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The Transmembrane Glycoprotein of Human Immunodeficiency Virus Type 1 Induces Syncytium Formation in the Absence of the Receptor Binding Glycoprotein

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To study the intracellular transport and biological properties of the human immunodeficiency virus type 1 (HIV-1) transmembrane glycoprotein (TM; gp41), we constructed a truncated envelope gene in which the majority of the coding sequences for the surface glycoprotein (SU; gp120) were deleted. Transient expression of this truncated *env* gene in primate cells resulted in the biosynthesis of two proteins with M_rs of 52,000 and 41,000, respectively. Immunofluorescence studies with antibodies to the HIV-1 TM protein indicated that the intracellular and surface localization of these proteins were indistinguishable from those of the native HIV-1 gp120-gp41 complex. These results indicate that the oligosaccharide processing and cell surface transport of the HIV-1 TM protein were not dependent on the presence of the receptor binding subunit, gp120. Syncytium formation was readily detected upon expression of the deleted HIV-1 *env* gene into COS and CD4$^+$ HeLa cell lines, suggesting that in the absence of gp120, the TM protein retained biological activity. This observation was confirmed by infection of primate and mouse cell lines with a recombinant vaccinia virus (vvgp41) expressing the truncated HIV-1 *env* gene. These results strongly suggest that (i) the two biological activities of the HIV-1 envelope glycoprotein can occur independently and (ii) the association of the two glycoprotein subunits may restrict the fusion activity of the transmembrane component to CD4$^+$ cells.

Human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS, is the prototypical member of the lentivirus subfamily of retroviruses. As with other replication-competent retroviruses, HIV-1 codes for a single high-mannose oligosaccharide glycoprotein precursor, gp160. Once synthesized on the rough endoplasmic reticulum, the gp160 is transported to the Golgi complex where selected oligosaccharide chains are modified to those of the complex type (29). The Env precursor is then proteolytically cleaved into a heavily glycosylated surface glycoprotein subunit known as gp120 (SU) and a transmembrane subunit known as gp41 (TM) (1, 4, 13, 21, 25, 26, 31). The exact intracellular site of cleavage is presently unknown, but studies have suggested that this event occurs in the *trans*-Golgi network (25). The two subunits of the gp120-gp41 complex are associated by noncovalent bonds in an oligomeric structure with the gp41 protein anchoring the complex to the viral membrane (2, 10, 37, 38). Infection by HIV is initiated by attachment of the gp120 glycoprotein to the first V region-like domain of the CD4 receptor on the target cell (20, 30). Subsequently, the transmembrane protein mediates a pH-independent membrane fusion event at the cell surface, permitting release of the viral nucleocapsid into the cell (14, 18, 24, 33). Previous studies have indicated that binding of the gp120 to the CD4 is essential to initiate membrane fusion and that domains other than the binding site on the CD4 molecule are necessary for the fusion event to take place (5, 30). Although the gp120 and gp41 subunits seem to have very well defined domains responsible for either receptor binding or membrane fusion (3, 9, 14, 20), the interactions between the two subunits necessary for membrane fusion are not well understood.

To more fully understand the biological activities of the gp41 protein in eukaryotic cells, a HIV-1 glycoprotein gene in which the majority of the receptor binding subunit has been deleted but retains those sequences essential for membrane glycoprotein biosynthesis and processing (signal sequence and the gp120-gp41 proteolytic cleavage site) has been constructed. We present evidence that this truncated envelope gene expresses a TM protein which is indistinguishable from the gp41 subunit of the native HIV-1 glycoprotein complex. Furthermore, the recombinant transmembrane protein is capable of being transported to the cell surface and inducing syncytium formation in several different mammalian cell lines, some of which do not express the CD4 receptor molecule. These results indicate that the fusion activity associated with the HIV-1 envelope protein is an independent event that does not require expression of the gp120 subunit or the presence of the CD4 cell receptor.

MATERIALS AND METHODS

Nomenclature. SU and TM refer to the surface and transmembrane glycoprotein subunits of the HIV-1 glycoprotein (they are also known as gp120 and gp41), respectively (21). Nucleotide numbers are based on the published HIV-$1_{BRU/LAI}$ DNA sequence (39); the first nucleotide of R corresponds to nucleotide number 1.

Expression vectors and construction of the truncated enve-

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lope glycoprotein gene. The plasmids pwtENV and pME241 were gifts of M. Emerman at the Fred Hutchinson Cancer Research Center, Seattle, Wash. pwtENV is a variant of the HIV-$1_{BRU/LAI}$ infectious clone pBRU-1 with the following modifications: pwtENV contains a deletion between nucleotides 989 and 4098 that removes the majority of the HIV-$1_{BRU/LAI}$ *gag* and *pol* sequences, and the simian virus 40 (SV40) origin of replication has been placed adjacent to the HIV long terminal repeat (LTR). In COS-1 cells, this construction expresses the HIV-1 *env*, *tat*, and *rev* gene products by using the viral LTR promoter. The plasmid pME241 expresses only the HIV-1 *tat* product from the SV40 early promoter (12). The SU sequences were deleted from pwtENV by using the following procedure. The fragment *Sal*I-*Bam*HI, containing two-thirds of the HIV-$1_{BRU/LAI}$ *env* coding sequences, was separated from the expression plasmid pwtEnv and subcloned into pBR322. This subclone was digested with the restriction enzymes *Kpn*I and *Bgl*II to remove the SU sequences. The large *Kpn*I-*Bgl*II fragment was isolated, digested with T4 DNA polymerase and mung bean nuclease to remove the protuding ends, and self-ligated with T4 DNA ligase. Chain termination DNA sequencing of this subclone confirmed that the remaining *env* sequences were in the same ribosomal reading frame. The *Sal*I-*Bam*HI fragment without the SU sequences was removed from the pBR322 clone and used to reconstruct the HIV-1 *env* gene in the resulting expression plasmid ptrEnv.

Transfections. Cells grown to 70 to 80% confluence in 60-mm plates were transfected with 7 μg of plasmid ptrENV or pwtENV per plate by the DEAE-dextran procedure described by Emerman et al. (12). At 48 h posttransfection, the HIV glycoprotein expression was monitored by Western immunoblot analysis with the commercial kit Protoblot (Promega Corporation, Madison, Wis.) or by indirect immunofluorescence staining of cells grown on coverslips.

Antibodies. The HIV-1-positive human sera were obtained locally, and the reactivity of the sera to the HIV-1 glycoproteins was characterized by Western blot analysis. The monoclonal antibody to HIV-1 gp41 (50-69) was obtained from Susan Zolla-Pazner through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. The mouse monoclonal antibody to the HTLV-IIIB gp41 protein was obtained from NEN/Dupont (NEA-9303). The mouse monoclonal antibody Chessie 8 against the HTLV-IIIB gp41 protein was a contribution of George Lewis through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases.

Immunofluorescence studies. The intracellular localization of the wild-type and truncated forms of the HIV-1 envelope glycoprotein was determined by indirect immunofluorescence of permeabilized cells by using either human or mouse antibodies against the HIV-1 *env* gene products as the primary antibody and the corresponding fluorescein isothiocyanate (FITC)-conjugated anti-immunoglobulin as the second antibody. Cells grown on coverslips were washed with phosphate-buffered saline (PBS) and fixed with 5% acetic acid and 95% ethanol for 30 min at −20°C. The cell preparations were incubated with a 10^{-2} dilution of the primary antibody for 30 min and then washed with PBS–0.1% bovine serum albumin (BSA). An FITC-labeled anti-mouse or anti-human antibody was used as the secondary antibody. Cell surface expression was detected in a similar way, with the sole difference being that cells were not fixed until the incubation with the second antibody was completed.

Multinucleate activation of β-galactosidase indicator $CD4^+$ HeLa cells. A complete characterization of the $CD4^+$ HeLa indicator cell line that expresses the *Escherichia coli* β-galactosidase in the nucleus upon HIV-1 Tat transactivation was recently published (17). Briefly, expression of the HIV-1 glycoproteins and the Tat protein in this cell line induces the formation of multinucleated cells; the nuclei of these syncytia can be selectively stained because of the high contents of β-galactosidase activity. For this purpose cells were fixed in 1% formaldehyde–0.2% glutaraldehyde in PBS. Syncytium formation was visualized by staining for 50 to 120 min with a PBS solution containing 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, and 2.5 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at 37°C.

Construction of the recombinant vaccinia virus vector. We utilized the polymerase chain reaction (PCR) techniques to specifically amplify and molecularly clone the truncated HIV-1 glycoprotein into vaccinia virus-based vectors for expression studies. Briefly, oligonucleotide primers that corresponded to the 5′ end of the gp120 (primer A: 5′-GGCAATGAGAGTGAAGG-3′) and the 3′ end of the *env* gene including the termination codon (primer B: 5′-GCCAC CCATCTTATAGC-3′) were synthesized. These primers were used to amplify the truncated *env* gene sequence from plasmid ptrENV in a polymerase chain reaction by using the conditions suggested by the manufacturer for thermostable DNA polymerase isolated from *Thermus aquaticus*. Thirty cycles of denaturation (92°C, 1 min), annealing (55°C, 3 min), and primer extension (72°C, 3 min) were used in the amplification process. The PCR product, approximately 1.4 kb in length, was separated by agarose gel electrophoresis and the DNA was isolated by electroelution techniques. The isolated gene was molecularly cloned by ligation into the *Sma*I site of the vaccinia virus recombination vector, pEB-1. This vector is similar to the widely used pSC11 except that it utilizes two late p11 promoters instead of the p7.5 and p11 promoters previously described in the construction of pSC11 (7). Potential recombinants were then screened for plasmids with appropriate size inserts and confirmed by DNA hybridization to a probe derived from the gp41 gene. The complete sequence of one recombinant, pHIVgp41, was determined to ensure that no nucleotide errors that would lead to any deletions or the generation of premature termination codons had been introduced into the gene product (32). No premature codons were found from DNA sequence analysis. The pHIVgp41 plasmid was used to generate recombinant vaccinia viruses as previously described (7, 34, 36). A recombinant vaccinia virus, designated vvgp41, was isolated and used to prepare stocks for expression studies (16).

Labeling and immunoprecipitation of the truncated *env* gene products. HeLa cells were infected with either vvgp41 or vvIHD-J (wild-type vaccinia virus) at a multiplicity of infection of 10. At 15 h postinfection, infected cells were starved for leucine for an hour and radiolabeled with [^{3}H]leucine (500 μCi/10^6 cells in 500 μl of leucine-free minimum Eagle's medium) for 15 min. The radiolabel was removed and chased for a period (0 to 120 min) in nonradioactive complete medium. Cells were then washed with ice-cold Tris-buffered saline, and cell lysates were prepared in 1 ml of radioimmunoprecipitation buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate). The nuclei were removed by centrifugation, and cell lysates were incubated with a human monoclonal antibody against the HIV-1 gp41 glycoprotein for 15 h at 4°C. The immune

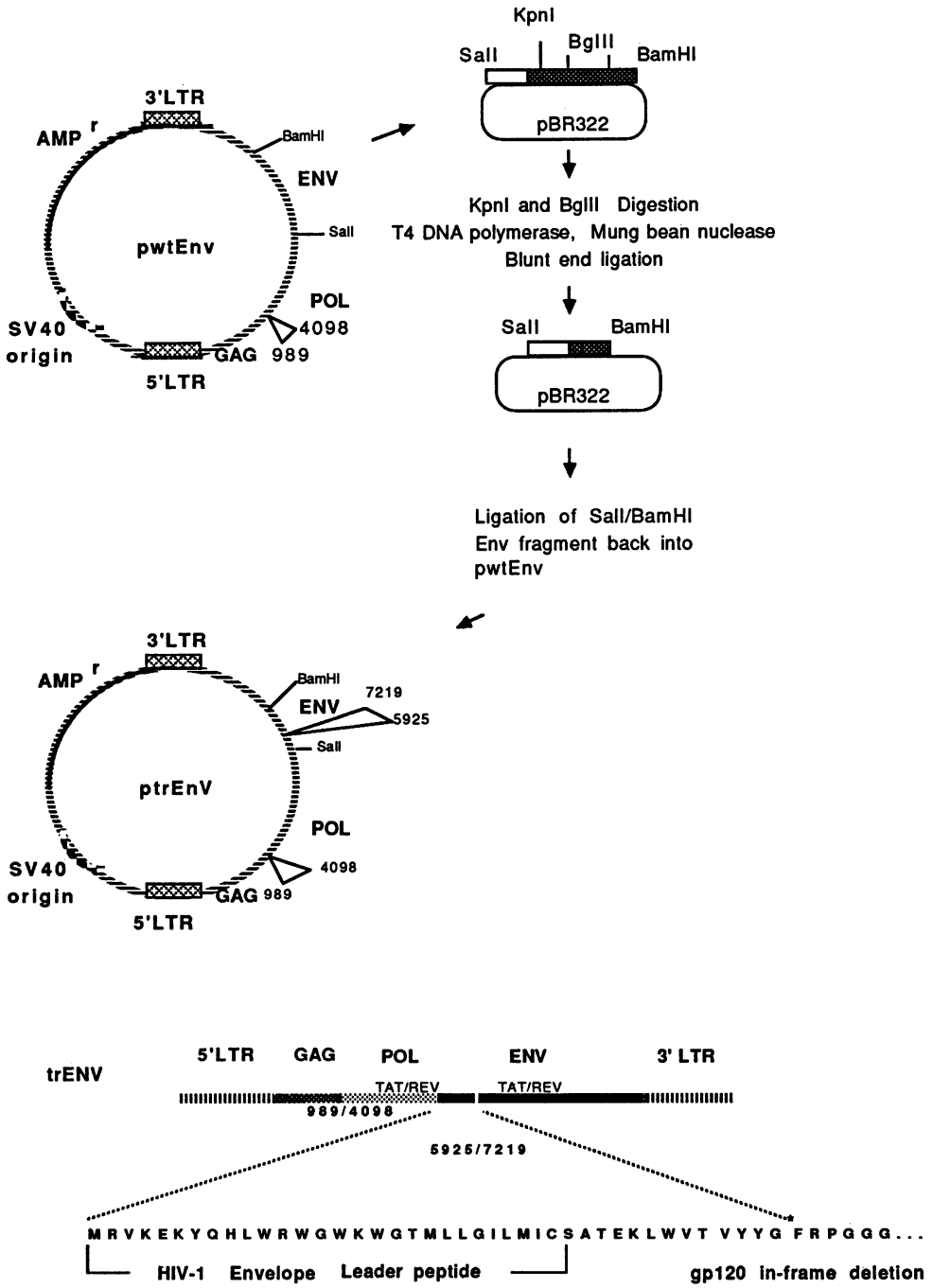

FIG. 1. Schematic diagram of the construction of a vector expressing the HIV-$1_{BRU/LAI}$ TM protein. The fragment *Sal*I-*Bam*HI, containing two-thirds of the HIV-$1_{BRU/LAI}$ *env* coding sequences, was separated from the expression plasmid pwtEnv and subcloned into pBR322. This subclone was digested with the restriction enzymes *Kpn*I and *Bgl*II to remove the SU sequences. The large *Kpn*I-*Bgl*II fragment was isolated, digested with T4 DNA polymerase and mung bean nuclease to remove the protruding ends, and self-ligated with T4 DNA ligase. Chain termination DNA sequencing of this subclone confirmed that the remaining *env* sequences were in the same ribosomal reading frame. The *Sal*I-*Bam*HI fragment without the SU sequences was removed from the pBR322 clone and used to reconstruct the HIV-$1_{BRU/LAI}$ *env* gene in the resulting expression plasmid ptrEnv.

complexes were collected on protein A-Sepharose, and the HIV-1 proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19). The proteins bands were visualized by standard autoradiographic techniques.

RESULTS

Construction of a vector that expresses the TM protein in the absence of the SU glycoprotein. To investigate the intracellular transport and biological properties of the HIV-1

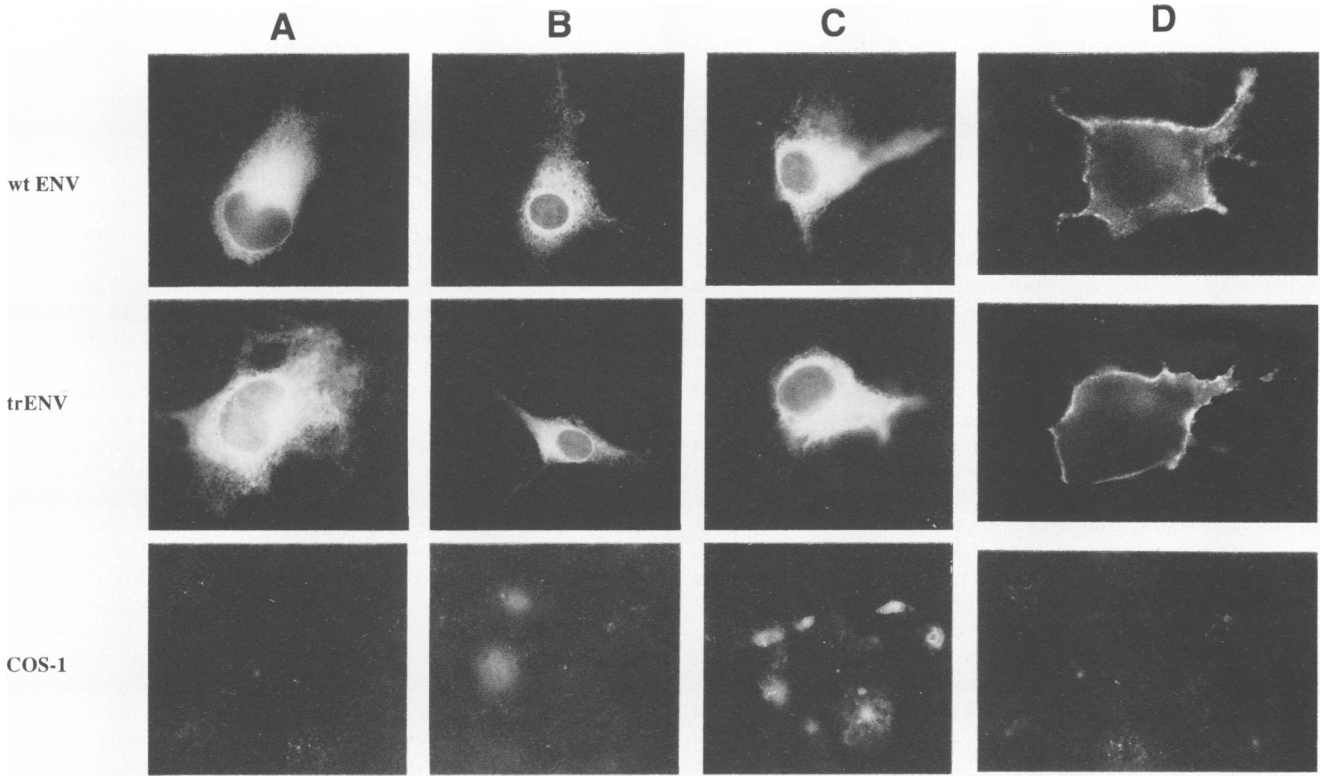

FIG. 2. Indirect immunofluorescence staining of COS-1 cells transiently expressing the HIV-$1_{BRU/LAI}$ *env* genes. COS cells grown on coverslips were transfected with the plasmids trENv and wtENV by using DEAE-dextran. At 48 h posttransfection, coverslips were washed with PBS and fixed in ethanol-acetic acid for 30 min at −20°C. The fixed cell preparations were incubated with a 10^{-2} dilution of the primary antibody for 30 min at 18°C, washed with PBS–0.1% BSA, and incubated with a FITC-labeled secondary antibody. (A) HIV-1-positive human serum; (B) anti-gp41 human monoclonal antibody 50-69; (C) anti-gp41 mouse monoclonal antibody NEA 9303 NEN/Dupont. wtENV, cells transfected with the pwtENV plasmid; trENV, cells transfected with the ptrENV; COS-1, mock-transfected COS-1 cells. (D) Cell surface expression of the HIV-$1_{BRU\ LAI}$ *env* gene products. COS-1 cells were transfected with either ptrENV or pwtENV as described in Materials and Methods. At 48 h posttransfection, cells grown on coverslips were washed with cold PBS and incubated with HIV-1-positive human serum followed by a FITC-labeled anti-human antibody at 4°C. Cells were fixed on the coverslips and mounted for observation. wtENV, cells transfected with the pwtENV plasmid; trENV, cells transfected with pwtENV plasmid; COS-1, mock-transfected COS-1 cells.

transmembrane glycoprotein in the absence of the receptor binding glycoprotein, the plasmid ptrENV was constructed as outlined in Fig. 1. The rationale behind the design of this truncated *env* gene was to obtain a primary translation product with the gp41 amino acid sequence that utilized the signals for protein translocation, intracellular transport, and proteolytic processing present in the native HIV-1 gp160 precursor. If the signals for translocation and N-linked glycosylation are recognized, a precursor glycoprotein with an M_r of 47,000 to 48,000 could be expected with the gp41 protein as the final product.

Intracellular and cell surface expression of the truncated HIV-1 envelope glycoprotein. Indirect immunofluorescence was used to monitor the level of expression of the native and truncated HIV-1 envelope glycoprotein gene products in transiently transfected COS cells. As shown in Fig. 2, the pattern of intracellular immunofluorescence in cells transfected with plasmids expressing the unmodified HIV gp120-gp41 or truncated HIV-1 glycoproteins were similar with human HIV-1-positive serum (Fig. 2A) and two monoclonal antibodies specific for the HIV-1 gp41 protein (Fig. 2B and C). Similarly, comparable levels of cell surface immunofluorescence were observed in cells transfected with plasmids expressing either the native or truncated envelope gene products (Fig. 2D). These results indicate that removal of the majority of the sequences encoding the receptor binding glycoprotein affected neither the level of expression nor the intracellular transport of the HIV-1 transmembrane glycoprotein.

The truncated HIV-1 *env* gene expresses two protein species in COS cells. The biosynthesis and processing of the primary translation product from the HIV-1 truncated *env* gene was compared with that of the native HIV-1 *env* gene product by Western blot analysis. When COS cells were transfected with pwtENV, protein bands corresponding to the precursor (gp160), the SU protein (gp120), and the TM protein (gp41) were detected with an HIV-1-positive human serum (Fig. 3A, lane 2). In contrast, a mouse monoclonal antibody specific for TM detected gp41 and more weakly the precursor gp160 but not gp120 (Fig. 3B, lane 2). Transfection of COS cells with the ptrENV vector resulted in the detection of two proteins having M_rs of 52,000 and 41,000 by using the HIV-1-positive human serum (Fig. 3A, lane 1), whereas the mouse monoclonal antibody specific for the TM detected a band with an M_r of 41,000 (Fig. 3B, lane 1). A protein with an M_r of 43,000 was also detected with human HIV-1-positive serum in cells expressing both envelope genes (Fig. 3A). This protein was less reactive with TM-specific monoclonal antibodies and not detected in mock-transfected cells. We have not ruled out the possibility that this protein species

corresponds to a form of the TM glycoprotein in which the N-linked oligosaccharides were processed differently. These data suggest that the truncated gene is initially translated as a protein precursor having an M_r of 52,000 that is subsequently processed to a polypeptide with an M_r of 41,000. With the antibodies tested this protein is antigenically indistinguishable from the gp41 subunit of the HIV-1 envelope glycoprotein complex.

The product of the truncated envelope gene is biologically active in different mammalian cell lines. Immunofluorescence studies revealed that approximately 10% of the COS cells transiently expressing the truncated *env* gene were multinucleated. Because syncytium formation is not usually observed in CD4-negative cells expressing the complete HIV-1 *env* gene, we decided to further study the apparent biological activity of this truncated Env product. To accomplish this, a HeLa cell line that expresses the human CD4 receptor and a transactivable *E. coli* β-galactosidase enzyme was used as an indicator of the fusion activity (17). In this system, the transfected plasmid that expresses the *env* gene also encodes the Tat protein which transactivates β-galactosidase expression. Because the β-galactosidase protein has been modified to contain a nuclear location signal, this enzyme is transported to the nucleus, where it is easily detected (17). In the first set of experiments, COS cells expressing the truncated and unmodified HIV-1 *env* genes were overlaid with 2×10^5 to 4×10^5 indicator HeLa cells. Fusion between the HeLa indicator cells and COS cells was readily detectable (>200 blue syncytia per 60-mm plate) when the complete envelope protein was expressed in these cells (Fig. 4A), whereas no fusion was detectable when COS cells were transfected with an unrelated pBR322 plasmid (Fig. 4B). These results contrast with those obtained with the truncated *env* protein (Fig. 4C and D). Transfection of COS cells with the ptrENV plasmid followed by immunofluorescence staining with a mouse monoclonal antibody for gp41 revealed the presence of multinucleated COS cells (Fig. 4D). However, when these cells were overlaid with the HeLa indicator cell line, fusion between the two cell lines was not observed (Fig. 4C). In a second set of experiments, the HeLa indicator cell line was transfected with either pwtENV or ptrENV expression vectors. Syncytium formation was detected in cells expressing either the truncated (Fig. 5C) or the complete (Fig. 5A) HIV-1 *env* gene product. In this set of experiments, even when the efficiencies of transfection were similar (3 to 5%), differences were observed in the number and size of the induced syncytia. When the entire envelope glycoprotein gene was expressed, about 30% of the total number of cells with blue nuclei were multinucleated (≥4 nuclei), whereas in cells transfected with ptrENV, 12% of the cells with blue nuclei were associated with the syncytia. Only blue mononucleated cells were observed when a Tat plasmid expression vector was transfected into the HeLa indicator cells (Fig. 5D). To rule out the possibility that the biological activity observed with trENV was the result of aberrant proliferation of the gp41 expressing cells, we determined the ability of antibodies to the HIV-1 glycoprotein to inhibit trENV-induced syncytium formation. As shown in Table 1, two HIV-1-positive human sera and a monoclonal antibody to gp41 were able to block the induction of syncytium formation by trENV and wtENV in this HeLa indicator cell line, demonstrating that the syncytia were the result of gp41-induced cell-to-cell fusion. From these results, we conclude that the transmembrane protein expressed from the truncated *env* gene is biologically active. The fusion activity of the TM protein is independent of the CD4-gp120 interactions. However, the process seems to require a critical distance between cell membranes which is facilitated by the gp120-CD4 receptor interaction.

Expression of the truncated envelope glycoprotein by using a recombinant vaccinia virus vector. Because of the SV40 T antigen dependence of the ptrENV vector in the previous experiments, it became necessary to utilize another vector system to study the expression of the transmembrane glycoprotein in other cell lines. Recombinant vaccinia virus vectors are a well-characterized system that has been used to study the biosynthesis, processing, and biological activity of several retroviral glycoproteins (6, 10, 11, 34, 36). For these reasons, we constructed a recombinant vaccinia virus vector expressing the truncated envelope glycoprotein. Infection of CV-1 cells with the vvgp41 indicated the presence of high levels of intracellular and cell surface expression of the transmembrane protein as detected by immunofluorescence studies (data not shown). Western blot analyses detected the presence of two proteins having apparent M_rs of 52,000 and 41,000 in cells infected with vvgp41 but not in cells infected with a wild-type vaccinia virus (Fig. 6A). The sizes of these proteins were indistinguishable from those of proteins detected in COS cells transiently expressing the ptrENV plasmid. Pulse-chase analysis revealed that a protein with an M_r of 52,000 was chased into proteins with an M_r of 41,000 to 43,000 (Fig. 6B). The recombinant vaccinia virus vvgp41 induced syncytium formation in approximately 40% of the fluorescent HeLa cells (Fig. 7A). Surprisingly, infection of the mouse fibroblast cell lines (NIH 3T3 and BLK-SV) with vvgp41 also resulted in the formation of a significant number of multinucleated cells (35 to 45% of the fluorescent cells), as determined by indirect immunofluorescence (Fig. 7B and C). These results confirm those obtained with the plasmid ptrENV and support our previous conclusion that the truncated envelope glycoprotein gene resulted in the expression of a biologically active TM protein. Moreover, the finding that the TM protein is fusion competent in mouse cells implies that if there is a cell receptor-TM interaction involved in the HIV-1 glycoprotein-mediated fusion process, this molecule must be also present on the membrane of mouse cells.

DISCUSSION

The entry of enveloped viruses into susceptible cells is mediated by the interaction of the virally encoded glycoprotein(s) with receptors on the cell surface. This interaction begins with the attachment of the receptor binding viral glycoprotein to the appropriate cell receptor; next is fusion of the viral and cellular membranes. Virus-cell membrane fusion reactions have been classified as being either low pH dependent or pH independent (40). For bunyaviruses, orthomyxoviruses, rhabdoviruses, togaviruses, and some retroviruses, fusion is pH dependent and occurs in the acidic environment of endosomal vesicles after receptor-mediated endocytosis. The most extensively studied virus exhibiting low-pH-dependent fusion has been influenza virus (40). With influenza virus, the viral hemagglutinin (HA) glycoprotein mediates both attachment and fusion processes. Evidence from biochemical and biophysical studies indicates that the mildly acidic pH of the endosome induces an irreversible change in the conformation of the HA molecule (41). This conformational change results in increased hydrophobicity because of the "unmasking" of the fusion domain located at the HA_2 molecule, which permits the fusion reaction to proceed (41). With members of other virus families, such as

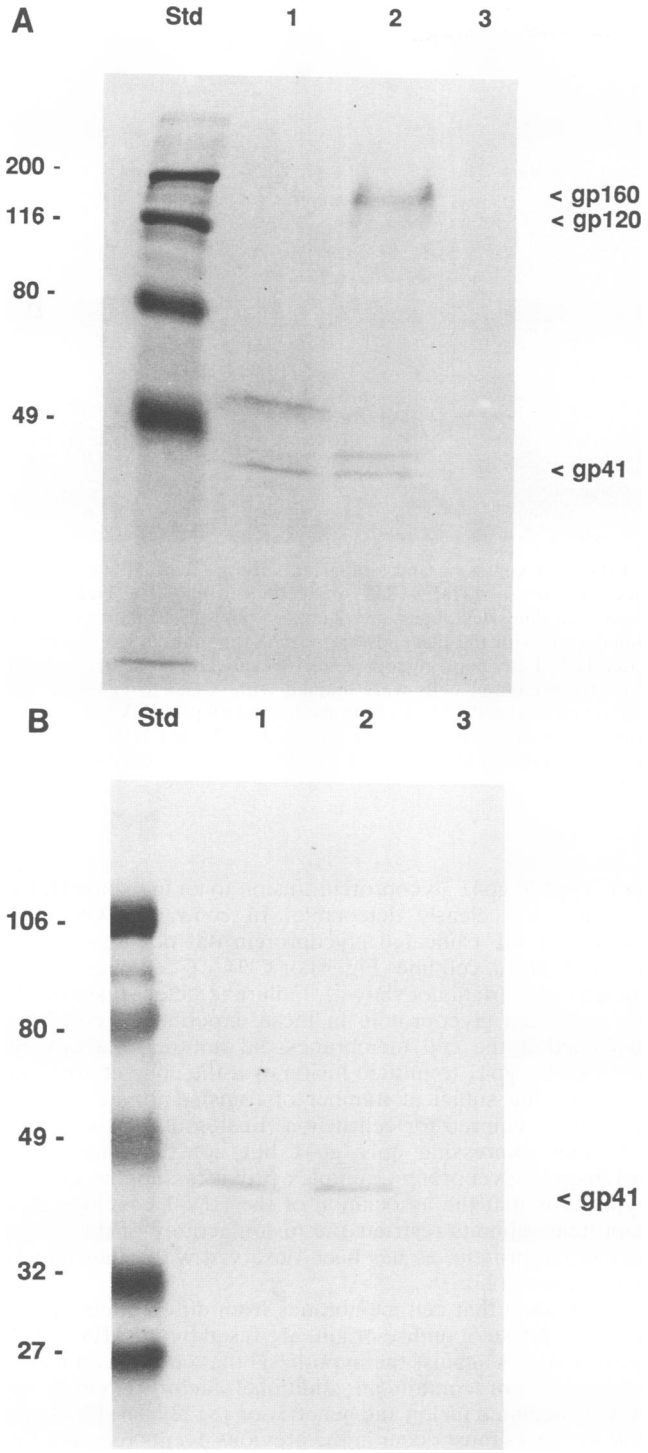

FIG. 3. Western blot analysis of HIV-1 glycoprotein expression in COS-1 cells. COS-1 cells were transfected with either ptrENV or pwtENV, and lysates were prepared at 48 h posttransfection. Aliquots of cell lysates were separated by SDS-PAGE, and the proteins were electrotransferred to nitrocellulose filters for immunoblotting. Nitrocellulose filters were incubated with antibodies specific for the HIV-1 glycoproteins, and the blots were developed with the Protoblot kit. Lane 1, HIV proteins detected from cells transfected with the ptrENV plasmid; lane 2, HIV proteins detected from COS-1 cells transfected with plasmid pwtENV; lane 3, mock-transfected COS-1 cell control; Std, prestained molecular mass markers (in kilodaltons). (A) HIV-1-positive human serum; (B) mouse monoclonal antibody against HIV-1 gp41.

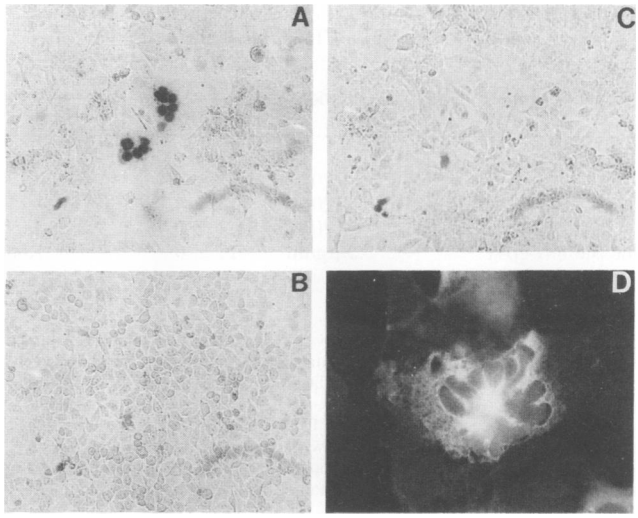

FIG. 4. Detection of biological activity of the native and truncated HIV glycoproteins in COS-1 cells. COS-1 cells were transfected with the pwtENV or ptrENV plasmid as described in Materials and Methods. At 48 h posttransfection, COS-1 cells were overlaid with the HeLa $CD4^+$ indicator cell line. After 24 h of cocultivation, syncytium formation was detected by the enzymatic assay described in Material and Methods. (A) COS-1 cells transfected with the pwtENV plasmid; (B) mock-transfected COS-1 cells. (C) COS-1 cells transfected with the ptrENV plasmid; (D) COS-1 cells transfected with the ptrENV plasmid and stained for the presence of the truncated envelope glycoprotein by using a mouse monoclonal antibody against HIV-1 gp41.

the paramyxoviruses, coronaviruses, herpesviruses, and retroviruses (e.g., HIV-1), virus-cell membrane fusion has been shown to be pH independent and can occur at the cell surface or within endosomal vesicles (23, 35, 40). The mechanism through which these viruses cause membrane fusion has yet to be elucidated.

In this investigation we examined the expression, intracellular transport, and biological properties in mammalian cells of the transmembrane subunit of HIV-1 glycoprotein from an envelope glycoprotein gene lacking the majority of the gp120 coding sequences. To mimic the gp160 precursor, the truncated glycoprotein contains those sequences corresponding to the leader peptide signal sequence and the gp120-gp41 proteolytic cleavage site. Our data demonstrate that these signals are sufficient for the proper biosynthesis, intracellular transport, and cell surface localization of the HIV-1 gp41 glycoprotein. If properly synthesized by using the host cell machinery for membrane protein transport, a molecule with an M_r of 52,000 (gp52) should be detectable within infected cells. Support for the gp52 being the precursor of the truncated glycoprotein comes from several lines of evidence. First, HIV-1-positive human serum and gp41 monoclonal antibodies detect both the gp52 and gp41 molecules in Western blot analyses (Fig. 3 and 6). Second, pulse-chase analyses demonstrate that the gp52 but not the gp41 is labeled during short radiolabeling periods (Fig. 6B). When the radiolabel is chased in the presence of excess unlabeled leucine, the gp52 appears to be chased into a major cleavage product having an M_r of 41,000 (Fig. 6B). This protein is indistinguishable in size from the gp41 synthesized from cells expressing the unmodified glycoprotein gene. Third, Western blot analysis with a monoclonal antibody

TABLE 1. Antibody neutralization of wtEnv and trEnv-induced cell fusion in HeLa CD4 LTR β-Gal cells[a]

Antibody type[b]	HIV status	Dilution	% Syncytia[c]	
			wtEnv	trEnv
Human serum				
87	+	1:20	15.5 ± 2.4	0.45 ± 0.17
244	+	1:20	12 ± 4.7	0
418	−	1:20	21.2 ± 0.95	9.2 ± 2.09
Chessie 8 α gp41 monoclonal		1.2 μg/ml	15 ± 6	1.7 ± 1.08
None			21.7 ± 5.3	12.7 ± 3.4

[a] Cell monolayers were transfected with 5 μg of DNA per 60-mm plate and incubated immediately in antibody-containing medium. Two days later, cells were fixed and stained for β-galactosidase activity as described in Materials and Methods.

[b] All sera were heat inactivated at 56°C for 1 h before use, and their specific reactivities to the HIV-1 glycoprotein were tested by immunoblot.

[c] % Syncytia, number of cells with four or more blue nuclei/total number of cells with blue nuclei. Values were obtained from triplicate experiments.

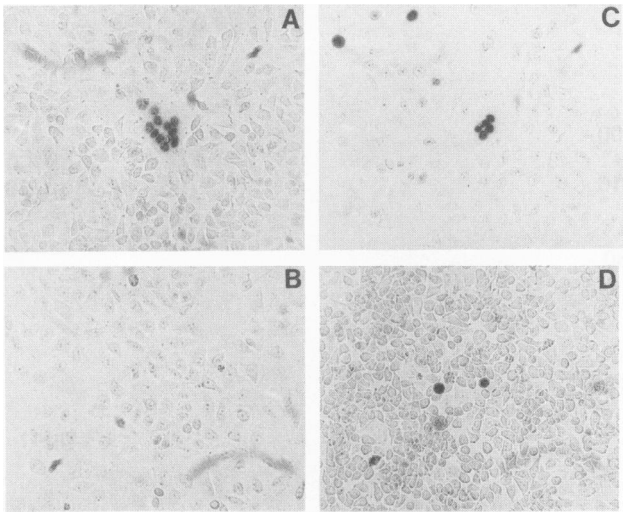

FIG. 5. Expression and biological activity of the HIV envelope glycoproteins in a HeLa-CD4$^+$ indicator cell line. The HeLa indicator cell line developed by Kimpton and Emerman (17) was transfected with the plasmids expressing the unmodified and truncated HIV-1 envelope glycoproteins by using DEAE-dextran. At 48 h posttransfection, cells were stained with X-Gal to visualize the multinucleated cells. (A) Cells transfected with pwtENV; (B) mock-transfected cells; (C) cells transfected with ptrENV; (D) cells transfected with a plasmid, pME241, that expresses only the HIV-1 Tat protein.

specific for the carboxyl terminus of gp120 detected gp52 but not gp41, which is consistent with gp52 being the precursor glycoprotein (data not shown). Thus, it appears that the majority of gp120 is dispensable for proper intracellular transport and oligosaccharide processing of the TM protein. Additionally, deletion of the majority of gp120 does not impair the proteolytic processing of this gp52 precursor into mature gp41. These results indicate that the protein conformation of the primary translation product from this truncated envelope glycoprotein gene is compatible with the structural requirements of the cellular protease involved in gp160 cleavage (4, 13, 25).

The precise mechanism by which the HIV-1 envelope glycoprotein induces syncytium formation has yet to be completely elucidated. In the most common route of HIV-1 infection, the high-affinity interaction between the gp120-gp41 glycoprotein complex and the V1 region of the CD4 receptor is a prerequisite for the pH-independent fusion of the cellular and viral membranes (5, 27, 28). It has been postulated that the fusion domain present at the amino terminus of the gp41 protein is not exposed until the viral and cellular membranes are in close apposition (15, 25, 28). In addition, studies have shown that CD4$^+$ chimpanzee cells, which will bind gp120, do not demonstrate fusion, suggesting that interaction with protein domains other than the CD4 V1 binding site are required for the initiation of fusion (5). Furthermore, other studies have suggested that the gp120 subunit dissociates from the virus particle following gp120-CD4 interactions, leading to the exposure of the fusogenic domain of gp41 (28). Taken together, these results indicate that interaction of gp120 with the CD4 receptor results in the dissociation of the gp120-gp41 glycoprotein complex and/or induces a conformational change that would be the initial stage in virus-cell or cell-cell fusion (5, 27, 28). Therefore, if HIV glycoprotein-mediated membrane fusion can only occur when the fusogenic domain of gp41 is free to interact with other membranes, successful surface expression of only the gp41 should trigger the fusion process. We present evidence that gp41 is capable of inducing syncytium formation in the absence of CD4-gp120 interactions. Fusion could be detected in several primate (both CD4-positive and -negative) and mouse cell lines transfected with ptrENV or infected with recombinant vaccinia virus vectors (Fig. 5 and 7). When COS cells were transfected with the vector expressing the native gp120-gp41 glycoprotein, fusion to an indicator HeLa cell line was clearly detectable. In contrast, COS cells expressing the truncated glycoprotein did not fuse to the indicator HeLa cell line (Fig. 4) or CD4$^+$ T-cell lines during cocultivation (data not shown). Failure to detect fusion with the truncated glycoprotein in these experiments could be explained if the cell membranes did not reach a critical distance for gp41 to initiate fusion or if the contact area did not have the sufficient number of transmembrane protein molecules required for cell fusion. Fusion of CD4-negative cell lines expressing only gp41 but not the unmodified gp120-gp41 glycoprotein complex provides support for the hypothesis that the association of the HIV-1 envelope glycoprotein subunits restricts the fusion activity of the transmembrane protein, as has been observed with other enveloped viruses (23).

The finding that cell membranes from different cell lines, including those of mouse origin, are fused by the HIV-1 TM protein argues against the hypothesis that certain cell membranes do not contain an additional factor required for HIV-1-mediated fusion and penetration (8, 22). The blockage in those cases must occur in the previous events required for the activation of the fusion domain. Thus, the biological activity of the HIV-1 TM protein is similar to that of the influenza virus HA glycoprotein, which mediates fusion with virtually any type of biological or artificial membrane in the absence of the viral receptor (23, 40).

The construction of vectors expressing the transmembrane component of the HIV-1 glycoprotein on the surface of infected cells should permit the independent study of events involved in the viral entry process after the receptor binding step. It may also contribute to the design of chemotherapeutic agents that block the fusion process and therefore viral infection. In addition, the vaccinia recombinant

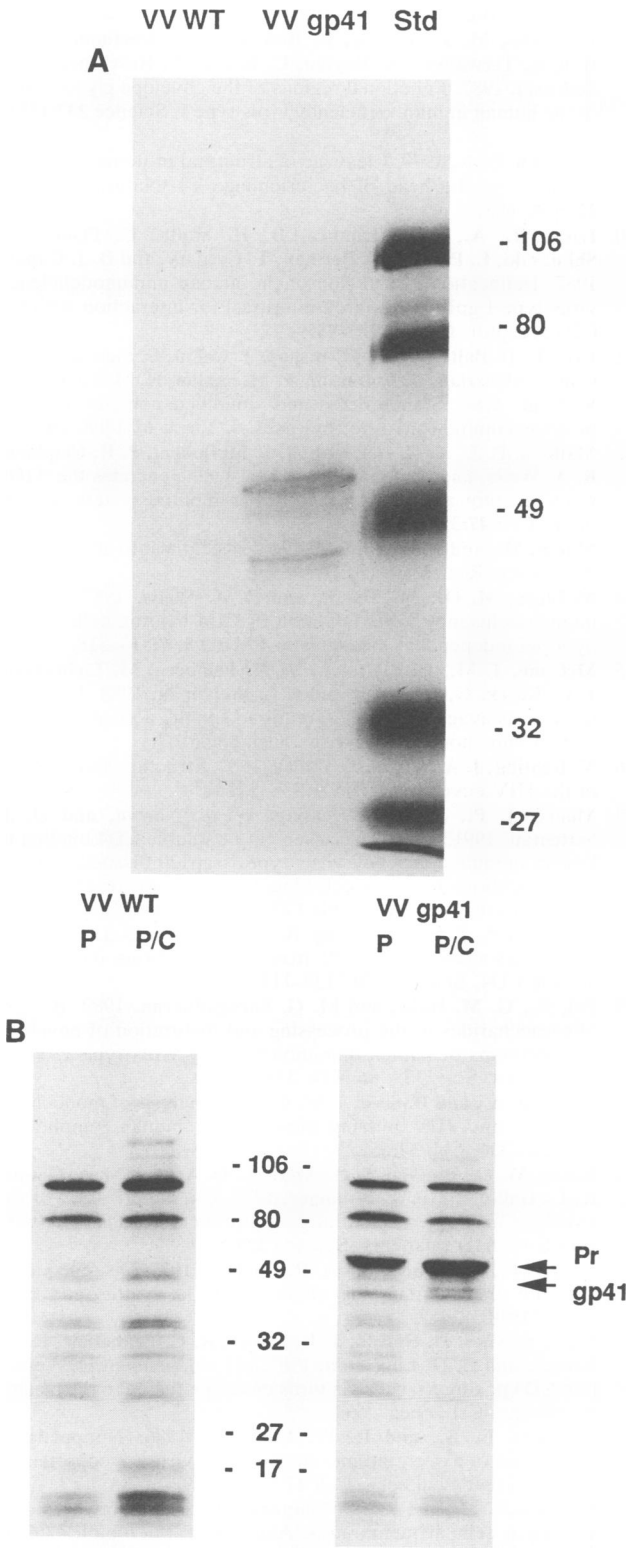

FIG. 6. (A) Expression of the truncated envelope glycoprotein by a vaccinia recombinant virus. Monolayers of HeLa cells were infected with vvgp41 or wild-type vaccinia virus at a multiplicity of infection of 10. At 15 h after infection, cells were harvested and lysed. Aliquots of each cell lysate were separated by SDS-PAGE (10% gel), and the proteins were electrotransferred to nitrocellulose filters for immunoblotting. The blot was incubated with a mouse monoclonal antibody against gp41 and developed with the Protoblot kit as described by the manufacturer. vvWT, HIV-1 proteins detected from cells infected with vaccinia wild-type virus; vvgp41, HIV-1 proteins detected from cells infected with vvgp41; Std, prestained molecular mass markers (in kilodaltons). (B) Pulse-chase analysis of the truncated *env* gene products expressed from vvgp41-infected cells. HeLa cells were infected with vvgp41 or wild-type vaccinia virus (multiplicity of infection, 10) as described above. At 15 h postinfection cells were pulse-labeled with 500 μCi of [^{3}H]leucine for 15 min. The radioactive label was chased for various periods (lane P, 0 min; lane P/C, 120 min) in medium containing an excess of nonradioactive leucine. Cell extracts were prepared in radioimmunoprecipitation assay buffer, and the nuclei were removed by centrifugation. Lysates were incubated with a human monoclonal antibody against the HIV-1 gp41 glycoprotein, and immunoprecipitates were collected on protein A-Sepharose. The immunoprecipitated proteins were separated by SDS-PAGE and visualized by autoradiography. The positions of molecular mass markers (in kilodaltons) are indicated.

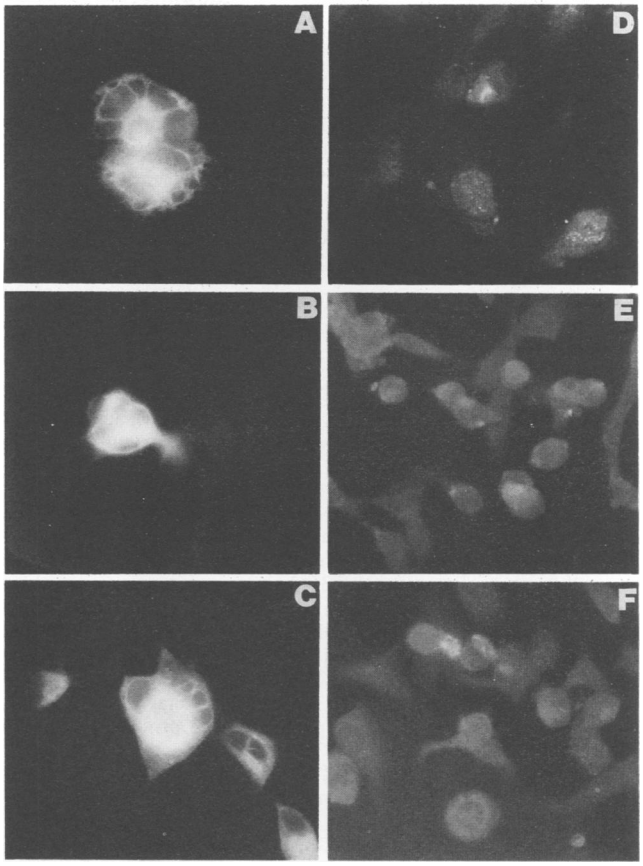

FIG. 7. Expression and biological activity of the truncated envelope glycoprotein by vvgp41 in mouse and HeLa cells. Cells grown on coverslips were infected with the recombinant vvgp41 (A, B, and C) or the wild-type vaccinia virus (D, E, and F) for 15 h at a multiplicity of infection of 0.1. Cells were fixed with ethanol-acetic acid and incubated with a mouse monoclonal antibody against the HIV-1 TM protein and then by a FITC-labeled goat anti-mouse immunoglobulin G. (A and D) HeLa cells; (B and E) BLK-SV mouse fibroblasts; (C and F) NIH 3T3 mouse fibroblasts.

virus described here may prove to be useful to determine additional targets for humoral and cell-mediated responses against HIV with the final goal of finding an efficient vaccine against AIDS.

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