VIROLOGY 220, 461–472 (1996)
ARTICLE NO. 0333

Mutational Analysis of HIV-1 gp160-Mediated Receptor

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Received February 8, 1996; accepted April 15, 1996

Formation of CD4–gp160 intracellular complexes represents an important mechanism leading to the induction of receptor interference. Previous studies have demonstrated that cells coexpressing gp160 and CD4 formed complexes of CD4 and gp160 which became blocked within the endoplasmic reticulum (ER), preventing CD4 from reaching the cell surface. In this report we have investigated the domains and residues of CD4 and gp160 involved in intracellular interaction. Accordingly, we have introduced mutations in both CD4 and gp160 at sites previously shown to disrupt CD4–gp120 interactions at the cell surface. Using a T7-vaccinia virus transient expression system, we expressed these gp160 and CD4 mutants in HeLa cells and analyzed their effects on intracellular complex formation and CD4 surface modulation. We observed that a number of gp160 mutants which failed to interact with CD4 at the cell surface also failed to bind and trap CD4 within the ER as expected. However, mutations at a critical residue, W427, did not abrogate intracellular CD4 binding. These gp160 mutants continued to interact with intracellular CD4 and inhibit CD4 transport to the cell surface, although gp120 produced from these mutants did not bind CD4 at the cell surface as expected. A number CD4 mutants also continued to form intracellular complexes with gp160, resulting in the loss of CD4 surface expression. Again, these CD4 mutants did not bind to gp120 at the cell surface, consistent with earlier reports. These results demonstrate that intracellular interactions between gp160 and CD4 in the ER may utilize different contact sites compared to those used during CD4 and gp120 binding at the cell surface. The data provide further evidence that the environment in which CD4 and the HIV-1 envelope glycoprotein interact can have a significant effect on their interaction.

INTRODUCTION

The down-regulation of CD4 cell surface expression following HIV infection, known as receptor interference, is required for induction of superinfection immunity, which is associated with the establishment of a persistent, noncytopathic infection and can result in the loss of functional T cells without cell death. Despite its potential importance, little is known of the molecular events governing this process. This down-regulation of CD4 has been shown to occur by several mechanisms acting at the level of transcription, translation, and posttranslation (Hoxie *et al.,* 1986; Stevenson *et al.,* 1988; Yuille *et al.,* 1988). Mechanisms which act posttranslationally are the best characterized to date. Studies have shown that two viral proteins, Nef and gp160, act independently to down-regulate CD4 from the surface of infected cells (Anderson *et al.,* 1993; Kawamura *et al.,* 1989). Nef-mediated CD4 down-regulation is independent of effects on transcription or translation and does not affect the normal maturation or transport of CD4 to the cell surface. Instead, Nef acts on CD4 present at the cell surface to mediate its internalization and lysosomal degradation (Bour *et al.,* 1995; Sanfridson *et al.,* 1994). Nef sequences important for CD4 down-regulation have not been precisely defined; however, N-terminal myristolation and the first 20 amino acids of Nef have been shown to be essential (Greenway *et al.,* 1994). Conversely, mutational analysis of CD4 has demonstrated that the membrane-proximal 20 amino acids of the CD4 cytoplasmic domain, including a dileucine motif at positions 413 and 414, are necessary and sufficient for CD4 internalization by Nef (Aiken *et al.,* 1994; Bour *et al.,* 1995).

In contrast to Nef, posttranslational down-regulation of CD4 by gp160 is mediated by blocking the normal maturation and transport of CD4 to the cell surface. The envelope glycoprotein binds to CD4 within the endoplasmic reticulum (ER) soon after synthesis, with a $t_{1/2}$ of approximately 15 min (Earl *et al.,* 1991a). This complex becomes blocked within the ER, resulting in the depletion of CD4 at the cell surface. In HIV-infected cells, CD4 trapped in the ER is further degraded by Vpu, another HIV accessory protein, thus ensuring that the blocked CD4 does not escape and reach cell surface (Earl *et al.,* 1991b; Willey *et al.,* 1992a,b). This loss of CD4 surface expression can be seen following productive infection of T cell and monocytic cell lines *in vitro* and is accompanied by the accumulation of intracellular complexes of viral glycoproteins and newly synthesized CD4 (Geleziunas *et al.,* 1991; Hoxie *et al.,* 1986; Stevenson *et al.,*

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0042-6822/96 $18.00

1987). The extent of CD4 surface depletion caused by the formation of such complexes directly correlates with levels of viral activation and production of gp160 (Bour *et al.,* 1995; Kawamura *et al.,* 1989). The role of gp160 in the down-modulation of CD4 has been confirmed by a number of laboratories. Lymphocytic and monocytic cells, engineered to express gp160, contain intracellular CD4–gp160 complexes and show reduced levels of CD4 expression at the cell surface (Geleziunas *et al.,* 1994; Stevenson *et al.,* 1988). Studies by us and others have shown that HeLa cells transfected with both CD4 and gp160 expressing constructs produce intracellular CD4–gp160 complexes in the ER, which are blocked in transport (Crise *et al.,* 1990; Jabbar and Nayak, 1990; Kawamura *et al.,* 1989).

Although much information is available about the nature of the gp120–CD4 interaction at the cell surface, little is known about the nature of intracellular interaction. Based on results from several labs, it is now accepted that the majority of residues important for binding CD4 are located in conserved regions within the C-terminal half of gp120, from C2 to C4 domains (Lasky *et al.,* 1987; Pollard *et al.,* 1992). Mutations of single amino acids in the C3 and C4 domains profoundly impair CD4 binding and viral infectivity. Both conservative and nonconservative substitutions of a typtophane residue at position 427 (C4 domain) have been shown to completely abrogate binding (Cordonnier *et al.,* 1989), while substitutions at amino acids 368, 370 (C3 domain), and 457 (C4 domain) severely impair CD4 binding without disrupting the conformation of gp120 (Olshevsky *et al.,* 1990).

Similarly, a great deal of work has been done to elucidate the gp120 binding site on CD4. It has been shown that the membrane distal domain of CD4, D1, contains all the residues required for binding to gp120. Further analysis suggests that the majority of important residues are within the CDR2 domain (aa 26–57) of D1 (Richardson *et al.,* 1988). Epitope mapping of CD4 substitution mutants with monoclonal antibodies confirms that amino acids 41–52 are involved in gp120 binding (Sattentau *et al.,* 1989). In addition, substitution of amino acids from the mouse CD4 (L3T4) D1 domain onto human CD4 demonstrates that residues 38–57 are indispensable for gp120 binding at the cell surface (Landau *et al.,* 1988). Recently, more precise mutagenesis studies have confirmed that substitution and insertions between residues 41 and 59 result in decreased binding to gp120 without disrupting CD4 conformation (Arthros *et al.,* 1989). In this report we have examined if the residues of CD4 and gp160 involved in their interaction at the cell surface are also involved in their intracellular interaction in the ER.

MATERIAL AND METHODS

Cells, viruses, and antibodies

HeLa cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in Minimal Essential Media (MEM, Gibco-BRL) supplemented with 10% fetal bovine serum (FBS), 250 U of penicillin, and 250 μg of streptomycin per milliliter. HeLa T4 cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Richard Axel (Maddon *et al.,* 1986) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. HeLa env c cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Ruth Ruprecht (Gama Sosa *et al.,* 1989).

Recombinant vaccinia virus (vTF7.3) encoding the bacteriophage T7 RNA polymerase was kindly provided by B. Moss, NIH.

OKT4 ascites fluid was obtained from C57 black mice injected with OKT4 hybridoma cells provided by Dr. William Clark, UCLA. Mouse anti-gp41 monoclonal antibody was purchased from NEN research products (Boston, MA). Sheep anti-gp120 serum was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Michael Phelan.

Mutagenesis

Construction of plasmids encoding gp160 (pGENV-7N) and CD4 (pGCD4) has been described previously (Jabbar and Nayak, 1990). In order to introduce mutations, pGCD4 and pGENV-7N encoding CD4 and gp160, respectively, were restricted with *Eco*RI and *Bam*HI and 1.7- and 2.8-kb fragments were subcloned into M13 mp18. Mutations were introduced by site-directed mutagenesis using the Sulptor *in vitro* mutagenesis system (Amersham) and the following synthetic oligonucleotides. For gp160 mutants, 257T-R: GCCATTTAACAGCAGTTGCCTTGACACTATTGGCC; 368D-R: GCATTACAATTTCTGGGCGCCCTCCTGAGG; 370E-R: GCATTACAAATTCTTGGGTCCCCTCC; 457D-A: CATTTGTACCACCAGCTCTTGTTAATAGCAGCCCTGTAATATTTG; 427W-S: CCTACTTCCTGACTATGTTTAT; 427W-V: CCTACTTCCTGAACCATGTTTAT; 427ΔMWQ: GCTTTTCCTACTTCGTTTATAATTTGTTTTA.

For CD4 mutants, 43F-V: CCTTTAGTTAAGACGGAGCCCTGATTT; 52[F]53: GCTTCTTCTTGAGTCAGCGGATCAAAATTCAGCAG; 55A-F: GGCTTCTTCTTGAGTCAAAGCGATCATTC.

Following mutagenesis, RF from M13 mp18 clones were restricted with *Eco*RI and *Bam*HI and fragments encoding CD4 and gp160 were subcloned into pGEM-3 (Promega) under the control of the T7 polymerase promoter. All DNA constructs were sequenced to confirm the mutations and two independent clones of each mutated CD4 and gp160 fragment were used to ensure that spontaneous mutations distant from the desired mutations were not responsible for the observed phenotypes.

Expression, pulse–chase, immunoprecipitation, and endoglycosidase H (endo H) treatment

Approximately, 2×10^6 HeLa cells were layered on to 60-mm culture dishes the night before. These dishes were then infected with recombinant vaccinia virus vTF7.3 at a multiplicity of infection (m.o.i.) of 1 in 0.5 ml of phosphate-buffered saline (PBS) containing 0.68 m*M* Ca^{2+} and 0.50 m*M* Mg^{2+} (PBS^+) for 1 hr at 37°. The inoculum was then removed, and the cells washed once with PBS^+. The cells were then transfected with 15 μg of each plasmid DNA in 2 ml of MEM supplemented with 2.5% FBS using Lipofectin reagent (Gibco-BRL). At 14–16 hr posttransfection (hpt), cells were washed twice with MEM lacking cysteine and methionine, incubated in same medium for 30 min, and labeled for times indicated with 150 μCi/ml Translabel and 100 μCi/ml [^{35}S]-cysteine (ICN) in 1 ml MEM lacking cysteine and methionine. Cells were then washed twice with MEM, 2.5% FBS containing an excess of unlabeled methionine and cysteine and incubated in the same medium at 37° for the times indicated.

At times indicated, cells were washed once with ice-cold PBS^+ and lysed in 0.5 ml of lysis buffer [50 m*M* Tris–HCl (pH 7.6), 150 m*M* NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate] for 5 min on ice and nuclei were removed by centrifugation at 12,000 *g* for 3 min. Sodium dodecyl sulfate (SDS) was added to a concentration of 0.1% and the lysate precleared with 50 μl of Pansorbin (Calbiochem) on ice for 20 min. Antibodies (3 μl) were added to the clarified cytoplasmic lysates and incubated at 4° with shaking for 1 hr. Protein G–Sepharose (20 μl, Pharmacia) was added and the samples were incubated for 1 hr at 4° with shaking. Sepharose beads containing immune complexes were precipitated and washed three times with lysis buffer containing 0.1% SDS and 1 *M* NaCl. After the final wash, 20 μl of 0.1% SDS was added to the beads and boiled for 2 min, followed by addition of 180 μl water and an additional 2 min of boiling. Sepharose beads were removed by centrifugation, and each sample was split in half. One-half was incubated with 10 mU of endoglycosidase H (endo H, Boehringer-Mannheim) overnight at 37° and the other half was mock-treated. Immunoprecipitates were concentrated by Speed-Vac and analyzed by electrophoresis on SDS (0.1%) polyacrylamide (8%) electrophoresis gels (SDS–PAGE) (Laemmli, 1970).

Indirect immunofluorescence (IF)

HeLa cells (2×10^5) were grown on glass chamber slides (Nunc, Inc.) in MEM containing 10% FBS at 37° overnight. Cells were then washed with PBS^+ and incubated with vTF7.3 (m.o.i. 1) in PBS^+ for 1 hr at 37°. The cells were then transfected with 7.5 μg of each plasmid DNA in 2 ml of MEM, 2.5% FBS using Lipofectin Reagent (Gibco-BRL) and incubated for 12–14 hr. Cells were then washed twice with PBS^+, fixed in 4% formaldehyde solution for 10 min, and used for surface staining. For internal staining, fixed cells were washed with PBS^+ and permeabilized with 1% NP-40 for 5 min. Slides were incubated overnight at 4° in PBS^+ containing 0.2% gelatin (PBS-G). Cells were incubated with OKT4 or anti-gp41 (1:100) in PBS–G for 1 hr at 37°, and washed three times with PBS-G, followed by incubation with fluorescein-conjugated goat anti-mouse antibody (Cappel) diluted 1:100 in PBS-G for 1 hr at 37°. The slides were then washed three times in PBS-G and mounted in Gelvatol (Monsanto)–glycerol solution containing DABCO (Sigma). Transmission fluorescence microscopy was performed using a Nikon optiphot microscope (Nippon Kogaka K.K.).

CD4–gp120 binding assays

For determining the binding of mutant gp120, gp160 mutant constructs were transfected into HeLa cells (4×10^6) in 60-mm dishes. At 16 hpt cells were labeled with 150 μCi/ml of Translabel (ICN) and 100 μCi/ml of [^{35}S]-methionine for 2 hr at 37°. The 2 ml culture media containing labeled gp120 was collected and concentrated to approximately 100 μl using a Centricon-30 microconcentrator (Amicon) to remove free radioactive amino acids. Samples were then diluted to 500 μl with PBS^+ and incubated for 2 hr at 4° on HeLa cells with rocking. HeLa cells that were transfected with the wild-type (WT) CD4 encoding plasmid the day before expressed high levels of CD4. The unbound gp120 in the medium was removed, the dishes were washed twice with PBS^+, and the cell-bound gp120 was immunoprecipitated with sheep anti-gp120 and analyzed by SDS–PAGE as described above.

For determining the gp120 binding to mutant CD4, the CD4 mutant constructs were transfected into HeLa cells as described above. At 16 hpt the CD4 expressing cells were washed and incubated with radiolabeled wild-type gp120, which had been prepared as described above. Following a 2-hr incubation at 4° with rocking, the cells were then washed twice with PBS^+ and the bound gp120 was immunoprecipitated with sheep anti-gp120 and analyzed as described.

Syncytium assays

HeLa T4 or HeLa env c cells (2×10^6/60-mm dish) were grown overnight. Cells were then infected with vTF7-3 at an m.o.i. of 1 as described above. HeLa T4 or HeLa env c cells were then transfected with plasmids (15 μg) encoding gp160 mutants or CD4 mutants, respectively, using Lipofectin. Number of syncytia per high-powered field (HPF) was then measured at selected times. An average of five fields was recorded for comparison.

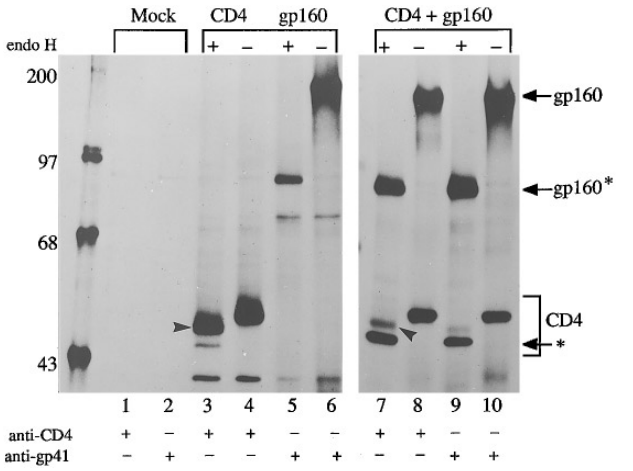

FIG. 1. Coexpression and coprecipitation of CD4 and gp160. HeLa cells were infected with recombinant vaccinia virus vTF7-3 (m.o.i. 1) and transfected with cDNAs encoding CD4 and gp160 or mock transfected. At 16 hpt cells were pulse-labeled for 30 min and then incubated in chase media for 3 hr prior to lysis. Cell lysates were divided in half and immunoprecipitated with OKT4 (lanes 1, 3 and 4, 7 and 8) or anti-gp41 (lanes 2, 5 and 6, 9 and 10). Immunoprecipitates were divided in half, and digested with 10 mU of endo H (+) or mock digested (−). Samples were analyzed by SDS–PAGE followed by fluorography. *Indicates endo H-sensitive proteins. Arrowheads indicate position of partially endo H-resistant CD4. Molecular weight markers (in thousands) are shown on the left. The DNAs used to transfect the cells are indicated above the lanes.

RESULTS

CD4 and gp160 form intracellular complexes

Previous studies have shown that intracellular CD4–gp160 complexes become blocked within the ER, preventing the transport of CD4 to the Golgi (Crise *et al.,* 1990; Jabbar and Nayak, 1990). To confirm these results, we expressed CD4 and gp160 alone or together in HeLa cells using the recombinant vaccinia virus T7 polymerase expression system (Fuerst *et al.,* 1987). Plasmid DNAs encoding gp160 and CD4 under the control of the bacteriophage T7 RNA polymerase promoter were transfected into HeLa cells that had been previously infected with recombinant vaccinia virus encoding the bacteriophage T7 RNA polymerase (1 m.o.i.). We observed that this system yields high levels of protein expression. Transfection efficiency was approximately 75–85%, and when plasmids encoding two different proteins are cotransfected using this procedure, there is greater than 95% coexpression (Crise *et al.,* 1990; Jabbar and Nayak, 1990).

HeLa cells transfected with plasmids encoding CD4 or gp160 or both gp160 and CD4 were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine for 30 min and then incubated in the presence of excess unlabeled cysteine and methionine for 3 hr. The cell lysates were immunoprecipitated with OKT4 (anti-CD4) or anti-gp41 antibody. OKT4 was used because it recognizes an epitope outside of the site of CD4–gp120 interaction. For this and all subsequent immunoprecipitations the amount of antibody used was determined to be in excess. Immunoprecipitations were then divided in half. One half was digested with endo H, the other half mock-treated, and the samples were analyzed by SDS–PAGE (Fig. 1).

Following 3 hr chase, the majority (>80%) of the CD4 expressed alone became endo H resistant as expected, indicating that CD4 was transported efficiently to the medial Golgi. CD4 has two N-linked glycans, one of which normally acquires resistance to endo H digestion due to the addition of complex sugars (Fig. 1, compare lanes 3 and 4). HIV gp160 expressed alone was also precipitated from lysates (Fig. 1, lanes 5 and 6). As expected, gp160 was sensitive to endo H digestion. The envelope glycoprotein has many N-linked glycans, and some of them attain resistance to endo H at the same time gp160 is cleaved to form gp120 and gp41.

When gp160 and CD4 were coexpressed, both proteins could be coprecipitated as a complex by either antibody to CD4 or gp160 (Fig 1, lanes 7–10). In addition, CD4 remained mostly endo H sensitive, indicating that CD4 transport into the Golgi was inhibited (compare lanes 7–10 with lanes 3 and 4). In these and subsequent experiments, unless stated otherwise, 15 μg of each DNA was used for transfection as this amount of DNA was found optimum for coexpression and complex formation using the WT constructs.

Mutations in gp160 and CD4 result in loss of gp120–CD4 binding in soluble assays

Mutations were made in both gp120 and CD4 at residues which have been shown previously to disrupt the interaction between CD4 and gp120 (Cordonnier *et al.,* 1989; Olshevsky *et al.,* 1990; Landau *et al.,* 1988; Arthros *et al.,* 1989). Mutations in gp160 included: a threonine to arginine substitution at amino acid 257, 257T-R (C2 domain); an aspartic acid to arginine substitution at amino acid 368, 368D-R (C3 domain); a glutamic acid to arginine substitution at amino acid 370, 370E-R (C3 domain); a tryptophan to serine and valine substitution at amino acid 427, 427W-S, 427W-V, and a deletion of MWQ, 427ΔMWQ (C4 domain), and an aspartic acid to alanine substitution at amino acid 457, 457D-A (C5 domain). For cell surface binding, we transfected plasmids encoding these gp160 mutants into HeLa cells which were labeled for 2 hr at 16 hpt. The labeled gp120, shed into the medium, was collected and used for binding to cells expressing CD4 on the surface as described under Materials and Methods. The results show that each of the mutant and WT gp160 plasmids shed large amounts of labeled gp120 into the medium. However, only the WT gp160 bound significantly (~30%) to CD4$^+$ HeLa cells. None of the other gp120 mutants bound to cell surface CD4 (Fig. 2A) as reported previously (Olshevsky *et al.,* 1990).

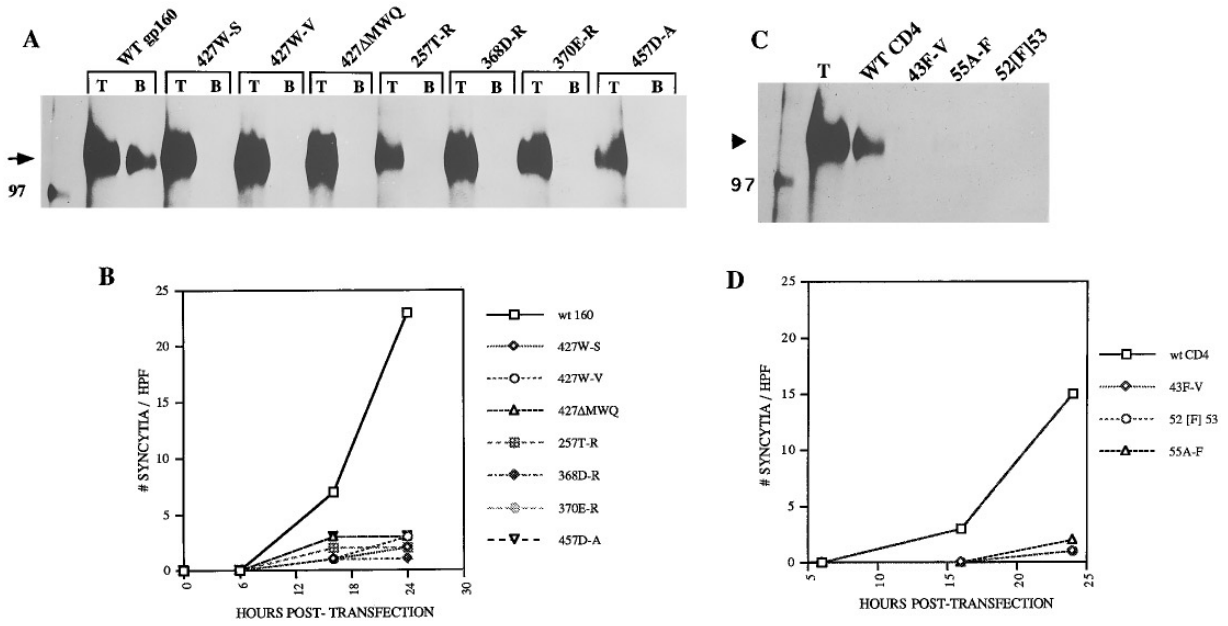

FIG. 2. Cell surface binding and syncytium formation of wild-type and mutant gp120 and CD4. (A) HeLa cells were infected with vTF7-3 (m.o.i. 1) and transfected with pGCD4. At 16 hpt the cells were washed twice with PBS^+ and incubated for 2 hr at 4° with PBS^+ solution containing labeled wild-type gp120 or mutant gp120 molecules prepared as described under Material and Methods. The unbound labeled gp120 was removed, and the cells washed twice with PBS^+, lysed, and immunoprecipitated for bound (B) gp120. Parallel samples were used for determining total (T) gp120. The position of gp120 is indicated by the arrow. (B) HeLa T4 cells were infected with vTF7-3 and transfected with plasmids encoding wild-type gp160 or each of the mutated gp160 molecules. At the indicated times, the number of syncytia were counted for 5 high-powered fields (HPF) and an average was plotted. (C) HeLa cells were infected with vTF7-3 and transfected with constructs expressing the WT or mutated CD4. At 16 hpt cells were washed twice with PBS^+ and incubated for 2 hr at 4° with 0.5 ml of a PBS^+ solution containing radiolabeled wild-type gp120 prepared as described under Material and Methods. The unbound labeled gp120 was removed, and cell lysates containing the bound (B) gp120 were immunoprecipitated. "T" represents the total gp120 (0.5 ml) used for the binding assay. CD4 molecules expressed in the dishes are indicated above the lanes. The arrowhead indicates the position of gp120. (D) HeLa env c cells were infected with vTF7-3 and transfected with plasmids encoding the WT or mutant CD4 molecules. At the indicated times the number of syncytia in 5 HPF were counted and the average was plotted.

Mutations in CD4 included a phenylalanine to valine substitution at amino acid 43, (43F-V), a phenylalanine insertion between amino acids 52 and 53 (52[F]53), and an alanine to phenylalanine substitution at amino acid 55 (55A-F), as these mutations have been shown to affect gp120 binding (Arthros *et al.,* 1989). To test the ability of these CD4 mutants to bind WT gp120, plasmids encoding CD4 mutants were transfected into HeLa cells. At 16 hpt, transfected cells were incubated with the radiolabeled WT gp120, prepared as described above. The bound gp120 was determined by immunoprecipitation with anti-gp120 antibody, and SDS–PAGE analysis (Fig. 2C). Again, only cells expressing the WT CD4 at the cell surface were able to bind the radiolabeled gp120, and none of the CD4 mutants at the cell surface bound gp120 (Fig. 2C), consistent with previous reports (Arthros *et al.,* 1989). All CD4 mutants were shown to express at the surface of transfected cells (Fig. 6).

Since syncytium formation requires interaction of cell surface CD4 with gp120, we evaluated the ability of these mutant glycoproteins to mediate syncytium formation (Figs. 2B and 2D). HeLa cells which constitutively express CD4 (HeLa $T4^+$) or gp160 (HeLa env c) were transfected with plasmids encoding gp160 mutants or CD4 mutants, respectively. At various times, the number of syncytia per HPF was noted for each of the constructs. The results demonstrated that only the WT gp120 and CD4 combination produced syncytia, while mutants were unable to induce syncytia, consistent with lack of CD4–gp120 interaction at the cell surface (Figs. 2B and 2D).

Mutations of gp160 that disrupt intracellular interaction with CD4

To determine if mutations made in gp160 affected the intracellular binding of CD4, HeLa cells were cotransfected with the WT CD4 expression plasmid, pGCD4, and with one of the mutant gp160 expression plasmids, 257T-R, 368D-R, 370E-R, or 457D-A. At 16 hpt the cells were pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine for 30 min, and chased for 3 hr. Aliquots of cell lysates were immunoprecipitated with either anti-CD4 or anti-gp41 antibodies. Precipitates were digested with or without endo H and analyzed by SDS–PAGE. The results demonstrated that these mutations in gp160 severely interfered with their ability to form CD4–gp160 complexes in the cytoplasm of cotransfected cells. For 257 T-R, 368 D-R, and 370 E-R mutants neither

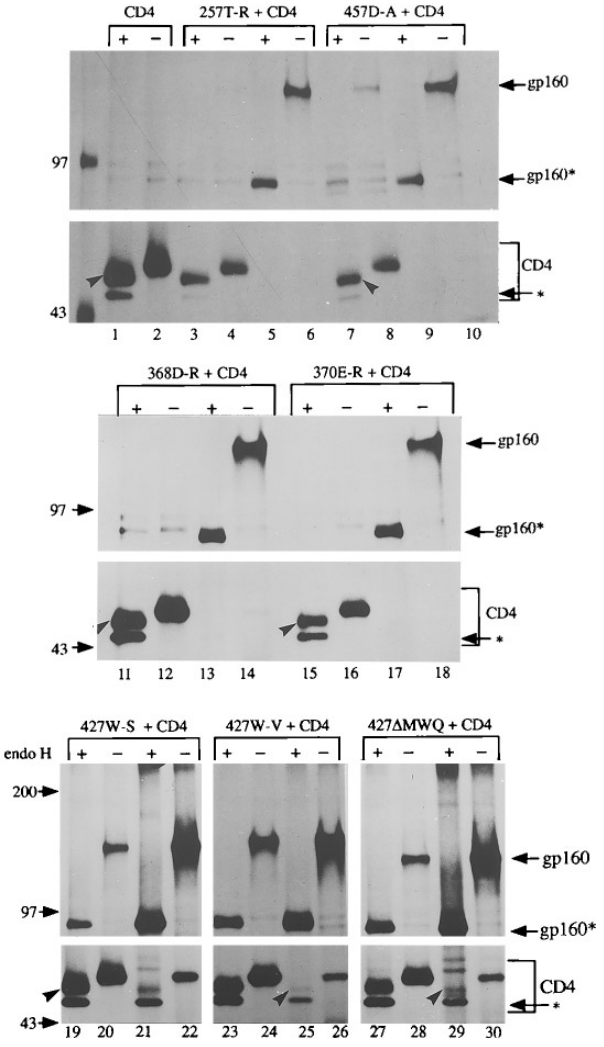

FIG. 3. Coexpression if mutant gp160 with wild-type CD4. Hela cells were infected with vTF7-3 (m.o.i. 1) and then transfected with constructs encoding CD4 alone or cotransfected with CD4 and gp160 mutants. At 16 hpt, cells were pulse-labeled for 30 min and incubated in chase media for 3 hr. Cell lysates were divided and immunoprecipitated using OKT4 (lanes 1–4, 7 and 8, 11 and 12, 15 and 16, 19 and 20, 23 and 24, 27 and 28) or anti-gp41 (lanes 5 and 6, 9 and 10, 13 and 14, 17 and 18, 21 and 22, 25 and 26, and 29 and 30) antibodies. Precipitates were divided in half and digested with 10 mU endo H for 16 hr at 37° (+) or mock digested (−). *Indicates endo H-sensitive proteins. Positions of molecular weight markers (in thousands) are shown at the right. The DNAs used to transfect the cells are indicated above the lanes. Arrowheads indicate position of partially endo H-resistant CD4.

anti-CD4 nor anti-gp41 antibodies coprecipitated gp160 or CD4, respectively (Fig. 3). For 457 D-A, anti-CD4 antibodies coprecipitated a small amount of gp160, but anti-gp41 antibodies did not coprecipitate detectable amounts of CD4 (Fig. 3). In addition, the CD4 precipitated in these cotransfected cells was found to be predominantly endo H resistant, suggesting that CD4 was efficiently transported. This lack of complex formation was not because the same cells were not cotransfected by both CD4 and mutant gp160 plasmids, as greater than 90% of cells were cotransfected and also differing the ratios of plasmid DNA in cotransfection did not affect the outcome. On the other hand, WT CD4 and gp160 cotransfection always demonstrated complex formation in these experiments (data not shown).

To determine if this lack of binding also resulted in the increased expression of CD4 at the cell surface, we investigated CD4 surface expression by indirect immunofluorescence (IF) (Fig. 4). HeLa cells grown on slides were transfected with plasmids encoding gp160 mutants alone or in combination with pGCD4. At 16 hpt, cells were fixed and then stained with OKT4 and anti-gp41 with and without permeabilization. Each of the mutant gp160 constructs, transfected alone or cotransfected with CD4, gave expression patterns and intensity of staining similar to those of the WT gp160 after permeabilization (data not shown), while the surface expression of CD4 upon cotransfection varied. CD4, expressed alone, resulted in strong staining at the cell surface (Fig. 4A), whereas CD4 coexpressed with the WT gp160 exhibited decreased surface expression as expected (Fig. 4B). However, the internal expression of CD4 in the cotransfected cells as determined by IF staining of permeabilized cells was not affected (data not shown). Coexpression of CD4 with each of the above gp160 mutants did not inhibit the surface expression of CD4 (Figs. 4C–4F). Taken together, these results suggest that residues 257, 368, 370, and 457 are critical for both cell surface and intracellular interaction between gp160 and CD4.

Mutations of gp160 at amino acid 427 do not completely disrupt gp160–CD4 intracellular complex formation

Tryptophane at position 427 (427W) is considered to be a crucial residue for gp120:CD4 interaction, as both conservative and nonconservative changes of this residue were shown to abrogate the ability of gp120 to interact with CD4 (Cordonnier *et al.,* 1989; Olshevsky *et al.,* 1990). We therefore made two substitutions (427W-S and 427W-V) and one deletion mutant at residue 427 (427ΔMWQ) and tested for their ability to bind and block CD4 within the ER. When these mutant gp160 constructs were coexpressed with the WT CD4 in HeLa cells, we observed that mutations at position 427 did not completely abrogate intracellular complex formation (Fig. 3). In each case, treatment with either OKT4 antibody (Fig. 3 lanes 19 and 20, 23 and 24, and 27 and 28) or anti-gp41 antibody (Fig. 3 lanes 21 and 22, 25 and 26, and 29 and 30) resulted in coprecipitation of CD4–gp160 complexes and the CD4 in the complex was predominantly endo H sensitive, suggesting that the complexed CD4 was blocked in transport (Fig. 3 lanes 21 and 22, 25 and 26, and 29 and 30). However, a significant fraction of the CD4 immunoprecipitated by CD4 antibody exhibited endo H resistance after 3 hr chase, indicating that some CD4 was able to reach the Golgi (Fig. 3 lanes 19

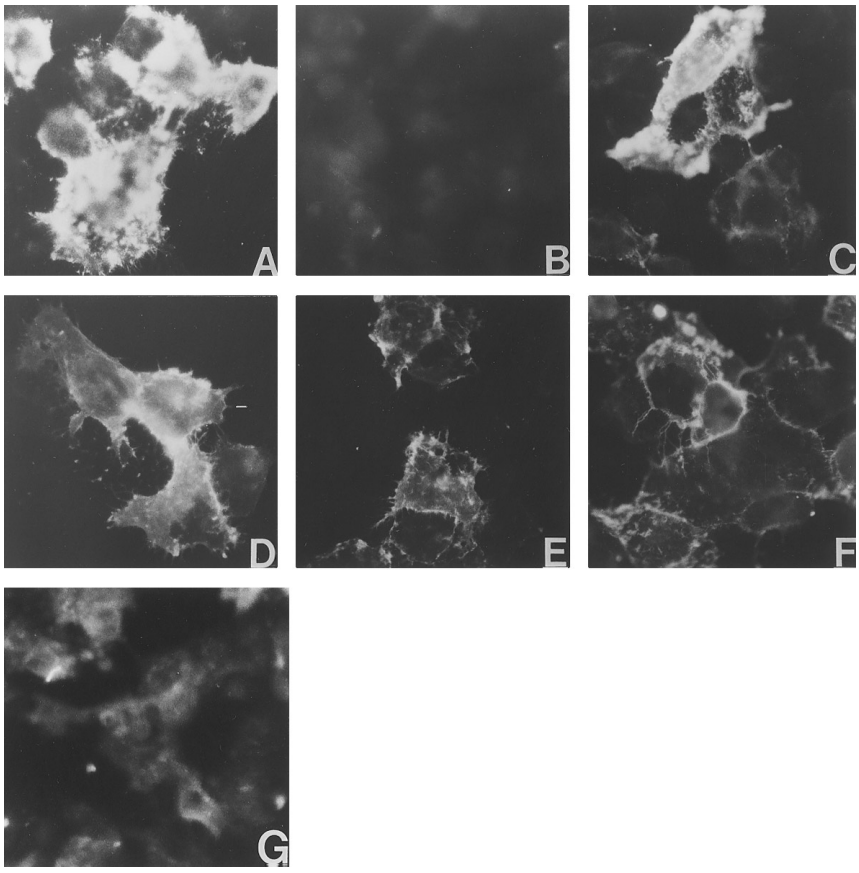

FIG. 4. Cell surface staining of CD4 in HeLa cells coexpressing CD4 and gp160 mutants. HeLa cells were grown on chamber slides for at least 12 hr. Cells were then infected with vTF7-3 and cotransfected with cDNAs encoding CD4 and the WT gp160 (A) or mutant gp160 (B–F). At 16 hpt, cells were fixed and surface stained for CD4 as described under Materials and Methods. (A) pGCD4; (B) pGCD4 + PGENV-7N; (C) pGCD4 + 257T-R; (D) pGCD4 + 368 D-R; (E) pGCD4 + 370E-R; (F) pGCD4 + 457 D-A; (G) pGCD4 + 427 W-V.

and 20, 23 and 24, and 27 and 28). Indirect immunofluorescence staining also confirmed that CD4 surface expression in these coexpressing cells was considerably reduced, but still a significant amount of CD4 was observed at the cell surface when compared to cells coexpressing WT gp160 and CD4 (Fig. 4G, 427 W-V). Similar results were obtained for 427 W-S and 427ΔMWQ (data not shown). The reason for this increased cell surface expression of CD4 on these coexpressing cells remains unclear. The possibility exists that the intracellular binding of the mutant gp160 was less efficient or less stable (compare Figs. 1 and 3) or that CD4 expression was more efficient, causing some CD4 to escape gp160 binding in these experiments. However, repeated experiments and varying the ratios of cotransfected DNAs yielded similar results.

Despite this increased cell surface CD4, it is clear that unlike lack of any interaction of these gp120 mutants with CD4 at the cell surface (Figs. 2A and 2B), a significant amount of intracellular complex formation was occurring between these gp160 gp427W mutants and WT CD4. These results demonstrate that variation exists between the intracellular complex formation and interactions at the cell surface. Whereas some residues are critical for both intracellular and cell surface binding, others are required predominantly for cell surface binding and not for intracellular interactions within the ER.

Mutations in CD4 do not disrupt intracellular binding

Three changes were made in the D1 domain of CD4 at positions which have been shown previously to be critical for binding to gp120, a phenylalanine to valine substitution at residue 43 (43 F-V), a phenylalanine insertion between residues 52 and 53 (52[F]53), and an alanine to phenylalanine substitution at position 55 (55A-F) (Arthros *et al.,* 1989; Landau *et al.,* 1988; Richardson *et al.,* 1988; Sattentau *et al.,* 1989). We also confirmed that these CD4 mutants were unable to bind the WT gp120 at the cell surface or cause syncytium formation (Figs. 2C and 2D). For determining their role in intracellular complex formation, these mutants were coexpressed with the WT gp160 constructs in HeLa cells, and at 16 hpt these cells were pulse-labeled for 30 min and chased 3 hr. Lysates were immunoprecipitated with either OKT4

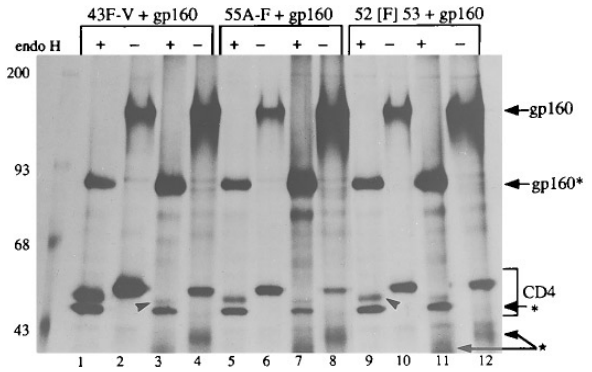

FIG. 5. Coexpression of mutated CD4 and wild-type gp160. HeLa cells were infected with recombinant vaccinia virus vTF7-3 (m.o.i. 1) and cotransfected with cDNAs encoding wild-type gp160 and mutated CD4. At 16 hpt, cells were pulse-labeled for 30 min chased for 3 hr prior to lysis. Cell lysates were divided in half and immunoprecipitated with OKT4 (lanes 1 and 2, 5 and 6, and 9 and 10) or anti-gp41 (lanes 3 and 4, 7 and 8, and 11 and 12). Immunoprecipitates were divided in half, and digested with 10 mU of endo H (+) or mock digested (−). Samples were analyzed by SDS–PAGE followed by fluorography. *Indicates endo H-sensitive proteins. Molecular weight markers (in thousands) are shown on the left. The DNAs used to transfect the cells are indicated above the lanes. Arrowheads indicate positions of partially endo H-resistant CD4. *Indicate gp41 molecules.

or anti-gp41 antibodies, treated with endo H, and analyzed by SDS–PAGE. The results demonstrated that unlike the complete lack of interaction at the cell surface, these CD4 mutants were unable to disrupt the ability of CD4 to interact with WT gp160 intracellularly (Fig. 5). In each case, CD4 and gp160 complexes could be precipitated with OKT4 or anti-gp41. Endo H treatment revealed that CD4 in the complex remained primarily endo H sensitive throughout the chase period, suggesting that CD4 mutants were blocked in the ER.

These results were confirmed by indirect IF. The CD4 mutants were expressed alone or coexpressed with WT gp160 in HeLa cells grown on glass slides. At 16 hpt the slides were fixed and stained with OKT4 antibody followed by fluorescein-conjugated goat anti-mouse antibody. The WT and mutant CD4 expressed alone reached the cell surface (Figs. 6A, 6C, 6E, and 6G). However, when coexpressed with the WT gp160, surface expression of the WT and mutant CD4 was markedly reduced (Figs. 6B, 6D, 6F, and 6H) although the intracellular staining of CD4 in these coexpressing cells was essentially similar (data not shown). These results were consistent with the formation of intracellular gp160–CD4 complexes leading to the modulation of CD4 surface expression. Taken together, these experiments indicate that mutations in CD4 that are critical for cell surface binding of gp120 do not disrupt intracellular binding with gp160.

Truncation of constructs containing mutations at amino acid 427 results in loss of intracellular binding

The result that mutations at position 427 of gp160 did not disrupt intracellular binding (Fig. 3) was surprising in light of the results obtained by others (Olshevsky *et al.,* 1990) and by us (Fig. 2). The possibility existed that the difference was due to the fact that previous studies were based on assays involving the interaction of membrane-bound CD4 with soluble gp120, and not between two membrane bound proteins as was the case within the ER. In an attempt to address this question, we introduced stop codons into each of the gp160 constructs at the junction between the ectodomain and the transmem-

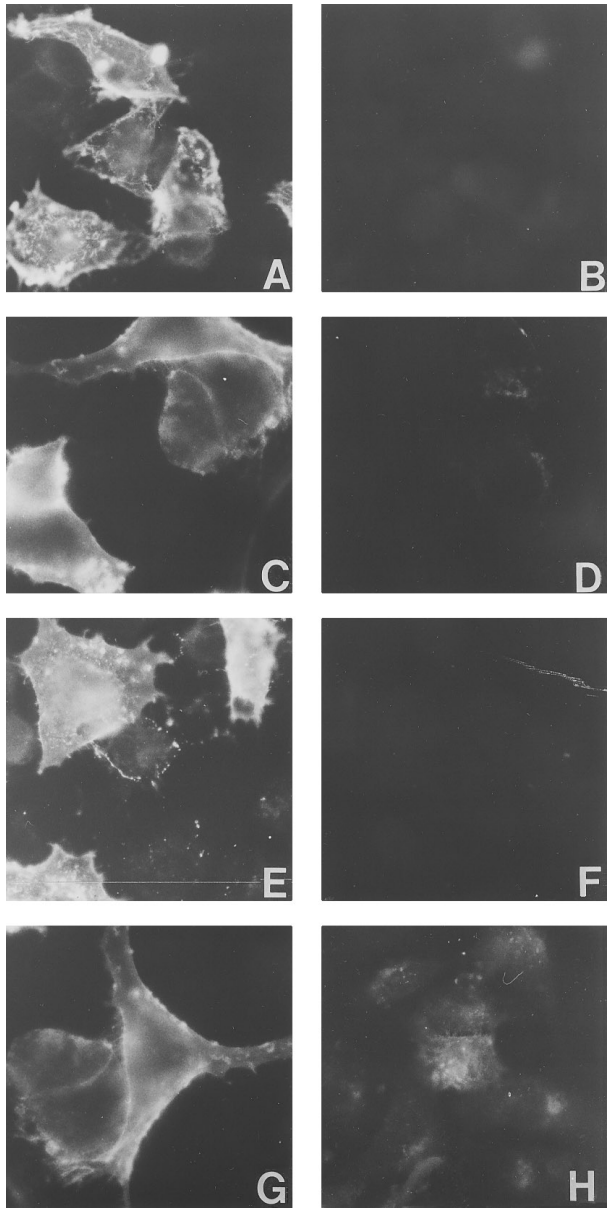

FIG. 6. Surface expression of CD4 mutants. HeLa cells were grown on chamber slides, infected with vTF7-3, and transfected with plasmids encoding WT and CD4 mutants alone (A, C, E, G) or cotransfected with WT gp160 plasmids (B, D, F, H). The cells were fixed with 4% formaldehyde and stained using OKT4 antibody, followed by FITC goat anti-mouse. (A) pGCD4, (B) pGCD4 + pGENV-7N, (C) 52[F]53, (D) 52[F]53 + pGENV-7N, (E) 55A-F, (F) 55A-F + pGENV-7N, (G) 43F-V, (H) 43F-V + pGENV-7N.

brane domain or at the junction between gp120 and gp41. These constructs are designated ΔTM and Δ41, respectively. ΔTM constructs encode gp120 plus the ectodomain of gp41; Δ41 constructs lack all gp41 sequences and therefore express only gp120.

Wild-type gp160Δ41 or gp160ΔTM constructs were cotransfected with pGCD4 encoding WT CD4 into HeLa cells for 16 hr. The cells were then pulse-labeled for 30 min and chased for 3 hr. Cell lysates were immunoprecipitated with OKT4 and anti-gp120 followed by endo H treatment. The results demonstrated that the WT CD4 and the truncated gp160 proteins formed complexes, albeit reduced, that can be coprecipitated by either antibody (Fig. 7A). gp160 constructs containing mutations at amino acid 427 were also truncated similarly and expressed with the WT CD4. Truncation of these mutants at the transmembrane domain (427W-SΔTM, 427W-VΔTM, and 427ΔMWQΔTM) did result in reduction of intracellular binding to CD4 (Fig. 7B), although in most cases some CD4–gp160 complexes could be precipitated from cell lysates. Therefore lack of membrane anchoring of gp160 mutants could be a factor in reduced interaction with, and transport block of, CD4 in the ER.

A more dramatic effect was seen when these gp160 mutants were truncated at gp41. 427W-SΔ41, 427W-VΔ41, and 427ΔMWQΔ41 express gp120 mutated at residue 427 as described earlier. When these constructs were coexpressed with CD4, gp120–CD4 complexes could not be precipitated by either OKT4 or anti-gp120 (Fig. 7C). These results suggest that one reason for the discrepancies observed in binding of gp160 and CD4 intracellularly versus extracellularly was due to the possible influence of gp41 sequences and/or membrane anchoring on gp120 conformation within the ER.

Truncation of CD4 mutants does not abrogate intracellular interactions

To determine if the influence of transmembrane or cytoplasmic domains was responsible for the results obtained with the CD4 mutants, we truncated the WT and mutant CD4 constructs. Stop codons were introduced into CD4 constructs at the junction between the D4 domain and the transmembrane region. These soluble CD4 constructs were cotransfected with WT gp160 constructs, and complexes precipitated as described (Fig. 8A). Truncation of the mutant CD4 molecules did not inhibit intracellular complex formation between CD4 and gp160. Complexes of soluble CD4 mutant proteins and WT gp160 were precipitated from cell lysates, and CD4 remained essentially endo H sensitive throughout the chase period. Thus, the presence or absence of transmembrane and cytoplasmic sequences of CD4 does not appear to explain the differences in results between extracellular and intracellular binding of these mutants.

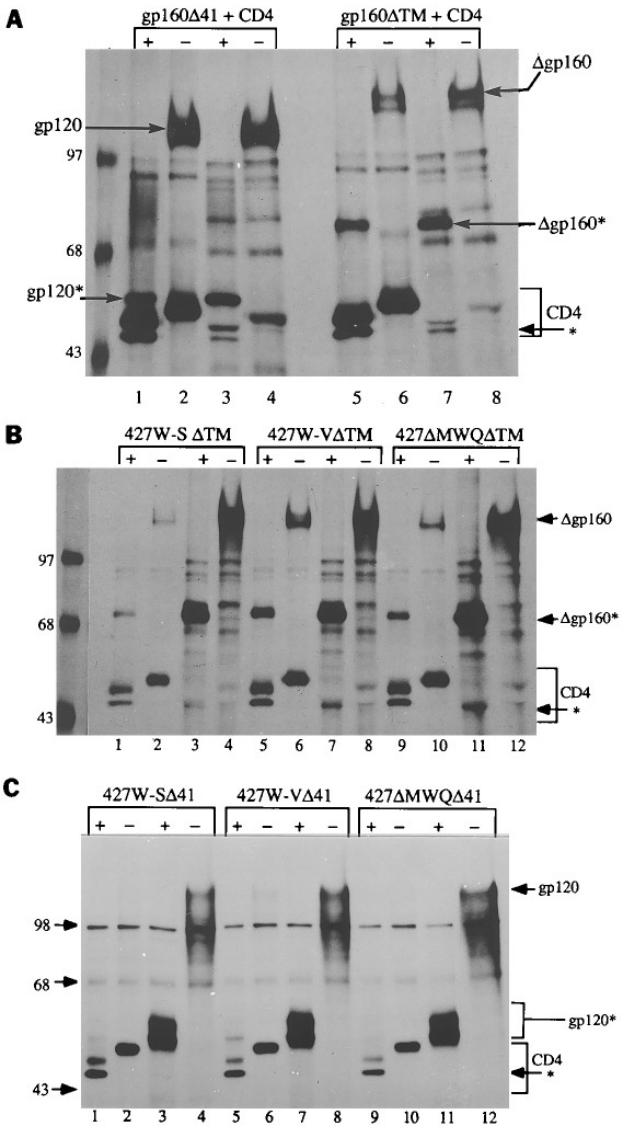

FIG. 7. (A) Coprecipitation of CD4 and soluble gp160 molecules. HeLa cells were infected with recombinant vaccinia virus vTF7-3 (m.o.i. 1) and transfected with cDNAs encoding CD4 and gp160Δ41 or gp160ΔTM. At 16 hpt, cells were pulse-labeled for 30 min and then incubated in chase media for 3 hr prior to lysis. Cell lysates were divided in half and immunoprecipitated with OKT4 (lanes 1 and 2, 5 and 6) or anti-gp120 (lanes 3 and 4, 7 and 8). (B) Coexpression and coprecipitation of the wild-type CD4 and soluble gp160 mutants with changes at amino acid 427. HeLa cells were infected with vTF7-3 and cotransfected with cDNAs encoding CD4 and soluble gp160 mutants (427W-SΔTM, 427W-VΔTM, 427ΔMWQΔTM). (C) Coexpression and coprecipitation of wild-type CD4 and soluble gp120 mutants (427W-SΔ41, 427W-VΔ41, 427ΔMWQΔ41). For panels B and C, cells were pulse-labeled for 30 min at 16 hpt, cell lysates were prepared, divided in half, and immunoprecipitated with OKT4 (lanes 1 and 2, 5 and 6, 9 and 10) or anti-gp120 (lanes 3 and 4, 7 and 8, and 11 and 12). Immunoprecipitates were divided in half and digested with 10 mU endo H (+) or mock digested (–). All samples were analyzed by SDS–PAGE followed by fluorography. *Indicates endo H-sensitive proteins. Molecular weight markers (in thousands) are shown on the left. The DNAs used to transfect the cells are indicated above the lanes.

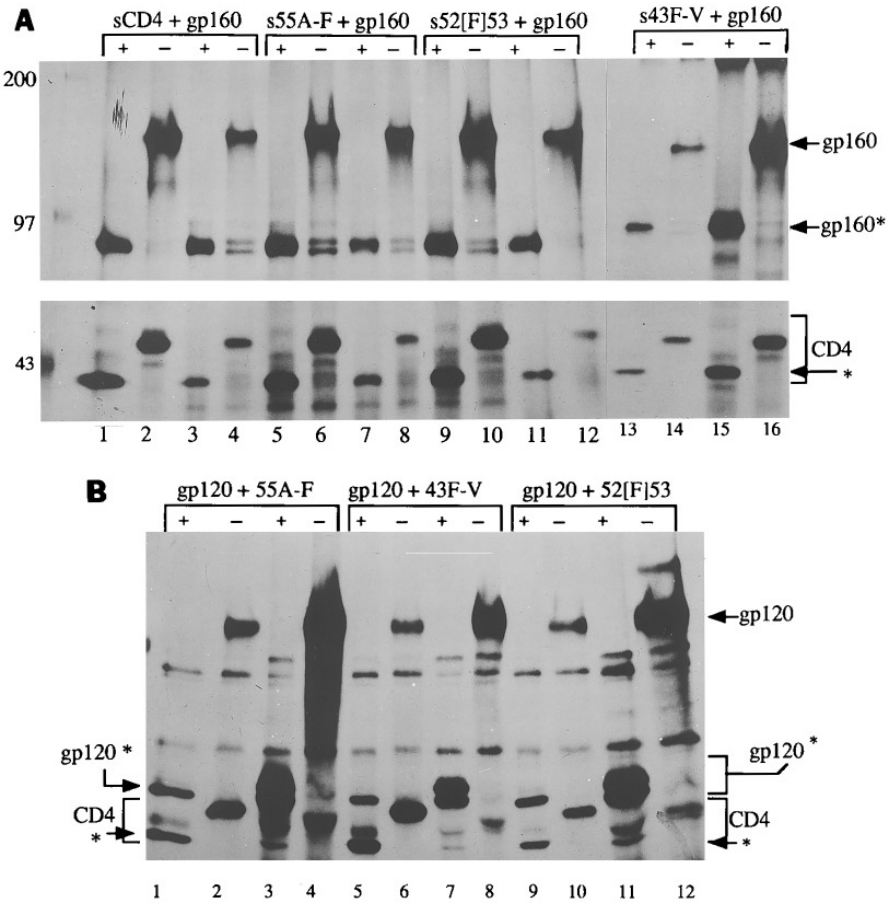

FIG. 8. (A) Coprecipitation of soluble CD4 mutants and wild-type gp160. HeLa cells were infected with recombinant vaccinia virus vTF7-3 (m.o.i. 1) and cotransfected with cDNAs encoding wild-type gp160 and soluble CD4 mutants. At 16 hpt, the cells were pulse-labeled for 30 min and then incubated in chase media for 3 hr prior to lysis. Cell lysates were divided in half and immunoprecipitated with OKT4 (lanes 1 and 2, 5 and 6, 9 and 10, and 13 and 14) or anti-gp41 (lanes 3 and 4, 7 and 8, 11 and 12, and 15 and 16). (B) Coprecipitation of CD4 mutants and wild-type gp120. HeLa cells were infected with recombinant vaccinia virus vTF7-3 (m.o.i. 1) and cotransfected with cDNAs encoding WT gp120 and CD4 mutants. At 16 hr posttransfection, the cells were pulse-labeled for 30 min. Cell lysates were divided in half and immunoprecipitated with OKT4 (lanes 1 and 2, 5 and 6, 9 and 10) or anti-gp120 (lanes 3 and 4, 7 and 8, and 11 and 12). Immunoprecipitates were divided in half and digested with 10 mU of endo H (+) or mock digested (−). Samples were analyzed by SDS–PAGE followed by fluorography. *Indicates endo H-sensitive proteins. The DNAs used to transfect the cells are indicated above the lanes. Molecular weight markers (in thousands) are shown on the left.

GP120 forms intracellular complexes with CD4 mutants

Last, we examined the ability of soluble gp120 to form intracellular complexes with each of the full-length CD4 mutants. We found that coexpression of the CD4 mutants with soluble gp120 resulted in the formation of intracellular complexes which can be precipitated by OKT4 or anti-gp120 (Fig. 8B). Thus, the ability of the CD4 mutants to form intracellular complexes with gp160 does not appear to be due to the differences between gp160 and gp120.

DISCUSSION

In this report we have examined whether mutations known to disrupt gp120–CD4 extracellular interactions can also disrupt gp160–CD4 intracellular interactions. We introduced mutations at selected sites in both CD4 and gp160 which were shown previously to abrogate gp120–CD4 binding and tested the ability of these mutants to bind in an intracellular assay. The results presented in this report suggest that there are differences in the way CD4 and gp160 interact within the ER when compared to CD4–gp120 binding at the cell surface.

We observed that mutations at residues 257, 368, 370, and 457 of gp160 disrupted both extracellular and intracellular interactions between gp160 and WT CD4. However, previous results by others have shown that both conservative and nonconservative mutations in the HIV envelope glycoprotein at position 427 result in the loss of CD4 binding in soluble assays (Cordonnier *et al.,* 1989; Olshevsky *et al.,* 1990). We confirmed these results, but we also found that mutation of this highly conserved tryptophan residue did not result in the abrogation of intracellular CD4–gp160 complex formation. Although we found some loss of intracellular CD4 binding with these mutants, CD4–gp160 complexes could be clearly

and specifically coprecipitated from the lysates of HeLa cells expressing both molecules (Fig. 3).

Similarly, when CD4 mutations known to disrupt CD4–gp120 interactions were tested, we found that these mutations did not result in the loss of gp160 binding within the cell. Taken together, these results suggest that the residues required for binding of gp120 to CD4 at the cell surface may not be as critical for CD4–gp160 interactions within the ER.

The reason for these differences is unclear at this time. In the case of gp160, it appears that gp41 sequences may influence the way in which gp120 binds to CD4. Many of the previous studies used soluble gp120 to evaluate the ability of mutants to bind CD4, an approach now considered to have significant limitations (Eiden and Lifson, 1992). When we expressed HIV envelope glycoprotein with mutations at amino acid 427 as soluble gp120 molecules, the ability of mutant gp120 to bind CD4 intracellularly was inhibited, consistent with earlier studies (Fig. 7C). Thus, we speculate that one explanation for the finding that changes at residue 427 do not have as great an effect on intracellular binding is due to the presence of gp41 sequences which can influence the conformation of gp120. Support for this hypothesis comes from the recent report that a single amino acid change in gp41 results in subtle conformational changes in epitopes present on gp120 (Thali *et al.,* 1994). This change resulted in the emergence of viral variants which were resistant to neutralization by sera that neutralized the parental virus. Additional support comes from reports which demonstrate that membrane binding via gp41 can impose conformational constraints on gp120, influencing its ability to bind CD4 (Moore *et al.,* 1992). Primary isolates, in contrast to culture-adapted strains, are resistant to neutralization by soluble CD4 (sCD4). This difference is due, in part, to decreased affinity of gp120 on primary isolates for sCD4. However, soluble gp120 (sgp120) from both types of isolates bind sCD4 with similar affinities. Thus, this difference in sCD4 binding between sgp120 and intact virions indicates that most of the variation in sCD4 binding was related to differences in the structure of 120 bound to intact virions via gp41 (Moore *et al.,* 1992). In addition, sgp120 exists primarily as a monomer whereas mature HIV envelope glycoproteins exist on the membrane surface as tetramers, bound together by sequences within the ectodomain of gp41 which have been shown to be required for assembly of higher-order structures within the endoplasmic reticulum. Our experiments demonstrated that membrane-bound as well as soluble gp160 aa427 mutants, which can form oligomers (Earl *et al.,* 1991a), bound to CD4 within the cell. However, soluble gp120 W427 mutant molecules, which cannot form oligomers, were no longer able to bind CD4 intracellularly. These results suggest that oligomerization of the HIV glycoprotein may have an important influence on both the conformation and the intracellular CD4 binding site. Support for the influence of gp160 oligomerization on CD4 binding comes from a report by Mulligan *et al.* (1992), who demonstrated that the high affinity of an HIV-2 envelope glycoprotein for sCD4 was dependent on the multimeric presentation of the gp120 glycoprotein on the surface of the virus or virus-infected cell. Taken together, these results support the hypothesis that gp41 sequences present in gp160 may influence the binding site for CD4 within the ER.

The lack of significant effect of CD4 mutations on intracellular complex formation, however, cannot be explained on the basis of soluble vs membrane-bound molecules. Soluble CD4 mutants continued to bind gp160 within the ER, blocking their transport (Fig. 8A). Thus, the continued binding of these mutants appears to suggest that alternative sites are available for binding within the ER. This more promiscuous binding may be due to the fact that proteins within the ER are immature. As such, more sites could be available to bind gp120, making the known sites of mature CD4 molecules less critical for intracellular binding. Binding of CD4 to gp160 could also be influenced by the unique environment of the ER. The lumen of the ER provides environmental conditions optimized for the folding and oxidation of nascent proteins, including high calcium concentration, oxidizing redox potential, and high concentration of molecular chaperones and folding enzymes. All of these factors could influence the binding sites available for binding to gp120 (Doms *et al.,* 1993).

The ability of the HIV envelope glycoprotein to modulate the expression and transport of CD4 has been the focus of much research in recent years. However, the molecular mechanisms and *in vivo* implications of this phenomenon are unclear at this time. This report suggests that requirements for intracellular CD4–gp160 interactions may be different than those described for CD4–gp120. Further studies in defining the mechanism by which gp160 down-regulates its cellular receptor and its role in viral biology may provide important information for understanding viral pathogenesis, CD4 surface modulation, and viral persistence.

ACKNOWLEDGMENTS

These studies were partially supported by an Amgen fellowship and a Seed Grant from the UCLA AIDS Institute and by a Microbial Pathogenesis training grant from NIAID, NIH.

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