Site-specific Mutations in HIV-1 gp41 Reveal a Correlation between HIV-1-mediated Bystander Apoptosis and Fusion/Hemifusion*§

Received for publication, February 27, 2007, and in revised form, April 5, 2007 Published, JBC Papers in Press, April 6, 2007, DOI 10.1074/jbc.M701701200

Himanshu Garg‡, Anjali Joshi‡, Eric O. Freed§, and Robert Blumenthal‡

From the ‡Membrane Structure and Function Section, Center for Cancer Research Nanobiology Program, and §Virus Cell Interaction Section, HIV Drug Resistance Program, NCI, National Institutes of Health, Frederick, Maryland 21702

The loss of CD4+ T cells in HIV-1 infections is hypothesized to be caused by apoptosis of bystander cells mediated by cell surface-expressed HIV-1 Env glycoprotein. However, the mechanism by which Env mediates this process remains controversial. Specifically, the role of HIV-1 gp120 binding to CD4 and CXCR4 versus the fusion process mediated by gp41 remains unresolved. Env-induced apoptosis in bystander cells has been shown to be gp41-dependent and correlates with the redistribution of membrane lipids between Env-expressing cells and target cells (hemifusion). Using a rational mutagenesis approach aimed at targeting Env function via the gp41 subunit, we examined the role of HIV gp41 in bystander apoptosis. A mutation in the fusion domain of gp41 (V513E) resulted in a fusion-defective Env that failed to induce apoptosis. A mutation in the gp41 N-terminal helix (G547D) reduced cell fusion capacity and apoptosis; conversely, an Env mutant with a deletion of the gp41 cytoplasmic tail (Ct Del) enhanced both cell-to-cell fusion and apoptosis. Most significantly, an Env mutant containing a substitution in the loop region of gp41 (D589L) mediated transfer of lipids (hemifusion) to bystander cells but was defective in cell-to-cell and to a lesser degree virus-to-cell fusion. This mutant was still able to induce apoptosis in bystander cells. Hence, we have provided the first direct evidence that gp41-mediated hemifusion is both required and sufficient for induction of apoptosis in bystander cells. These results may help to explain the mechanism of HIV-1 Env-induced T cell depletion.

The mechanism by which HIV2 induces T cell depletion leading to immunodeficiency remains unresolved. As the number of CD4+ T cells lost during HIV infection far exceeds the number of infected cells (1), it is believed that HIV induces the death of uninfected bystander cells via apoptosis (2). The Env glycoprotein is a good candidate for inducing bystander cell death, as it is expressed on the surface of infected cells and can interact with bystander cells expressing the HIV receptor (CD4) and co-receptors (CXCR4/CXCR5). Furthermore, because the depletion of T cells is largely restricted to CD4+ cells, a role for the Env glycoprotein in either direct or indirect killing of bystander cells has been proposed (3).

The Env glycoprotein of HIV consists of a CD4- and CXCR4-binding gp120 subunit and a membrane fusion-inducing gp41 subunit. Although the role of the Env glycoprotein in inducing bystander cell death is well studied, it remains controversial whether gp120 or gp41 are major players in this process. It is well established that the fusion capacity of HIV gp41 contributes to HIV-induced pathogenesis both in vitro (4) and in vivo (5, 6). However, because HIV Env-induced syncytia formation is not a universal pathogenic feature of HIV infection, it is unclear how HIV gp41 could cause depletion of CD4+ cells in the absence of cell-to-cell fusion. It has been suggested that gp41-mediated autofusion may lead to the death of Env-expressing cells (7). However, autofusion explains only the death of Env-expressing or -infected cells and not bystanders, even though in HIV infection, the number of apoptotic/dying cells far exceeds the number of infected cells. Alternatively, it has been suggested that gp41-mediated hemifusion between Env-expressing and bystander cells may be involved in the induction of bystander apoptosis by a “kiss and run” mechanism (8, 9). Hemifusion is a phenomenon in which the outer leaflets of the membrane of the effector and target cells interact transiently without the formation of a fusion pore or progression to syncytia formation (10). This process has been well characterized for influenza hemagglutinin (11, 12) and has also been reported for HIV (8, 13, 14). Hemifusion may result in membrane destabilization, which can be measured by the transfer of lipophilic dyes from effector to target cells and may lead to apoptotic signaling.

The signaling of HIV-mediated bystander cell death shows signs of classical apoptosis involving caspase activation and mitochondrial depolarization (15–17). In the macaque model of acute HIV infection using simian immunodeficiency virus, apoptosis largely correlates with caspase-3 activation (18). Also, a recent study shows that bystander apoptosis mediated by HIV-1 in thymus tissue cultures is dependent on gp41 function and is mediated by caspase-3 (19). We have also observed previously that HIV gp41 mediates apoptosis via the mitochondrial pathway and can be inhibited by the caspase-3 inhibitor DEVD.
Mutational Analysis of HIV gp41-mediated Apoptosis

(9). Furthermore, the role of the apoptotic mitochondrial pathway in HIV infection has been suggested by others both in vitro and in vivo (20–22). We have also demonstrated that apoptosis mediated by HIV gp41 can be inhibited by the protease inhibitor Nelfinavir, which has been shown to inhibit the adenosine nucleotide translocator in the mitochondria, thereby preventing mitochondrial depolarization (23).

For gp41-mediated apoptosis, it is essential that there be a cell-to-cell interaction between infected and uninfected bystander cells. In the context of HIV infection, the interactions between HIV Env-expressing and uninfected T cells leads to the formation of an actin-dependent virological synapse that facilitates the direct transfer of virus between T cells (24). In fact, a recent study suggests that the majority of HIV transmission occurs via cell-to-cell interaction across the virological synapse (25). Although this interaction supports HIV replication, it may come at the expense of bystander cell death mediated by gp41. However, we do not know whether fusion or hemifusion is required for the induction of apoptosis via this synaptic type of cell-to-cell contact.

HIV gp41 consists of several functional domains, including an N-terminal fusion domain, N- and C-terminal helices joined by a loop region, a transmembrane region, and a cytoplasmic tail. Conformational changes induced by gp120 binding to the receptor/co-receptor induce a sequence of events that involve insertion of the gp41 fusion domain into the target cell membrane followed by folding of the helical regions into the 6-helix bundle finally resulting in fusion (26). Mutational studies indicate that the different regions of gp41 work cooperatively in this fusion reaction (27). However, a mutational study of HIV Env-mediated apoptosis has not been conducted. Hence, in this study, we have examined the effect of point mutations in gp41 that alter cell-to-cell fusion capacity on the apoptosis-inducing activity of Env glycoprotein. Using various mutants, we have shown a correlation between HIV gp41-mediated cell-to-cell fusion/hemifusion and the induction of apoptosis. With the help of a hemifusion-restricted mutant (D589L), we have provided the first direct evidence that gp41-mediated bystander cell death is hemifusion-dependent. Virus replication studies suggest an inverse correlation between virus replication and bystander apoptosis-inducing capacity of different mutants. These findings are further supported by the observation that replication of syncytia inducing the wild type (WT) (but not the non-syncytia inducing the W596M mutant) can be altered via a caspase-3 inhibitor. Our findings indicate that, even under conditions of virus replication, bystander cell apoptosis induced by HIV-1 Env may be gp41 fusion/hemifusion-dependent and caspase-3-mediated.

EXPERIMENTAL PROCEDURES

Cells and Reagents—SupT1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin streptomycin (5000 units/ml). HeLa and TZM (National Institutes of Health (NIH) AIDS Research and Reference Reagent Program) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin streptomycin (5000 units/ml). Fusion inhibitors C34, AMD3100, and protease inhibitor Nelfinavir were provided by the NIH AIDS Research and Reference Reagent Program. Caspase-3 inhibitor Ac-DEVD-CHO was obtained from Calbiochem.

Plasmid Constructs—Molecular clones of HIV-1 LAI and NL4–3 were obtained from the NIH AIDS Research and Reference Reagent Program. EcoRI-XhoI fragments from the LAI molecular clone were cloned into pcDNA3.1 expression vector (Invitrogen) making the WT LAI Env construct. The fragment contains open reading frames of env, tat, and rev genes. Point mutations were introduced in the WT Env construct using the QuikChange site-directed mutagenesis kit (Stratagene). The mutants were numbered based on the Env sequence of the HXB2 reference strain. All mutant Env regions were also introduced into the NL4–3 molecular clone using the EcoRI-XhoI fragments to generate infectious molecular clones containing mutant LAI Env.

Expression of Env Glycoprotein—HeLa cells were transfected in 6-well plates using ExGen 500 (Fermentas Life Science) transfection reagent according to the manufacturer’s instructions. 24 h post-transfection, the cells were collected and used for either cell-to-cell fusion assay or bystander apoptosis induction in co-culture experiments with target cells.

Cell-to-Cell Fusion Assay—HeLa cells transfected with a different Env expression vector (which also expresses HIV–1 Tat) were seeded in 96-well plates at 2 × 10⁴ cells/well. TZM cells expressing CD4 and CXCR4 as well as the Tat-dependent luciferase reporter gene were added at the same amount. The cells were co-cultured for 6 h, following which the luciferase activity was measured by Steadyglo luciferase substrate (Promega).

Apoptosis Induction—For induction of apoptosis, co-culture between Env-transfected HeLa cells and SupT1 cells was performed. HeLa cells transfected with either WT or mutant Env constructs were seeded in 24-well plates at 10⁵ cells/well. The cells were allowed to adhere for 4–6 h. Subsequently, the medium was removed, and SupT cells at 0.5–1 × 10⁶ cells were added. Different inhibitors were added at the time of co-culture. The cells were co-cultured for 24 h, following which the suspension cells were collected, stained with Annexin V (BD Biosciences), and analyzed by flow cytometry using a FACS Calibur flow cytometer (BD Biosciences). For some assays, apoptosis was detected by staining with mitochondrial potential-sensitive dye DiOC₆(10 µM) (Calbiochem) followed by flow cytometry. At least 10,000 events were collected and analyzed using Cellquest software.

Dye Transfer Assay—Env-transfected HeLa cells were labeled with either cytoplasmic dye CMTMR (10 µM) or lipophilic membrane dye DiI (Vybrant cell-labeling kit, Molecular Probes). The cells were washed and plated in 24-well plates at 10⁵ cells/well. After 4–6 h, unlabeled SupT1 cells were added and co-cultured for 24 h. Subsequently, the non-adherent target cells were collected and stained with Annexin V for detection of apoptosis and then analyzed by flow cytometry. Transfer of dye (cytoplasmic or membrane) was acquired in the red channel (FL-2), and the green channel (FL1) was used for apoptosis detection via Annexin V fluorescence isothiocyanate. This assay determined whether the apoptotic target cells seen in our system had taken up cytoplasmic or membrane dyes from the effector cells.
Mutational Analysis of HIV gp41-mediated Apoptosis

**RESULTS**

**Fusion-defective Env Glycoprotein Fails to Induce Bystander Cell Death**—To determine whether gp41 function is required for apoptosis or whether gp120-binding activity is sufficient for apoptosis induction, a point mutation in the gp41 fusion domain (V513E) was introduced that abolishes the fusion activity (28). Using this mutant, we found that V513E mutation is not only defective in cell-to-cell (Fig. 1A) and virus-to-cell fusion (Fig. 1C) but is also completely deficient in bystander apoptosis-inducing capacity (Fig. 1B). This effect was unlikely because of the expression or processing of Env glycoprotein, which was similar to the WT (supplemental Fig. 1). As shown previously (9), apoptosis mediated via WT Env was also inhibited by fusion inhibitor C34 and CXCR4 antagonist AMD3100 to the same levels as V513E or control-transfected cells. This suggests that the fusion capacity of gp41 is essential for the induction of bystander cell death.

**Bystander Apoptosis-inducing Potential Correlates with Cell-to-Cell Fusion Capacity but Not Virus-to-Cell Fusion**—To further characterize the role of gp41 fusion capacity in apoptosis induction, we examined mutations in gp41 that are known to affect cell-to-cell fusion but have a limited effect on virus infection. One such mutation, G547D, is defective in cell fusion and is also associated with resistance to the fusion inhibitor Enfuvirtide or T20 as well as other fusion-inhibitory peptides such as C34 (29, 30). On the other hand, deletion of the cytoplasmic tail of gp41 (Ct Del) has been known to enhance cell-to-cell fusion capability (31). Consistent with previous work, we found that, although G547D was defective in cell fusion, Ct Del showed enhanced fusion in our assays compared with WT (Fig. 2A). Furthermore, although G547D was less potent at virus fusion than WT, Ct Del was considerably weaker (Fig. 2C) because of poor incorporation of the Env glycoprotein in the virus, as reported previously (32) (supplemental Fig. 2). Interestingly, the cell-to-cell fusion capacity of the mutants correlated with the bystander apoptosis-inducing function of gp41 (Fig. 2B) further validated the hypothesis that HIV Env-mediated apo-
Mutational Analysis of HIV gp41-mediated Apoptosis

ptosis is gp41 function-dependent. We also found that both G547D and Ct Del are less sensitive than WT to the fusion inhibitor C34, consistent with published results (29, 31), but not to the CXCR4 antagonist AMD3100 (Fig. 2 D). This has implications for the T20-resistant mutants that arise in patients receiving T20 therapy, suggesting that they may have an altered fusion capacity and therefore have a different pathogenesis.

Cell-to-Cell Fusion Is Not Required for HIV-1 gp41-mediated Apoptosis—Previously, we and others (8, 9) have shown that gp41-mediated bystander cell apoptosis correlates with gp41-mediated hemifusion. Although these findings are based on dye transfer assays using WT Env, direct evidence of gp41-mediated apoptosis in the absence of syncytia formation (cell-to-cell fusion) is missing. A recent report by Alizon et al. (13) describes certain mutations in the loop region that are restricted at the hemifusion state. To address the issue that Env-mediated apoptosis is hemifusion-dependent, we utilized the loop mutations to induce apoptosis in bystander cells. Two mutations in the loop region, W596M and D589L, were used in the study.

FIGURE 2. Apoptosis correlates with cell-to-cell fusion activity. A, cell-to-cell fusion mediated by HeLa cells expressing WT, G547D, or Ct Del Env in TZM cell co-culture. B, apoptosis mediated by WT, G547D, or Ct Del Env expressing HeLa cells in SupT1 target cells. C, single round infection of WT, G547D, or Ct Del Env containing virus in TZM indicator cells. D, G547D and Ct Del are resistant to peptide inhibitor C34 in cell-to-cell fusion assay. Cell-to-cell fusion was performed in the presence of various concentrations of either C34 (left) or AMD3100 (right). Data were normalized to no drug control for each mutant. RLU, relative light units.

FIGURE 3. Cell-to-cell fusion is not required for apoptosis induction. A, cell-to-cell fusion mediated by HeLa cells expressing WT, D589L, or W596M Env in TZM cell co-culture. B, apoptosis mediated by WT, D589L, or W596M Env-expressing HeLa cells in SupT1 target cells. C, single round infection of WT, D589L, or W596M Env containing virus in TZM indicator cells. RLU, relative light units.
gp41-dependent, as it was inhibited by C34, hence providing the first direct evidence that gp41-mediated apoptosis does not require cell-to-cell fusion or syncytia formation. Although both W596M and D589L have been suggested to be associated with hemifusion, we did find considerable differences between these mutants, which have also been reported by Alizon et al. (13). D589L was relatively defective in virus infection (Fig. 3C) compared with W596M, suggesting that it is different from W596M. Furthermore, W596M has been reported to replicate efficiently without causing cytopathic effects in cell lines (33). Hence, under our co-culture conditions, D589L was the predominantly hemifusion-restricted Env and therefore used in subsequent experiments.

**HIV gp41-mediated Apoptosis Correlates with Hemifusion**—Hemifusion is a phenomenon whereby the outer leaflets of opposing membranes interact without the formation of a fusion pore. This process can be differentiated from complete fusion by labeling either the membrane or cytoplasm of effector and/or target cells. To determine whether apoptosis mediated via D589L Env is hemifusion-dependent, we utilized a dye transfer assay where the Env-expressing (effector) cells were labeled with either a lipophilic membrane dye (DiI) or cytoplasmic dye (CMTMR). The cells were co-cultured with unlabeled SupT1 cells and apoptosis determined via Annexin V staining. Flow cytometry was used to determine the transfer of lipid or aqueous dye from the effector cells to the apoptotic target cells. As seen in Fig. 4, co-culture of SupT1 cells with Env-expressing cells resulted in apoptosis (Annexin V+) that was seen in either DiI+ (Fig. 4A) or CMTMR− (Fig. 4B) cell populations. Transfer of DiI (and not CMTMR) to the apoptotic target cells suggests that there was lipid mixing between the effector and target cells but not cytoplasmic content mixing. This phenomenon was seen in both WT and D589L, although the extent of apoptosis was limited in D589L (30%) compared with WT. Furthermore, both apoptosis and dye transfer for WT as well as D589L were inhibited by C34, suggesting that it was gp41-mediated and not non-specific transfer of dye. This confirms that cell-to-cell hemifusion without progression to complete fusion or syncytia formation is sufficient for bystander apoptosis induction.

**Hemifusion-induced Apoptosis Is Inhibited by Nelfinavir and Caspase-3 Inhibitor**—Previously (9), we have observed that bystander apoptosis mediated by WT HIV Env is inhibited by caspase-3 inhibitor DEVD as well as by the HIV protease inhibitor Nelfinavir via its action on the mitochondrial pathway of apoptosis. In co-culture experiments using WT Env, a limited number of syncytia are formed, which complicates the assertion that hemifusion-mediated apoptosis is inhibited by Nelfinavir or caspase-3 inhibitor. We wished to address this issue by using the D589L mutant, which mediates gp41-dependent apoptosis in the absence of cell-to-cell fusion or syncytia formation. Interestingly, we found that D589L-mediated apoptosis is also inhibited by both DEVD and Nelfinavir, similar to WT (Fig. 5). This suggests that the apoptotic signaling by WT Env is the same as hemifusion-restricted D589L. Because D589L does not cause cell-to-cell fusion, our data suggest that, even with fusion-competent Env, the apoptosis is likely mediated by the hemifusion phenomenon.

**Virus Replication Correlates Inversely with Apoptosis Potential of Env Glycoprotein**—The fact that the mutants used in this study showed varied cell-to-cell fusion activity, although having significantly less effect on virus cell entry, encouraged us to perform virus replication studies to determine whether the bystander cell death-inducing property had an effect on virus replication. SupT1 cells infected with equal RT values of virus
Mutational Analysis of HIV gp41-mediated Apoptosis

FIGURE 5. Inhibition of apoptosis by caspase-3 inhibitor DEVD and Nelfinavir. Apoptosis by WT (A) or D589L (B) Env-expressing cells was induced in SupT1 co-culture. Either DEVD (40 μm) or Nelfinavir (10 μm) was used to inhibit apoptosis. Apoptosis was determined as phosphatidyl serine exposure by Annexin V staining or mitochondrial depolarization by DiOC6 staining.

FIGURE 6. Replication of various Env mutant viruses in SupT1 cells. Equal RT values of different viruses were used to infect SupT1 cells. Supernatants were collected at the indicated time points and analyzed for virus replication by RT assay. The experiment was repeated three times with similar results. cpm, counts/min.

were cultured for 24 days with virus replication determined by RT activity in culture supernatant every alternate day (Fig. 6). Interestingly, WT, W596M, and G547D, although having a different infectivity in a single round of infection, showed efficient replication, suggesting that a threshold Env fusion activity is sufficient for replication. Based on the day of peak replication, an inverse correlation was found between replication kinetics and bystander cell death. This observation suggests that a threshold Env fusion activity is necessary for the induction of apoptosis by HIV Env. We hypothesize that the slower replication kinetics of WT compared with W596M observed in the previous experiment is likely because of the induction of bystander cell apoptosis, thereby reducing the effective number of cells available for infection across the infected-uninfected cell synapse. This suggests that inhibition of bystander apoptosis may alter the replication kinetics of WT virus. To test this hypothesis, we infected SupT1 cells with either WT or W596M virus and cultured the cells in the presence or absence of caspase-3 inhibitor DEVD to prevent bystander apoptosis. As shown in Fig. 7A, the presence of caspase-3 inhibitor DEVD altered the replication kinetics of WT virus with peak virus replication seen at days 6–8 compared with days 8–10 for the no treatment group. The differences were more significant at day 6, suggesting that only early lag phase of replication may be altered. These early differences were reproducible and also in agreement with a previous report showing that WT virus causes early single cell death compared with W596M (33) and another study showing that caspase inhibition can alter HIV replication (35). Interestingly, DEVD treatment did not have any effect on the rate of replication of W596M, consistent with our suggestion that it does not cause bystander apoptosis. Furthermore, this effect was not due to syncytia formation, as the syncytia formed by WT virus in the presence or absence of DEVD were the same (Fig. 7B). Also evident from Fig. 7B is the fact that W596M replicates in the absence of any cell-to-cell fusion that may result in syncytia formation. This experiment suggests that gp41 fusion activity mediates both syncytia formation (fusion) and bystander cell death (hemifusion) and that these two activities, although interdependent, may have a separate effect on bystander cells and virus replication.

DISCUSSION

Env glycoprotein is a major determinant of HIV-1 pathogenesis that results in the development of AIDS. However, the mechanism via which HIV Env mediates bystander cell death remains controversial. In this study, using a hemifusion-restricted mutant (D589L), we have provided the first direct evidence that cell-to-cell hemifusion is both sufficient and necessary for the induction of apoptosis by HIV Env. The membrane-fusing potential of HIV Env has long been suspected as the cause of apoptosis induction both in vitro and in vivo (5, 7). However, the role of gp41 in bystander cell death is complicated by the fact that syncytia formation in vivo is rare. An alternate hypothesis is that gp41-mediated hemifusion may lead to the induction of apoptosis in the absence of syncytia formation. Previous in vitro studies both by us and others (8, 9) have suggested that hemifusion mediated by WT HIV-1 Env in fact correlates with bystander cell death. However, these stud-
Mutational Analysis of HIV gp41-mediated Apoptosis

Recent studies suggest an important role of the virological synapse in the transfer of virus from infected cells to uninfected cells (24, 39, 40). This interaction between an infected and uninfected cell could result in three outcomes, 1) successful transmission of virus to the target cell, 2) the target cell could fuse with the infected cell to form syncytia, or 3) the target cell undergoes hemifusion-mediated apoptosis. In the case of W596M, because of its lack of bystander apoptosis-inducing potential, there is efficient transmission of virus to neighboring cells. With WT virus infection, hemifusion-induced apoptosis limits the number of cells available for infection, thereby resulting in slightly delayed replication kinetics. Our finding that the caspase-3 inhibitor DEVD enhances the replication kinetics of WT (but not W596M) by inhibiting bystander cell death is consistent with this notion as well as with previous studies (33, 35). However, DEVD treatment does not alter syncytia formation, suggesting that the increased replication kinetics is likely because of inhibition of hemifusion-induced bystander apoptosis and not syncytia formation.

We have also found that an Enfuvirtide-resistant mutant, G547D, is limited in its apoptosis-inducing potential. Others have reported that the common mutations seen in gp41 in patients receiving Enfuvirtide therapy are associated with reduced cell-to-cell fusion capacity (29). Our data suggest that this reduction in fusion capacity could be associated with a reduced bystander cell death induction. Consistent with this hypothesis, a recent clinical study (41) of Enfuvirtide-treated patients shows that certain Enfuvirtide resistance mutations arising in vivo are associated with immunological success and progressive rise in CD4+ T cell counts, even after virological failure, possibly as a result of reduced fusion/hemifusion-dependent bystander apoptosis. Hence, the hypothesis that gp41-mediated hemifusion results in bystander cell death may not be just an in vitro effect but also correlates with in vivo clinical findings and has implications for Enfuvirtide therapy.

We have also found that apoptosis mediated via hemifusion-restricted D589L is similar to WT, as evident from inhibition by Nelfinavir as well as caspase-3 inhibitor DEVD. This suggests that the mechanism of apoptosis by WT and D589L is the same, and as D589L only causes hemifusion but not fusion, apoptosis mediated by WT should also be hemifusion-dependent. In fact, as suggested by Chernomordik et al. (14), most fusion events progress through a hemifusion intermediate, and the rate of successful fusion compared with hemifusion is determined by the density of the surface expression of viral fusion proteins (36). Hence, all fusion proteins are also capable of inducing hemifusion under the right conditions.

The mechanism by which hemifusion results in apoptosis induction is a matter of further investigation. Both our current study and previous findings suggest a role of early caspase-3 activation in this process. We have previously shown that this process does not involve caspase-8 activation and is independent of p53 or p38 activity (9). Fusion proteins, such as HIV-1 gp41, drive hemifusion by producing bending stresses in bilayers (10). We suggest that membrane-associated caspase-3 may be activated in the target cell as a result of gp41-mediated membrane perturbations resulting in apoptosis similar to type II fas signaling (37). However, the role of membrane lipid components in initiating apoptosis cannot be ruled out.

We have also found that mutants that lack cell-to-cell fusion capacity are deficient in apoptosis induction but not necessarily in virus infection or virus replication. In fact, our data suggest that there is a negative correlation between virus replication and bystander cell death, at least in the cell line used in this study. The mutant W596M replicates faster and to higher levels than WT, even though it is significantly less infectious in a single round infection. Hence, a threshold Env activity is sufficient for virus replication but not for bystander apoptosis induction. This may be due to fewer successful Env molecule interactions required for viral entry, as suggested by Yang et al. (38), compared with the induction of cell-to-cell fusion or hemifusion. This also suggests that bystander cell death is most likely not virus replication-dependent. This is further supported by our observation that the kinetics of WT (but not W596M) virus replication can be altered by inhibiting apoptosis by the caspase-3 inhibitor DEVD.

ies were conducted using fusion-competent WT Env, and a limited number of syncytia are formed under these conditions. Hence, without the use of a fusion-defective or hemifusion-restricted Env, the mechanism of gp41-mediated apoptosis cannot be differentiated from syncytia formation. Using a hemifusion-restricted mutant (D589L) that does not mediate cell-to-cell fusion, yet is capable of mediating apoptosis in a gp41-dependent manner, we have provided the first direct evidence that cell-to-cell hemifusion is both required and sufficient for induction of apoptosis.

We have also found that apoptosis mediated via hemifusion-restricted D589L is similar to WT, as evident from inhibition by Nelfinavir as well as caspase-3 inhibitor DEVD. This suggests that the mechanism of apoptosis by WT and D589L is the same, and as D589L only causes hemifusion but not fusion, apoptosis mediated by WT should also be hemifusion-dependent. In fact, as suggested by Chernomordik et al. (14), most fusion events progress through a hemifusion intermediate, and the rate of successful fusion compared with hemifusion is determined by the density of the surface expression of viral fusion proteins (36). Hence, all fusion proteins are also capable of inducing hemifusion under the right conditions.

A fusion-competent WT Env, and a limited number of syncytia are formed under these conditions. Hence, without the use of a fusion-defective or hemifusion-restricted Env, the mechanism of gp41-mediated apoptosis cannot be differentiated from syncytia formation. Using a hemifusion-restricted mutant (D589L) that does not mediate cell-to-cell fusion, yet is capable of mediating apoptosis in a gp41-dependent manner, we have provided the first direct evidence that cell-to-cell hemifusion is both required and sufficient for induction of apoptosis.

We have also found that apoptosis mediated via hemifusion-restricted D589L is similar to WT, as evident from inhibition by Nelfinavir as well as caspase-3 inhibitor DEVD. This suggests that the mechanism of apoptosis by WT and D589L is the same, and as D589L only causes hemifusion but not fusion, apoptosis mediated by WT should also be hemifusion-dependent. In fact, as suggested by Chernomordik et al. (14), most fusion events progress through a hemifusion intermediate, and the rate of successful fusion compared with hemifusion is determined by the density of the surface expression of viral fusion proteins (36). Hence, all fusion proteins are also capable of inducing hemifusion under the right conditions.

A fusion-competent WT Env, and a limited number of syncytia are formed under these conditions. Hence, without the use of a fusion-defective or hemifusion-restricted Env, the mechanism of gp41-mediated apoptosis cannot be differentiated from syncytia formation. Using a hemifusion-restricted mutant (D589L) that does not mediate cell-to-cell fusion, yet is capable of mediating apoptosis in a gp41-dependent manner, we have provided the first direct evidence that cell-to-cell hemifusion is both required and sufficient for induction of apoptosis.

We have also found that apoptosis mediated via hemifusion-restricted D589L is similar to WT, as evident from inhibition by Nelfinavir as well as caspase-3 inhibitor DEVD. This suggests that the mechanism of apoptosis by WT and D589L is the same, and as D589L only causes hemifusion but not fusion, apoptosis mediated by WT should also be hemifusion-dependent. In fact, as suggested by Chernomordik et al. (14), most fusion events progress through a hemifusion intermediate, and the rate of successful fusion compared with hemifusion is determined by the density of the surface expression of viral fusion proteins (36). Hence, all fusion proteins are also capable of inducing hemifusion under the right conditions.

A fusion-competent WT Env, and a limited number of syncytia are formed under these conditions. Hence, without the use of a fusion-defective or hemifusion-restricted Env, the mechanism of gp41-mediated apoptosis cannot be differentiated from syncytia formation. Using a hemifusion-restricted mutant (D589L) that does not mediate cell-to-cell fusion, yet is capable of mediating apoptosis in a gp41-dependent manner, we have provided the first direct evidence that cell-to-cell hemifusion is both required and sufficient for induction of apoptosis.

A fusion-competent WT Env, and a limited number of syncytia are formed under these conditions. Hence, without the use of a fusion-defective or hemifusion-restricted Env, the mechanism of gp41-mediated apoptosis cannot be differentiated from syncytia formation. Using a hemifusion-restricted mutant (D589L) that does not mediate cell-to-cell fusion, yet is capable of mediating apoptosis in a gp41-dependent manner, we have provided the first direct evidence that cell-to-cell hemifusion is both required and sufficient for induction of apoptosis.

A fusion-competent WT Env, and a limited number of syncytia are formed under these conditions. Hence, without the use of a fusion-defective or hemifusion-restricted Env, the mechanism of gp41-mediated apoptosis cannot be differentiated from syncytia formation. Using a hemifusion-restricted mutant (D589L) that does not mediate cell-to-cell fusion, yet is capable of mediating apoptosis in a gp41-dependent manner, we have provided the first direct evidence that cell-to-cell hemifusion is both required and sufficient for induction of apoptosis.

A fusion-competent WT Env, and a limited number of syncytia are formed under these conditions. Hence, without the use of a fusion-defective or hemifusion-restricted Env, the mechanism of gp41-mediated apoptosis cannot be differentiated from syncytia formation. Using a hemifusion-restricted mutant (D589L) that does not mediate cell-to-cell fusion, yet is capable of mediating apoptosis in a gp41-dependent manner, we have provided the first direct evidence that cell-to-cell hemifusion is both required and sufficient for induction of apoptosis.

A fusion-competent WT Env, and a limited number of syncytia are formed under these conditions. Hence, without the use of a fusion-defective or hemifusion-restricted Env, the mechanism of gp41-mediated apoptosis cannot be differentiated from syncytia formation. Using a hemifusion-restricted mutant (D589L) that does not mediate cell-to-cell fusion, yet is capable of mediating apoptosis in a gp41-dependent manner, we have provided the first direct evidence that cell-to-cell hemifusion is both required and sufficient for induction of apoptosis.

A fusion-competent WT Env, and a limited number of syncytia are formed under these conditions. Hence, without the use of a fusion-defective or hemifusion-restricted Env, the mechanism of gp41-mediated apoptosis cannot be differentiated from syncytia formation. Using a hemifusion-restricted mutant (D589L) that does not mediate cell-to-cell fusion, yet is capable of mediating apoptosis in a gp41-dependent manner, we have provided the first direct evidence that cell-to-cell hemifusion is both required and sufficient for induction of apoptosis.
Mutational Analysis of HIV gp41-mediated Apoptosis

...ural progression of HIV disease results in the selection of more pathogenic (42) and probably more fusogenic Env proteins as a selection for viral fitness. However, the use of Enfuvirtide therapy to select less fusogenic and consequently less pathogenic viruses may be beneficial to patients, even after failure of Enfuvirtide therapy to control virus replication. Also of clinical significance is the finding that Nelfinavir may inhibit HIV Env-mediated bystander cell death. This finding may also hold true in vivo as a recent study shows that Nelfinavir has a beneficial effect on CD4+ T cell counts in patients by inhibiting mitochondrial apoptosis (43).

Overall, we have provided the first mutational evidence of bystander apoptosis induction via HIV gp41 that is related to its hemifusion capacity. Our findings may help explain previous in vivo observations in a macaque model where pathogenesis is related to the fusogenic potential of the Env glycoprotein (6). Also, they may help explain the recent observation that certain Env mutations provide new insights into the controversial issue of HIV-mediated bystander cell death. This finding may also hold true in vivo observations in a macaque model where pathogenesis is related to its fusogenic potential of the Env glycoprotein (6).

ACKNOWLEDGMENTS—We are grateful to the National Institutes of Health AIDS Research and Reference Reagent Program for supplying valuable reagents. We also thank Dr. Dimiter Dimitrov for critical reading of the manuscript.

REFERENCES

1. Finkel, T. H., Tudor-Williams, G., Banda, N. K., Cotton, M. F., Curiel, T., Monks, C., Baba, T. W., Ruprecht, R. M., and Kupfer, A. (1995) Nat. Med. 1, 129–134
2. Gougeon, M. L., and Montagnier, L. (1993) Science 260, 1269–1270
3. Perfettini, J. L., Castedo, M., Roumier, T., Andreau, K., Nardacci, R., Piacentini, M., and Kroemer, G. (2005) Cell Death. Differ. 12, Suppl. 1, 916–923
4. LaBonte, J. A., Patel, T., Hofmann, W., and Sodroski, J. (2000) J. Virol. 74, 10690–10698
5. Etemad-Moghadam, B., Rhone, D., Steenbeke, T., Sun, Y., Manola, J., Gelman, R., Fenton, J. W., Racz, P., Tenner-Racz, K., Asthelm, M. K., Letvin, N. L., and Sodroski, J. (2001) J. Virol. 75, 5646–5655
6. Etemad-Moghadam, B., Sun, Y., Nicholson, E. K., Fernandes, M., Liou, K., Golmia, R., Lee, J., and Sodroski, J. (2000) J. Virol. 74, 4433–4440
7. Cao, I., Park, I. W., Cooper, A., and Sodroski, J. (1996) J. Virol. 70, 1340–1354
8. Blanco, I., Barretina, J., Ferrer, K. F., Jacobot, E., Gutierrez, A., Armand-Ugon, M., Cabrera, C., Kroemer, G., Cloget, B., and Este, J. A. (2003) Virology 305, 318–329
9. Garg, H., and Blumthalf, R. (2006) J. Leukocyte Biol. 79, 351–362
10. Blumthalf, R., Clague, M. J., Durell, S. R., and Epand, R. M. (2003) Chem. Rev. 103, 53–69
11. Kemble, G. W., Daniell, T., and White, J. M. (1994) Cell 76, 383–391
12. Chernomordik, L. V., Frolov, V. A., Leikina, E., Bronk, P., and Zimmerberg, J. (1998) J. Cell Biol. 140, 1369–1382
13. Bar, S., and Alizon, M. (2004) J. Virol. 78, 811–820
14. Chernomordik, L. V., and Kozlov, M. M. (2005) Cell 123, 375–382
15. Biard-Piechaczyk, M., Robert-Hebmann, V., Richard, V., Roland, J., Hipskind, R. A., and Devaux, C. (2000) Virology 268, 329–344
16. Roggero, R., Robert-Hebmann, V., Harrington, S., Roland, J., Vergne, L., Jaleco, S., Devaux, C., and Biard-Piechaczyk, M. (2001) J. Virol. 75, 7637–7650
17. Ferri, K. F., Jacotot, E., Blanco, J., Este, J. A., and Kroemer, G. (2000) Ann. N. Y. Acad. Sci. 926, 149–164
18. Li, Q., Duan, L., Estes, J. D., Ma, Z. M., Rourke, T., Wang, Y., Reilly, C., Carlis, J., Miller, C. J., and Haase, A. T. (2005) Nature 434, 1148–1152
19. Meissner, E. G., Zhang, L., Jiang, S., and Su, L. (2006) J. Virol. 80, 11019–11030
20. Castedo, M., Macho, A., Zamzami, N., Hirsch, T., Marchetti, P., Uriel, J., and Kroemer, G. (1995) Eur. J. Immunol. 25, 3277–3284
21. Macho, A., Castedo, M., Marchetti, P., Aguilar, J. J., Decaudin, D., Zamzami, N., Girard, P. M., Uriel, J., and Kroemer, G. (1995) Blood 86, 2481–2487
22. Castedo, M., Perfettini, J. L., Andreau, K., Roumier, T., Piacentini, M., and Kroemer, G. (2003) Ann. N. Y. Acad. Sci. 1010, 19–28
23. Weaver, J. G., Tarze, A., Moffat, T. C., Lebras, M., Deniaud, A., Brenner, C., Bren, G. D., Morin, M. Y., Phenix, B. N., Dong, L., Jiang, S. X., Sim, V. L., Zurakowski, B., Lallier, J., Hardin, H., Wettstein, P., van Heeswijk, R. P., Douen, A., Kroemer, R. T., Hou, S. T., Bennett, S. A., Lynch, D. H., Kroemer, G., and Badley, A. D. (2005) J. Clin. Investig. 115, 1828–1838
24. Jolly, C., Kashefi, K., Hollinshead, M., and Sattentau, Q. J. (2004) J. Exp. Med. 199, 283–293