CD4 Is Retained in the Endoplasmic Reticulum by the Human Immunodeficiency Virus Type 1 Glycoprotein Precursor

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Received 12 June 1990/Accepted 14 August 1990

We analyzed coexpression of the human immunodeficiency virus type 1 glycoprotein precursor, gp160, and its cellular receptor CD4 in HeLa cells to determine whether the two molecules can interact prior to transport to the cell surface. Results of studies employing coprecipitation, analysis of oligosaccharide processing, and immunocytochemistry showed that newly synthesized CD4 and gp160 form a complex prior to transport from the endoplasmic reticulum (ER). CD4 expressed by itself was transported efficiently from the ER to the cell surface, but the complex of CD4 and gp160 was retained in the ER. This retention of CD4 within the ER is probably a consequence of the very inefficient transport of gp160 itself (R. L. Willey, J. S. Bonifacino, B. J. Potts, M. A. Martin, and R. D. Klausner, *Proc. Natl. Acad. Sci. USA* 85:9580-9584, 1988). Retention of CD4 in the ER by gp160 may partially explain the down regulation of CD4 in human immunodeficiency virus type 1-infected T cells. Inhibition of CD4 transport appears to be a consequence of the interaction of two membrane-bound molecules, because a complex of CD4 and gp120 (the soluble extracellular domain of gp160) was transported rapidly and efficiently from the ER.

The envelope glycoprotein of the human immunodeficiency virus type 1 (HIV-1) (2, 10) initiates infection by binding to the cellular surface glycoprotein CD4 and mediating the fusion of viral and cellular membranes (22, 23, 33). Following HIV-1 infection, there is a progressive loss of CD4-positive lymphocytes that severely compromises the host immune system and leads to the development of the acquired immune deficiency syndrome (AIDS).

CD4 is a cell surface glycoprotein found mainly on T-helper cells and monocytes (30, 38). Infection of T cells with HIV-1 leads to a reduction in the cell surface expression of CD4 (4, 11, 14). CD4 down regulation may be caused by more than one mechanism. Reduced levels of CD4 mRNA and protein as well as reduced cell surface expression have been reported previously (11, 42). Recently, expression of the HIV-1 glycoprotein alone, in the absence of other HIV-1 proteins, has been shown to reduce the level of CD4 expression on the cell surface (13). Intracellular binding of CD4 by gp160 was suggested as a possible mechanism of CD4 down regulation (11, 13, 36).

The surface glycoprotein of HIV-1 is synthesized as a heavily glycosylated precursor (gp160) that is inefficiently transported to the cell surface. In fact, the majority of the protein is retained in the endoplasmic reticulum (ER) or degraded in lysosomes. Only 5 to 15% of the total protein synthesized is cleaved to form the mature, heterodimeric gp120/41 molecule and expressed on the cell surface (41). In other studies, we have found that CD4, in contrast to gp160, is transported rapidly and efficiently from the ER through the Golgi apparatus to the cell surface. We therefore undertook the work reported here to determine whether CD4 might interact with gp160 in the exocytic pathway and whether CD4 transport was blocked or retarded through interaction with the poorly transported gp160 molecule. We report that a specific interaction of CD4 and gp160 does occur in the ER and that CD4 bound to gp160 is blocked in transport to the

cell surface. Other concurrent studies in this laboratory have established that a soluble form of CD4 bearing a signal for retention in the ER can interact with the HIV glycoprotein and block its exit from the ER (3a). Possible roles for the interaction between CD4 and gp160 in HIV pathogenesis are discussed.

MATERIALS AND METHODS

Construction of plasmids. The construction of the plasmids encoding gp160, gp120, and CD4 under the control of the bacteriophage T7 promoter (pBS-gp160, pBS-gp120, and pBS-CD4, respectively) has been described previously (3a, 34). A plasmid encoding vesicular stomatitis virus G protein under T7 promoter control (pBSG) was constructed by excising the entire coding region of pSVGL3 (31) by using the restriction endonucleases *Xho*I and *Bam*HI. This fragment was isolated and cloned into Bluescript SK⁺ (Stratagene, San Diego, Calif.).

Expression, radiolabeling, and immunoprecipitation of proteins. HeLa cells (approximately 5×10^5 cells per 6-cm dish) were infected with a recombinant vaccinia virus encoding T7 polymerase (vTF7-3) (9) at a multiplicity of 10 to 25 in 0.5 ml of Dulbecco modified Eagle medium (DMEM) without serum for 30 min at 37°C. The inoculum was then removed, and cells were transfected with 5 µg of plasmid DNA in 1.5 ml of DMEM, lacking serum, by using a liposome-mediated procedure similar to that described by Felgner et al. (7) but employing dimethyldioctadecyl ammonium bromide as the cationic lipid (J. Rose, L. Buonocore, and M. Whitt, submitted for publication). Transfected cells were incubated for 3 to 4 h, washed once with phosphate-buffered saline (PBS) (10 mM NaH₂PO₄, 10 mM Na₂HPO₄, 150 mM NaCl [pH 7.4]), and labeled for the times indicated (see legends to Fig. 1-3 and 6) with [³⁵S]methionine in DMEM lacking methionine. Cells were then washed once with ice-cold PBS and further incubated in DMEM with 5% fetal bovine serum and excess methionine at 37°C for various times.

Prior to immunoprecipitation, cells were washed once with PBS and lysed in 1 ml of a solution (lysis buffer)

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containing 1% Nonidet P-40, 0.4% deoxycholate, 66 mM EDTA, and 10 mM Tris hydrochloride, pH 7.4. Nuclei were removed by centrifugation at $10,000 \times g$ for 1 min. For CD4 immunoprecipitations, sodium dodecyl sulfate (SDS) was added to a concentration of 0.1% to the lysate and 100 μ l of OKT4 hybridoma (American Type Culture Collection) culture supernatant was then added. After incubation at 4°C for 60 min, antibody-antigen complexes were precipitated with fixed *Staphylococcus aureus* (Calbiochem-Behring) and washed three times. OKT4 recognizes an epitope other than the gp160-binding site (17) and is capable of binding CD4 complexed to gp160. Immunoprecipitations of gp160 and gp120 were performed in lysis buffer plus 0.2% SDS by using 1 μ l of sheep anti-gp120 serum (AIDS Research and Reagent Program reagent number 288). After incubation at 37°C for 30 min, antigen-antibody complexes were precipitated and washed as described above. Immunoprecipitates were analyzed on 10% polyacrylamide gels containing SDS (16), and the gels were prepared for fluorography, dried, and exposed to preflashed X-ray film by the method of Bonner and Laskey (3). Endoglycosidase H (endo H [37]) treatments of immunoprecipitations were performed by the method of Rose and Bergmann (31).

Indirect immunofluorescence. Localization of CD4 was performed on infected cells that were transfected with either pBS-CD4 (5 μ g) or a mixture of pBS-CD4 and pBS-gp160 in which pBS-gp160 DNA was in fivefold excess (0.8 and 4.2 μ g, respectively). This DNA ratio gave two to three times more gp160 than CD4 expression as judged by metabolic labeling (data not shown). Three hours after the transfection, the transfection medium was removed and replaced with 2 ml of DMEM containing 5% fetal bovine serum, and the resulting medium was incubated for an additional 3 h. Cycloheximide (Sigma Chemical Co.) was then added to the medium at a concentration of 100 μ g/ml, and the cells were incubated for an additional 2 h before being washed once with PBS and fixed. The fixation procedure was modified from a procedure described by McLean and Nakane (25). The fixative was prepared by combining 6 ml of H₂O with 6 ml of 100 mM Na₂HPO₄ (pH 7.8) containing 220 mg of lysine. NaIO₄ (30 mg) was added to this solution and vortexed thoroughly before the addition of 3.66 ml of 8% paraformaldehyde plus 8 mM NaOH. Cells were incubated in fixative for 2 h at room temperature, washed once with PBS plus 10 mM glycine (PBS-G), and soaked overnight in PBS-G before being permeabilized in 1% Triton X-100. Cells were incubated with OKT4 hybridoma supernatant or sheep anti-gp120 serum (diluted 1:200 in PBS-G) for 30 min at room temperature and washed in PBS-G, followed by incubation with rhodamine-conjugated goat anti-mouse (Cappel) or fluorescein-conjugated rabbit anti-sheep (Jackson Immuno-research Laboratories) antibodies. Cells were observed with a Nikon Microphot-FX microscope by epifluorescence illumination with a 40 $\times$ oil immersion objective. Photographs were taken with Ilford XP1 film developed by C41 processing.

RESULTS

CD4 and gp160 form a complex when expressed in the same cell. To determine if a CD4-gp160 complex could be detected when both proteins were expressed together, we used the vaccinia-T7 hybrid expression system described by Fuerst et al. (9). Plasmid DNAs encoding gp160 and CD4 under T7 promoter control were transfected into HeLa cells that had first been infected with a recombinant vaccinia virus,

FIG. 1. Coexpression and coprecipitation of CD4 and gp160. HeLa cells (5×10^5 cells per plate) infected with a recombinant vaccinia virus, vTF7-3, were transfected with cDNAs encoding CD4 (pBS-CD4 [5 μ g]) or gp160 (pBS-gp160 [5 μ g]) or with both pBS-CD4 and pBS-gp160 (1.25 and 3.75 μ g, respectively) or left untransfected. Cells were pulse-labeled for 30 min with 50 μ Ci of [³⁵S]methionine in 0.5 ml of methionine-free medium and incubated in chase medium containing excess methionine for 30 min before lysis. Cell lysates were divided in half and immunoprecipitated with monoclonal antibody (OKT4) to CD4 (odd-numbered lanes) or with sheep anti-gp120 serum (even-numbered lanes), and immunoprecipitates were analyzed by SDS-PAGE followed by fluorography. The DNAs used to transfect the cells are indicated above the lanes. Positions of molecular weight markers (in thousands) are shown on the left. The positions of CD4 and gp160 are indicated on the right.

vTF7-3. This virus encodes bacteriophage T7 RNA polymerase, which transcribes the cytoplasmic plasmid DNA and yields a high level of protein expression. When plasmids encoding two different proteins were cotransfected, there was greater than 95% coexpression, as determined by double-label immunofluorescence (data not shown).

HeLa cells expressing gp160, CD4, or gp160 and CD4 were metabolically labeled with [³⁵S]methionine for 30 min and then incubated in the presence of excess unlabeled methionine for an additional 30 min. Cells were lysed in detergent, and the lysates were divided in half and immunoprecipitated with antibodies to gp120 or CD4 (Fig. 1). The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The results show that CD4 and gp160 were expressed and specifically precipitated by the antibodies (Fig. 1, lanes 3 and 6, respectively). In cells cotransfected with cDNAs encoding gp160 and CD4, both proteins were expressed and were precipitated as a complex either by antibody to CD4 or gp120 (note that gp160 was present in the immunoprecipitate prepared with anti-CD4 antibodies and CD4 was present in the immunoprecipitate prepared with anti-gp120 antibodies [lanes 7 and 8]).

We also performed an experiment to determine if CD4 and gp160 expressed in separate cultures would form a complex after detergent lysis. Two plates of cells, one expressing CD4 and one expressing gp160, were labeled as above. The cells were then removed from the dishes and mixed before lysis and immunoprecipitation. No complex formation was detected (data not shown), suggesting that the association detected after coexpression was occurring during coexpression in the same cell.

FIG. 2. Rate of intracellular binding of CD4 and gp160. (A) Seven plates of HeLa cells (5×10^5 cells per plate) infected with vTF7-3 and transfected with pBS-CD4 (1.25 μ g) and pBS-gp160 (3.75 μ g) DNAs were pulse-labeled with 50 μ Ci of [35 S]methionine in 0.5 ml of methionine-free medium for 10 min and then incubated in chase medium for the times indicated. Cells were lysed at the times indicated, and cell lysates were divided in half and immunoprecipitated with either OKT4 (even-numbered lanes) or sheep anti-gp120 serum (odd-numbered lanes) and analyzed by SDS-PAGE followed by fluorography. Background bands (labeled VV) precipitated from vTF7-3 infected, but untransfected, HeLa cells are indicated in control lanes 13 and 14 (120-min chase). These bands are also seen in lanes 9 through 12. Positions of molecular weight markers (in thousands) are shown at the left. (B) The percentage of maximal CD4 and gp160 binding over time was quantitated from densitometry of the data shown in panel A. The ratios of CD4 to gp160 (□) in immunoprecipitations prepared with OKT4 antibody or the ratios of gp160 to CD4 (◆) in immunoprecipitations prepared with anti-gp120 serum are expressed as percentages of the highest ratio observed.

Rate of gp160 and CD4 association. To determine the rate of association between gp160 and CD4, HeLa cells expressing both CD4 and gp160 were pulse-labeled with [35 S]methionine and incubated in the presence of unlabeled methionine for various times (Fig. 2A). Detergent lysates of the cells were divided into two equal parts, immunoprecipitated with antibodies to gp120 or CD4, and analyzed by SDS-PAGE (Fig. 2A). Immediately after the pulse (time zero), there was no association of newly synthesized CD4 and gp160 as judged by lack of coprecipitation (lanes 1 and

2). By 30 min, some association was evident (lanes 5 and 6). Quantitation of the autoradiogram (Fig. 2B) showed that maximal association occurred after about 60 min and that the half time for binding was approximately 30 to 40 min. Precipitation of two labeled vaccinia virus proteins occurred at later time points (Fig. 2A, bands marked VV). These proteins are not derived from CD4 or gp160, because they were present when the cells were not transfected with DNAs encoding gp160 or CD4 (lanes 13 and 14).

Our data are consistent with an earlier study indicating

that gp120 expressed by itself in Chinese hamster ovary cells became competent to bind a soluble form of CD4 *in vitro*, with a half time of about 30 min (8), indicating that folding of gp120 is required before binding of CD4. The long lag before binding observed here (Fig. 2) is probably also due to a requirement for folding prior to binding.

Coexpression of gp160 blocks oligosaccharide processing on CD4. Association of CD4 and gp160 with a half time of 30 min suggested that the association might be occurring early in the exocytic pathway. To obtain information on where the association was occurring and to analyze the effect of gp160 expression on CD4 transport, we examined the processing of the N-linked oligosaccharides on CD4 in the presence or absence of gp160 expression. CD4 has two N-linked glycans, one of which acquires resistance to the enzyme endo H. The high-mannose oligosaccharides added in the ER become resistant to cleavage by endo H after the sequential action of *N*-acetylglucosamine transferase and mannosidase II (15). The *N*-acetylglucosamine transferase is located in the medial Golgi compartment, suggesting that this is the site where endo H resistance is acquired (6, 15).

Transfected HeLa cells expressing CD4 alone or CD4 and gp160 were pulse-labeled with [³⁵S]methionine and then incubated with excess unlabeled methionine for various times. CD4 was then immunoprecipitated from cell lysates. Half of each immunoprecipitate was digested with endo H, and the remainder was left untreated (Fig. 3). The oligosaccharides on CD4 expressed alone became endo H resistant, with a half time of approximately 45 min (Fig. 3A). It is evident that only one of the two N-linked oligosaccharides on CD4 became endo H resistant, because the resistant band migrated halfway between the fully cleaved and uncleaved material.

Coexpression of CD4 and gp160 resulted in a dramatic inhibition of oligosaccharide processing on CD4 (Fig. 3B). Also, the association of CD4 with gp160 is evident from the coprecipitation occurring at later times. Quantitation of the results in Fig. 3A and 3B is shown in Fig. 3C. Only about 15% of the CD4 expressed with gp160 obtained endo H resistance even after 3 h. It is also evident that the oligosaccharides of gp160 (which precipitated with CD4) remained sensitive to endo H treatment (Fig. 3B). The shift in gp160 mobility to an apparent molecular mass of 90 kilodaltons is consistent with removal of all of the oligosaccharides (29). Even when gp160 was expressed alone, we did not observe processing of its oligosaccharides (data not shown). This result is consistent with an earlier report that only 5 to 15% of gp160 is transported (41).

Specific inhibition of CD4 processing by gp160. To determine if the effect of gp160 on CD4 processing was specific or due to a general inhibition of exocytosis by gp160, we expressed an unrelated protein, the surface glycoprotein of vesicular stomatitis virus (VSV) G protein, in the presence and absence of gp160 (Fig. 4). Cells were pulse-labeled with [³⁵S]methionine and incubated in the presence of unlabeled methionine for various times. The oligosaccharides of VSV G protein expressed alone in HeLa cells became endo H resistant, with a half time of about 30 min. This rate was unchanged in the presence of gp160. We have noted that VSV G protein processing in HeLa cells is somewhat slower than in other cells, where the half times are typically 15 to 20 min (28, 40). Immune precipitation with anti-gp120 antibodies and anti-VSV antibodies indicated that gp160 was expressed at levels equivalent to VSV G protein in this experiment (data not shown). We conclude that the effect of

gp160 on CD4 processing is specific and not due to a general blockade of exocytosis by gp160.

CD4 bound to gp160 is retained in the ER. The results of the metabolic labeling experiments followed by endo H treatment indicated that the CD4 bound to gp160 contained unprocessed oligosaccharides (Fig. 3). This lack of CD4 processing could be explained in two ways. CD4 bound to gp160 could have been retained at a site prior to the medial Golgi compartment or, alternatively, gp160 binding to CD4 might have had a more direct effect on oligosaccharide processing, such as inhibiting the access of processing enzymes to the CD4 oligosaccharides. To decide between these alternatives, we used indirect immunofluorescence microscopy to examine the localization of CD4 synthesized in the presence or absence of gp160.

HeLa cells expressing CD4, gp160, or CD4 and gp160 were treated with cycloheximide for 2 h and fixed with a modified paraformaldehyde fixative (25) to maintain the epitope recognized by the OKT4 antibody. Cycloheximide treatment blocks protein synthesis without disrupting protein transport through the exocytic pathway (12). This treatment allowed sufficient time for CD4 molecules made in the absence of gp160 to reach the cell surface. After fixation, the cells were made permeable with detergent (to allow entry of antibody) and incubated with OKT4 antibody followed by a rhodamine-conjugated second antibody. Figure 5A shows that CD4 expressed alone reached the plasma membrane (note that the outline of cell is labeled). When cells were not permeabilized, only the surface outline was seen. In permeabilized cells, CD4 was also seen in a punctate pattern which may represent endocytic vesicles (27). When CD4 was expressed with gp160, CD4 was not detectable at the cell surface and was seen only after permeabilization (Fig. 5C). CD4 appeared in a reticular pattern throughout the cytoplasm and in the nuclear membrane (ring stain around the nucleus). This pattern is typical of proteins localized in the ER (26, 31). Localization of gp160 expressed alone (Fig. 5B) gave a similar pattern of fluorescence, indicating that much of the gp160 remains in the ER during the cycloheximide treatment. This localization is consistent with the lack of oligosaccharide processing on gp160. The localization of CD4 in cells expressing gp160 and the lack of oligosaccharide processing on CD4 bound to gp160 indicates that CD4 bound to gp160 is blocked from exiting the ER.

CD4-gp120 complexes are not retained in the ER. During transport of gp160 to the cell surface, the molecule is cleaved to form two noncovalently associated subunits, gp120 and gp41 (1). gp41 is derived from the C terminus of gp160 and contains the membrane-spanning domain, while gp120 comprises most of the extracellular portion and contains the binding site for CD4 (4, 24). We had observed that gp120 expressed alone (from a mutated clone encoding only the soluble gp120 portion of gp160) was also transported inefficiently from the ER (3a). Therefore, we wanted to determine if gp120-CD4 complexes would form in the ER and be transported or retained.

We first expressed gp120 alone and examined its secretion (Fig. 6A). HeLa cells expressing gp120 were pulse-labeled with [³⁵S]methionine, and then gp120 was immunoprecipitated from the cell lysates or medium at various times after the addition of unlabeled methionine. A small amount of gp120 was detectable in the medium after 1 h, and about 15% was secreted after 3 h. We were unable to detect any endo H-resistant oligosaccharides on the intracellular gp120 at any time (Fig. 6A, lane 19). In contrast, all gp120 in the medium contained some resistant oligosaccharides (indicating Golgi

FIG. 3. Oligosaccharide processing on CD4 expressed in the presence and absence of gp160. Nine plates of HeLa cells (infected and transfected as described in the legend to Fig. 2) expressing CD4 (A) or CD4 and gp160 (B) were labeled for 10 min with 50 μ Ci of [35 S]methionine in 0.5 ml of methionine-free medium and then incubated in chase medium containing excess unlabeled methionine for the times indicated. Immunoprecipitates of cell lysates were prepared with OKT4 antibody, divided in half, and digested with endo H (even-numbered lanes) or left undigested (odd-numbered lanes). Samples were analyzed by SDS-PAGE and autoradiographed (A and B). The positions of CD4 and gp160 with oligosaccharides (+CHO) and after oligosaccharide cleavage (-CHO) are indicated. The two background bands also seen in nontransfected cells (Fig. 2) appear here and are labeled VV. The fraction of CD4 with endo H-resistant oligosaccharides at each time point was quantified by scanning densitometry of a fluorographed gel (C).

processing), since it ran as a broad band after endo H digestion at a position between the undigested and fully digested material (lane 17). The lack of processed oligosaccharides on cell-associated gp120 suggests that secretion is very rapid once the protein reaches the medial Golgi compartment.

To determine whether intracellular CD4-gp120 complexes formed in the cells and whether they were transported, we coexpressed gp120 at about a twofold molar excess over CD4 in HeLa cells. Under these conditions, nearly all of the CD4 associated with gp120 and approximately half of the gp120 molecules remained soluble and not associated with

CD4. Cells were then labeled with [35 S]methionine, chased for various times, and immunoprecipitated with antibody to CD4. Half of the immunoprecipitate from each time point was treated with endo H, and the remainder was left untreated (Fig. 6B). Analysis of the samples by SDS-PAGE showed that association of gp120 with CD4 occurred while the oligosaccharides on both proteins were still sensitive to cleavage by endo H, suggesting binding in the ER (Fig. 6B, lanes 5 to 8). The rate of association was similar to that observed with gp160 and CD4 (Fig. 2).

The CD4 associated with gp120 acquired endo H resistance with a half time very similar to that of CD4 expressed

FIG. 4. Processing of oligosaccharides on vesicular stomatitis virus (VSV) G protein expressed in the presence and absence of gp160. Five plates of HeLa cells (5×10^5 cells per plate) infected with vTF7-3 and transfected with pBS-G alone or both pBS-G and pBS-gp160 were labeled for 10 min with 50 μ Ci of [35 S]methionine in 0.5 ml of methionine-free medium and incubated in chase medium containing excess unlabeled methionine for the times indicated. Immunoprecipitates of cell lysates were prepared from each time point with rabbit anti-VSV serum and incubated in the presence or absence of endo H. The percent of endo H-resistant oligosaccharides on G protein at each time point was quantitated from a fluorographed gel and plotted. Symbols: ◆, -gp160; □, +gp160.

alone (~45 min), indicating that its transport was not blocked or slowed by association with gp120. We also noted that the gp120 bound to CD4 attained endo H resistance. The fully endo H-sensitive form of gp120 migrated slightly slower than fully glycosylated CD4 (7 and 8), whereas gp120 with endo H-resistant oligosaccharides migrated as a broad band, presumably due to heterogeneous processing of its multiple oligosaccharides (Fig. 6B, lanes 8, 10, 12, 14, and 16). This heterogeneity made it difficult to quantitate the amount of endo H-resistant gp120 directly, but on the basis of the rate of disappearance of the endo H-sensitive band of gp120, we estimated that the gp120 associated with CD4 was being processed at the same rate as CD4. This result would be expected for two molecules passing through the exocytic pathway as a complex. The gp120 associated with CD4 also showed an increase in apparent molecular weight at later time points, presumably due to the addition of terminal sialic acid on its oligosaccharides (lanes 9, 11, 13, and 15). By carrying out a second round of immunoprecipitations on the same samples with antibody to gp120, we were also able to follow the rate of processing and secretion of the gp120 that was not associated with CD4 (about 50% of the total). This material appeared to be processed like gp120 expressed alone. The cell-associated protein showed no endo H resistance, while a small endo H-resistant fraction (10 to 15%) was found in the medium after 3 h (data not shown).

These results indicate that CD4 transport is not slowed by association with gp120. Instead, it appears that gp120-CD4 complexes are transported at the normal CD4 rate, much faster than gp120 expressed by itself.

DISCUSSION

The results presented here show that specific binding of the HIV-1 precursor glycoprotein (gp160) to the CD4 receptor occurred within the ER when both proteins were expressed in the same cell. Binding was observed after a short

FIG. 5. Detection of CD4 and gp160 by indirect immunofluorescence. HeLa cells (5×10^5 cells per plate) plated on cover slips were infected with vTF7-3 and transfected with pBS-CD4, pBS-gp160, or a mixture of both plasmids (4.2 and 0.8 μ g, respectively). Cells were treated with cycloheximide (100 μ g/ml) 6 h posttransfection for 2 h and subjected to fixation (see Materials and Methods). The cells were then permeabilized by treatment with 1% Triton X-100 and treated with either hybridoma supernatant containing OKT4 antibody (A and C) or with sheep anti-gp120 serum (B), followed by treatment with fluorescent secondary antibodies (rhodamine-conjugated anti-mouse or fluorescein-conjugated anti-sheep antibodies). Two typical examples are shown in each case.

lag period and occurred with a half time of about 30 min. This lag period probably represents the time required for one or both proteins to fold and display their respective binding sites. An earlier study of the time required for gp120 (the portion of gp160 containing the CD4-binding site) to fold *in vivo* and then bind CD4 *in vitro* also determined the half time to be 30 min (8). The folding of gp120 may therefore be the rate-limiting step in the association. The topology of CD4 binding to gp160 in the ER is not entirely clear. One normally envisions the extracellular domains of CD4 and the HIV-1 glycoprotein interacting from separate membranes during virus-cell or cell-cell interactions. Our results indicate that these extracellular (luminal) domains can also interact within the membranes of the ER. It is not clear if the interaction occurs between molecules that are next to each other or on opposite sides of ER cisternae.

We also observed a profound inhibition of CD4 transport to the cell surface when CD4 was expressed in the presence of gp160. Greater than 95% of CD4 expressed alone was transported efficiently from the ER through the Golgi apparatus, where it acquired an endo H-resistant oligosaccharide (half time, ~45 min). In contrast, when expressed in the presence of excess gp160, 85 to 90% of CD4 did not acquire any endo H-resistant oligosaccharides and was not expressed on the cell surface. Indirect immunofluorescence of CD4 expressed with excess gp160 showed that CD4 was retained by gp160 inside the cells in a pattern typical of the ER.

FIG. 6. Transport and secretion of gp120 expressed in the presence and absence of CD4. (A) Nine plates of HeLa cells (5×10^5 cells per plate) expressing gp120 were pulse-labeled with [35 S]methionine for 10 min and incubated in chase medium for the times indicated. Cell lysates or chase medium (C and M, respectively) were immunoprecipitated with anti-gp120 antibodies. Immunoprecipitates of cellular and secreted gp120 (after incubation for 3 h in chase medium) were treated with endo H (lanes 17 and 19) or left untreated (lanes 16 and 18). Positions of molecular weight markers (in thousands) are indicated. (B) Eight plates of HeLa cells (5×10^5 cells per plate) were infected with vTF7-3 and transfected with both gp120 and CD4 (1.2 μ g of pBS-gp120 and 3.8 μ g of pBS-CD4 per plate). These cells expressing gp120 and CD4 were pulse-labeled with [35 S]methionine for 10 min and incubated in chase medium for the times indicated. Immunoprecipitates were prepared with OKT4 and incubated in the presence (even-numbered lanes) or absence (odd-numbered lanes) of endo H. Endo H-resistant forms of CD4 and gp120 (gp120 Endo H^r and CD4 Endo H^r) are indicated. The positions of gp120 with oligosaccharides (+CHO) and without oligosaccharides (-CHO) are shown. All samples (A and B) were analyzed by SDS-PAGE followed by fluorography.

The blockade of CD4 transport observed here appears to result from CD4 association with the very slowly and inefficiently transported gp160 molecule (41). In agreement with this earlier study on gp160 transport (41), we find that most (90 to 95%) of gp160 synthesized is not delivered to the cell surface but is degraded inside the cell or accumulates in the ER (unpublished results). An inhibition of surface expression of CD4 as a consequence of HIV-1 infection (4, 11, 14, 36) or of expression of gp160 alone (13) has been observed previously. In the latter study, the surface expression of CD4 was reduced after expression in gp160 was initiated. The results presented here indicate that the inhibition of CD4 transport from the ER by gp160 can be as great as 10-fold. In fact, CD4 that escapes the gp160 blockade may be doing so in cells that are not coexpressing gp160 in excess over CD4. During an HIV-1 infection, if the rate of synthesis of gp160 exceeds that of CD4, the excess gp160 may effectively block transport of newly synthesized CD4 to the cell surface. This mechanism of down regulation of the receptor might prevent rebinding of virus to infected cells.

The disappearance of CD4⁺ T lymphocytes is a hallmark of AIDS. Several mechanisms through which CD4⁺ T lymphocytes are depleted have been proposed. Formation of

T-cell syncytia leading to cell death (19, 20, 35) or direct killing of infected or uninfected cells (with shed gp120 bound to CD4) by the host immune system (21) may be responsible for the disappearance of CD4-positive cells. A blockade of CD4 transport by gp160 might partially explain decreases in CD4⁺ T lymphocytes without cell killing by HIV-1. Also, depletion of CD4 from the surface of lymphocytes would result in loss of adhesion (5) and signaling (32, 39) functions of CD4 and probably interfere with the normal immune response.

In addition to studying the interaction of CD4 with the HIV-1 glycoprotein precursor gp160, we also examined the interaction of CD4 with gp120 expressed by itself. We found that this soluble portion of the gp160 molecule was secreted from cells, as expected from earlier studies (18). The secretion of gp120, like the transport of gp160, was slow and inefficient, with only about 15% being released from cells after 3 h. Like gp160, soluble gp120 was also able to bind CD4 in the ER when the two proteins were coexpressed. Interestingly, binding of the soluble gp120 molecule to CD4 in the ER did not inhibit CD4 transport at all. Judging from the rate of carbohydrate processing on both molecules, the complex of CD4 and gp120 (which contained about half of

the gp120 molecules) was transported efficiently through the Golgi apparatus as fast as CD4 expressed by itself (half time, ~45 min). Thus, gp120 transport was greatly accelerated by virtue of association with the more rapidly transported, membrane-bound CD4 molecule.

An important unanswered question is why is CD4 transport blocked by association with membrane-associated gp160 but not by association with soluble gp120. The answer does not appear to be differences in rates or efficiencies of gp120 and gp160 transport, since both molecules are transported with similar inefficiency. Possibly the complex of two membrane proteins, gp160 and CD4, forms cross-bridges within the ER cisternae. Such complexes might not be able to be incorporated into transport vesicles budding from the ER or might even inhibit vesicle formation. It is unlikely that association of CD4 with the soluble gp120 would cross-link the ER, and gp120 would be expected to be transported while complexed to the membrane-bound CD4 at the normal CD4 rate.

We considered the possibility that gp160-CD4 complexes are capable of inhibiting all protein transport from the ER by interfering with the normal ER structure. Such complexes could then be directly responsible for T-cell killing. We have not, however, detected any effect of gp160-CD4 complexes in the ER on transport of a third protein, the VSV G protein, from the ER. We are now investigating the possibility that higher levels of gp160-CD4 complexes would have general effects on protein transport from the ER.

It should be noted that the mechanism by which gp160 retains CD4 in the ER is probably unrelated to the retention of the transported fraction gp120 or gp160 by the sCD4-KDEL construct that we described recently (3a). This soluble CD4 molecule was designed with a specific signal for retention of soluble proteins in the ER (26).

ACKNOWLEDGMENTS

We thank D. Brown, L. Chong, A. Shaw, M. Whitt, and P. Zagouras for helpful suggestions and comments on the manuscript.

This work was supported by Public Health Service grant AI-24345 from the National Institutes of Health. The AIDS Research and Reference Reagent Program of the National Institute of Allergy and Infectious Diseases provided the anti-gp120 serum (Michael Phelan, contributor) used in these studies.

ADDENDUM IN PROOF

Conclusions very similar to those presented here were reached independently by M. A. Jabbar and D. P. Nayak, *J. Virol.*, in press.

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