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Human Immunodeficiency Virus Type 1 Glycoprotein Precursor Retains a CD4-p56^{lck} Complex in the Endoplasmic Reticulum

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The cell surface glycoprotein, CD4, is the receptor for human immunodeficiency virus (HIV) in T lymphocytes. Following HIV infection, there is reduced expression of CD4 on the cell surface, and this downregulation probably results, at least in part, from the formation of complexes containing the HIV type 1 (HIV-1) glycoprotein precursor (gp160) and CD4 that are not transported from the endoplasmic reticulum (ER). At the plasma membrane of T cells, CD4 is tightly associated with a cytoplasmic tyrosine kinase (p56^{lck}) that is involved in T-cell activation. Using a transient expression system with HeLa cells, we show by pulse-labeling and immunoprecipitation that newly synthesized CD4 can associate with p56^{lck} before CD4 is transported from the ER. In the presence of HIV-1 gp160, a ternary complex of gp160-CD4 and p56^{lck} forms in the ER. Using confocal immunofluorescence microscopy, we observed complete retention of p56^{lck} in the ER. Such mislocalization of a tyrosine kinase to the cytoplasmic face of the ER could play a role in lymphocyte killing caused by HIV infection or expression of gp160 alone.

CD4 is an integral membrane glycoprotein found on the plasma membrane of helper T lymphocytes, monocytes, and other cell types, and it serves as the receptor for human immunodeficiency virus (HIV) (22, 35, 38). The cytoplasmic domain of CD4 is bound to a cytoplasmic tyrosine protein kinase, p56^{lck} (28, 29, 36), and this complex is thought to play an important role in T-cell activation (1, 8, 27).

HIV infection can exert a cytopathic effect upon single T cells in culture (31, 33) through a mechanism independent of syncytium formation (19, 20, 30). Although the mechanism of single-cell killing is not known, it may be critical for understanding the devastation of the immune system seen in patients with AIDS. The HIV envelope gene encoding the HIV glycoprotein precursor, gp160, is thought to be responsible for the cytopathic effect (32). In addition, expression of gp160 alone can kill CD4$^+$ but not CD4$^-$ T cells, suggesting that cell killing might be mediated through an interaction of gp160 with CD4 (16).

Infection of T lymphocytes by HIV type 1 (HIV-1) causes reduced expression of CD4 on the cell surface (6, 10, 14), and expression of only gp160 is sufficient to cause this downregulation (13). In earlier studies, we and others have shown that newly synthesized gp160 and CD4 form a complex which is retained in the endoplasmic reticulum (ER) (5, 11). Although the reason that these gp160-CD4 complexes are retained is not known, it may be related to the inefficient transport of gp160 itself (37). We show here that gp160 is also capable of retaining CD4-p56^{lck} complexes in the ER. Redistribution of CD4-p56^{lck} complexes may contribute to T-lymphocyte pathology during the course of HIV infection.

MATERIALS AND METHODS

Expression, metabolic labeling, and immunoprecipitation of proteins. Hela cells (approximately 5×10^5 cells per 35-mm-diameter dish) were infected with a recombinant vaccinia virus encoding T7 polymerase (vTF7-3) (7) at a multiplicity of infection of 10 to 25. Infections were carried out in 0.5 ml of Dulbecco's modified Eagle's medium (DMEM) without serum for 30 min at 37°C. The inoculum was then removed, and the cells were transfected with a total of 5 μg of plasmid DNA in 1.5 ml of DMEM, lacking serum, by using TransfectACE (Life Sciences, Inc.) according to the procedure described by Rose et al. (26). Plasmids encoding CD4, gp160, and p56^{lck} under control of the T7 promoter have been described previously (3, 29). Transfected cells were incubated for 3 to 4 h, washed once with DMEM lacking methionine, and labeled with 50 μCi of [^{35}S]methionine in 0.5 ml of methionine-free DMEM for 15 min at 37°C. Cells were then washed and incubated further in DMEM with excess methionine at 37°C for various times.

Prior to immunoprecipitation, cells were washed once with phosphate-buffered saline and lysed in 1 ml of a solution (lysis buffer) 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 2 min. For CD4 immunoprecipitations, sodium dodecyl sulfate (SDS) was added to a concentration of 0.1% to the lysate, and 100 μl 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 the precipitate was washed three times. Immunoprecipitations of p56^{lck} from metabolically labeled cells were performed in lysis buffer plus 0.2% SDS with 2 μl of rabbit anti-p56^{lck} serum. After incubation at 37°C for 60 min, antigen-antibody complexes were precipitated and washed as described above. Rabbit antibodies to p56^{lck} were raised against a synthetic peptide of p56^{lck} (amino acids 39 to 58) coupled to keyhole limpet hemocyanin. Immunoprecipitates were analyzed on 9% polyacrylamide gels containing SDS (17), and the gels were prepared for fluorography, dried, and exposed to preflashed X-ray film by the method of Bonner and Laskey (2). Endoglycosidase H (endo H [34]) treatments of immunoprecipitations were performed as described previously (25).

In vitro kinase assays. Detergent lysates of the cells were divided equally and immunoprecipitated with anti-gp160

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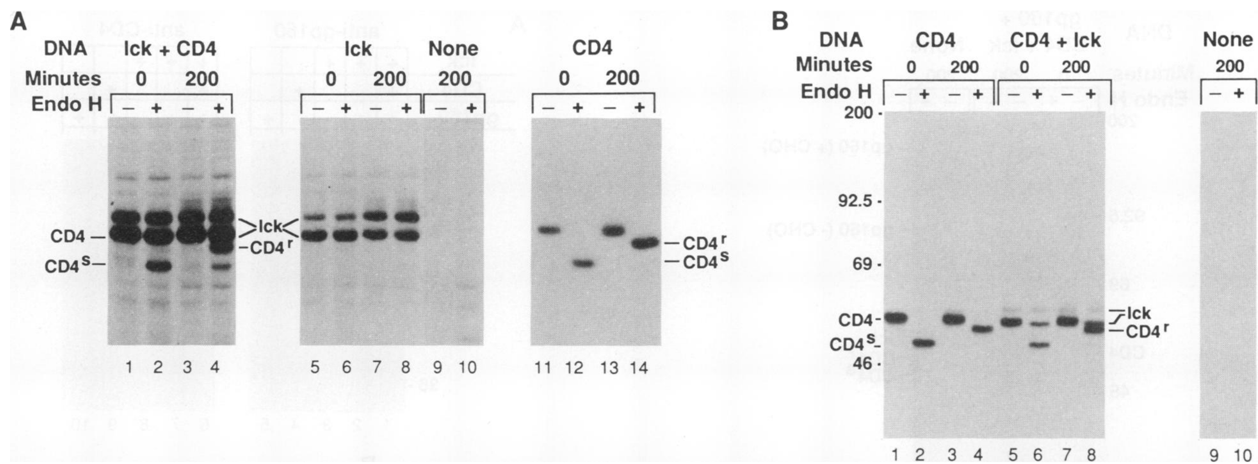

FIG. 1. Rapid intracellular association of p56^{lck} and CD4. (A) Newly synthesized CD4 binds p56^{lck}. HeLa cells (5×10^5 per 35-mm-diameter dish) were infected with a recombinant vaccinia virus that expresses T7 RNA polymerase, vTF7-3 (7). Infected cells were subsequently transfected (26) with plasmids encoding p56^{lck} and CD4 under the control of the T7 promoter (29) as indicated above the lanes. The cells were then labeled for 15 min with 50 μCi of [^{35}S]methionine in 0.5 ml of methionine-free DMEM and either lysed immediately or incubated in the presence of excess unlabeled methionine for 200 min as indicated. Immunoprecipitations from detergent lysates of cells were performed by adding rabbit anti-p56^{lck} serum (lanes 1 to 10) or OKT4 hybridoma supernatant (lanes 11 to 14). Immunoprecipitates were washed, divided in half, and either digested with endo H (even-numbered lanes [+]) or left untreated (odd-numbered lanes [−]). Proteins were analyzed by electrophoresis in 9% polyacrylamide gels containing SDS. (B) Newly synthesized p56^{lck} binds CD4. The experiment was performed as described for Fig. 1A. CD4 was immunoprecipitated from detergent lysates by using OKT4 after 0 or 200 min of chase in the presence of unlabeled methionine. The immunoprecipitates were divided in half and either digested with endo H (even-numbered lanes [+]) or left untreated (odd-numbered lanes [−]). The positions of untreated CD4, CD4 with one oligosaccharide (CD4^r), CD4 lacking oligosaccharides (CD4^s), and p56^{lck} (lck) are indicated. The positions of molecular weight markers (in thousands) are indicated on the left.

(AIDS Research and Reagent Program reagent no. 188), anti-CD4 (OKT4), or anti-p56^{lck} antibodies. The immunoprecipitates were washed and then incubated in a 50-μl in vitro kinase reaction buffer containing 10 μCi of [γ-^{32}P]ATP. Cell lysis, immunoprecipitation, and in vitro kinase assay conditions were as described by Rudd et al. (28). The proteins phosphorylated in vitro were analyzed by electrophoresis in 10% polyacrylamide gels containing SDS (17). The phosphorylated proteins (from a duplicate experiment) which migrated at approximately 55 kDa (see Fig. 3A) were analyzed by partial proteolysis by using V8 protease along with in vitro ^{32}P-labeled p56^{lck} (29). The peptides were resolved by electrophoresis in 15% polyacrylamide gels containing SDS (17).

Confocal indirect immunofluoresence microscopy. HeLa cells were infected with vTF7-3 for 30 min and subsequently transfected with plasmid DNAs (as indicated in Fig. 4). At 6 h posttransfection, the cells were treated with cycloheximide (100 μg/ml) for 40 min at 37°C and fixed with paraformaldehyde. Cells were made permeable with Triton X-100 to allow entry of anti-CD4, anti-p56^{lck} (as described above), and anti-gp120 (AIDS Research and Reagent Program reagent no. 288) antibodies. After immunostaining, confocal imaging of the cells was performed with a Bio-Rad MRC-600 system mounted on a Zeiss Axiovert 10 with a 63× objective. Images were photographed from the microscope video monitor.

RESULTS

Rapid association of p56^{lck} with newly synthesized CD4. To determine whether newly synthesized CD4 associates with p56^{lck}, HeLa cells expressing CD4 in the presence of p56^{lck} were pulse-labeled with [^{35}S]methionine, and then cell lysates were immunoprecipitated with antibody to p56^{lck}. This analysis is complicated by the fact that the majority of p56^{lck} comigrates with fully glycosylated CD4 in SDS-polyacrylamide gel electrophoresis. However, after CD4 was treated with endo H (34), it was possible to visualize the CD4 which coprecipitated with p56^{lck} because of the increase in mobility caused by removal of oligosaccharides from CD4. Both glycans on CD4 are initially sensitive to endo H, and subsequently, one glycan becomes resistant with a half-time of 45 min as CD4 is transported through the Golgi apparatus (5).

After a 15-min pulse-label of cells expressing both CD4 and p56^{lck}, antibody to p56^{lck} precipitated two bands corresponding in mobility to p56^{lck} expressed alone (Fig. 1A, lanes 1 and 5). The major p56^{lck} band comigrated with CD4 expressed alone (lane 11). After treatment with endo H, the CD4 that coprecipitated with p56^{lck} or the CD4 expressed alone migrated faster than p56^{lck} (lanes 2 and 12). After the 200-min chase period, approximately the same amount of CD4 precipitated with p56^{lck}, but it had a slower mobility after endo H digestion because of processing of one of the glycans (lane 4). Similar processing was observed for CD4 expressed alone (lanes 13 and 14). These results indicated that the newly synthesized CD4 was associated with p56^{lck} within 15 min. The amount of labeled CD4 complexed with p56^{lck} remained constant as the complex was transported from the ER through the Golgi apparatus to the cell surface. Given the fact that CD4 remains stably associated with p56^{lck} during immunoprecipitation in vitro, it seems likely that the association is also stable during transport to the cell surface. Note that the oligosaccharides on CD4 expressed with p56^{lck} were not processed as extensively as was CD4 expressed alone (compare lanes 4 and 14), suggesting that the binding of p56^{lck} to the cytoplasmic domain of CD4 might slow its transport. A portion of p56^{lck} also shifted to a more slowly migrating form (lanes 5 to 8) after the chase, suggest-

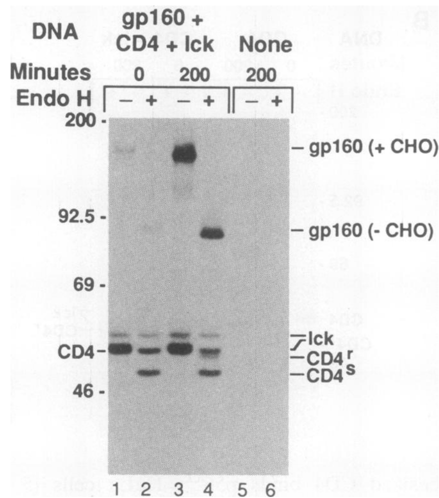

FIG. 2. CD4 association with p56^{lck} and gp160. The experiment was performed as described in the legend to Fig. 1. Transfection was done with plasmids encoding p56^{lck}, CD4, and gp160 (3) as indicated. CD4 was immunoprecipitated from detergent lysates of the labeled cells. The immunoprecipitates were divided in half and either digested with endo H (even-numbered lanes [+]) or left untreated (odd-numbered lanes [−]). The positions of p56^{lck} (lck) and gp160 with (+ CHO) and without (− CHO) oligosaccharides are indicated. CD4, CD4 with one oligosaccharide (CD4^r), and CD4 without oligosaccharides (CD4^s) are indicated. The numbers on the left represent the positions of molecular weight markers (in thousands).

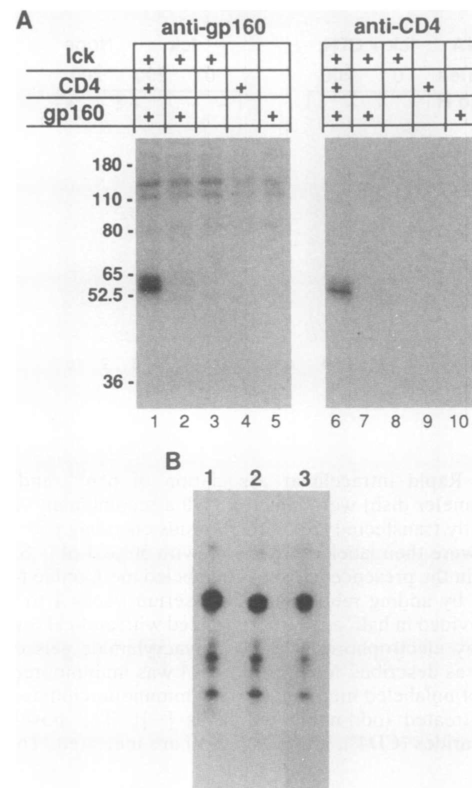

FIG. 3. Detection of a p56^{lck}-CD4-gp160 complex. (A) HeLa cells infected with vTF7-3 were transfected with plasmids encoding p56^{lck}, CD4, and gp160, as indicated above the lanes. Detergent lysates of the cells were divided equally and immunoprecipitated with anti-gp160 (lanes 1 to 5) or anti-CD4 (lanes 6 to 10) antibodies. The immunoprecipitates were washed and then incubated in an in vitro kinase reaction buffer containing 10 μCi of [γ-^{32}P]ATP. Cell lysis, immunoprecipitation, and in vitro kinase assay conditions were as described previously (28). The proteins phosphorylated in vitro were analyzed by electrophoresis in 10% polyacrylamide gels containing SDS. The positions of molecular weight markers (in thousands) are indicated on the left. (B) The phosphorylated proteins (from a duplicate experiment) which migrated at approximately 55 kDa (Fig. 3A, lanes 1 and 6) were analyzed by partial proteolysis by using V8 protease along with in vitro ^{32}P-labeled p56^{lck} (lanes 1, 2, and 3, respectively) (29). The peptides were resolved by electrophoresis in 15% polyacrylamide gels containing SDS.

ing increased phosphorylation with time. In other similar experiments, we were able to detect CD4 in anti-p56^{lck} immunoprecipitates immediately after a 5-min pulse with [^{35}S]methionine (data not shown).

Rapid association of CD4 with newly synthesized p56^{lck}. In the experiment for which the results are shown in Fig. 1A, we could not determine whether newly synthesized p56^{lck} associated rapidly with CD4, because in that experiment CD4 might have associated only with unlabeled p56^{lck} synthesized prior to the pulse period. Therefore, we expressed CD4 alone or p56^{lck} in the presence of CD4 and immunoprecipitated with anti-CD4 monoclonal antibodies (Fig. 1B). Immediately after the pulse period, p56^{lck} could be resolved from CD4 after endo H treatment because it remained at the position on the gel previously occupied by CD4 (Fig. 1B, lanes 1, 2, 5, and 6). This result indicates that newly synthesized p56^{lck} binds CD4 rapidly. At 200 min of chase, the CD4 with one endo H-resistant glycan (CD4^r) migrated faster than the smaller form of p56^{lck} (lanes 3, 4, 7, and 8). The amount of p56^{lck} bound to CD4 did not change after the 200-min chase period, indicating that the association was complete within 15 min.

HIV gp160 prevents intracellular transport of CD4-p56^{lck} complexes. Coexpression of HIV gp160 with CD4 leads to retention of the majority of CD4 in the ER (5, 11). To determine whether p56^{lck} associates with CD4 when both are expressed in the presence of excess gp160, we performed the pulse-chase experiment for which the results are shown in Fig. 2. Cells were transfected with DNAs encoding gp160, CD4, and p56^{lck} and pulse-labeled with [^{35}S]methionine. Cell lysates were then immunoprecipitated with antibody to CD4. Immunoprecipitates were either digested with endo H so that p56^{lck} could be visualized or left undigested. The results (Fig. 2, lanes 1 and 2) showed that p56^{lck} was bound to CD4, and some gp160 also precipitated with CD4. After 200 min of chase, the majority of CD4 (90%) was still fully sensitive to endo H. The amount of p56^{lck} associated with unprocessed CD4 did not change during the course of the chase period. Also, at the 200-min time, CD4 was also associated with an increased amount of gp160 (Fig. 2, lanes 3 and 4). These results suggested that complexes of CD4 and p56^{lck} were retained in the ER after association with gp160 because of the lack of processing of the CD4 oligosaccharides.

Identification of gp160-CD4-p56^{lck} complexes. To obtain additional evidence of a gp160-CD4-p56^{lck} complex, we carried out the experiment, for which the results are shown in Fig. 3A, in which gp160, CD4 and p56^{lck} were expressed in the indicated combinations. The samples were immunoprecipitated with anti-gp160 serum or with antibody to CD4 and then incubated with [γ-^{32}P]ATP in an in vitro kinase assay for detecting the presence of p56^{lck} by autophosphorylation (28). Only when all three molecules were expressed

together did antibodies to gp160 precipitate two candidate p56^{lck} bands that became phosphorylated in the in vitro assay (Fig. 3A, lane 1). Antibody to CD4 was also able to precipitate the same bands (Fig. 3A, lane 6), as expected.

To verify that the material phosphorylated in vitro was ^{32}P-labeled p56^{lck}, we carried out an analysis by partial proteolysis (4). As shown in Fig. 3B, the partial proteolysis products of candidate p56^{lck} bands that precipitated with antibody to gp160 or CD4 were identical to each other and to authentic p56^{lck} precipitated with anti-p56^{lck} antibodies (Fig. 3B).

CD4-p56^{lck} complexes are retained in the ER by HIV gp160. The retention of unprocessed oligosaccharides on CD4 bound to gp160 and p56^{lck} suggested that the entire complex was probably retained in the ER. To obtain direct evidence for the localization of the complex, we used confocal immunofluorescence microscopy. We first expressed CD4 and p56^{lck} to determine whether these proteins colocalize. HeLa cells expressing CD4 and p56^{lck} were treated with cycloheximide for 40 min and fixed with paraformaldehyde. Cycloheximide treatment inhibits protein synthesis without disrupting protein transport through the exocytic pathway (12). This treatment allowed sufficient time for the CD4 molecules to reach the cell surface. After fixation, the cells were immunostained for p56^{lck} and CD4. Confocal immunofluorescence microscopy showed that some CD4 (red) and p56^{lck} (green) were located at the plasma membrane, as indicated by the visible outline of the cell (Fig. 4A and B, respectively). The images were then superimposed to assess the degree of colocalization of CD4 and p56^{lck}. The extensive colocalization of CD4 and p56^{lck} is shown in yellow (Fig. 4C).

To determine whether expression of excess gp160 was capable of retaining the CD4-p56^{lck} complex in the ER, we examined HeLa cells expressing p56^{lck}, CD4, and gp160. Cells were incubated for 40 min with cycloheximide prior to fixation and immunostained for CD4 (red) and p56^{lck} (green). In contrast to the pattern observed for CD4 and p56^{lck} expressed without gp160, both molecules were now localized to a reticular pattern within the cell, including staining of the nuclear membrane. This pattern is indicative of proteins localized to the ER (Fig. 4D and E). CD4 and p56^{lck} largely colocalize, as seen in the extensive overlap of the fluorescence (Fig. 4F). With all cells expressing CD4 and p56^{lck}, there was no detectable p56^{lck} expression near the plasma membrane, indicating quantitative retention in the ER.

To control for the possibility that gp160 expression might have a direct effect on the localization of p56^{lck}, we coexpressed gp160 and p56^{lck} without CD4. Localization of gp160 (green) and p56^{lck} (red) in these cells showed that p56^{lck} had distinct surface localization (Fig. 4G) in contrast to the cytoplasmic localization of gp160 (Fig. 4H and I). This pattern is identical to that observed for p56^{lck} expressed alone (not shown). We conclude that the reticular cytoplasmic localization of p56^{lck} in cells expressing p56^{lck}, CD4, and gp160 results from retention of a gp160-CD4-p56^{lck} complex in the ER.

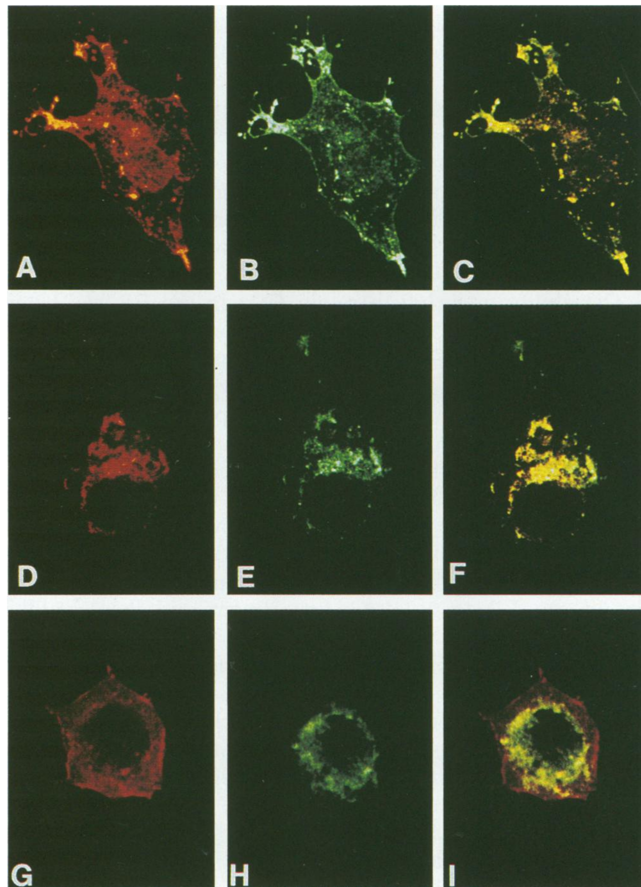

FIG. 4. Localization of p56^{lck}, CD4, and gp160 in HeLa cells by confocal immunofluorescence microscopy. HeLa cells were infected with vTF7-3 for 30 min and subsequently transfected, fixed, and immunostained as described below. Images representing the rhodamine fluorescence (left panels), fluorescein fluorescence (center panels), or the two images superimposed (right panels) are shown. (A to C) Cells cotransfected with plasmids encoding p56^{lck} and CD4 were incubated with rabbit anti-p56^{lck} serum (diluted 1:200) and OKT4 hybridoma supernatant (undiluted) and then with rhodamine-conjugated goat anti-mouse and fluorescein-conjugated goat anti-rabbit antibodies (Cappel). (D to F) Cells transfected with plasmids encoding p56^{lck}, CD4, and gp160 were incubated with antibodies as described above. (G to I) Cells transfected with plasmids encoding p56^{lck} and gp160 were incubated with rabbit anti-p56^{lck} serum and sheep anti-gp120 serum (diluted 1:200) and then with rhodamine-conjugated donkey anti-rabbit antibodies and fluorescein-conjugated donkey anti-sheep antibodies (Jackson Laboratories). Appropriate controls showed that the anti-rabbit and anti-mouse secondary antibodies used in these experiments were specific for their respective primary antibodies. Cells were fixed with 3% paraformaldehyde according to the procedures of Rose and Bergman (25) (panels G to I) or by a modified paraformaldehyde fixation procedure of McLean and Nakane (23) (panels A to F) as described previously (5).

DISCUSSION

We have shown that the association between CD4 and p56^{lck} can occur at the cytoplasmic face of the ER immediately after synthesis of both proteins when both are expressed in HeLa cells. We know this because pulse-labeled CD4 containing immature oligosaccharides is readily immunoprecipitated with p56^{lck}, using antiserum recognizing p56^{lck}. Pulse-chase experiments suggested that the complex remained associated as the two molecules were transported through the Golgi complex and eventually to the plasma membrane.

Using confocal immunofluroescence microscopy, we also observed a plasma membrane distribution of some of the p56^{lck} expressed by itself, and CD4 can certainly be transported to the cell surface in the absence of p56^{lck}. It is,

therefore, likely that p56^{lck} can bind to CD4 that is already localized at the plasma membrane, although this has not been demonstrated directly. In an earlier study (29), it was reported that a chimeric protein containing the extracellular and transmembrane domains of the vesicular stomatitis virus glycoprotein fused to the cytoplasmic domain of CD4 would bind to p56^{lck} soon after synthesis. Although the complexes were not localized in that study, the results are consistent with that which we have reported here on the association of CD4 and p56^{lck} in the ER.

The ability of gp160 to retain CD4 in the ER has been shown previously (5, 11). Here we observed that a ternary complex of gp160, CD4, and p56^{lck} forms in cells expressing all three proteins and that it is apparently fully retained in the ER. By immunofluorescence microscopy, we observed no p56^{lck} localized at the plasma membrane when cells were also expressing CD4 and gp160. All of the p56^{lck} appeared to colocalize with CD4 in an intracellular membrane network characteristic of the ER. Although we favor the idea that the complex is permanently retained in the ER, we cannot exclude the possibility that it might actually be transported out of the ER to a later compartment and then rapidly recycled back. Such a mechanism of retention was first described for proteins containing the specific KDEL retention signal (24).

In two T-lymphocyte cell lines which have been examined, at least 95% of p56^{lck} is associated with CD4 (21). Thus, it is likely that during an HIV infection the disappearance of CD4 from the cell surface would be accompanied by the eventual redistribution of the majority of p56^{lck} from the inner surface of the plasma membrane to the cytoplasmic face of the ER.

Other viral proteins have been shown to cause alterations in intracellular signaling. For example, association of the Friend spleen focus-forming virus glycoprotein with the erythropoietin receptor, occurring largely in the ER, results in cellular transformation (18, 39). Also, inappropriate expression of a tyrosine kinase, the Abelson murine leukemia virus-transforming gene product, is toxic in certain cell types (40).

Others have examined the effects of binding gp120 (the major extracellular portion of the HIV-1 glycoprotein) or HIV-1 virus itself to CD4 at the plasma membrane of T cells and have not detected the generation of intracellular signals (9). Intracellular association of gp160 with CD4-p56^{lck} complexes may have different effects on intracellular signaling pathways compared with the effect of exogenously bound gp120 at the cell surface. The retention of the CD4-p56^{lck} complex in the ER that we have observed here would be expected to abolish the signaling potential of the CD4-p56^{lck} complex at the plasma membrane and might contribute to cell killing by giving p56^{lck} access to novel intracellular substrates. Recent work has shown that gp160 expression in a CD4$^+$ T-cell line rapidly blocks nuclear import prior to cell killing (15), and we suggest that this blocking might involve tyrosine phosphorylation by p56^{lck} of critical substrates around the nuclear pore complex. The nuclear membrane is continuous with the ER, and thus, some of p56^{lck} would likely be retained in close proximity to the nuclear pores. It should be possible to examine a role for p56^{lck} in cell killing and nuclear import blocking by deriving T-cell lines lacking p56^{lck} and examining their sensitivities to killing by HIV.

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