JOURNAL OF VIROLOGY, May 1990, p. 2250–2259
0022-538X/90/052250-10$02.00/0

Vol. 64, No. 5

Functional Contribution of Cysteine Residues to the Human Immunodeficiency Virus Type 1 Envelope

ERWIN TSCHACHLER,† HARTMUT BUCHOW,‡ ROBERT C. GALLO, AND MARVIN S. REITZ, JR.*

Laboratory of Tumor Cell Biology, National Cancer Institute, Building 37, Room 6A09, Bethesda, Maryland 20892

Received 6 September 1989/Accepted 5 January 1990

Although the envelope gene of human immunodeficiency virus type 1 shows considerable strain variability, cysteine residues of the envelope protein are strongly conserved, suggesting that they are important to the envelope structure. We constructed and analyzed mutants of a biologically active molecular clone of human immunodeficiency virus type 1 in which different cysteines were replaced by other amino acids in order to determine their functional importance. Substitution of cysteines 296 and 331, on either side of a region recognized by type-specific neutralizing antibodies, or on either side (residues 418 and 445) of a region important for CD4 binding, resulted in noninfectious mutants. These mutants were blocked early in the viral life cycle. Their gp160 envelope precursor polypeptides were poorly cleaved, and CD4 binding was also strongly impaired. Similar substitutions in the first variable region (residue 131) or between the first and second variable regions (residue 196) also gave noninfectious mutant virus, but here the block was late in the virus life cycle; these mutants were defective for syncytium formation. Substitution of *cys*386, between the neutralization and CD4 binding regions, resulted in a virus which retained infectivity but which spread much more slowly than the wild type. As with the *cys*131 and *cys*196 mutants, the *cys*386 mutant appeared to be defective in syncytium formation. These results show that all seven of the tested cysteines are vital for envelope function and suggest that this is likely true for all envelope cysteines. The results further show that regions important for CD4 binding, proteolytic cleavage recognition, and syncytium formation are all multiple and distributed over a relatively large part of the gp120 and therefore are likely dependent on protein tertiary structure.

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent for acquired immunodeficiency syndrome. Different isolates of HIV-1 are characterized by relatively high levels of genetic diversity. The *env* gene is the most variable (1, 10, 12, 31, 32, 35), with the deduced amino acid sequences of the envelope proteins of different strains differing from each other by as much as 30% of their residues. Despite this divergence, there are 21 cysteine residues in the HIV-1 envelope which are completely conserved in all reported isolates. Even more striking is the conservation of the same Cys residues within the envelope proteins of simian immunodeficiency virus (3, 8, 13) and HIV-2 (11), which have only 30 to 40% amino acid identity with HIV-1. This suggests that Cys residues are extremely important to envelope function, probably at least in part through the contribution of disulfide bridges to the tertiary structure of the envelope protein.

The envelope proteins of HIV-1, like those of other retroviruses, are synthesized as a precursor polypeptide (gp160) (2, 34) in the rough endoplasmic reticulum. The gp160 is transported to the cell surface through the Golgi complex, during which it is glycosylated, modified by glycosyl-trimming enzymes, and cleaved proteolytically to the mature gp120 outer membrane protein and the gp41 transmembrane protein. The envelope proteins are then incorporated into budding virions, where they form the outermost part of the virus. The gp120 binds to the CD4 molecule on the surface of the target cell (5, 15, 23) and, acting in concert with the gp41, fuses with the target cell membrane, causing the appearance of syncytia (20, 21, 30, 33). After binding to CD4, the virion is internalized by fusion, either at the plasma membrane (33) or within endocytosed vacuoles (21). After the virion is uncoated, viral DNA is synthesized. In order to better understand the relationship of each of these steps to the viral life cycle and the contribution of various Cys residues in maintaining the necessary envelope protein structure, we constructed a series of HIV-1 envelope mutants in which Cys residues were replaced by other amino acids and we characterized the mutants with respect to their biological activity.

MATERIALS AND METHODS

Cell culture. cos-1 cells (9), CD4-expressing HeLa cells (21), the monocytoid cell line U937, and the T-cell lines CEM, H9, and MT-2, the latter infected with human T-cell leukemia virus type I, were grown in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum, 2 mmol of L-glutamine per liter, 100 U of penicillin per ml, and 100 μg of streptomycin per ml in a humid atmosphere at 37°C in the presence of 5% CO_2. Virus transmission studies were carried out by either incubating the respective U937, CEM, H9, or MT-2 cells for 24 h with supernatant harvested from cos-1 cells 48 h after transfection or by cocultivation for 24 h of the respective cell lines with transfected cos-1 cells. After 24 h, cells were washed and maintained in culture medium as detailed above.

DNA transfection. The biologically active molecular clone pHXB2D (7) or the molecular clones derived from it which contain the different point mutations in the envelope gene described here were transfected into cos-1 or HeLa CD4

* Corresponding author.
† Present address: Department of Dermatology I, University of Vienna Medical School, A-1090 Vienna, Austria.
‡ Present address: Universitatskinderklinik, Rudolf Virchow, 1000 Berlin 65, Federal Republic of Germany.

cells by electroporation (4). Briefly, 30 μg of plasmid DNA was used per transfection of 10^7 cells; the DNA was suspended in 0.8 ml of sucrose electroporation buffer (272 mM sucrose, 7 mM sodium phosphate buffer [pH 7.4], 1 mM $MgCl_2$). For electroporation, the Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) was used at a voltage setting of 0.3 kV for cos-1 cells and 0.25 kV for HeLa CD4 cells. Capacitance setting for both cell lines was 25 μF. After transfection, cells were suspended in 20 ml of complete medium and plated either in T75 tissue culture flasks (Costar, Cambridge, Mass.) or in Lab-Tek chamber slides.

RT assays. Magnesium-dependent reverse transcriptase (RT) activity in the supernatant of transfected cos-1 cells was assessed by using a standard protocol (25). Either $poly(A)_npoly(dT)_{12-18}$ was used as the template primer or $poly(dA)_npoly(dT)_{12-18}$ as a negative control. The polymerase reaction was carried out in the presence of 7.5 mM Mg^{2+}.

Radioimmunoprecipitation assays. Radioimmunoprecipitation assays of transfected cos-1 cells were performed by standard methods. Briefly, at 24 to 36 h after transfection tissue culture flasks were washed once with 50 ml of phosphate-buffered saline, and culture medium was replaced by 10 ml of methionine- and cysteine-free medium containing 10% dialyzed fetal calf serum and [^{35}S]cysteine and [^{35}S]methionine (1,200 Ci/mmol) to a final concentration of 100 μCi/ml. After an incubation time of 12 h, medium was removed and cells were exposed to lysis buffer (0.5% Nonidet P-40, 50 mM Tris [pH 8.0], 150 mM NaCl, 5 mM EDTA [pH 8.0], 0.1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide). The lysates were precleared overnight by incubation with rabbit serum and protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). One milliliter of labeled lysate was then incubated for 16 h at 4°C with either 10 μl of a mixture of sera from three HIV-infected patients or a goat anti-HIV-1 gp41 antiserum and 30 μl of a 50% suspension of protein A-Sepharose beads. Immunoprecipitates were collected by centrifugation, washed repeatedly in lysis buffer, suspended in Laemmli sample buffer (17), heated for 3 min at 100°C, and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. For radioimmunoprecipitation of proteins of virions produced by transfected cos-1 cells, the medium from cells radiolabeled as described above was spun at 2,000 rpm for 10 min to remove cells and cell debris. Lysis buffer (4× concentrated) was added in a 1:4 ration to 10 ml of supernatant, and immunoprecipitation was performed as described for cell lysates except that the volumes of antisera and protein A-Sepharose beads were increased threefold.

CD4 binding assay. Recombinant soluble CD4 (29) was covalently linked to CNBr-activated Sepharose beads (Pharmacia) at a ratio of 1 mg of CD4 per ml of beads under conditions specified by the supplier. To either 500 μl of radioactive lysate or 4 ml of culture supernatant of cos-1 cells (equivalent to 3×10^6 cells) transfected with either pHXB2D or the different mutant clones, 2 μl of CD4-Sepharose was added and incubated on a rotating wheel overnight at 4°C. In some experiments, 10 μg of soluble CD4 was added to the lysate 2 h before incubation with the Sepharose-CD4 beads. After the beads were washed extensively in lysis buffer, samples were denaturated in Laemmli buffer and analyzed by SDS-polyacrylamide gel electrophoresis.

Syncytium formation assay. HeLa CD4 cells were transfected with pHXB2D, the cysteine substitution mutants, or a noninfectious mutant defective in the integrase gene, which was generated by site-directed mutagenesis (H. Buchow, unpublished data). Transfected cells were plated in Lab-Tek chamber slides. At 12 to 24 h after transfection, the slides were fixed in methanol-acetone (1:2, vol/vol) and subsequently stained for HIV-1 protein expression in an indirect immunofluorescence assay, using serum from an HIV-1-infected individual as the first-step reagent. HeLa CD4 cells expressing HIV-1 proteins and containing ≥4 nuclei were considered syncytia.

Immunofluorescence assays. For surface immunofluorescence assays, cos-1 cells were plated after transfection in Lab-Tek tissue culture chamber slides and after 24 to 48 h were exposed without fixation to either a monoclonal antibody against gp120 (28) at a concentration of 1 μg/ml in phosphate-buffered saline–1% fetal calf serum–0.1% sodium azide or to control ascites fluid. After incubation at room temperature for 15 min, the chamber slides were washed extensively with phosphate-buffered saline-bovine serum albumin-sodium azide and then reacted with fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (1:10; Tago Inc., Burlingame, Calif.). After extensive washes, slides were fixed for 2 min in methanol-acetone (1:2) and examined under a Zeiss immunofluorescence microscope. For immunofluorescence detection of total HIV-1 antigens on transfected HeLa CD4 cells, sera from HIV-1 seropositive individuals were used. Serum from seronegative individuals served as a negative control. After incubation of cells with the respective sera, slides were washed extensively and reacted with fluorescein isothiocyanate-labeled goat anti-human immunoglobulin G (1:20; Cappel, Organon Teknika Corp., West Chester, Pa.). Transmission of HIV-1 from transfected cos-1 cells to U937, CEM, H9, or MT-2 cells was monitored in a similar way once a week.

Mutagenesis. Site-directed mutagenesis was performed for the single Cys substitution mutants, using the method of Zoller and Smith (36), by subcloning either a 2.7-kilobase (kb) *Sal*I-*Bam*HI or a 0.6-kb *Bgl*II fragment of the gp120 coding region of pHXB2D (7) into M13. After mutagenized fragments were recloned into pHXB2D, successful mutagenesis was confirmed by DNA sequence analyses, using the chain termination method (27) with [^{35}S]dATP (600 C/mmol) (New England Nuclear Corp., Boston, Mass.) and the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio). The oligonucleotides used for mutagenesis and their respective positions within pHXB2D are listed in Table 1. Restriction enzymes, DNA ligase, and polynucleotide kinase were purchased from various commercial sources and used under conditions recommended by the manufacturers.

The double mutants were prepared by utilizing a polymerase chain reaction (PCR) step with a Perkin-Elmer Cetus GeneAmp kit and DNA thermal cycler under conditions recommended by the manufacturer. The *cys*296/331 mutant was prepared from the *cys*296 mutant with a PCR reaction, using a wild-type primer M61 (see Table 1) and the *cys*331 mutagenic primer, which introduces a unique *Hpa*I site. The PCR product was digested with *Bgl*II plus *Hpa*I, giving a 0.18-kb fragment which was coligated with the contiguous 0.4-kb *Hpa*I-*Bgl*II fragment of the *cys*331 mutant into the 2.7-kb *Sal*I-*Bam*HI subclone of wild-type pHXB2D from which the central 0.6-kb *Bgl*II fragment had been removed. The resultant doubly mutagenized *Sal*I-*Bam*HI fragment was then substituted by ligation for the analogous wild-type fragment in pHXB2D. The *cys*418/445 double mutant was prepared in a similar manner, except that the *cys*445 mutant was used as a template with the *cys*418 and M43 (Table 1) primers in the PCR reaction. The *cys*418 primer introduces

TABLE 1. Oligonucleotides used for mutagenesis

Mutant or primer	Nucleotide position[a] and sequence	Direction
*cys*131	6188 G TTA AAG GGC ACT GAT TTG 6206 (TGC)	Sense
*cys*196	6379 TG ACA AGT GTT AAC ACC TC 6397 (TGT)	Sense
*cys*296	6698 GGG TCT TGT AAC ATT AAT TTC TAC 6675 (ACA)	Antisense
*cys*331	6802 CT AAT GTT AAC ATG TGC 6786 (ACA)	Antisense
*cys*386	6945 G TGT TGA ATT AAC GTA GAA AAA 6966 (ACA)	Antisense
*cys*418	7062 T TAT TCT AGA TGG GAG GG 7045 (GCA)	Antisense
*cys*445	7146 T ATT TGA TGA GCC TCT AAT TTG TC 7123 (ACA)	Antisense
M43	7239 CCA ATT GTC CCT CAT ATC TCC TCC T 7215	Antisense
M61	6587 GGC AGT CTA GCA GAA GAA GAG G 6608	Sense

[a] Nucleotide positions are derived from reference 26. The codons as they appear in the original sequence are put in parentheses.

an *Xba*I site. The PCR product was digested with *Bgl*II and *Xba*I, and the resultant 0.14-kb fragment was coligated with the contiguous 0.4-kb *Bgl*II-*Xba*I fragment of the *cys*418 mutant into the 2.7-kb *Sal*I-*Bam*HI fragment described above. The resultant doubly mutagenized fragment was then substituted into pHXB2D. The mutations were verified in the final construct by direct sequencing.

RESULTS

Construction and identification of Cys substitution mutants. HIV-1 mutants in which Cys residues in the envelope protein gp120 were replaced by other amino acids were constructed by subcloning either of several restriction endonuclease fragments from the biologically active molecular clone pHXB2D (7) into M13, performing site-directed mutagenesis, and then inserting the mutagenized fragment back into pHXB2D, as described in Materials and Methods. Clones were identified by differential hybridization with the appropriate mutagenic oligomers. The mutagenesis was also designed to introduce or eliminate restriction endonuclease sites so that successful mutagenesis could be further analyzed by Southern blotting and substitutions of additional cysteines could be generated by PCR techniques. The final constructs were all verified by direct DNA sequence analysis, using plasmid DNA. The positions of the substituted Cys residues and the amino acids substituted in their stead are indicated in Fig. 1.

Biological activity of Cys substitution mutants. Plasmids representing the Cys substitution mutants were transfected into cos-1 cells. At 48 h after transfection, RT activity was assayed in the supernatant of transfected cos-1 cells. All mutant proviruses were able to release RT into the medium. Analyses by transmission electron microscopy of the transfected cos-1 cells revealed the presence of mature and immature viral particles in the cell medium or budding from the cell membrane (data not shown).

Infectivity of the resultant mutant viruses was tested by cocultivation of the cos-1 cells with several different target cells and by incubation of the same cell types with cell-free medium from the cos-1 cultures. Virus transmission was measured by immunofluorescence assays for viral antigen expression by the target cells. Figure 2 shows a typical transmission experiment of the different mutant viruses from cos-1 cells to H9 cells by cocultivation. With the exception of the *cys*386 mutant, none of the mutant viruses was demonstrably transmissible. The *cys*386 mutant could be transmitted by cocultivation and cell-free virus to the T-cell lines MT-2 and CEM but not to the monocytoid cell line U937, in contrast to the wild-type virus. The rate of spread to the T-cell lines, however, was considerably slower with

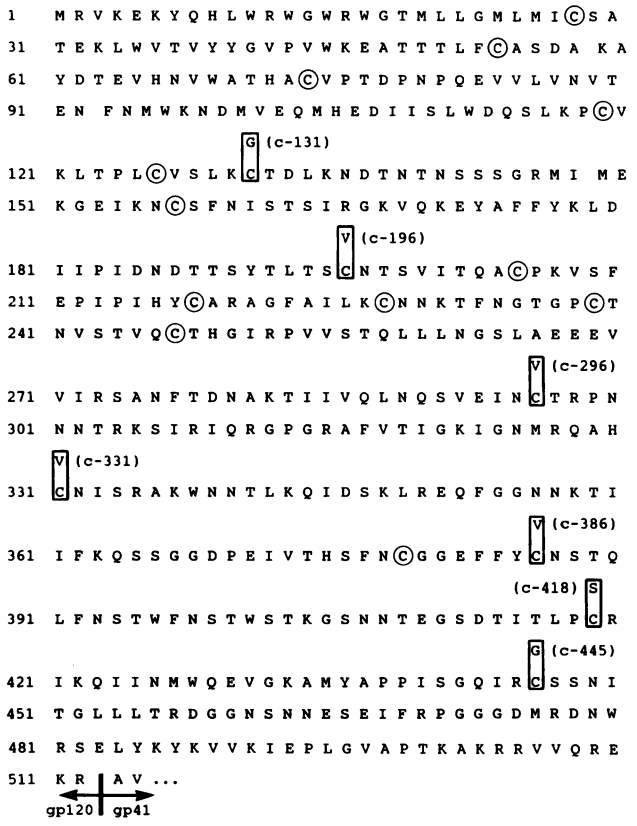

FIG. 1. Locations of cysteine residues within the gp120 of HIV-1. The amino acid sequence shown is deduced from the DNA sequence of HIV-1 published by Ratner et al. (26), starting with the first methionine residue. Mutated cysteine residues and the respective substituted amino acids are boxed. The respective position within the sequence appears in parentheses.

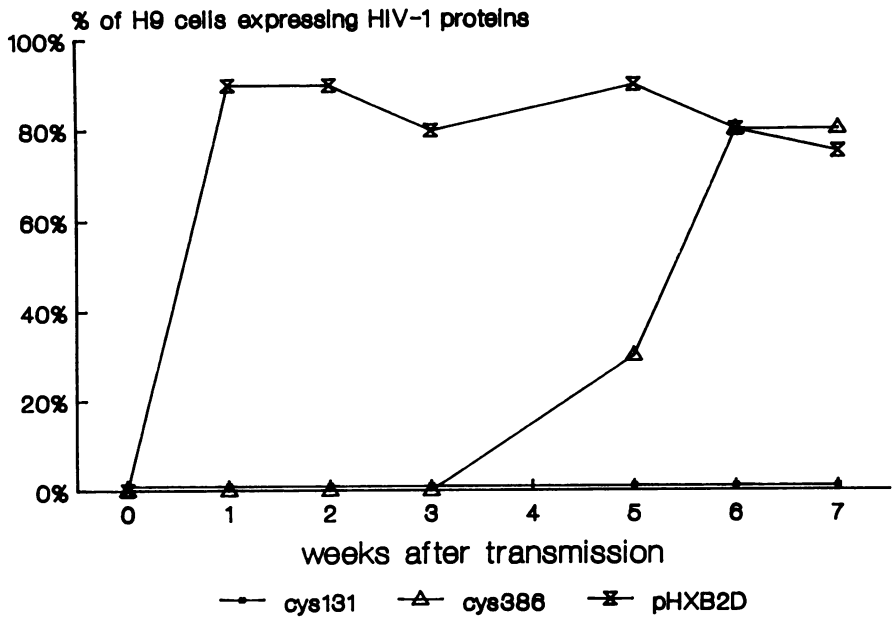

FIG. 2. Transmission of wild-type and mutant viruses from cos-1 cells to H9 cells. Either wild-type pHXB2D or the respective Cys mutant plasmids were transfected into cos-1 cells by electroporation. After 48 h, cos-1 cells were cocultivated for 24 h with 2×10^6 H9 cells. Transmission of virus was monitored weekly by indirect immunofluorescence, using defined sera of HIV-1 seropositive patients as first-step reagents as specified in Materials and Methods.

the *cys*386 mutant than with the parental virus. In typical transmission experiments with the human T-cell leukemia virus type I-infected T-cell line MT-2 as a target, only 20% of the cells were positive by immunofluorescence two weeks after cell-free transmission of the *cys*386 mutant, whereas in the same experiment, positivity reached 90% within 3 days of infection with the wild type (data not shown). Infection of H9 cells was 90% at 1 week after infection with the wild-type virus, but was only 20 to 30% by 4 weeks after infection with the *cys*386 mutant (Fig. 2). After 2 months in culture, infection of H9 cells with *cys*386 was ~90% and a marked increase in cytopathology was evident. The progeny virus could now rapidly infect H9 cells but were still unable to infect U937 cells. All other mutants were completely negative in this experiment. The data with the *cys*131 mutant are shown in Fig. 2 as a representative example.

Envelope precursor peptide expression and cleavage by the Cys substitution mutants. After transfection into cos-1 cells, the Cys substitution mutants were analyzed for protein expression by radioimmunoprecipitation assays, using defined sera from HIV-1-seropositive individuals. As is evident from Fig. 3A and B, the *gag* protein p24 and precursor proteins pr39 and pr55 were detectable at comparable levels in cos-1 cell lysates transfected with each of the Cys substitution mutants except for *Cys*131. Proteolytic processing of the envelope precursor molecule gp160, however, was substantially impaired in *cys*296, *cys*331, *cys*296/331, *cys*418, *cys*445, and *cys*418/445. Virtually no gp120 was detected in the cell lysates of five of these mutants, although the precursor molecule was identical in size to the gp160 in wild-type virus. Some gp120 could be detected in lysates of cells producing the *cys*445 mutant, but gp160 was vastly predominant, indicating a strong impairment in the cleavage process. The amount of both gp160 and gp120 was strongly reduced in lysates of cells transfected with the *cys*386 mutation.

Immunoprecipitation of viral proteins from the supernatant of the transfected cells (Fig. 3C and D) generally confirmed the data obtained from the cell lysates. There were comparable amounts of p24 present in the media of cells transfected by each of the mutants except *cys*131, but extracellular gp120 was readily detectable only with the mutants for which cell-associated gp120 was demonstrated. Surprisingly, however, cells transfected with the *cys*386 mutant, which contain relatively low amounts of cell-associated gp120 and gp160, expressed levels of extracellular gp120 comparable to those seen with wild-type virus. This was also true for long-term cultures of MT-2 cells infected with *cys*386, even though virus from these cultures had become capable of rapid infection. On a prolonged exposure of gels containing radioimmunoprecipitates from the medium of cos-1 cells transfected with the *cys*296, *cys*331, *cys*296/331, and *cys*418 mutants, trace amounts of gp120 were evident, indicating that cleavage of gp160 was vastly reduced but not completely abrogated. This was also confirmed by radioimmunoprecipitation assays of the same cells with an antiserum specific for gp41, which detected low but measurable amounts of the smaller envelope cleavage product in the cell lysate (Fig. 4). No gp120 was detectable in the medium of cells expressing the *cys*418/445 double mutant, even after extended exposures of radioimmunoassays (Fig. 3D). No gp160 could be detected in the medium of cells transfected with either the wild-type virus or any of the mutants, confirming that cleavage of the envelope precursor protein is normally an obligatory step preceding its incorporation into virions (H. G. Guo, F. diMarzo Veronese, E. Tschachler, R. Pal, V. S. Kalyanaraman, R. C. Gallo, and M. S. Reitz, Jr., Virology, in press).

To find out whether the defect in processing of the gp160 by Cys mutants *cys*296, *cys*331, and *cys*418 was due to retention of the proteins in intracellular compartments or to a failure to reach the cell surface, we performed immunostaining experiments on viable cos-1 cells transfected with the respective mutants, using two different monoclonal

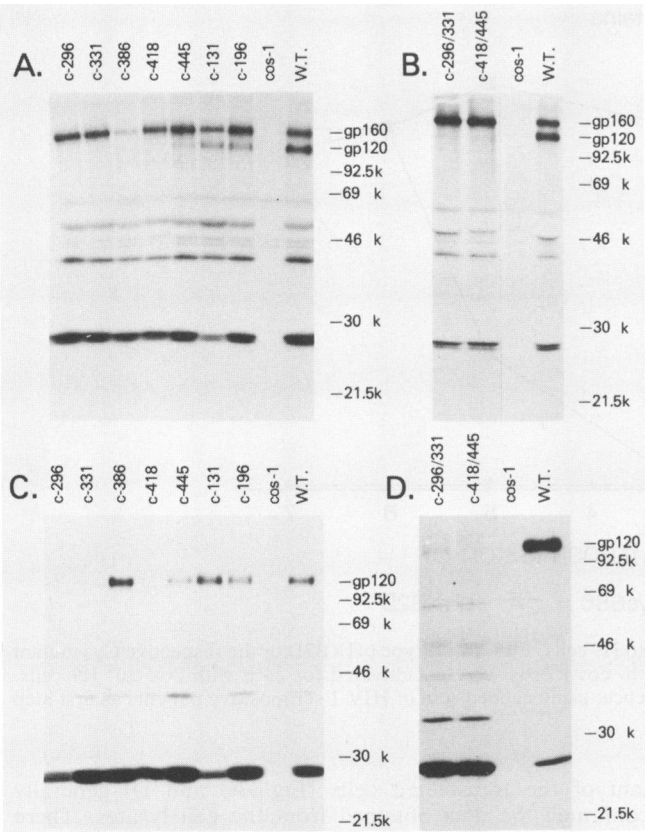

FIG. 3. Immunoprecipitation of viral proteins produced by cos-1 cells after transfection of wild-type (W.T.; pHXB2D) or mutant HIV-1 genomes. pHXB2D or the different Cys mutant plasmids were transfected into cos-1 cells by electroporation. After 24 h, cells were cultured in cysteine- and methionine-free medium substituted with [^{35}S]cysteine and [^{35}S]methionine for 12 h. Cells were lysed in a Nonidet P-40-containing lysis buffer as described in Materials and Methods, and the cell lysate was preadsorbed overnight with protein A-Sepharose beads and normal rabbit serum. Immunoprecipitation was carried out by incubating the cell lysate with 10 μl of a mixture of sera from three HIV-1-seropositive patients followed by protein A-Sepharose beads. Immunoprecipitates were analyzed on a 10% (panel A) or 11% (panel B) polyacrylamide-SDS gel. The supernatant of transfected, radiolabeled cos-1 cells was cleared of residual cells by centrifugation, and concentrated lysis buffer (4×) was added in a 1:4 ratio. Immunoprecipitation was performed as described above except that the volume of antisera and protein A-Sepharose was increased threefold. Immunoprecipitates were analyzed on a 10% (panel C) or 11% (panel D) polyacrylamide-SDS gel. The positions of gp120 and gp160 are indicated, as are the positions of marker proteins of known molecular weights.

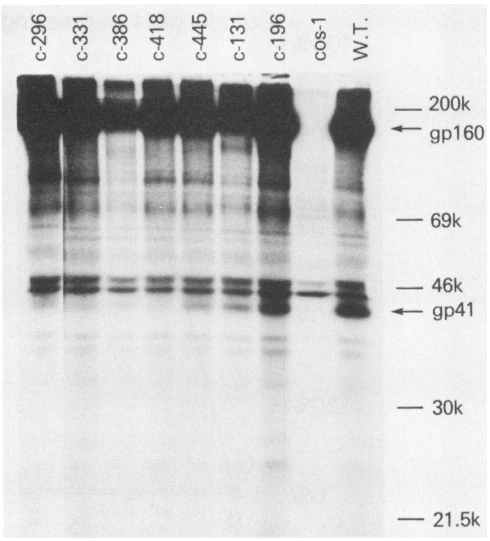

FIG. 4. Immunoprecipitation of HIV-1 gp160 and gp41 produced by cos-1 cells after transfection of pHXB2D or Cys mutant plasmids. Transfected cos-1 cells were labeled with [^{35}S]cysteine and [^{35}S]methionine, and cell lysates were prepared as described in the legend to Fig. 2. Instead of human antiserum, a specific polyclonal rabbit anti-gp41 antiserum was used for immunoprecipitation. Immunoprecipitates were analyzed on a 10% polyacrylamide-SDS gel. For the first lane (*cys*296), a shorter exposure of the same gel was used to normalize to comparable amounts of gp160.

antibodies directed against defined linear epitopes on gp120 (28; F. diMarzo-Veronese, personal communication). As shown in Fig. 5, the gp120 antigenic moieties of the uncleaved gp160 were detectable at comparable levels on the surfaces of cos-1 cells transfected with the *cys*296, *cys*331, *cys*296/331, *cys*418, and *cys*418/445 mutants or pHXB2D. The apparently unaltered ability of these monoclonal antibodies to recognize their binding sites in all of the mutants suggests that the introduced changes leave the gross protein structure intact.

CD4 binding by mutant envelope proteins. To test the ability of the mutant viral envelopes to bind to CD4, we used an assay system which detects binding of both gp160 and gp120 derived from lysates of cos-1 cells transfected with pHXB2D to recombinant soluble CD4 (29). Both gp160 and gp120 from wild-type virus readily bound to CD4 immobilized on CNBr-Sepharose beads (Fig. 6, W.T., lane a). Binding of HIV-1 envelope proteins to CD4-Sepharose beads was completely blocked when excess (10 μg) soluble CD4 (lanes b) or OKT-4A (10 μg) monoclonal antibody (data not shown) was added. The gp160 and gp120 of mutants *cys*131 and *cys*196 also readily bound to CD4. In contrast, no binding of gp160 to CD4 was detectable in mutants *cys*296, *cys*331, *cys*296/331, *cys*418, and *cys*418/445. The gp120 proteins of the *cys*386 and *cys*445 mutants were able to bind to CD4 but, in contrast to the wild-type virus and the *cys*131 and *cys*196 mutants, no binding (*cys*386) or strongly reduced binding (*cys*445) of the gp160 was evident. For mutants *cys*296/331, *cys*386, and *cys*445, we also assayed the binding of gp120 released in the culture medium to CD4 (Fig. 7). Although gp120s of all three mutants were able to bind to CD4, we observed differences in the degree of binding. gp120 of *cys*386 bound to CD4 at a level comparable to that of the wild-type envelope; in contrast, binding of the gp120 of *cys*296/331 and in particular of *cys*445 was reduced.

Capacity of envelope mutants to form syncytia. To test the ability of the mutant viruses to form syncytia, the Cys mutant plasmids were transfected into HeLa CD4 cells. Syncytium formation was evaluated 12, 24, and 48 h post-transfection. Wild-type pHXB2D readily formed large syncytia, containing up to 15 nuclei by these time points. As a control for the ability of the HIV-1 envelope proteins to induce syncytia in the absence of a spreading infection, as is the case with most of the Cys mutants, we used a mutant rendered defective in the endonuclease coding region of the *pol* gene by the introduction of a premature termination codon. This virus was incapable of establishing a productive infection in target cells (not shown), but it expressed all of the viral structural proteins in transfected cells, including a

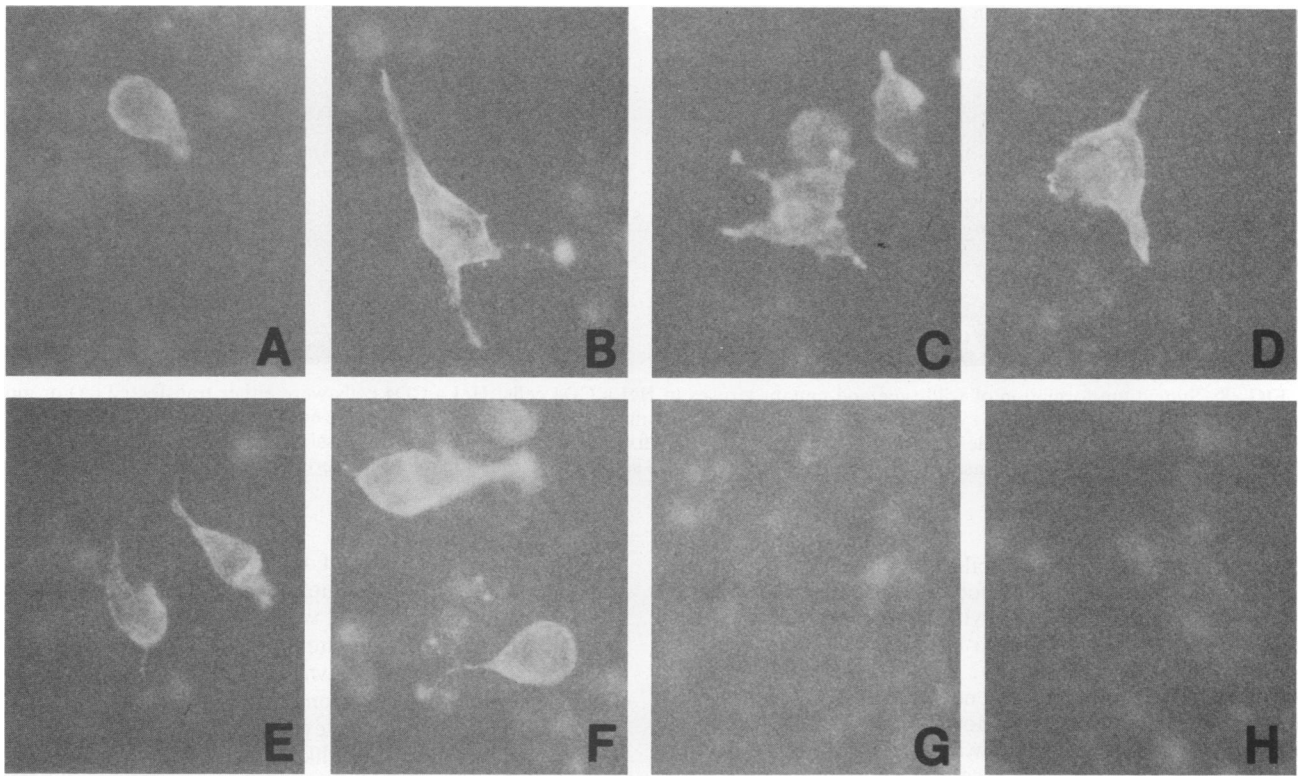

FIG. 5. Surface expression of gp120 antigen moieties by Cys mutants defective in envelope cleavage. cos-1 cells were transfected by electroporation with *cys*296 (A), *cys*331 (B), *cys*296/331 (C), *cys*418 (D), *cys*418/445 (E), or wild-type pHXB2D (F) plasmid DNA, or they were mock transfected (G). Cells were then cultured in chamber slides for 48 h. Viable adherent cells were stained in an indirect immunofluorescence assay, using a monoclonal anti-gp120 antibody or mouse control ascites (H) as a first-step reagent followed by a fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G antiserum. After extensive washes, cells were fixed in methanol-acetone prior to examination under an immunofluorescence microscope.

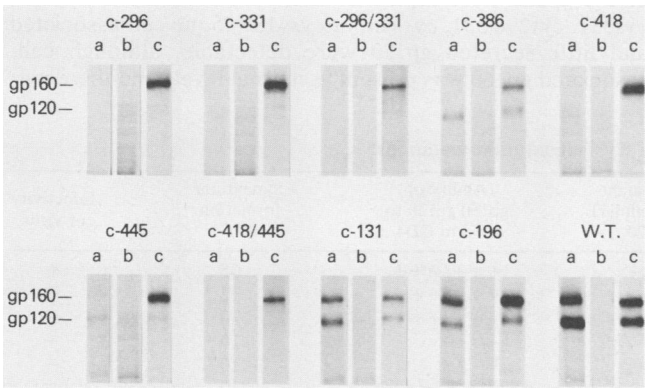

FIG. 6. Binding of cell-associated wild-type (W.T.) and Cys mutant envelope proteins to CD4. Cell lysates of radiolabeled cos-1 cells transfected with pHXB2D or the respective Cys mutants were prepared as described in Materials and Methods. Soluble CD4 immobilized on CNBr-Sepharose beads was added to equal amounts of cell lysates in the absence of free soluble CD4 (lanes a) or after preincubation of the lysates with 10 μg of soluble CD4 per ml (lanes b) and incubated for 16 h. After extensive washes in lysis buffer, samples were denatured by boiling them in Laemmli buffer for 3 min and then they were analyzed on an 8% polyacrylamide-SDS gel. Lanes c show the immunoprecipitation of envelope proteins remaining in the lysate after the reactions for lanes b were carried out.

wild-type envelope gene. As shown in Fig. 8D, this mutant induced the formation of large syncytia comparable to those of wild-type pHXB2D (panel A), showing that syncytium formation indeed does not depend on the spread of virus

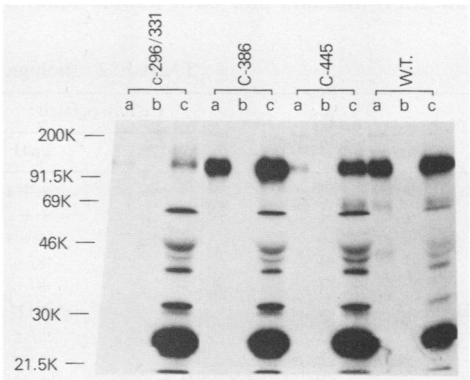

FIG. 7. Binding of wild-type (W.T.) and Cys mutant envelope proteins derived from culture supernatants to CD4. CD4-Sepharose beads were reacted with equal volumes of supernatant of cos-1 cells transfected with the different mutants and incubated for 20 h in medium substituted with [^{35}S]methionine and [^{35}S]cysteine in the absence (lanes a) or presence (lanes b) of 10 μg of soluble CD4 per ml. Lanes c show the results of immunoprecipitation experiments from identical volumes of supernatant, using sera of HIV-1-infected patients.

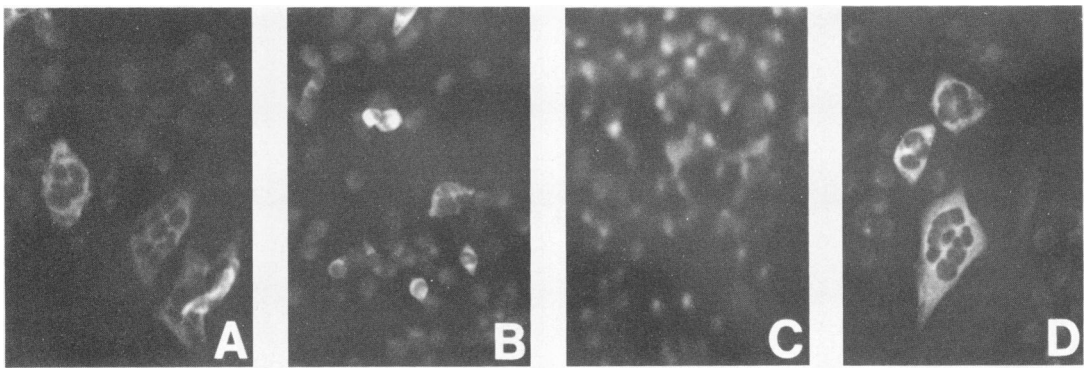

FIG. 8. Syncytium formation of wild-type and mutant viruses in HeLa CD4 cells. HeLa CD4 cells were either transfected (A) or mock transfected (C) with pHXB2D, the *cys*386 mutant (B), or a mutant plasmid containing a stop codon in the endonuclease gene (D). Transfected cells were cultured in chamber slides for 12 to 24 h and subsequently fixed in methanol-acetone. Expression of HIV-1 proteins was detected by indirect immunofluorescence, using a defined serum of an HIV-1-seropositive individual and a fluoresceinated goat anti-human immunoglobulin antiserum.

throughout the culture. The ability of all seven of the single Cys mutant viruses and of both double mutants to form syncytia was vastly reduced with respect to both size and number (Table 2), even in the case of the *cys*386 mutant, which retains some infectivity. Rarely were syncytial cells with more than four nuclei encountered with any of the mutant viruses. Figure 8B shows representative fields of HeLa CD4 cells transfected with *cys*386. Panel C shows mock-transfected HeLa CD4 cells used as a negative control.

DISCUSSION

We have created and characterized HIV-1 mutants which are defective in different steps of the infection process. These mutants were made by substituting other amino acids for different Cys residues in the outer envelope protein gp120 of the biologically active molecular clone of HIV-1, HTLV-III_{HXB2D}. The critical nature of Cys residues in normal envelope function, suggested by their conservation in HIV-1, HIV-2, and simian immunodeficiency virus, was confirmed by our data, which show that six of the seven single Cys substitution mutants and both double mutants lacked the ability to infect any of the target cells used in these studies. The remaining mutant, in which *cys*386 was replaced by Val, could infect various T-cell lines, although it showed a greatly reduced rate of transmission relative to that of wild type. When *cys*386 was cultured for several months in MT-2 cells, its transmission rate to new target cell cultures increased greatly, as did its cytopathic effect. This reversion to a wild-type phenotype likely involves a compensatory mutation for the altered Cys. Its exact nature is currently under investigation.

Analyses of the proteins produced by the Cys substitution mutants showed that they all produced a 160-kilodalton envelope precursor glycoprotein. They varied, however, in the ability to proteolytically process gp160. Replacement of either of two cysteines in the N-terminal portion of gp120 (*cys*131 and *cys*196) had little effect on the processing of gp160 to gp120 and gp41. A profound effect was evident, however, when different cysteines in the C-terminal portion of the gp120 were replaced. After replacement of *cys*296, *cys*331, *cys*296/331, *cys*418, or *cys*418/445, no cell-associated and little secreted gp120 were detectable, although cell-associated gp160 was present at normal levels and there was

TABLE 2. Biological properties of Cys substitution mutations[a]

Virus	RT production	Cell associated		Supernatant (extracellular) gp120	Ability of gp160/gp120 to bind to CD4	Syncytium formation (%)[b]	Infectivity of virus
		gp120	gp41				
pHXB2d	+	+++	+++	+++	+++/+++	65	+
*cys*131	+	++	++	+++	+++/+++	12	−
*cys*196	+	+++	+++	+++	+++/+++	<10	−
*cys*296	+	−[c]	+/−	+/−	−/	<10	−
*cys*331	+	−	+/−	+/−	−/	<10	−
*cys*296/331	+	−	ND[d]	+/−	−/++	<10	−
*cys*386	+	+/−	+/−	+++	−/+++	14	+[e]
*cys*418	+	−	+/−	+/−	−/	<10	−
*cys*445	+	++	++	++	+/+	<10	−
*cys*418/445	+	−	ND	−[c]	−/	<10	−

[a] −, Negative; +/−, slightly positive; +, positive; ++, very positive; +++, highly positive.

[b] Syncytium assays were performed as described in Materials and Methods. At 24 h after transfection, the expression of HIV-1 proteins by HeLa CD4 cells was as assessed by indirect immunofluorescence. Approximately 2 to 5% of all HeLa CD4 cells were positive for viral proteins. Those cells expressing viral proteins and which contained more than four nuclei were considered syncytia. The percentage of syncytia within the total population of virus antigen-positive cells, as calculated from a representative experiment, is listed in this table.

[c] gp120 was not detectable by radioimmunoprecipitation, using defined sera of HIV-1-seropositive patients.

[d] ND, Not done.

[e] Transmission of the *cys*386 mutant was considerably slower than that of wild-type pHXB2D (see Fig. 2).

no evidence of gp160 degradation. These substitutions, thus, almost completely abolish gp160 cleavage. Some impairment of cleavage was also noted when another Cys residue in the carboxy region of the gp120 (*cys*445) was replaced, but the impairment was not complete. The abrogation of gp160 cleavage by any of these mutants was not due to retention of envelope proteins in intracellular compartments, since they were readily detectable by immunostaining on the surfaces of transfected viable cells. An alteration of the primary sequence recognized by the responsible cellular protease can probably be excluded, since the site for cleavage is situated at residues 511 and 512 (34), 100 to 200 amino acids downstream of the mutagenized Cys residues. Our data suggest either of the following two possibilities. One is that the protease not only recognizes a short linear sequence of amino acids, but also recognizes a specific tertiary structure of the gp160. Alternatively, it may be that elimination of either Cys residue results in a gp160 in which the cleavage site is no longer accessible to the processing protease. In either case, the lack of Cys sulfhydryl groups could affect the folding pathway leading to the final tertiary structure or maintenance of the final structure itself.

The interpretation that the gp160 of these mutants have undergone considerable changes in tertiary structure is supported by the finding that the gp160s of the *cys*296, *cys*331, *cys*296/331, *cys*418, and *cys*418/445 mutants are not only unable to be processed to gp120 and gp41, but also lack the ability to bind to CD4, the receptor for HIV-1. This inability is not directly linked to impairment of cleavage since the gp160 of the wild-type virus as well as the gp160 of a mutant virus in which we altered the primary sequence of the envelope cleavage site (Guo et al., in press) binds efficiently to CD4. In contrast, the gp160 of *cys*386, which cleaved the envelope precursors efficiently, failed to bind to CD4. The *cys*386 gp120, however, binds normally to CD4. Similarly, the trace amount of gp120 present in the culture supernatant of the *cys*296/331 mutant was able to bind efficiently to CD4. The differential binding observed between the gp160 and gp120 of these mutants suggests that the uncleaved gp41 affects the tertiary structure of gp160 in such a way that it destabilizes the proper conformation for CD4 binding and that only after cleavage is an optimal CD4 binding site formed and stabilized.

An interesting finding is that the replacement of cysteine residues at the C and N termini of a putative disulfide loop (residues 418 and 445) which contains a site important for CD4 binding (18) leads to phenotypically different envelope proteins. Replacement of the N-terminal cysteine of this loop (*cys*418), as mentioned above, abrogates both cleavage and CD4 binding. Replacement of the C-terminal cysteine (*cys*445), in contrast, gives a gp160 which is processed, although not as efficiently as that of the wild type. The *cys*445 gp120 is easily detectable in the medium of transfected cells, although to a lesser extent than with wild-type envelope. Similarly, the ability of the gp160 and gp120 to bind to CD4 is retained to a detectable degree in the *cys*445 mutant. The different effect of substituting these two cysteine residues suggests that they are not partners in a disulfide bond, although an alternative explanation would be that with the two mutants the remaining cysteines find a different new binding partner during protein folding. At the least, the data indicate that a disulfide loop in this region is not absolutely necessary for CD4 binding.

Replacement of cysteines in the N-terminal region of the gp120 (*cys*131 and *cys*196) results in viruses which synthesize a gp160 that is efficiently cleaved to a gp120 and gp41. As is true for the wild-type virus, both species bind to CD4. This is consistent with the data of others (16, 18), which suggest that the regions important in CD4 binding are located toward the C terminus of gp120, although it has also been reported that deletion of the N-terminal 164 amino acids eliminates CD4 binding (6).

The loss of the capacity of the envelope proteins to bind to CD4 is brought about by mutations which are quite distant from each other in the primary structure of the envelope protein, i.e., at *cys*296, *cys*331, *cys*418, and partially at *cys*445. This supports the idea that binding requires a specific tertiary structure which is formed or maintained by disulfide bonding and which involves a relatively large portion of the carboxy part of the gp120. Additional support for the importance of disulfide bonds in forming or maintaining the proper tertiary structure of the HIV-1 envelope for the envelope-CD4 interaction comes from data by McDougal et al. (24), who found that reduction and alkylation of gp120 abrogate its capacity to bind to CD4. Furthermore, the difficulty in these and other studies (14, 19) in defining a single region within the CD4 molecule which interacts with gp120 suggests that more than one site of interaction is necessary for the functional binding of these two molecules.

One aspect of the expression of the envelope protein by the *cys*396 mutant is puzzling. Although normal amounts of *gag* proteins are produced by this mutant, relatively small amounts of gp160 and gp120 are found within cell lysates. In the medium of cells transfected with this mutant, however, a normal-sized gp120 is present in amounts comparable to those present with the wild type. The same pattern is seen in T-cell lines infected with this mutant (data not shown). The apparently enhanced release of gp120 does not seem to be due to a reduced association with the transmembrane protein gp41, since the amount of cell-associated gp41 is also vastly reduced. This is also the case for the rapidly infectious *cys*386 obtained by extended culture in MT-2 cells.

All of the mutants described here, including the early infectious *cys*386 mutant, show a greatly diminished ability to form syncytia. This defect is not due to a lack of virus spread, since we showed that a mutant with a premature stop codon in the endonuclease open reading frame, which is unable to establish a productive infection, was able in our assay to induce syncytia similar in size and number to those of the wild-type virus. The inability of the *cys*296, *cys*331, *cys*296/331, *cys*418, *cys*445, and *cys*418/445 mutants to form syncytia is probably merely secondary to their defects in envelope cleavage and CD4 binding. Both steps are thought to be necessary for syncytium formation (16, 20, 22). It is, however, rather surprising that replacement of *cys*131 and *cys*196 in the N-terminal part of gp120 also strongly reduces the ability to form syncytia in HeLa CD4 cells, even though neither cleavage of the gp160 nor CD4 binding of these mutants is impaired. These mutants, however, cannot productively infect target cells and are not able to form syncytia comparable in size and number to those formed by wild-type virus. Since the endonuclease-negative mutant, as mentioned above, is able to induce syncytia even though it is not able to infect, it is likely that *cys*131 and *cys*196 indeed have a primary defect in envelope fusogenesis. However, since they lack even the low-level infectivity of the *cys*386 mutant, which is also deficient in syncytium formation, they may also be blocked at a later post-CD4 binding step, such as internalization or uncoating of the viral core.

In summary, the results presented in this report suggest that Cys residues within the HIV-1 envelope play a crucial role in generating or maintaining the proper tertiary structure

for several different steps of the viral replicative cycle. It appears that conservation of the proper tertiary structure is essential for cleavage into gp120 and gp41 and that cysteines located at the carboxy-terminal portion of gp120 stabilize the protein conformation necessary for this process. In addition, cysteines in the carboxy portion are important to a functional CD4 binding site. At least some cysteines located toward the amino terminal region of the HIV-1 gp120 seem not to be involved in forming the CD4 binding site and are not necessary for proper envelope cleavage but are important for fusogenic activity.

LITERATURE CITED

1. **Alizon, M., S. Wain-Hobson, L. Montagnier, and P. Sonigo.** 1986. Genetic variability of the AIDS virus: nucleotide sequence analysis of two isolates from African patients. Cell **46**:63–74.
2. **Allan, J. S., J. E. Coligan, F. Barin, M. F. McLane, J. G. Sodroski, C. A. Rosen, W. A. Haseltine, T. H. Lee, and M. Essex.** 1985. Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III. Science **228**:1091–1094.
3. **Chakrabarti, L., M. Guyader, M. Alizon, M. D. Daniel, R. C. Desrosiers, P. Tiollais, and P. Sonigo.** 1987. Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. Nature (London) **328**: 543–547.
4. **Chu, G., H. Hayakawa, and P. Berg.** 1987. Electroporation for the efficient transfection of mammalian cells with DNA. Nucleic Acids Res. **15**:1311–1326.
5. **Dalgleish, A. G., P. C. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature (London) **312**:763–767.
6. **Dowbenko, D., G. Nakamura, C. Fennie, C. Shimasaki, L. Riddle, R. Harris, T. Gregory, and L. Lasky.** 1988. Epitope mapping of the human immunodeficiency virus type 1 gp120 with monoclonal antibodies. J. Virol. **62**:4703–4711.
7. **Fisher, A. G., M. B. Feinberg, S. F. Josephs, M. E. Harper, L. M. Marselle, G. Reyes, M. A. Gonda, A. Aldovini, C. Debouk, R. C. Gallo, et al.** 1986. The trans-activator gene of HTLV-III is essential for virus replication. Nature (London) **320**:367–371.
8. **Franchini, G., C. Gurgo, H. G. Guo, R. C. Gallo, E. Collalti, K. A. Fargnoli, L. F. Hall, F. Wong-Staal, and M. S. Reitz, Jr.** 1987. Sequence of simian immunodeficiency virus and its relationship to the human immunodeficiency viruses. Nature (London) **328**:539–543.
9. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
10. **Gurgo, C., H. G. Guo, G. Franchini, A. Aldovini, E. Collalti, K. Farrell, F. Wong-Staal, R. C. Gallo, and M. S. Reitz, Jr.** 1988. Envelope sequences of two new United States HIV-1 isolates. Virology **164**:531–536.
11. **Guyader, M., M. Emerman, P. Sonigo, F. Clavel, L. Montagnier, and M. Alizon.** 1987. Genome organization and transactivation of the human immunodeficiency virus type 2. Nature (London) **326**:662–669.
12. **Hahn, B. H., M. A. Gonda, G. M. Shaw, M. Popovic, J. A. Hoxie, R. C. Gallo, and F. Wong-Staal.** 1985. Genomic diversity of the acquired immune deficiency syndrome virus HTLV-III: different viruses exhibit greatest divergence in their envelope genes. Proc. Natl. Acad. Sci. USA **82**:4813–4817.
13. **Hirsch, V., N. Riedel, and J. I. Mullins.** 1987. The genome organization of STLV-3 is similar to that of the AIDS virus except for a truncated transmembrane protein. Cell **49**:307–319.
14. **Jameson, B. A., P. E. Rao, L. I. Kong, B. H. Hahn, G. M. Shaw, L. E. Hood, and S. B. Kent.** 1988. Location and chemical synthesis of a binding site for HIV-1 on the CD4 protein. Science **240**:1335–1339.
15. **Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J. C. Gluckman, and L. Montagnier.** 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature (London) **312**:767–768.
16. **Kowalski, M., J. Potz, L. Basiripour, T. Dorfman, W. C. Goh, E. Terwilliger, A. Dayton, C. Rosen, W. Haseltine, and J. Sodroski.** 1987. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. Science **237**:1351–1355.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Lasky, L. A., G. Nakamura, D. H. Smith, C. Fennie, C. Shimasaki, E. Patzer, P. Berman, T. Gregory, and D. J. Capon.** 1987. Delineation of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. Cell **50**:975–985.
19. **Lifson, J. D., M. B. Feinberg, G. R. Reyes, L. Rabin, B. Banapour, S. Chakrabarti, B. Moss, F. Wong-Staal, K. S. Steimer, and E. G. Engleman.** 1986. Induction of CD4-dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. Nature (London) **323**:725–728.
20. **Lifson, J. D., K. M. Hwang, P. L. Nara, B. Fraser, M. Padgett, N. M. Dunlop, and L. E. Eiden.** 1988. Synthetic CD4 peptide derivatives that inhibit HIV infection and cytopathicity. Science **241**:712–716.
21. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47**:333–348.
22. **McCune, J. M., L. B. Rabin, M. B. Feinberg, M. Lieberman, J. C. Kosek, G. R. Reyes, and I. L. Weissman.** 1988. Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus. Cell **53**:55–67.
23. **McDougal, J. S., M. S. Kennedy, J. M. Sligh, S. P. Cort, A. Mawle, and J. K. Nicholson.** 1986. Binding of HTLV-III/LAV to T4+ T cells by a complex of the 110K viral protein and the T4 molecule. Science **231**:382–385.
24. **McDougal, J. S., J. K. Nicholson, G. D. Cross, S. P. Cort, M. S. Kennedy, and A. C. Mawle.** 1986. Binding of the human retrovirus HTLV-III/LAV/ARV/HIV to the CD4 (T4) molecule: conformation dependence, epitope mapping, antibody inhibition, and potential for idiotypic mimicry. J. Immunol. **137**: 2937–2944.
25. **Poiesz, B. J., F. W. Ruscetti, A. F. Gazdar, P. A. Bunn, J. D. Minna, and R. C. Gallo.** 1980. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc. Natl. Acad. Sci. USA **77**:7415–7419.
26. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, et al.** 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature (London) **313**:277–284.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
28. **Skinner, M. A., R. Ting, A. J. Langlois, K. J. Weinhold, H. K. Lyerly, K. Javaherian, and T. J. Matthews.** 1988. Characteristics of a neutralizing monoclonal antibody to the HIV envelope glycoprotein. AIDS Res. Hum. Retroviruses **4**:187–197.
29. **Smith, D. H., R. A. Byrn, S. A. Marsters, T. Gregory, J. E. Groopman, and D. J. Capon.** 1987. Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen. Science **238**: 1704–1707.
30. **Sodroski, J., W. C. Goh, C. Rosen, K. Campbell, and W. A. Haseltine.** 1986. Role of the HTLV-III/LAV envelope in syncytium formation and cytopathicity. Nature (London) **322**:470–474.
31. **Srinivasan, A., R. Anand, D. York, P. Ranganathan, P. Feorino, G. Schochetman, J. Curran, V. S. Kalyanaraman, P. A. Luciw, and R. Sanchez-Pescador.** 1987. Molecular characterization of human immunodeficiency virus from Zaire: nucleotide sequence analysis identifies conserved and variable domains in the envelope gene. Gene **52**:71–82.
32. **Starcich, B. R., B. H. Hahn, G. M. Shaw, P. D. McNeely, S. Modrow, H. Wolf, E. S. Parks, W. P. Parks, S. F. Josephs, R. C. Gallo, and F. Wong-Staal.** 1986. Identification and characteriza-

tion of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. Cell **45**:637–648.

33. **Stein, B. S., S. D. Gowda, J. D. Lifson, R. C. Penhallow, K. G. Bensch, and E. G. Engleman.** 1987. pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. Cell **49**:659–668.
34. **Veronese, F. D., A. L. DeVico, T. D. Copeland, S. Oroszlan, R. C. Gallo, and M. G. Sarngadharan.** 1985. Characterization of gp41 as the transmembrane protein coded by the HTLV-III/LAV envelope gene. Science **229**:1402–1405.
35. **Willey, R. L., R. A. Rutledge, S. Dias, T. Folks, T. Theordore, C. E. Buckler, and M. A. Martin.** 1986. Identification of conserved and divergent domains within the envelope gene of the acquired immunodeficiency syndrome retrovirus. Proc. Natl. Acad. Sci. USA **83**:5038–5042.
36. **Zoller, M. J., and M. Smith.** 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. DNA **3**:479–488.