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Intracellular Interaction of Human Immunodeficiency Virus Type 1 (ARV-2) Envelope Glycoprotein gp160 with CD4 Blocks the Movement and Maturation of CD4 to the Plasma Membrane

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The envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1) plays a major role in the down-regulation of its receptor, CD4. Using a transient-expression system, we investigated the interaction of the HIV-1 envelope glycoprotein with CD4 during their movement through the intracellular membrane traffic. In singly transfected cells, the envelope glycoprotein gp160 was synthesized, glycosylated, and localized predominantly in the endoplasmic reticulum. Only a minor fraction of gp160 was proteolytically cleaved, producing gp120 and gp41, and gp120 was secreted into the medium. On the other hand, the CD4 molecule, when expressed alone, was properly glycosylated and transported efficiently to the cell surface. However, when gp160 and CD4 were coexpressed in the same cell, the cell surface delivery of CD4 was greatly reduced. In coexpressing cells, CD4 formed a specific intracellular complex with gp160 as both proteins could be immunoprecipitated by antibodies against either the gp160 or CD4 (OKT4) but not by OKT4A, a blocking antibody against CD4. The specific gp160-CD4 complex was localized predominantly in the endoplasmic reticulum, and the CD4 in the complex did not acquire endoglycosidase H resistance. The present studies demonstrated that a specific intracellular interaction between gp160 and CD4 was responsible for the cell surface down-regulation of CD4 in cells expressing both the envelope glycoprotein of HIV-1 and its receptor, CD4.

Human immunodeficiency virus type 1 (HIV-1), an etiological agent of acquired immunodeficiency syndrome (AIDS), encodes gp160, an integral membrane glycoprotein (1, 2, 4, 31). The endoproteolytic processing of gp160 into gp120 and gp41 is essential for the production of infectious virions (29). gp120, the extracellular glycoprotein of the virion, binds to CD4-bearing cells and initiates the infection process, whereas gp41, the transmembrane component (43), mediates virus-to-cell fusion as well as cell-to-cell fusion of infected cells (19, 22, 23, 25, 28, 40, 41).

Since the infectious process requires a productive interaction of gp120 with CD4 at the cell surface, both proteins must be targeted correctly to the plasma membrane (5, 12, 15, 21, 32, 35, 45). Recent studies have shown that in HIV-1-infected cells, only a small fraction of gp160 is processed into gp120 and gp41, delivering gp120 to the cell surface, whereas the majority of gp160 is sorted to the lysosome and degraded in the compartment (44). Similar studies on CD4 have shown that the CD4 molecule is an integral membrane glycoprotein and exists predominantly as a monomer (20, 25, 39; Jabbar and Nayak, unpublished data) and that the cell surface expression of CD4 is essential for the productive HIV-1 infection (3, 6, 25, 27, 36). However, in HIV-1-infected cells, the CD4 molecule has been shown to be down-regulated (7, 11, 16), suggesting that a viral component(s) might play a role in the disappearance of CD4 at the cell surface. However, the mechanism, by which CD4 is down-regulated in HIV-1-infected cells, is poorly understood. Recent studies (14, 42) have shown that the cell surface expression of CD4 is substantially reduced in cells expressing both the HIV-1 envelope glycoprotein and CD4 and suggested a possible role for the envelope glycoprotein in CD4 down-regulation.

The present study was undertaken to determine whether the interaction of HIV-1 envelope glycoprotein with CD4 while in transit through the membrane traffic compartments, was responsible for the down-regulation of CD4 on the cell surface. Our study shows that in singly transfected cells, the majority of gp160 remained blocked in the endoplasmic reticulum (ER), acquiring predominantly the endoglycosidase H (endo-H)-sensitive high-mannose oligosaccharides. Only a minor fraction of gp160 was proteolytically cleaved into gp120 and gp41, delivering gp120 to the extracellular medium. On the other hand, CD4, when expressed alone, was delivered efficiently to the cell surface undergoing complex sugar modifications. However, in cells coexpressing both gp160 and CD4, the expression of CD4 molecule was greatly reduced on the cell surface; it remained predominantly localized in the ER and existed as a gp160-CD4 complex, without undergoing complex oligosaccharide modifications. The formation of specific gp160-CD4 binary complex was, therefore, responsible for blocking the intracellular transport and the productive delivery of CD4 to the plasma membrane.

For studies reported here, a transient expression system using vaccinia virus T7 RNA polymerase (10) in HeLa cells was employed. The plasmid pARV-7A/2 carrying the *Eco*RI permuted ARV-2 (24) was cut with restriction enzymes *Sst*I and *Kpn*I, and the *Sst*I-*Kpn*I fragment (3.2 kb) was cloned into pGem3 (Promega Corporation, Madison, Wis.) to generate a recombinant plasmid, pGENV-7. The plasmid pGENV-7 was cut with *Eco*RI and *Bam*HI to obtain a 3.2-kb DNA fragment encoding the envelope glycoprotein, which was cloned into mp18 vector. An *Eco*RI site was generated by in vitro mutagenesis by using a primer (TTA TTC TAT GAA TTC ACTTGTCTA) 47 bp upstream of the envelope

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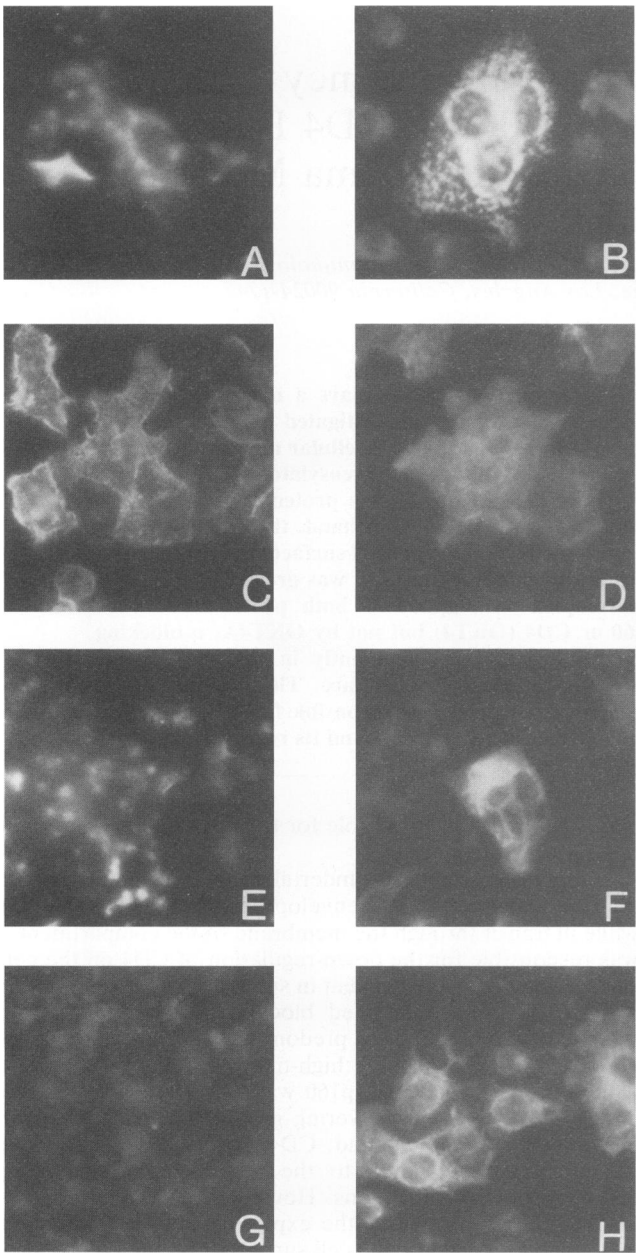

FIG. 1. Expression of the HIV-1 envelope glycoprotein and CD4 molecule in HeLa cells. HeLa cells (5×10^5) were grown on glass cover slips in Dulbecco modified Eagle medium containing 10% fetal bovine serum for 1 day prior to lipofectin-mediated transfection. The vaccinia virus (vTF7.3) expressing the bacteriophage T7 RNA polymerase (10) was adsorbed to HeLa cells at 37°C for 1 to 2 h in a 5% CO_2 incubator. After removal of the viral inoculum, fresh Dulbecco modified Eagle medium containing 2.5% fetal bovine serum was added to the infected cells, and the lipofectin (30 μg)-plasmid DNA (15 μg) mix was layered onto the vaccinia virus-infected cells. For cotransfection assays, the plasmids pGENV-7N (10 to 20 μg) and pGCD4 (10 to 20 μg) were mixed together, and the DNA mixture was added to the lipofectin (20 to 30 μg) for the transfection of vTF7.3 vaccinia virus-infected HeLa cells. At 16 h posttransfection, transfected HeLa cells were analyzed for the expression of glycoproteins both at the cell surface (A, C, E, and G) as well as in the intracellular compartment (B, D, F, and H) according to the procedure of Koch et al. (17). (A and B) HeLa cells transfected with the plasmid pGENV-7N encoding the HIV-1 envelope glycoprotein and analyzed by the anti-gp41 antibody. (C and D) HeLa cells transfected with the plasmid pGCD4 encoding the CD4 molecule and analyzed by the OKT4 (anti-CD4) antibody. (E and F) HeLa cells, cotransfected with the plasmids pGENV-7N and pGCD4, were analyzed by the anti-gp41 antibody. (G and H) HeLa cells, cotransfected with the plasmids pGENV-7N and pGCD4, were analyzed by the OKT4 antibody.

glycoprotein ATG (38) to delete a major portion of the noncoding nucleotides, and the new *Eco*RI-*Bam*HI DNA fragment was cloned into pGem3. The recombinant plasmid was named pGENV-7N and used in the studies reported here. For the construction of the vector expressing the CD4 molecule, the plasmid pMV7-T4 (26; obtained from R. Axel of Columbia University) was digested with *Eco*RI and *Bam*HI to generate a 1.8-kb fragment that encodes the full-length CD4 molecule. The 1.8-kb *Eco*RI-*Bam*HI fragment was subcloned into pGem3 to place the gene under the control of T7 promoter. The recombinant plasmid pGCD4 was used for expression.

A mouse monoclonal antibody against HIV-1 gp41 (NEA-9303) was purchased from Dupont Company, Wilmington, Del. Monoclonal antibodies OKT4 and OKT4A (39) were bought from Orthodiagnostics, Raritan, N.J. A hyperimmune human AIDS antiserum (CDC 9) was kindly provided by P. G. Nishanian, University of California at Los Angeles School of Medicine, Los Angeles, Calif. Fluorescein-conjugated anti-mouse antibodies and rhodamine-conjugated anti-human antibodies were purchased respectively, from the Caltag Laboratories, South San Francisco, Calif., and Accurate Antibodies, Westbury, N.Y. Lipofectin was purchased from the Bethesda Research Laboratories, Gaithersburg, Md. The vaccinia virus vTF7.3, expressing T7 RNA polymerase, was a kind gift of B. Moss, National Institutes of Health, Bethesda, Md.

By using the vaccinia virus T7 polymerase expression system (10), HeLa cells were transfected with individual plasmids or cotransfected with both CD4 and gp160 plasmids, and the transfected cells were analyzed for the synthesis and intracellular localization of the glycoproteins by indirect immunofluorescence assays. Specific monoclonal antibodies were used to label the expressed proteins both at the cell surface and in the intracytoplasmic membrane compartments. Results showed that the intracellular staining of the HIV-1 envelope glycoprotein was intense and the protein was predominantly present in the perinuclear region (Fig. 1B). Cell surface staining of the protein was much less intense (Fig. 1A), suggesting that the major fraction of the HIV-1 envelope glycoprotein was blocked in the intracellular membrane compartment, possibly in the ER. The CD4 molecule, on the other hand, was transported efficiently to the cell surface (Fig. 1C). OKT4, a monoclonal antibody against CD4, stained both the cell surface and intracytoplasmic CD4 with almost equal intensity (Fig. 1C and D).

To determine whether coexpression would affect the intracellular transport of either gp160 or CD4, HeLa cells were cotransfected with the plasmids pGENV-7N and pGCD4, and expression of the glycoproteins was analyzed by the indirect immunofluorescence assay (Fig. 1E to H). As in the singly transfected cells, the majority of gp160 was absent on the cell surface (Fig. 1E) but present internally in cotransfected cells (Fig. 1F). However, contrary to the singly transfected cells, the cell surface expression of CD4 was markedly reduced in cotransfected cells (Fig. 1G), even though the intracytoplasmic staining of CD4 was pronounced in the coexpressing cells (Fig. 1H). To further

determine whether these two glycoproteins share the same membrane compartment in coexpressing cells, HeLa cells cotransfected with both plasmids pGENV-7N and pGCD4 were examined for colocalization of the glycoproteins by intracytoplasmic double staining. The results showed that both HIV-1 envelope glycoprotein and CD4 had a similar intracellular distribution, suggesting that they were present in the same intracellular compartments in coexpressing cells (data not shown).

The above results suggested that in coexpressing cells, CD4 might interact with gp120 in an intracellular compartment during transport through the secretory pathway. To determine that the interaction between the two glycoproteins was specific involving the receptor-binding domain of CD4, OKT4A (a blocking monoclonal antibody; 39) was used to label CD4 and the CD4-gp160 complex in HeLa cells expressing either CD4 alone or both gp160 and CD4 (Fig. 2). The indirect immunofluorescence data showed that the OKT4A antibody stained both the cell surface as well as the intracellular CD4 in singly transfected cells (Fig. 2A and B) but failed to recognize either the intracellular or the cell surface CD4 in cells expressing both gp160 and CD4 (Fig. 2C and D). Thus, both OKT4A (Fig. 2C) and OKT4 (Fig. 1G) antibodies failed to stain the CD4 molecule on the cell surface of coexpressing cells, suggesting that the cell surface delivery of CD4 was markedly reduced in cells coexpressing gp160 and CD4. However, OKT4 exhibited an intense intracellular staining (Fig. 1H), whereas OKT4A failed to decorate the intracellular CD4 (Fig. 2D) in coexpressing cells, suggesting that the binding domain of CD4 was inaccessible to OKT4A. Taken together, these immunofluorescence data showed that in coexpressing cells, CD4 was blocked during intracellular transport and that the intracellular interaction was specific involving the binding domain of CD4.

To further analyze the nature of the interaction between gp160 and CD4 and its effect on intracellular transport of proteins, pulse-chase analyses were performed and the kinetics of glycosidic modifications were determined in both singly transfected and cotransfected HeLa cells. In singly transfected cells, the precursor polyprotein gp160 was synthesized and remained endo-H sensitive throughout the chase period of 5 h (Fig. 3A, arrowheads labeled b and d), suggesting that the gp160 was localized predominantly in the ER or pre-Golgi region. During the chase, a minor fraction of gp160 underwent proteolytic cleavage, generating gp120 and gp41 (Fig. 3A and B), and the extracellular gp120 appeared in the medium at about 2.5 h of chase (Fig. 3B, arrow a). However, the secreted gp120 did not undergo extensive complex sugar modifications and was only partially resistant to endo-H, displaying a heterogeneous mobility on gels (Fig. 3B, arrow b). In addition, a 39,000-molecular-weight protein was secreted with a similar kinetics as that of the gp120 and was immunoprecipitated by the AIDS sera (Fig. 3B and 4B, △). The identity of the protein is unknown at present, although it appeared to possess endo-H-resistant oligosaccharides. The cell-associated gp41 remained endo-H sensitive throughout the 5-h chase period (Fig. 3A, arrowheads e and f). Furthermore, a high-molecular-mass species (>200 kDa) was immunoprecipitated in the transfected HeLa cell lysates by the anti-gp41 antibody (Fig. 3A and 4A, arrowhead a). The higher-molecular-weight protein species might represent an aggregate form of the HIV-1 envelope glycoprotein (9).

In singly transfected cells, the CD4 molecule, synthesized during a 30-min pulse, was glycosylated (Fig. 3C, arrowhead a′) and predominantly endo-H sensitive (Fig. 3C, arrowhead c′), with a minor fraction of CD4 acquiring a partial endo-H resistance (Fig. 3C, arrowhead b′). During the chase, however, the amount of endo-H-sensitive form progressively decreased with a corresponding increase in the amount of partially endo-H-resistant form, indicative of the CD4 movement through the *trans*-Golgi compartments in which the complex sugar modifications occur (18, 37). By the end of the 5-h chase, 90% of the CD4 became partially endo-H resistant ($t_{1/2} \approx 45$ min) and only a small fraction, which could not be resolved clearly in polyacrylamide gel electrophoresis, might have acquired complex modifications in both glycosylation sites (Fig. 3C, arrowhead d′), suggesting that

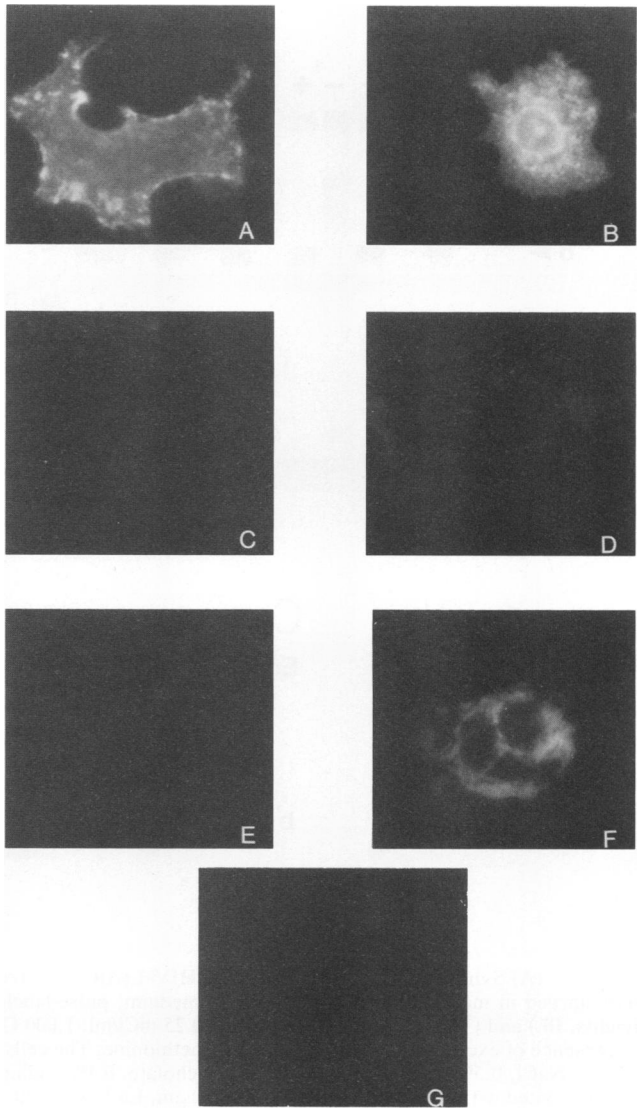

FIG. 2. Intracellular interaction of HIV-1 envelope glycoprotein with CD4 molecule. HeLa cells expressing either CD4 (A and B) or both HIV-1 envelope glycoprotein and CD4 (C, D, E, and F) were fixed and stained for the cell surface (A, C, E, and G) as well as intracellular (B, D, and F) antigens. The labeling by OKT4A antibody of singly (A and B) or doubly transfected (C and D) cells was performed. The anti-gp41 antibody was used to stain cells coexpressing the envelope glycoprotein and CD4 (E and F). No detectable staining by the anti-gp41 antibody of cells infected with vaccinia virus vTF7.3 was observed (G).

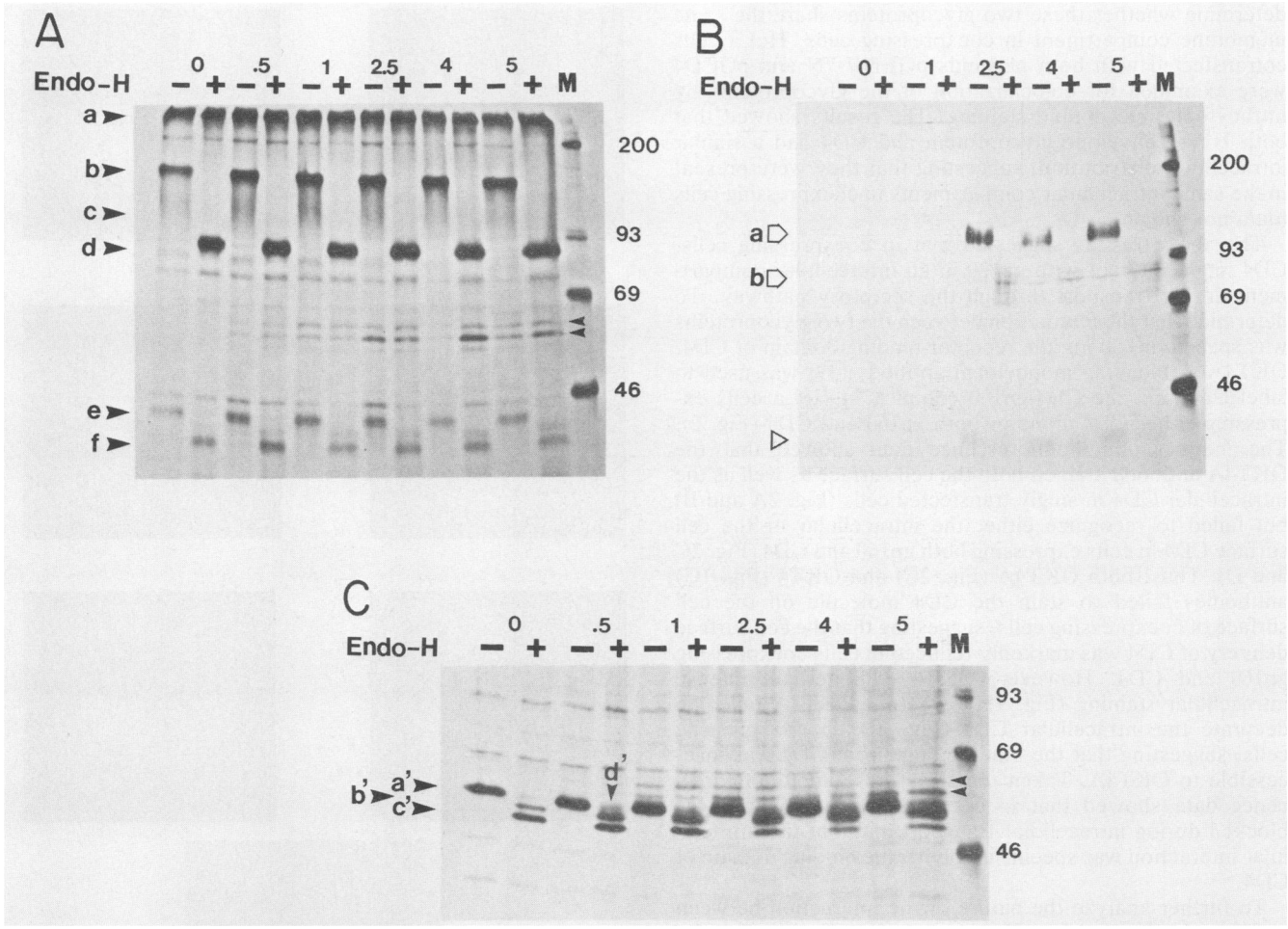

FIG. 3. (A) Synthesis and processing of the HIV-1 (ARV-2) envelope glycoproteins. At 16 h posttransfection, the transfected HeLa cells were starved in methionine and cysteine-free medium, pulse-labeled in [^{35}S]cysteine (0.25 mCi/ml, 1,000 Ci/mmol; Amersham, Arlington Heights, Ill.) and [^{35}S]methionine (Translabel; 0.25 mCi/ml; 1,000 Ci/mmol; ICN, Irvine, Calif.) for 30 min, and chased for different times in the presence of excess unlabeled cysteine and methionine. The cells were lysed in 0.5 ml of RIPA buffer (50 mM Tris hydrochloride [pH 7.6], 150 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride), and the lysates were incubated with 50 μl of Pansorbin (Calbiochem, La Jolla, Calif.) for 30 min on ice. The antibodies (4 μl, anti-gp41 or human AIDS serum; 5 μl, OKT4 or OKT4A) were added to the clarified cytoplasmic lysates and incubated on ice for 2 h. Protein A-Sepharose (5 μg) (Pharmacia, Piscataway, N.J.) mixed in RIPA buffer was added to the antigen-antibody complex and shaken overnight in the cold room. The immune complex was collected by spinning the Sepharose beads and washing them three times in the RIPA buffer containing 500 mM NaCl and treated with 10 mU of endo-H (+) or mock treated (−) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13). Arrowheads indicate the aggregate form of the HIV-1 envelope glycoprotein (a), gp160 (b), gp120 (c), deglycosylated gp160 (d), gp41 (e), and deglycosylated gp41 (f). M, Marker proteins in kilodaltons. The numbers at the top of the gel denote the time of chase in hours. (B) Secretion kinetics of the extracellular gp120. The transfected HeLa cells were pulse-labeled and chased at different times as described above. Cell culture medium was collected at each time point, filtered (Amicon Co., Danvers, Mass.) by spinning at 5,000 rpm (Beckman TJ-6) for 15 to 20 min, immunoprecipitated with the human AIDS serum, and treated (+) or not treated (−) with endo-H. To the left of the gel, a indicates secreted gp120 and b indicates heterogenous deglycosylated gp120. △, An unidentified 39,000-molecular-weight protein. The numbers at the top of the gel denote the time of chase in hours. (C) Synthesis and processing of the CD4 molecule. HeLa cells transfected with 20 μg of the plasmid pGCD4 were pulse-labeled for 30 min and chased at different time periods in the presence of excess unlabeled methionine and cysteine. The cytoplasmic lysates were immunoprecipitated with the OKT4 antibody, and the immunoprecipitates were treated with endo-H (+) or mock treated (−). The arrowheads indicate: glycosylated CD4 molecule (a′), partially endo-H-resistant CD molecule (b′), completely endo-H-sensitive CD4 molecule (c′), and completely endo-H-resistant CD4 molecule (d′). The two arrowheads on the right side indicate nonspecific, polypeptides that were coprecipitated either by the anti-CD4 or anti-gp41 antibodies (see also Fig. 4A and C and Fig. 5A, B, and C). M, Marker proteins. The numbers at the top of the gel denote the time of chase in hours.

only one of the two sites (26) predominantly acquired complex sugar modifications. However, even at the end of the 5-h chase, a minor fraction of CD4 (≈10%) still remained endo-H sensitive.

To study the in vivo interaction of HIV-1 envelope glycoprotein with CD4, doubly transfected HeLa cells were pulse-labeled, chased, and analyzed for complex formation. Results showed that HIV-1 envelope glycoprotein and CD4 formed specific heterocomplexes which were immunoprecipitated by either the anti-gp41 (Fig. 4A) or anti-CD4 (OKT4) antibodies (Fig. 4C) but not by OKT4A, the blocking antibody (Fig. 5C). The pulse-chase analyses showed that in cotransfected cells, the two glycoproteins interacted with each other, forming specific protein complexes, and

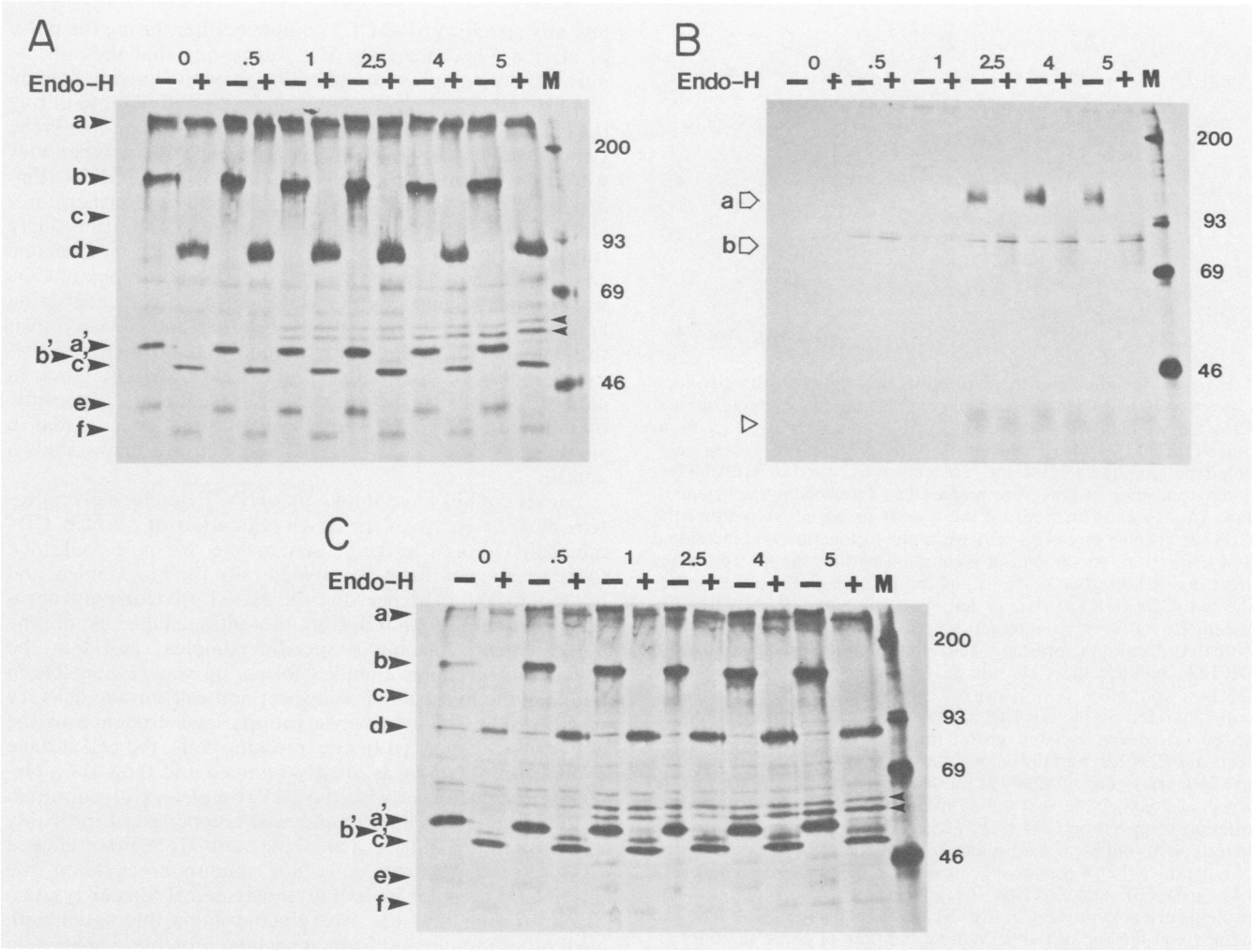

FIG. 4. (A) Analysis of the intracellular HIV-1 envelope glycoprotein-CD4 complex immunoprecipitated by the anti-gp41 antibody. HeLa cells cotransfected with 20 μg each of pGENV-7N and pGCD4 were pulse-labeled for 30 min and chased for different time periods as described in the legend to Fig. 3. The cytoplasmic lysates were immunoprecipitated with the anti-gp41 antibody, and the immunoprecipitates were treated with endo-H (+) or mock treated (−). Arrowheads indicate the aggregate form of the HIV-1 envelope glycoprotein (a), gp160 (b), gp120 (c), deglycosylated gp160 (d), glycosylated CD4 (a′), partially endo-H-resistant CD4 (b′), completely endo-H sensitive CD4 (c′), gp41 (e), and deglycosylated gp41 (f). M, Marker proteins in kilodaltons. The numbers at the top of the gel denote the time of chase in hours. (B) Secretion kinetics of the extracellular gp120 in coexpressing HeLa cells. HeLa cells transfected with the plasmids pGENV-7N and pGCD4 were pulse-labeled for 30 min and chased for different time periods as described in the legend to Fig. 3. The cell culture medium, collected at each time point, was immunoprecipitated with the AIDS serum, and treated (+) or not treated (−) with endo-H. To the left of the gel, a indicates secreted gp120 and b indicates heterogenous deglycosylated gp120. △, An unidentified 39,000-molecular-weight protein. M, Marker proteins. The numbers at the top of the figure denote the time of chase in hours. (C) Analysis of the intracellular HIV-1 envelope glycoprotein-CD4 complex molecule immunoprecipitated by the OKT4 antibody. One-half of the lysates, prepared for the experiment described in the legend to Fig. 4A, was immunoprecipitated with the OKT4 antibody, and the immunoprecipitates were treated with endo-H (+) or mock-treated (−) as described in the legend to Fig. 4A. The arrowheads indicate position of the aggregate form of the HIV-1 envelope glycoprotein (a), gp160 (b), gp120 (c), deglycosylated gp160 (d), glycosylated CD4 (a′), partially endo-H-resistant CD4 (b′), deglycosylated CD4 (c′), gp41 (e), and deglycosylated gp41 (f). M, Marker proteins. The numbers at the top of the gel denote the time of chase in hours.

that the gp160 in the complex remained endo-H sensitive (Fig. 4A, arrowheads b and d) as in singly transfected HeLa cells (Fig. 3A, arrowheads b and d). However, the intracellular transport and the glycosidic modifications of CD4 were dramatically altered in coexpressing cells. A major fraction (85 to 90%) of CD4 in the complex remained totally endo-H sensitive during the entire 5-h chase (Fig. 4A and C, arrowhead c′), suggesting that the majority of the CD4 molecules failed to traverse the secretory pathway in coexpressing cells and were predominantly localized in the ER. A minor fraction (5 to 10%) of CD4 in the complex, however, had acquired a partial endo-H resistance (Fig. 4A and C, arrowhead b′) compared with 90 to 95% of the CD4 molecule acquiring a partial endo-H resistance in singly transfected HeLa cells at the end of 5-h chase (Fig. 3C, arrowhead b′). Moreover, in addition to the precursor polyprotein gp160 and CD4 in the complex, the anti-gp41 or OKT4 antibodies also immunoprecipitated gp120-gp41 heterodimers (Fig. 4A and C, arrowheads c and e) as well as the aggregate forms of HIV-1 envelope glycoprotein that remained at the top of the gel (Fig. 4A and C, arrowhead a). Coprecipitation of gp120-gp41 with CD4 suggested that a fraction of CD4 might exist as gp120-gp41-CD4 ternary complexes (Fig. 4A and C, arrowheads c, a′, and e).

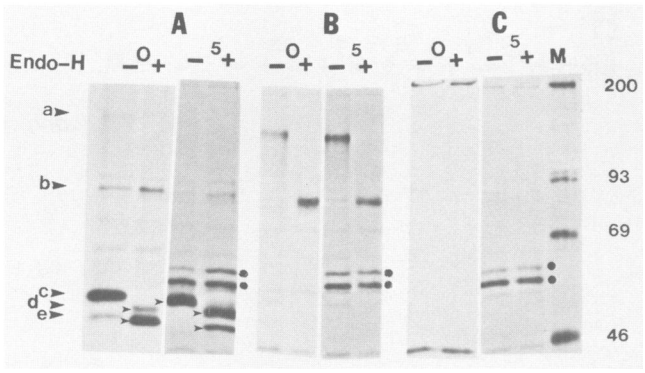

FIG. 5. Specificity of in vivo interaction between the envelope glycoprotein and CD4. HeLa cells expressing either the gp160 and CD4 individually or coexpressing gp160 and CD4 together were pulse-labeled (30 min) and chased in the presence of excess unlabeled methionine and cysteine. The cells were lysed in RIPA buffer, and cytoplasmic lysates were prepared as described in the legend to Fig. 3A. Samples (0.25 ml) of the lysates prepared from either the CD4- or gp160-expressing cells were mixed together and incubated at 4°C for 10 h. At the end of incubation period, the mixed lysates were divided into two portions and immunoprecipitated with either the anti-CD4 (OKT4) (A), or anti-gp41 antibody (B). To test the specificity of in vivo interaction, the cytoplasmic lysates were prepared from coexpressing cells and immunoprecipitated with OKT4A. Neither the CD4 nor gp160 was immunoprecipitated by OKT4A antibodies (C). However, nonspecific polypeptides were coprecipitated by the OKT4A antibodies. The arrowheads indicate gp160 (a), deglycosylated gp160 (b), CD4 (c), partially endo-H-resistant CD4 (d), and deglycosylated CD4 (e). M, Marker proteins in kilodaltons. The numbers at the top of the gel denote the time of chase in hours. Note that unlike the results in Fig. 4, only a small fraction of gp160 or CD4 (<10%) was immunoprecipitated by the heterologous antisera. Two solid circles on the right side indicate the polypeptides that were nonspecifically coprecipitated by anti-OKT4 (A), gp41 (B), and OKT4A (C) antibodies. Note that the same proteins are also present in Fig. 3A and C and Fig. 4A and C. The numbers at the top of the gel denote the time of chase in hours.

To determine whether interactions between the two glycoproteins were occurring in vitro (30) during the lysis and immunoprecipitation, labeled proteins from individually transfected cells were mixed in vitro and immunoprecipitated. Figure 5A and B show the results of immunoprecipitation of the in vitro mixed proteins by either anti-CD4 antibodies (Fig. 5A) or anti-gp41 (Fig. 5B). The anti-gp41 antibody precipitated predominantly gp160, with only a minor amount of CD4 coprecipitating with gp160 (Fig. 5B, arrowhead c). Similarly, in a reciprocal experiment, OKT4 antibody also precipitated mainly CD4, with only a small fraction of gp160 coprecipitating with CD4 (Fig. 5A, arrowhead a). Thus, unlike the predominant complex formation observed in vivo in coexpressing cells (Fig. 4A and C), the in vitro complex formation, under the conditions of lysis and immunoprecipitation used here, was very inefficient and represented only a minor fraction of both gp160 and CD4 (Fig. 5A and B). Therefore, in coexpressing cells, the complex formation between the two proteins must have occurred in vivo before the lysis of cells. Moreover, the difference in the maturation kinetics and glycosylation of CD4 in singly transfected (Fig. 3C) and cotransfected (Fig. 4A and C) cells could not be accounted for by the in vitro complex formation.

To further determine the specificity of in vivo complex formation, labeled proteins in doubly transfected HeLa cell lysates were analyzed by OKT4A. OKT4A did not precipitate any specific gp160-CD4 complex either during the pulse or after a 5-h chase (Fig. 5C), suggesting that the specific intracellular complex formation involved the binding domain of CD4 and that the complex remained tightly bound during the lysis of cells, thereby masking the OKT4A epitope on the CD4 molecule. However, the same nonspecific polypeptides were brought down by anti-gp41 (Fig. 5B) and OKT4 (Fig. 5A) as well as OKT4A (Fig. 5C) antibodies. Furthermore, OKT4A was able to immunoprecipitate CD4 from singly transfected cells (data not shown). Thus, these biochemical analyses have demonstrated that the transport block of CD4 was primarily due to a specific intracellular interaction between the HIV-1 envelope glycoprotein and CD4 in transit through the intracellular membrane compartments. However, under the conditions of the CD4 transport block in coexpressing cells, the kinetics of secretion and glycosidic modifications of gp120 were essentially the same as that in singly transfected cells (Fig. 4B and Fig. 3B, arrowheads a and b).

Earlier studies have shown that HIV-1 envelope glycoprotein may be involved in down-regulation of surface CD4 molecule (14, 42). In the present article, we have confirmed these results and further provided both the biochemical and immunological evidence that the HIV-1 envelope glycoprotein interacted with CD4 in the intracellular membrane compartment, forming a specific complex, and that the specific intracellular complex formation was responsible in blocking the intracellular transport and cell surface delivery of CD4. The following observations lend credence to the above conclusions. (i) In coexpressing cells, the cell surface expression of CD4 was greatly reduced and the CD4 molecule was colocalized with the HIV-1 envelope glycoprotein in the same intracellular membrane compartment (probably in the ER). (ii) OKT4A, a blocking antibody, neither labeled CD4 in coexpressing cells nor immunoprecipitated the gp160-CD4 complex in doubly transfected HeLa cell lysates, demonstrating that the two glycoproteins interacted with each other in vivo and formed specific protein complexes in the intracellular membrane compartment masking the OKT4A epitope of CD4 in the complex. (iii) CD4 formed intracellular heterocomplex with the HIV-1 envelope glycoprotein and the heterocomplex was immunoprecipitated either by the anti-gp41 or anti-CD4 (OKT4) antibodies. (iv) Since a major fraction of CD4 in the envelope glycoprotein-CD4 complex acquired predominantly the endo-H-sensitive high-mannose oligosaccharides, the primary transport block appeared to be in the ER, where the precursor polyprotein gp160 was predominantly localized. Furthermore, the reduction in complex sugar modification or cell surface transport of CD4 was not due to a global transport block or inhibition of complex glycosylation processes because neither transport nor glycosylation of vesicular stomatitis virus G protein was affected in gp160-expressing cells (data not shown). Taken together, these data show that the CD4 transport block was mainly due to the formation of specific complexes between gp160 and CD4 and that the gp160-CD4 binary complex remained predominantly localized in the ER, blocking the entry of CD4 into successive membrane compartments and thereby abrogating the delivery of CD4 to the cell surface.

It has been reported that in HIV-1-infected cells, the intracellular gp160 was routed to lysosomes and degraded in the compartment with a $t_{1/2}$ of 2 to 3 h (44). The pulse-chase analysis of the transiently expressed gp160, however, showed little or no intracellular degradation of gp160 either in the presence or absence of CD4 (Fig. 3A and Fig. 4A and

C). If the transiently expressed gp160 were sorted to the lysosomes at similar kinetics as in the case of HIV-1-infected cells without undergoing appreciable degradation, gp160 would have acquired at least partially endo-H-resistant oligosaccharides, indicative of the protein movement through the Golgi complex (18, 37). The failure to undergo such oligosaccharide modification indicates that gp160 was predominantly localized in the ER. Alternatively, it is possible that gp160 might be targeted to the lysosomes without undergoing extensive complex sugar modifications since the secreted gp120, which traversed the entire secretory pathway, was only partially endo-H resistant (Fig. 3B and 4B, arrow b). Thus, both gp160 and the gp160-CD4 binary complex were predominantly localized in a membrane compartment in which only high-mannose oligosaccharide modifications primarily occurred and did not undergo extensive degradation in lysosomes. The observed difference in the stability of gp160 in transfected cells compared with that in the HIV-1-infected cells might be due to the presence of other viral proteins as well as due to overexpression of gp160 in transfected cells.

The transmembrane glycoprotein E19 of adenovirus type 2, an ER resident protein (33, 34), binds to human leukocyte antigen class I antigens in the ER and blocks the intracellular movement and the cell surface expression of class I antigens (34). Adenovirus probably employs this mechanism to evade the immune surveillance by cytotoxic T cells in the infected host (34). Recently, Delwart and Panganiban (8) have shown that intracellular interaction of reticuloendotheliosis virus envelope glycoprotein with a cellular receptor in the ER rendered the infected or transfected cells refractory to superinfection by homologous virus strains. In the present study, we have provided evidence that the coexpression of gp160 with its receptor, CD4, dramatically altered the maturation and cell surface delivery of CD4. Further work is necessary to understand the molecular nature of CD4 block in the intracellular compartment as well as to understand the role of this phenomenon in the overall biology and pathogenesis of human immunodeficiency virus (e.g., superinfection interference and viral persistence).

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ADDENDUM IN PROOF

Conclusions very similar to those presented here were reached independently by B. Crise, L. Buonocore, and J. K. Rose (J. Virol. **64**:5585–5593, 1990).

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