitive MAbs (Fig. 1A). According to similar logic, this could have resulted from incomplete or asynchronous folding of gp160 or from varied affinities of the MAbs. This time we used the supernatant obtained after a first round of immunoprecipitation of a late-chase extract with a monomer-sensitive MAb for a second round of immunoprecipitation with the same MAb. No additional immunoprecipitation of gp160 occurred in the second round (data not shown), indicating that small amounts of incompletely folded gp60 had been removed during the first round. Thus, monomer- and oligomer-sensitive MAbs have distinct and nonoverlapping specificities.

The kinetics of oligomerization (16) and loss and gain of gp160 recognition by monomer- and oligomer-sensitive MAbs, respectively, were compared. The reactivity of monomer-sensitive MAbs was reduced by 50% after a chase time of about 30 min (Fig. 2A). At this time, the amount of monomeric protein was also reduced by 50% (Fig. 2B). Moreover, at 30 min, about half of the gp160 reacted with oligomer-sensitive MAbs (Fig. 2A) and half the gp160 was oligomeric (Fig. 2B).

To quantify the amounts of gp160 recognized by the different classes of MAbs, additional pulse-chase experiments were performed (Fig. 3). MAb M15 (monomer sensitive) and MAb D47 (V3 loop) reacted with similar amounts of gp160 imme-

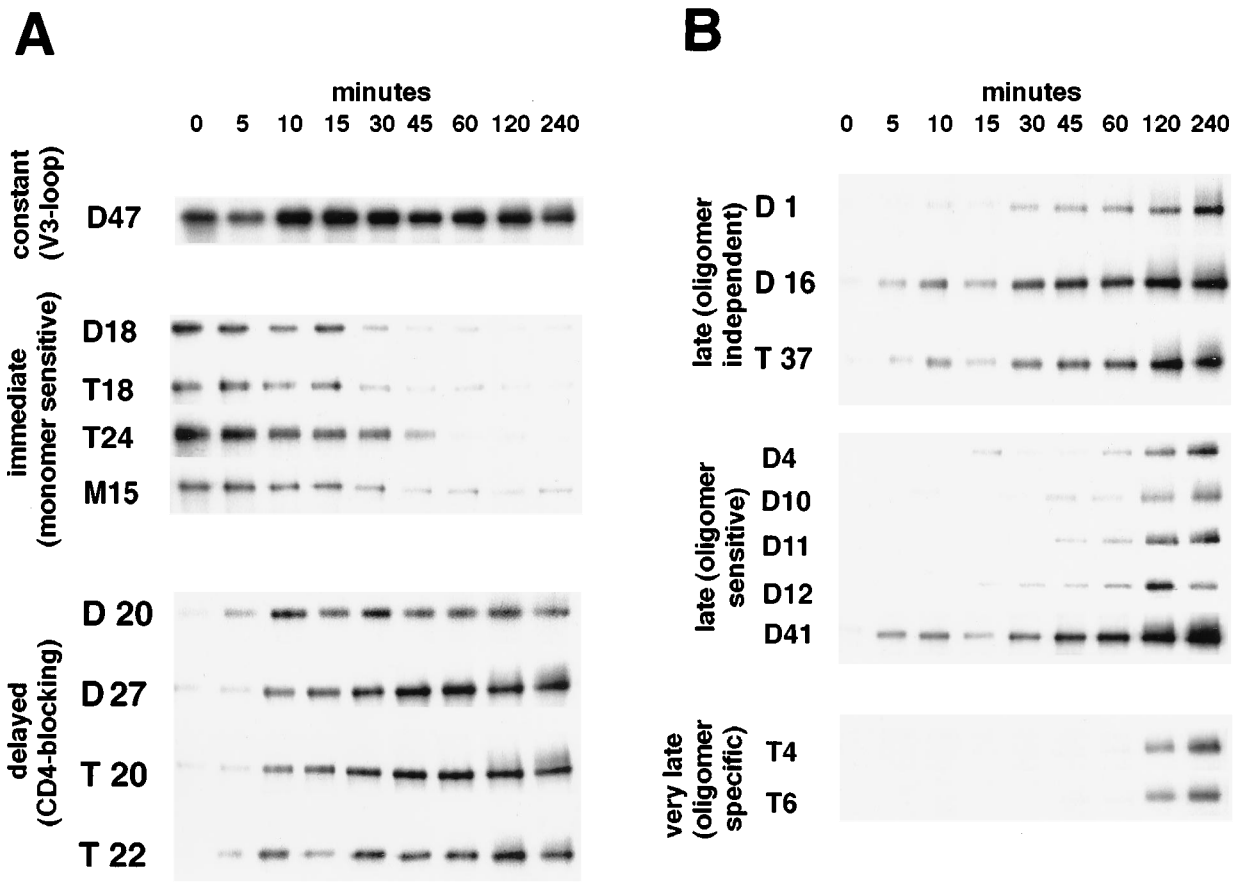

FIG. 1. Recognition of pulse-labeled gp160 by MAbs. BS-C-1 cells were infected with a recombinant vaccinia virus coding for a noncleavable HIV-1 Env molecule. At 16 h after infection, the cells were metabolically labeled with [^{35}S]methionine for 15 min and chased for the times indicated. At each time point, cells were harvested and extracts were prepared. Extracts were incubated with individual MAbs, and bound proteins were analyzed by SDS-PAGE. After autoradiography, a composite of the X-ray film strips, which were obtained from several different experiments, was made. (A) MAbs grouped as immediate and constant, immediate but transient, and delayed. (B) MAbs grouped as oligomer independent late, oligomer sensitive late, and very late.

diately after the pulse, whereas the other MAbs had low levels of reactivity at this time. The reactivities of all of the MAbs except MAb M15 increased during the chase. However, even after 4 h, much less of the gp160 reacted with the conformational MAbs T20 and D16 than with the V3 loop MAb D47. Only 20 to 30% as much gp160 reacted with the oligomer-dependent and -specific MAbs D10 and T6 as with D47. These data indicated that gp60 folding and maturation were incomplete.

To investigate whether the kinetics of MAb reactivities to Env were atypical because of our use of a noncleavable form of gp160, immunoprecipitations were also performed with extracts from pulse-chased, labeled cells expressing authentic gp160 after infection with the recombinant vaccinia virus vPE16. When the reactivities of MAbs D47, M15, T20, and T6 were tested, we observed the same recognition pattern as with the noncleavable gp160 (data not shown).

Binding of gp160 maturational intermediates to BiP/GRP78. Molecular chaperones, such as BiP/GRP78, can facilitate the folding of nascent proteins through a series of binding and ATP-mediated release steps (24, 37). BiP/GRP78 binds transiently to newly synthesized gp160 with half-maximal binding at approximately 25 min after pulse-labeling (19). To extend these observations, we used MAbs to identify the maturational intermediates of gp160 that are associated with BiP/GRP78. The association of BiP/GRP78 with proteins is weak and temperature dependent and can be abrogated by ATP (31, 32). To stabilize the complexes of BiP/GRP78 and gp160, the metabolically labeled cells were chilled and depleted of ATP by addition of hexokinase and glucose prior to lysis. The proteins bound to BiP/GRP78 were captured by using BiP/GRP78-directed MAbs and then specifically released with ATP, and the released proteins were incubated with gp160-directed MAbs (Fig. 4). Monomer-sensitive or oligomer-independent MAbs M15, T20, and D16 bound gp160 that had been released from BiP/GRP78. In contrast, the oligomer-sensitive MAb D10 and the oligomer-specific MAb T6 bound only trace amounts of the gp160 that had been released from BiP/GRP78. We concluded that there was little oligomeric gp160 bound to BiP/GRP78.

Cellular localization of gp160 maturational forms. The intracellular transport of Env was analyzed by indirect immunofluorescence staining of cells infected with recombinant vaccinia viruses or HIV-1. HeLa cells infected with a recombinant vaccinia virus expressing a noncleavable gp160 are shown in Fig. 5A to G. V3 loop-specific MAb D47, which reacted with gp160 in the pulse-chase experiments at all time points equally well, stained the ER and Golgi complexes (Fig. 5A). The monomer-sensitive MAb M15 stained the ER preferentially (Fig. 5B), as confirmed by colocalization with the ER-residential protein disulfide isomerase (Fig. 5C). The oligomer-sensitive MAb D12 stained both ER and Golgi compartments (Fig.

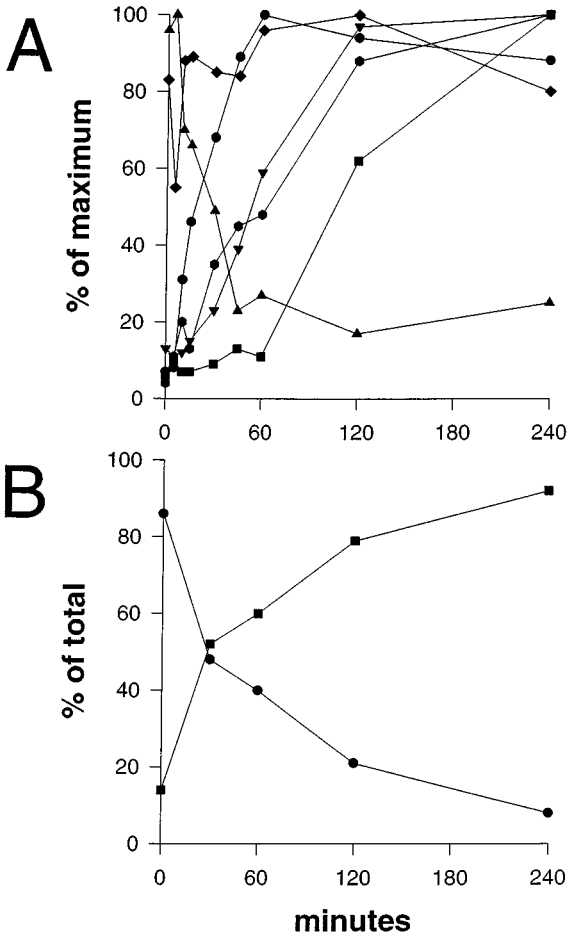

FIG. 2. Kinetics of gp160 folding and oligomerization. (A) Binding of gp160 to representative MAbs at successive intervals after pulse-labeling. The bands corresponding to those shown in Fig. 1 were quantitated with a PhosphorImager. In this figure, the reactivity pattern of one prototypic MAb of each group is shown: V3 loop-directed MAb D47 (◆), monomer-sensitive MAb M15 (▲), CD4-blocking MAb T20 (●), oligomer-independent gp41-directed MAb D16 (●), oligomer-sensitive MAb D10 (▼), and oligomer-specific MAb T6 (■). For each MAb, the maximum amount of immunoprecipitated gp160 was defined as 100% and the other values were calculated as percentages thereof. (B) Oligomerization of gp160 and recognition by oligomer- and monomer-sensitive MAbs. Extracts of pulse-chase-labeled BS-C-1 cells expressing uncleavable gp160 were analyzed by sucrose velocity gradient centrifugation followed by SDS-PAGE and autoradiography (15). The resulting bands were quantitated with a PhosphorImager. For each time point, the total amount of gp160 was defined as 100%. The amounts of monomeric (●) or oligomeric (■) gp160 were calculated on this basis.

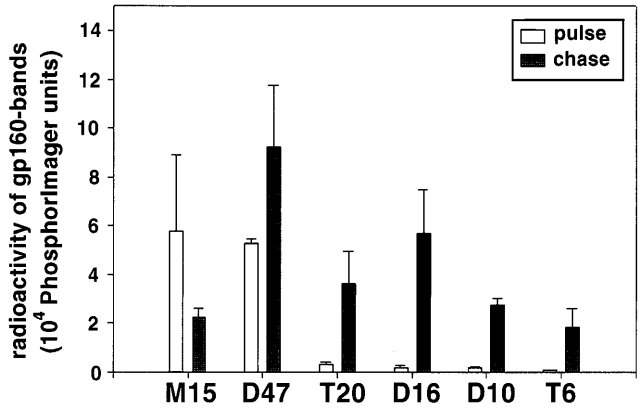

FIG. 3. Quantitation of gp160 immunoprecipitated by MAbs. Metabolic labeling was performed as for Fig. 1 with a 15-min pulse time and a 4-h chase. The data are from a single experiment. Most of the immunoprecipitations were carried out in triplicate with saturating amounts of MAbs. SDS-PAGE gels were analyzed with a PhosphorImager, and the data are presented with standard error bars.

5D). Most interestingly, the oligomer-specific MAb T6 stained the Golgi complex in a highly specific fashion (Fig. 5E), as confirmed by colocalization with the galactose-specific lectin RCA I (Fig. 5F).

The same MAb staining patterns were observed with HeLa cells infected with recombinant vaccinia virus expressing authentic gp160 as with cells expressing the noncleavable form. Thus, monomer-sensitive MAb M15 stained the ER preferentially (Fig. 5H) and oligomer-dependent MAb T6 stained the Golgi complex predominantly (Fig. 5J). In addition, the CD4-blocking MAb D20 stained both ER and Golgi compartments (Fig. 5I). We also demonstrated specific staining patterns in HIV-1-infected Jurkat lymphocytes, although their small size made them less suitable than HeLa cells for such studies. Colocalizations of MAb M15 staining with antibody to ER-residential disulfide isomerase are shown in Fig. 5K and L. Note that not all cells are HIV-1 infected and that the infected cells generally have a swollen appearance. Colocalization of MAb T6 binding with the Golgi complex-staining lectin RCA I is presented in Fig. 5N and O. Again, MAb D20 stained both ER and Golgi compartments (Fig. 5M).

Effect of BFA, an inhibitor of intracellular trafficking, on gp160 maturation. BFA blocks intracellular trafficking, causing disruption of the Golgi complex and reflux of materials into the ER (13, 30). Consequently, some events regularly occurring in the Golgi compartment may take place in the ER. Remarkably, MAb T6 staining of gp160-expressing cells treated with BFA showed an ER pattern (Fig. 5G) instead of the previous Golgi complex pattern (Fig. 5E). Immunoprecipitations of extracts of gp160-expressing cells that were labeled for 4 h in the presence of BFA confirmed the formation of the MAb T6-reactive epitope (data not shown, but similar results appear in Fig. 8, when gp160 was coexpressed with CD4).

Perturbation of gp160 maturation by low temperature. In-

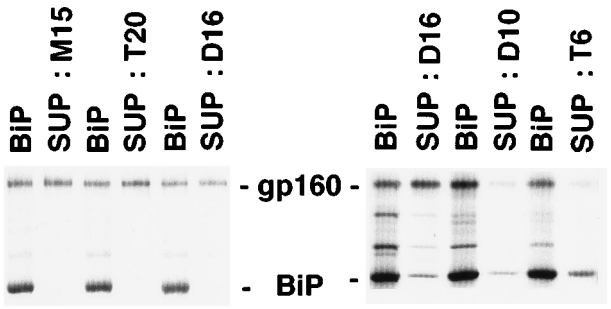

FIG. 4. BiP/GRP78 binding to gp160. Cells expressing gp160 were labeled for 4 h with [^{35}S]methionine. Cell extracts were prepared under ATP-depleting conditions at 4°C and then immunoprecipitated by BiP/GRP78-directed MAbs. The washed protein A-Sepharose pellets were incubated in the presence of ATP at 37°C. After this, the mixtures were centrifuged and the pellets were analyzed by SDS-PAGE (lanes BiP). The supernatants were used for a second immunoprecipitation, this time by MAbs directed to maturational intermediates of gp160. The bound proteins were analyzed by SDS-PAGE (lanes SUP:MAb). The assays in the left and right panels were carried out separately, and therefore immunoprecipitation with MAb D16 was done twice to have a common reference. Autoradiographs are shown.

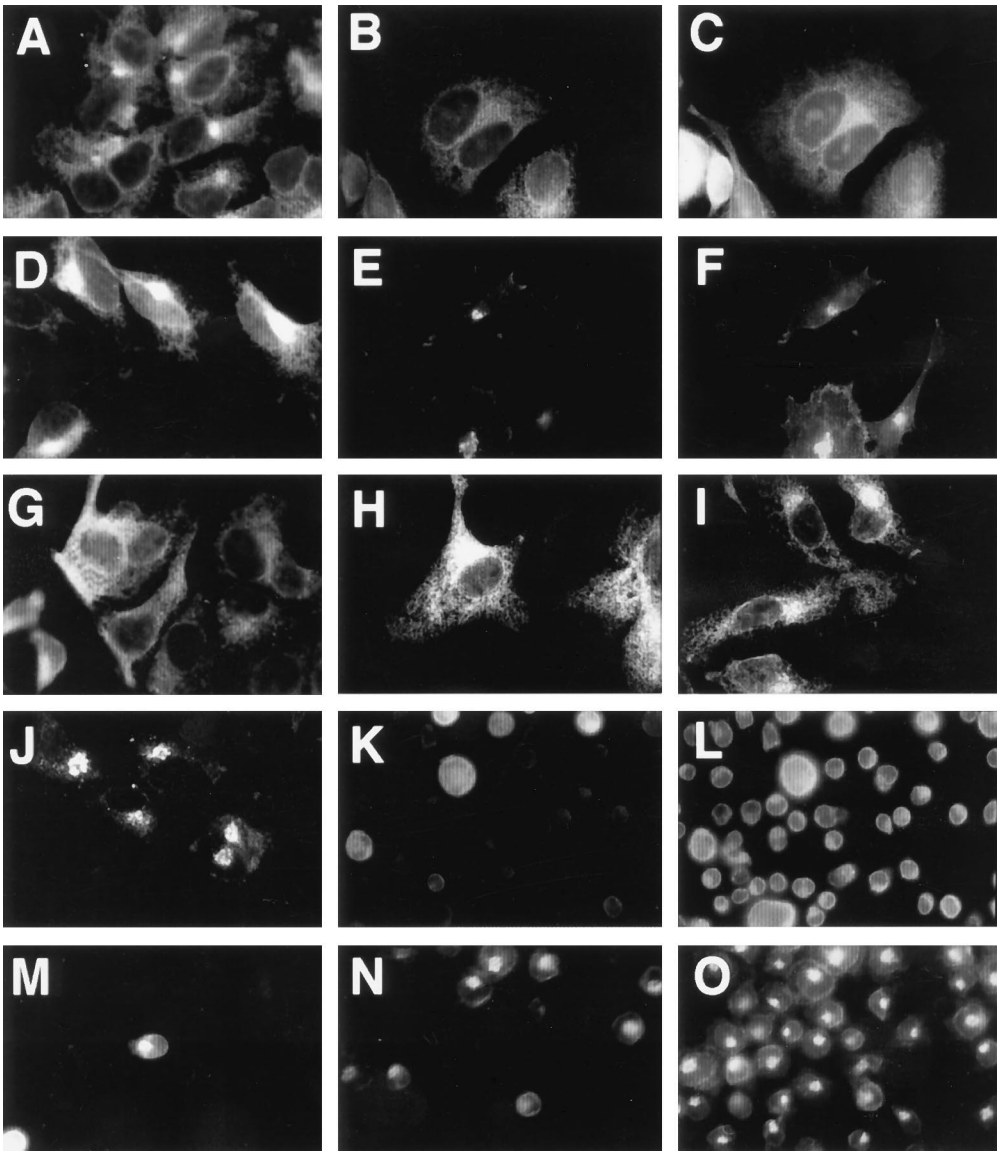

FIG. 5. Intracellular localization of gp160 maturational forms. Adherent HeLa cells (A to J) infected with 10 PFU of a recombinant vaccinia virus expressing uncleavable gp160 (A to G) or expressing authentic gp160 (H to J) per cell and HIV-1-infected Jurkat cells (K to O) were fixed with paraformaldehyde and incubated with gp160-directed MAbs followed by rhodamine-conjugated anti-mouse immunoglobulin G. (A) V3 loop-directed MAb D47; (B, H, and K) monomer-sensitive MAb M15; (C) antibody to the ER-residential protein disulfide isomerase to show colocalization with MAb M15 in panel B; (D) oligomer-sensitive MAb D12; (E, J, and N) oligomer-specific MAb T6; (F) Golgi-staining lectin RCA I conjugated to FITC to show colocalization with MAb T6 in panel E; (B) MAb T6 on BFA-treated cells; (I and M) CD4-blocking MAb D20; (L) antibody to the ER-residential protein disulfide isomerase to show colocalization with MAb M15 in panel K; (O) Golgi-staining lectin RCA I conjugated to FITC to show colocalization with MAb T6 in panel N.

cubation of cells at temperatures of 15°C or less retards the delivery of proteins from the ER into the *cis*-Golgi complex (38, 39, 44). We used this approach to obtain more information about the site of formation of the oligomer-specific epitope recognized by MAb T6. For this purpose, BS-C-1 cells expressing gp160 were pulse-labeled at 37°C and chased at 15°C. Under these conditions, MAb T6 did not recognize gp160 even after a processing time of 8 h, but MAbs M15, T20, D16, and D10 precipitated gp160 (Fig. 6). This result suggested that the epitope recognized by the oligomer-specific MAb T6 was formed in the Golgi complex. Alternatively, the failure of MAb T6 to detect its antigen could be caused by a slowing down or blocking of protein folding at the low temperature. To investigate the latter possibility, the low-temperature experiment was repeated in the presence of BFA. Under these conditions, gp160 was recognized by all of the MAbs, even T6 (Fig. 6). Evidently, low temperature per se did not prevent maturation of gp160, and the reflux of Golgi material in the presence of BFA allowed MAb T6 reactivity to occur at 15°C in the ER.

We also noted that the fraction of gp160 recognized by MAb M15 did not decrease as much during the chase at 15°C as it did at 37°C. This perturbation in folding could be a direct low-temperature effect. Alternatively, the blocking of protein transport to the Golgi complex might cause an accumulation of intermediates that overload the ER.

Requirements for disulfide bond formation. Disulfide bond formation is an early event in the maturation of gp160 (19). DTT has been used to alter the reducing environment of the

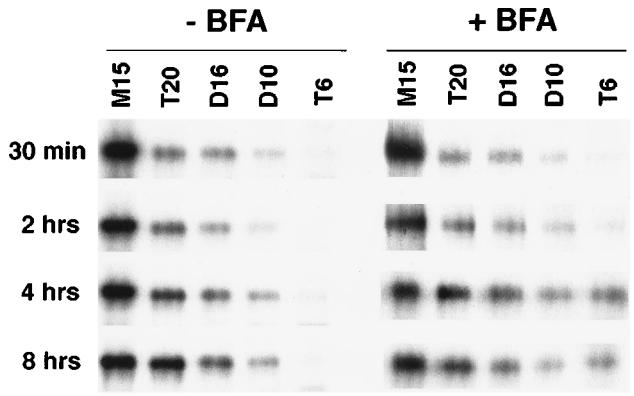

FIG. 6. Effect of low temperature on maturation of gp160. BS-C-1 cells expressing gp160 were incubated with (+) or without (−) BFA and labeled for 15 min with [^{35}S]methionine. The cells were then chased at 15°C for the times indicated on the left. Cell extracts derived from these assays were immunoprecipitated by the panel of MAbs shown above the lanes. Proteins were analyzed by SDS-PAGE and autoradiography.

secretory pathway in order to study the requirement for disulfide bond formation in folding and transport of glycoproteins (3, 33, 41, 45). We found that gp160 synthesized in the presence of DTT was recognized by the monomer-sensitive, gp120-directed linear MAb M15. This result was expected, since the relevant epitope is formed immediately after pulse-labeling and therefore prior to disulfide bond formation. However, the epitope for the oligomer-independent, gp41-directed conformational MAb D16 also formed in the presence of DTT. By contrast, epitopes for MAb T20, which partially blocks CD4 binding, and for MAbs D10 and T6, which are oligomer dependent, did not form under these conditions, even after a chase of 240 min (Fig. 7). Sucrose gradient analysis suggested that there was some aggregation of gp160 that formed in the presence of DTT (data not shown). In summary, these results suggest that some epitopes are more sensitive to DTT than others and are generally consistent with the importance of disulfide bond formation for the correct folding and oligomerization of gp160.

Binding of gp160 maturational intermediates to CD4. The majority of our experiments were carried out with BS-C-1 cells, which lack CD4. To determine which folding intermediates of gp160 bind CD4, Env and its receptor were expressed simultaneously. All MAbs except M15 coprecipitated CD4 bound to gp160 maturational intermediates (Fig. 8). Since immunofluorescence microscopy had shown that MAb M15 reacts with

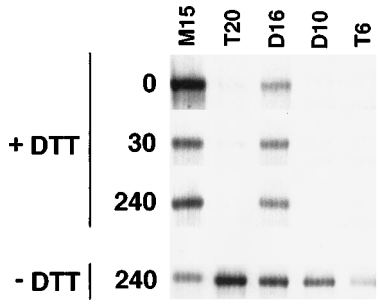

FIG. 7. DTT inhibits maturation of gp160. To BS-C-1 cells expressing gp160, 5 mM DTT was added 10 min before pulse-labeling with [^{35}S]methionine and maintained during the chase. Cell extracts produced after the pulse or after chases of 30 or 240 min were immunoprecipitated by the MAbs shown at the top and analyzed by SDS-PAGE and autoradiography.

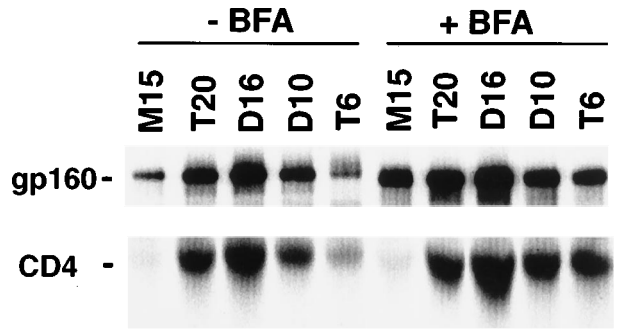

FIG. 8. Coprecipitation of gp160 and CD4 by gp160-directed MAbs. BS-C-1 cells coexpressing gp160 and CD4 were labeled continuously for 4 h with [^{35}S]methionine. To one set of infected cells, 5 μg of BFA was added per ml beginning 1 h before the labeling. After production of cell extracts, immunoprecipitations with the indicated MAbs were performed followed by SDS-PAGE and autoradiography.

gp160 only in the ER, we added BFA to prolong the opportunity for gp160 and CD4 to interact in that compartment. Even under these conditions, MAb M15 did not coprecipitate CD4, whereas all the other MAbs did (Fig. 8). Since the linear, monomer-sensitive epitope recognized by M15 is transient, it probably becomes inaccessible or altered during folding or oligomerization at the time gp160 acquires the capability to bind CD4. The possibility that CD4 masks the epitope recognized by MAb M15, however, has not been excluded.

MAb T6 ordinarily reacts with gp160 in the Golgi complex. The observation that MAb T6 coprecipitated some CD4 in the absence as well as in the presence of BFA (Fig. 8) implies that some gp160-CD4 complexes were transported out of the ER or that complexes between these molecules formed after they traveled independently to a post-ER compartment. In several experiments, BFA appeared to increase the amounts of gp160 and CD4 immunoprecipitated with MAb T6.

DISCUSSION

The intracellular synthesis and maturation of HIV-1 Env were analyzed previously by use of pulse-labeling protocols and polyclonal antibodies. Such studies indicated that processing of the Env precursor takes about 2 h and involves glycosyltransferases, chaperones, and proteases (19, 35, 40, 47). That approach, however, is limited by difficulties in identifying folding intermediates. MAbs can provide unique reagents for probing the formation and accessibility of linear and conformational epitopes. We sorted representatives of a large and diverse panel of MAbs (15) into five groups by determining their reactivities for gp160 at various times after pulse-labeling (Fig. 9). The specificities of the MAbs suggest a maturational process involving rapid folding of monomeric gp160 and a slower process of oligomerization and maturation. The $t_{1/2}$ of 30 min for nascent gp160 to lose and acquire competence to bind MAbs that exhibit a preference for monomers and oligomers, respectively, correlated well with the formation of oligomeric gp160, as determined by sucrose density gradient and cross-linking analyses (19). The $t_{1/2}$ of 30 min for gp160 oligomerization is shorter than the $t_{1/2}$ of 80 min for the assembly of the envelope glycoprotein of the Rous sarcoma retrovirus (21). However, the vesicular stomatitis virus G protein and the influenza virus hemagglutinin protein oligomerize more rapidly than gp160, with $t_{1/2}$s of 6 to 8 min (12) and 5 to 10 min (7, 23, 48), respectively. Binding of gp160 to some MAbs (T4 and T6) was detected at 1 to 2 h after pulse-labeling, suggesting a

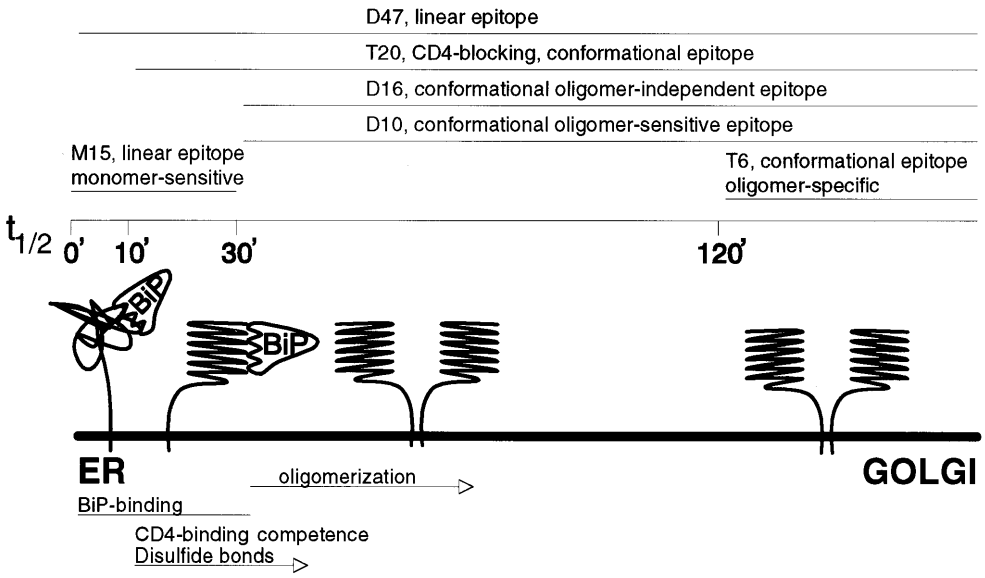

FIG. 9. Cinematic view of the aquisition and loss of MAb reactivities during maturation of gp160. The $t_{1/2}$ (in minutes) for the formation of epitopes recognized by the MAbs specific for folding intermediates of gp160 is shown. MAb D47, which binds to the V3 loop of gp120, recognized linear epitopes that formed rapidly and remained accessible throughout Env maturation. A group of MAbs exemplified by M15 (including D18, T18, and T24) recognized linear epitopes on gp160 that formed immediately but became masked during folding, with a $t_{1/2}$ of 30 min. A group of conformationally dependent MAbs exemplified by T20 (including D20, D27, and T22) which interfere with CD4 binding to various degrees bound to Env after a delay of about 10 min. A still longer folding period ($t_{1/2}$ of 30 min) was required for formation of epitopes that bound another group of conformational MAbs represented here by D16 (including D1 and T37) that exhibit no preference for the oligomeric state of gp160. A group of MAbs represented by D10 (including D4, D11, D12, and D41) that did preferentially bind to oligomers also recognized gp160, with a $t_{1/2}$ of 30 min. The oligomer-specific MAbs T4 and T6 bound gp160 starting at 1 to 2 h after synthesis.

requirement for postoligomerization maturational events. Maturation appeared inefficient, since the amount of gp160 recognized by the latter MAbs at 4 h was only about 20% of that recognized by a conformation-independent MAb.

We previously reported that gp160 associates with the chaperone BiP/GRP78, forms disulfide bonds, and acquires competence to bind the CD4 receptor during the first 15 min after synthesis (19). We devised specific assays to correlate these events with the folding intermediates identified by MAb binding. Nascent gp160 was captured on a solid support with antibody to BiP/GRP78, specifically released with ATP, and then tested for the ability to bind MAbs. One caveat with this approach is that Env could be altered during release from BiP/GRP78. We think this unlikely, since the step was carried out briefly and at a neutral pH and physiological temperature. The design of this experiment had two main advantages. First, analysis of ATP-released protein eliminated background due to nonspecific binding to BiP/GRP78 or to the solid support. Second, the epitopes required for MAb binding could not be masked by BiP/GRP78, since the protein was already released. We found that the released gp160 could bind MAbs that recognized linear epitopes on monomeric Env (e.g., M15) as well as MAbs that recognized certain conformational epitopes (e.g., T20 and D16). However, the released gp160 bound poorly to MAbs with a preference for oligomeric Env (e.g., D10 and T6). Since T6 reactivity is acquired at 1 to 2 h in a post-ER compartment, we did not expect such molecules to be associated with BiP/GRP78. However, even though gp160 binds to MAbs D16 and D10 at similar times after synthesis ($t_{1/2}$, 30 min), the former, oligomer-independent MAb recognized gp160 that had been associated with BiP/GRP78, whereas the latter, oligomer-sensitive MAb did not. Accordingly, dissociation of gp160 from BiP/GRP78 precedes oligomerization. We have also obtained evidence that two other chaperones, calnexin and calreticulin, bind gp160 soon after synthesis (35).

Nine pairs of intramolecular disulfide bonds have been demonstrated in mature gp120 molecules (28), and the gp41 subunit has two conserved cysteines that are required for cleavage (10, 42) but not for oligomerization (18). The timing of correct intramolecular disulfide bond formation relative to oligomerization varies for different viral proteins, occurring before oligomerization for the influenza virus hemagglutinin (45) and after oligomerization for the parainfluenza virus hemagglutinin (6). Short-term incubation with DTT has been used to prevent disulfide bond formation in newly synthesized proteins without appreciably affecting translation, translocation, or early covalent modifications, such as N-linked glycosylation and signal sequence removal (3, 45). Moreover, transport of proteins lacking or not requiring ER luminal disulfide bonds is relatively unaffected by DTT (33). The importance of disulfide bond formation for gp160 folding was tested with DTT and MAbs. We found that monomer-sensitive MAb M15 as well as the conformational oligomer-independent MAb D16 bound gp160 expressed by DTT-treated cells. However, other conformational MAbs, including the CD4-blocking MAb T20 and the oligomer-dependent MAbs D10 and T6, failed to recognize such gp160 molecules. Both the kinetics of detection of intramolecular disulfide bonds (19) and the effects of DTT on the acquisition of specific MAb reactivities indicate that correct disulfide bond formation normally precedes oligomerization of gp160.

For the majority of our experiments, non-CD4-expressing cell lines were used. However, CD4-gp160 coexpression experiments were carried out to correlate the acquisition of CD4-binding competence with specific epitope formation. We found that all of the MAbs tested, with the exception of the monomer-sensitive MAb M15, bound gp160 associated with CD4. The experimental result obtained with MAb M15 could arise from perturbation by CD4 of the epitope for MAb M15. Alternatively, the MAb M15 epitope may become altered or

inaccessible at the time gp160 acquires the capability of binding CD4. The latter explanation seems likely, as the epitope recognized by MAb M15 exists transiently even in cells not expressing CD4. It may seem paradoxical that MAb T20, referred to as a CD4 blocker, is able to bind gp160 that is already associated with CD4. However, MAb T20 does not block CD4 binding very efficiently (20). Moreover, antibody blocking can be achieved by steric hindrance without complete overlapping of binding sites or by altering the conformation or flexibility of gp160 prior to receptor binding. Also, as MAb T20 recognizes oligomeric gp160, it is possible that the gp160 oligomers bound to MAb T20 were not saturated with CD4.

Several groups have reported that formation of intracellular complexes between gp160 and CD4 leads to retention of both molecules in the ER (2, 8, 25). It has been proposed that this retention may be one cause for the depletion of CD4-positive cells in HIV-infected individuals. It was interesting that the oligomer-dependent MAb T6 bound gp160 that was associated with CD4. It seems unlikely that such complexes had been retained in the ER, since maturation of gp160 does not occur in this compartment except in the presence of BFA. Therefore, either CD4 associates with immature forms of gp160 in the ER and some of those complexes are then transported to the Golgi complex, where maturation of gp160 occurs, or CD4 and oligomeric gp160 associate after exiting the ER. It was previously reported that oligomeric gp160 can bind more than one molecule of CD4 (17). When the ratios of CD4 to gp160 were calculated from the SDS-PAGE gels, we noted that the values were highest with Env precipitated with oligomer-specific MAb T6; further work is needed to determine whether the binding or binding affinity increases during intracellular transport or maturation.

We carried out some inhibitor experiments that we felt were too preliminary for presentation here but which may be of some interest. At 30 mM, the monovalent ionophore monensin, which slows intracellular transport of proteins through the Golgi complex (43), did not prevent formation of epitopes that recognized representatives of each MAb group, including the oligomer-specific MAb T6 (data not shown). It may be that the inhibition was incomplete in our work, since Dewar et al. (11) showed that monensin inhibited late processing events, including endoproteolytic cleavage of gp160 produced by an adenovirus vector. The effects of several inhibitors of glycosylation also were tested for their ability to block maturation, as determined by MAb binding. We found that gp160 molecules synthesized in cells treated with inhibitors of ER α-glucosidase I and II (castanospermine or 1-deoxynojirimycin) or ER α-glucosidase II (bromoconduritol) or Golgi α-mannosidase II (swainsonine) had altered electrophoretic mobilities but were still recognized by the panel of prototypic MAbs consisting of M15, T20, D16, D10, and T6 (data not shown). Thus, these compounds did not prevent gp160 maturation to the stage at which it was recognized by MAb T6. The results of these experiments are difficult to interpret, as the inhibition may not have been complete and the results were analyzed only in a qualitative fashion.

There is controversy as to whether certain viral glycoproteins oligomerize in the ER or in post-ER compartments, and both may well be utilized, depending on the particular protein and its concentration. In our studies, the MAbs were used in conjunction with immunofluorescence microscopy to determine the intracellular compartments of Env folding intermediates. MAbs with a preference for monomeric gp160 localized primarily to the ER, whereas MAbs that were oligomer specific localized to the Golgi complex. Other MAbs, including those that preferentially bind oligomers, stained both the ER and the Golgi compartments. Although the oligomer-sensitive MAbs reacted with Env in the ER, we cannot conclude that oligomerization occurs in this compartment, since the specificity of these MAbs for oligomers (at least in immunoprecipitation assays) is not absolute.

Although most experiments were carried out with a noncleavable recombinant gp160, similar pulse-labeling and immunofluorescence results were obtained with a cleavable recombinant protein. Moreover, the ER or Golgi complexes of HIV-infected lymphocytes were also preferentially stained with the monomer-selective or oligomer-specific MAbs, respectively. We used several methods to perturb the intracellular trafficking of gp160. In the presence of BFA, oligomer-specific MAb T6 reacted with Env in the ER. Again, this does not prove that oligomerization occurred in the ER, since BFA induces retrograde transport of both resident and itinerant proteins from the *cis*- and medial Golgi compartments (13, 29). At 15°C, the transport of integral membrane and secretory proteins between ER and Golgi compartments is blocked (38, 39, 44). Under these conditions, folding of gp160 and perhaps oligomerization occurred, as the protein was recognizable by all MAbs except the oligomer-specific T6. The failure to acquire competence to react with MAb T4 or T6 was due to the transport block and not to some energy barrier to proper gp160 folding, since reactivity occurred upon addition of BFA at 15°C. The immunofluorescence, BFA, and low-temperature results cannot discriminate between oligomerization in the ER or post-ER compartments, but the requirements for reactivity with MAbs T4 and T6 point to some additional maturational event occurring in the Golgi compartment or in the ER when Golgi components are refluxed. Terminal glycosylation is a possibility, although several glycosylation inhibitors did not prevent reactivity and gp160 remains largely sensitive to digestion with endo-β-*N*-acetylglucosaminidase H even after several hours (19, 34, 40). It will be informative to determine whether the reactivities of MAbs T4 and T6 depend on folding, quaternary structure, or covalent modifications of HIV-1 Env.

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