Transdominant Inhibition of Wild-Type Human Immunodeficiency Virus Type 2 Replication by an Envelope Deletion Mutant

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The envelope glycoprotein of human immunodeficiency virus type 2 (HIV-2) is primarily responsible for virus attachment and entry into the target cell population. We constructed an HIV-2 mutant virus containing an in-frame deletion within the putative CD4-binding sequences of the envelope glycoprotein and confirmed that the mutant envelope is unable to bind CD4 and that the mutant virus is noninfectious. To investigate whether this mutant could dominantly interfere with wild-type replication, we coexpressed proviral DNAs of both wild-type and mutant viruses in cells and assayed the production of infectious HIV-2 virions. Interference with virus replication was indeed observed with mutant DNA, and a maximal effect was achieved with 10-fold excess mutant DNA over wild-type DNA in the cotransfection experiments. The transdominant effect on virus replication does not appear to be at the level of wild-type envelope expression or gp120-CD4 interaction. Rather, the interference may be at the level of mixed-oligomer formation during progeny virus assembly and may occur by either destabilizing the multimeric structure of gp120 or forming a defective mixed multimeric gp120 which is unable to complete the receptor binding and/or postbinding events needed for infection.

The generation of a dominant negative phenotype provides a novel approach for inhibiting the normal function of a gene (8). Dominant negative mutations directed at essential genes during virus replication have been examined for use in possible antiviral strategies against human immunodefi
ciency virus (HIV) infection. The two major regulatory proteins of HIV-1, Tat and Rev, have recently been targeted for such an approach (26). A mutational analysis of Tat has generated several transdominant mutants which can effect
tively inhibit wild-type Tat function on the HIV promoter in vitro (17, 21). A dominant negative phenotype of Rev has also been shown to repress wild-type Rev function when supplied in trans (15). In addition, mutations in the core structural protein Gag and the amino-terminal domain of the transmembrane glycoprotein gp41 of HIV-1 have generated mutants which can dominantly interfere with wild-type virus replication in vitro (6, 27). For the gp41 dominant negative mutant, a dramatic interfering effect on syncytium formation and infectivity of HIV-1 was seen, even in the presence of excess wild-type gp41. A study conducted in our laboratory characterized viruses containing mutations in a similar fusogenic sequence in the amino-terminal domain of gp41 of HIV-2 (25). Although these HIV-2 mutant viruses, which are severely impaired in their fusogenic function, did not domi
nantly interfere with virus infection, they did diminish the capacity of the wild-type virus to induce syncytia, suggesting that an interfering effect on syncytium formation was ex
ereted by the mutant gp41 glycoprotein (24). These results indicate that the env gene may be an appropriate target for generation of transdominant mutants.

The envelope glycoprotein of HIV is initially synthesized as a precursor (gp160) which is proteolytically cleaved into the envelope glycoprotein gp120 and the transmembrane glycoprotein gp41. Previous reports have shown that the envelope glycoprotein gp120 of HIV-1 and HIV-2 most probably organizes into multimeric structures before virion assembly (1, 3, 22, 30). Therefore, an envelope mutant may be able to interfere with the production of infectious wild-type virus by disrupting multimerization of gp120 or inactivating the polypeptide complex.

We have initiated a study to determine whether an HIV-2 mutant containing a deletion within the putative CD4-binding domain of the envelope glycoprotein could inhibit wild-type virus production. This mutant virus is completely noninfectious, and the mutant envelope protein is unable to bind to soluble CD4. The generation of wild-type virus was dominantly interfered with in the presence of increasing proportions of mutant to wild-type proviral DNA in cotrans
dected cells. The dominant phenotype is not accounted for by impairment of wild-type envelope expression or wild-type gp120-CD4 interaction. We propose that this interference most probably resides at the level of envelope multimerization and/or virion stability.

MATERIALS AND METHODS

Cell lines and DNA. CV-1, COS-1, and HeLa cells were maintained in Dulbecco’s minimal essential medium with 10% fetal calf serum. Human TK-143B cells were maintained in Dulbecco’s minimal essential medium containing 10% fetal calf serum and 20 mg of 5-bromodeoxyuridine per ml. H9 cells were maintained in RPMI 1640 medium with 10% fetal calf serum. The complete HIV-2SIbl/Sy provirus (5) was obtained as two subclones, pEOP and pKF-3, which yield infectious virus following ligat
don at a common SacI restriction site and transfection into host cells (7).

Mutagenesis and plasmid constructions. The deletion muta
tion of the env gene was performed with pKF-3. A 1,230-bp EcoRI-BamHI fragment containing the putative gp120 CD4-binding site was subcloned into M13mp19. Oli
gonucleotide site-directed mutagenesis was performed es
sentially as described by Kunkel et al. (10) to delete four amino acids from positions 7411 to 7422 within the putative CD4-binding region of gp120. Plasmid sequencing and phage DNA sequencing were done essentially as described by Sanger et al. (23) with Sequenase (United States Biochemical). The mutation was verified by sequence analysis and reinserted into pKF-3 as an EcoRI-BamHI fragment. Recombinant plasmids were also verified by sequencing before the generation of mutant viruses.

**Transfections and virus production.** The replication-competent HIV-2_{
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\text{env}} provirus was generated by a ligation procedure previously described by Hattori et al. (7). Briefly, plasmids pEGFP and pKF-3 were digested with SacI and ligated. The ligated DNA was purified by phenol-chloroform extraction, concentrated by ethanol precipitation, and transfected into COS-1 cells by a modified lipofection procedure (4). At 48 h posttransfection, COS-1 cell supernatants were monitored for virus expression by a simian immunodeficiency virus (SIV) enzyme-linked immunosorbent assay (ELISA) system for p26 antigen production (Coulter), and equivalent p26 units were used to infect H9 cells. For dominant-interference experiments, 1, 5, and 10 µg of mutant constructs were cotransfected into COS-1 cells with 1 µg of the wild type. The ratio of mutant to wild-type DNA was consistent with the microgram amounts of DNA used in the transfections. The total amount of DNA was kept constant by the addition of nonspecific plasmid DNA pSVL (Pharmacia). Infections with H9 cells were done as described above. For all transfections, an internal control was established by cotransfecting plasmid pCH110 (Pharmacia) and quantitating for β-galactosidase activity (16).

**Generation of recombinant vaccinia viruses.** Recombinant vaccinia viruses (VV_{\text{env}} and VV_{\text{ΔCD4env}}) expressing wild-type and mutant HIV-2 envelope glycoproteins were constructed essentially as described by Mackett et al. (14). A 3.1-kb SacI-KpnI fragment from pKF-3 was cloned into M13mp19, and site-directed mutagenesis was used to generate a BamHI site immediately upstream from the start of env translation. A BamHI-EcoRI fragment isolated from a partial digest was cloned into the vaccinia virus insertion vector pVV1 (29). For construction of a recombinant vaccinia virus expressing Gag polyprotein, a KpnI-EcoRI fragment was isolated and cloned into pVV1. Plasmid DNA clones were used to generate recombinant viruses by calcium phosphate precipitation of vaccinia virus-infected CV-1 cells. Recombinant viruses were identified by dot blot hybridization, plaqued purified three times, and grown in larger stocks on HeLa cells.

**Radioimmunoprecipitation analysis.** Envelope expression was analyzed by labeling 3 × 10^6 CV-1 cells infected at a multiplicity of infection of 20. Cells were washed twice with phosphate-buffered saline, and culture medium was replaced with methionine-free medium and infected with virus for 1 h. After incubation, the inoculum was removed and cells were labeled for 16 h with medium containing 1% dialyzed fetal calf serum and [35S]methionine at 100 µCi. Cell extracts were prepared by exposure of the cells to lysis buffer (1% Triton X-100, 50 mM Tris [pH 8.0], 100 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 1% sodium deoxycholate) for 30 min at 0°C. Aliquots of lysates were precleared with protein A-Sepharose (Pharmacia), and immunoprecipitated (11) overnight with serum from an individual infected with HIV-2 (provided by P. Kanki, Harvard School of Public Health). Immunoprecipitates were washed three times in lysis buffer, boiled for 3 min in loading buffer (100 mM Tris-Cl [pH 7.0], 40% glycerol, 0.004% bromophenol blue, 1% SDS, 250 mM β-mercaptoethanol), and subjected to SDS-polyacrylamide gel electrophoresis. For immunoprecipitations of envelope protein from the medium of infected cells, 500-µl aliquots of labeled medium were subjected to low-speed centrifugation to remove cell debris before the addition of antibody.

**sCD4-binding assays.** To assess the ability of mutant envelope glycoproteins to bind CD4, we performed soluble-CD4 (sCD4) binding assays essentially as described by Dedera and Ratner (2). CV-1 cells were infected with recombinant viruses expressing wild-type or mutant envelope proteins. After labeling and preparation of infected-cell lysates, sCD4 (Repligen) was coupled to protein A-Sepharose by using purified OKT4 antibody (Ortho) as follows. Antibody (5 µg) was mixed with an equal volume of protein A-Sepharose for 1 h at 4°C. Then 20 µg of sCD4 (50 µg/ml) was added for 2 h at 4°C, and the mixture was washed three times. Lysates (500 µl) of infected cells were precipitated with the sCD4-OKT4 beads and subjected to SDS-polyacrylamide gel electrophoresis.

**RESULTS**

**Expression, processing, transport, and CD4 binding of the mutant envelope glycoprotein.** To examine whether an HIV-2 envelope mutation in the putative CD4-binding site could induce a transdominant phenotype, we generated an in-frame deletion in the CD4-binding region of the env coding sequence of the HIV-2_{\text{env}} clone (Fig. 1). This mutation (ΔCD4_{\text{env}}) eliminates four amino acids in the HIV-2 env gp120 corresponding to residues critical for the interaction between HIV-1 env gp120 and the CD4 protein (12, 19). This mutation was confirmed by DNA sequence analysis, and the fragment containing the env CD4-binding region was subcloned into plasmid pKF-3.

We examined by radioimmunoprecipitation the expression, processing, and secretion of the mutant envelope glycoprotein to determine whether the mutation within the putative CD4-binding region affected expression of the envelope glycoprotein. To optimize the expression of HIV-2 envelope glycoproteins, both wild-type and mutant glycoproteins were first expressed in recombinant vaccinia viruses. Radioimmunoprecipitations were performed in parallel on CV-1 cells infected with viruses VV_{\text{env}} and VV_{\text{ΔCD4env}}. No apparent differences between wild-type and
mutant envelope glycoprotein synthesis and expression were observed (Fig. 2A). Although the mutant envelope may have undergone some conformational changes as a result of the deletion, it has remained immunogenic as evidenced by the radioimmunoprecipitation analysis.

The detection of the surface glycoprotein gp120 in the cell medium indicates proteolytic cleavage of the precursor envelope glycoprotein, gp160, into the products gp120 and gp41 and the subsequent transport of the mature envelope glycoproteins to the cell surface (9). To investigate the processing and transport of the present mutant envelope glycoprotein, we assayed for extracellular HIV-2 gp120 in the cell medium. The medium of 35S-labeled CV-1 cells infected with the HIV-2 recombinant vaccinia viruses was immunoprecipitated with HIV-2 patient sera and subjected to SDS-polyacrylamide gel electrophoresis analysis. gp120 was evident in the medium of the infected cells, indicating that the deletion within the CD4-binding domain did not affect processing and transport of the mature gp120 glycoprotein (Fig. 2B).

To demonstrate that the ability of the mutant envelope glycoprotein to bind CD4 has been eliminated by the deletion mutation, we performed radioimmunoprecipitations on VVenv and VVΔCD4env-infected COS-1 cell lysates by using scD4 coupled to OKT4-protein A-Sepharose. No binding between the ΔCD4env protein and scD4 was observed when compared with the wild-type envelope protein (see Fig. 4, lanes 2 and 3). Therefore, the same region of gp120 previously shown to be important for HIV-1 gp120-CD4 interaction also appears to be critical for HIV-2 gp120.

**Phenotypic analysis of the HIV envelope mutant genome.** A phenotypic analysis of the HIV-2 ΔCD4env mutant was tested by transfecting COS-1 cells with proviral DNA of the mutant in parallel with wild-type DNA and measuring for p26 antigen production. Whereas the p26 activity in COS-1 cell lysates were nearly equivalent for both viruses, cells transfected with ACD4env DNA unexpectedly but consistently produced 5- to 10-fold lower p26 activity in the supernatant than the wild-type DNA did (Table 1). To assess the relative infectivity of the ΔCD4env mutant virus, H9 cells were exposed to wild-type and mutant DNA-transfected cell supernatants adjusted for equivalent amounts of antigen and assayed for p26 activity over a 3-week period. No measurable p26 antigen was detected for the mutant virus, suggesting that the virus particles generated were noninfectious.

**HIV-2 Env mutant interferes with the replication of wild-type virus.** To determine whether the ΔCD4env mutant could dominantly interfere with wild-type virus production, we cotransfected COS-1 cells with wild-type proviral DNA and ΔCD4env mutant DNA at ratios of 1:1, 1:5, and 1:10. At 48 h posttransfection, we used COS cell supernatants to infect H9 cells. To evaluate the generation of infectious virus during a 3-week period, we assayed aliquots of H9 cell supernatants for p26 activity by the SIV ELISA system (Table 2). A moderate interfering effect on the production of infectious virus was seen when equivalent amounts of wild-type and mutant proviral DNA were used (Fig. 3). However, increasing the amount of mutant ΔCD4env DNA over wild-type DNA produced a dramatic inhibitory effect on the amount of infectious virus generated from the COS cells. This inhibition appears to be specific for HIV-2 p26 because, similar assays with a constant amount of the HIV-1 proviral clone pHXB2 in the presence of increasing amounts (up to 20-fold excess) of mutant ΔCD4env proviral DNA did not show any inhibition of virus replication (data not shown).

**Expression and sCD4 binding of wild-type envelope glycoprotein are not repressed in the presence of coexpressed mutant envelope glycoprotein.** We attempted to determine whether the basis of interference of wild-type virus infectivity was due to inhibition of CD4 binding. However, it was difficult to perform these analyses with the transfected COS cells since the amount of Env protein expressed is very small. We therefore used the vaccinia virus system for this purpose. A coexpression experiment, which involved infecting CV-1 cells with recombinant vaccinia viruses VVenv and VVΔCD4env, and

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**TABLE 1. Phenotypic analysis of the ΔCD4env mutant**

<table>
<thead>
<tr>
<th>DNA transfected</th>
<th>p26 activity in COS cell medium (pg/ml)</th>
<th>p26 activity in COS cell lysate (pg/ml)</th>
<th>p26 activity in H9 supernatant 3 weeks postinfection (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Wild type</td>
<td>996</td>
<td>446</td>
<td>1,989</td>
</tr>
<tr>
<td>ΔCD4env</td>
<td>140</td>
<td>512</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

* Supernatants from either transfected COS cells or infected H9 cells were monitored for virus expression by using an SIV ELISA system (Coulter) for p26 antigen production. Because of the divergence of HIV-2 and SIV, this assay is 1 to 2 log units less sensitive than a homologous assay system.

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**TABLE 2. p26 activity in the supernatant of H9 cells**

<table>
<thead>
<tr>
<th>Virus</th>
<th>WT/ΔCD4env ratio</th>
<th>p26 activity (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-2 WT* alone</td>
<td></td>
<td>2,100</td>
</tr>
<tr>
<td>HIV-2 WT + ΔCD4env</td>
<td>1:1</td>
<td>912</td>
</tr>
<tr>
<td>HIV-2 WT + ΔCD4env</td>
<td>1:5</td>
<td>346</td>
</tr>
<tr>
<td>HIV-2 WT + ΔCD4env</td>
<td>1:10</td>
<td>135</td>
</tr>
</tbody>
</table>

* Infectivity of H9 cells was measured as described in Table 1, with supernatant from transfected COS cells adjusted for a constant amount of viral protein p26. Expression of virus from the infected H9 cells was then monitored by using extracellular p26 over a 3-week period. The values for p26 activity given here represent the highest recorded activities from a four-point assay 21 day 21 postinfection. A similar experiment involving HIV-1 (HXB2) DNA plus ΔCD4env DNA cotransfection of COS cells followed by infection of H9 cells did not show any reduction in viral protein expression even at a 1:20 ratio.

* WT, wild type.
V_{ACD4env} at a multiplicity of infection of 10, was performed. It was expected that 100% of the cells would be infected by both viruses. In the presence of up to a 10-fold excess of V_{ACD4env} wild-type envelope expression from V_{env} was unaffected and the sCD4-binding ability was not affected or outcompeted by coexpression of the mutant envelope glycoprotein (Fig. 4).

Expression of Gag protein in the presence of mutant and wild-type envelope glycoproteins. The finding of a decrease in the level of extracellular Gag protein released from cells transfected by the envelope mutant virus was unexpected. Recently it has been shown that a direct interaction between envelope and Gag protein may exist during virus assembly and that this interaction may influence the site of virus release from the infected cell (20). One possible explanation for the decrease in the p26 activity with the ACD4env virus is the inefficient interaction between Gag and the mutant envelope glycoprotein during virus assembly and release. To investigate whether the ACD4env mutant is interfering with the release of Gag protein during processing and assembly, we constructed a recombinant vaccinia virus expressing HIV-2 Gag polyprotein (V_{gag}) and analyzed it for release of core antigen in the presence of wild-type and mutant envelope glycoproteins. CV-1 cells were infected for 12 h with V_{gag} alone, V_{gag} and V_{env} or V_{gag} and V_{ACD4env} and the efficiency of core particles released from recombinant vaccinia virus-infected cells was quantitated (p26 ELISA) along with the level of intracellular Gag polyprotein (Table 3). No dramatic decrease in core antigen release was seen with the coexpression of V_{gag} and V_{ACD4env} when compared with either V_{gag} alone or V_{gag} and V_{env}. A slight increase in the efficiency of core antigen release was observed in the presence of wild-type envelope expression when compared with V_{gag} alone. A radioimmunoprecipitation analysis of the above experiment also revealed no discernible difference between the production and release of core protein in the presence of the mutant envelope glycoprotein (data not shown). Thus, it appears that the ACD4env mutant does not interfere with Gag protein release into the supernatant.

**DISCUSSION**

We have generated an HIV-2 envelope mutant which is defective for CD4 binding and infectivity. This proviral DNA displays a potent transdominant effect on wild-type virus replication in T cells. A maximal interfering effect on the generation of infectious virus particles was observed when a 10-fold excess of mutant proviral DNA over wild-type DNA was used. This inhibition appears to be strain specific, since cotransfections of mutant DNA with proviral DNA of HIV-1 did not show any inhibitory effects on virus replication.

An analysis of the mutant envelope glycoprotein expressed in vaccinia virus did not show any defect in the level of expression when compared with the wild type. The processing and transport of the mutant glycoprotein were unaffected by the deletion mutation as evidenced by the presence of gp120 in the cell medium. Furthermore, the coexpression of both glycoproteins in CV-1 cells revealed no dominant interfering effect on the expression or CD4-binding ability of the wild-type envelope glycoprotein.

The basis for the interference with infectivity could be the formation of mixed envelope multimers containing both wild-type and mutant envelope monomers. Previous studies have shown that gp120 can form oligomeric structures (3, 22, 30). Specifically, the data of Chakrabarti et al. (1) suggest that the HIV-2_{env} envelope protein assembles posttranslationally into more highly ordered oligomeric forms. A 10-fold excess of mutant gp120 may interact with wild-type gp120, resulting in the formation of a virus which has an impaired ability to infect susceptible cells. Thus, viruses containing a
mixture of envelope glycoproteins may be noninfectious as a result of the mutation in the putative CD4-binding region of gp120. Although our data showed that wild-type envelope protein coexpressed with mutant envelope retains the capacity to bind sCD4, this essay probably detects gp120 binding as a monomeric unit. It is possible that envelope-receptor interactions are different as multimeric complexes. Recently it has been shown that the sCD4 interaction with soluble HIV-2 gp120 is different from its interaction with the assembled envelope complex on the virion surface (18). Layne et al. (13) also recently showed that HIV requires multiple gp120 molecules for CD4-mediated infection. One could envision insertion of defective gp120 molecules on the virion surface, which would reduce the affinity for receptor binding and result in failure to initiate the infection process. Therefore, insertion of a mutant gp120 glycoprotein into a highly oligomerized complex of protein monomers may inactivate the complex, thereby contributing to the loss of infectivity. This theory has been previously proposed for the HIV-1 Gag and transmembrane envelope proteins and the vesicular stomatitis virus G glycoprotein (6, 27, 31). Freed et al. (6) have recently shown that a polar substitution in the fusogenic peptide region of the HIV-1 envelope transmembrane glycoprotein could dominantly interfere with syncytium formation and the generation of infectious wild-type virus. Even in the presence of excess wild-type gp41, a dominant interfering effect was evident, suggesting that a highly oligomerized complex of gp41 transmembrane glycoproteins may be necessary for fusion and infection. Another possibility is that the deletion in the mutant gp120 causes some conformational changes that would destabilize a mixed multimeric complex. It will be of interest to further characterize this Env mutant to define the mechanism of transdominance.

We also observed a moderate reduction in extracellular p26 antigen levels in cells transfected with mutant proviral DNA. This is not due to an accidental mutation in Gag since the same 5′ half of the HIV-2_bilsky clone was used to generate both wild-type and mutant viruses. These results suggest that a defect in virus assembly, release, or both occurred with the envelope mutant virus. If so, this defect can also contribute to the negative transdominant phenotype. A recent study describes the possible interactions between Gag and envelope proteins in the polarized maturation of HIV at the basolateral membrane of epithelial cells (20). Extrapolating from this proposed interactive role of Gag and Env proteins in determining the site of virus release, we decided to examine the possibility of a transdominant envelope phenotype interfering with export of Gag protein and therefore with the process of virus release. However, our results did not reveal an interference in the release of Gag protein by the ΔCD4-env glycoprotein in cells infected by recombinant vaccinia viruses expressing these proteins. Thus, the basis for the reduction in the level of extracellular Gag protein remains to be determined.

Much research activity has focused on the use of the envelope protein of HIV as a subunit vaccine for prophylaxis or therapy. However, because interaction of gp120 with CD4 itself can potentially contribute to immunosuppressive effects, a preferred gp120 vaccine should be deleted in the CD4-binding site (28), provided that such a deletion would not destabilize the protein or significantly alter its immunogenicity. The study reported here suggests that the HIV-2 gp120 deleted in sequences within the putative CD4-binding site is stable, is processed and recognized by antibodies directed against the wild-type envelope protein, but is unable to bind CD4. Furthermore, such an env mutant is also capable of interfering with the replication of wild-type virus. Although high levels of the interfering protein are necessary to inhibit virus replication, a lower level may be sufficient to induce immunity. Thus, a mutant envelope gene which is expressed at a basal level but superinducible upon HIV infection may function dually as a vaccine and an antiviral compound. Refinement of such approaches may add to the arsenal of strategies against HIV.

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