Caveolin-1 Modulates HIV-1 Envelope-Induced Bystander Apoptosis through gp41

Xiao Mei Wang, Peter E. Nadeau, Yung-Tsun Lo, and Ayalew Mergia*

Department of Infectious Disease and Pathology, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32611

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HIV infection causes a progressive, severe, and irreversible depletion of CD4+ T cells, which is responsible for the development of AIDS (9). The mechanism through which HIV infection induces cell death involves a variety of processes (58). Among these processes, apoptosis is most likely responsible for T-cell destruction in HIV-infected patients (33), because active antiretroviral therapy has been associated with low levels of CD4+ T-cell apoptosis (7), and AIDS progression was shown previously to correlate with the extent of immune cell apoptosis (34). Importantly, bystander apoptosis of uninfected cells was demonstrated to be one of the major processes involved in the destruction of immune cells (58), with the majority of apoptotic CD4+ T cells in the peripheral blood and lymph nodes being uninfected in HIV patients (22).

Binding to uninfected cells or the entry of viral proteins released by infected cells is responsible for the virus-mediated killing of innocent bystander CD4+ T cells (2–4, 9, 65). The HIV envelope glycoprotein complex, consisting of gp120 and gp41 subunits expressed on an HIV-infected cell membrane (73), is believed to induce bystander CD4+ T-cell apoptosis (58). Although there is a soluble form of gp120 in the blood, there is no conclusive agreement as to whether the concentration is sufficient to trigger apoptosis (57, 58). The initial step in HIV infection is mediated by the Env glycoprotein gp120 binding with high affinity to CD4, the primary receptor on the target cell surface, which is followed by interactions with the chemokine receptor CCR5 or CXCR4 (61). This interaction triggers a conformational change in gp41 and the insertion of its N-terminal fusion peptide into the target membrane (30).

Next, a prehairpin structure containing leucine zipper-like motifs is formed by the two conserved coiled-coil domains, called the N-terminal and C-terminal heptad repeats (28, 66, 70). This structure quickly collapses into a highly stable six-helix bundle structure with an N-terminal heptad repeat inside and a hydrophobic C-terminal heptad repeat outside (28, 66, 70). The formation of the six-helix bundle leads to a juxtaposition and fusion with the target cell membrane (28, 66, 70). The fusogenic potential of HIV Env is proven to correlate with the pathogenesis of both CXCR4- and CCR5-tropic viruses by not only delivering the viral genome to uninfected cells but also mediating Env-induced bystander apoptosis (71). Initial infection is dominated by the CCR5-tropic strains, with the CXCR4-tropic viruses emerging in the later stages of disease (20). Studies have shown that CXCR4-tropic HIV-1 triggers more depletion of CD4+ T cells than CCR5-tropic strains (36).

Glycolipid- and cholesterol-enriched membrane microdomains, termed lipid rafts, are spatially organized plasma membranes and are known to have many diverse functions (26, 53). These functions include membrane trafficking, endocytosis, the regulation of cholesterol and calcium homeostasis, and signal transduction in cellular growth and apoptosis. Lipid rafts have also been implicated in HIV cell entry and budding processes (19, 46, 48, 51). One such organelle is the caveola, which is a small, flask-shaped (50 to 100 nm in diameter) invagination in the plasma membrane (5, 62). The caveola structure, which is composed of proteins known as caveolins, plays a role in various functions by serving as a mobile platform for many receptors and signal proteins (5, 62). Caveolin-1 (Cav-1) is a 22- to 24-kDa major coat protein responsible for caveola assembly (25, 47). This scaffolding protein forms a hairpin-like structure and exists as an oligomeric complex of 14 to 16 monomers (21). Cav-1 has been shown to be expressed by a variety of cell types, mostly endothelial cells, type I pneumocytes, fibroblasts, and adipocytes (5, 62). In addition, Cav-1 expression is evident in

* Corresponding author. Mailing address: Department of Infectious Disease and Pathology, College of Veterinary Medicine, University of Florida, Gainesville, FL 32611. Phone: (352) 294-4139. Fax: (352) 392-9704. E-mail: mergiaa@ufl.edu.

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immune cells such as macrophages and dendritic cells (38, 39). However, Cav-1 is not expressed in isolated thymocytes (49). Furthermore, Cav-1 and caveolar structures are absent in human or murine T-cell lines (27, 41, 68). Contrary to this, there has been one report showing evidence of Cav-1 expression in bovine primary cell subpopulations of CD4+, CD8+, CD21+, and IgM+ cells with Cav-1 localized predominantly in the perinuclear region (38). That report also demonstrated a membrane region staining with Cav-1-specific antibody of human CD21+ and CD26+ peripheral blood lymphocytes (PBLs). Recently, the expression of Cav-1 in activated murine B cells, with a potential role in the development of a thymus-independent immune response, was also reported (56). It remains to be determined whether Cav-1 expression is dependent on the activation state of lymphocytes. For macrophages, however, which are one of the main cell targets for HIV infection, Cav-1 expression has been clearly documented (38).

The scaffolding domain of Cav-1, located in the juxtamembranous region of the N terminus, is responsible for its oligomerization and binding to various proteins (5, 62, 64). It recognizes a consensus binding motif, \( \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Phi \Ph...
CA), anti-β-actin (Sigma-Aldrich, St. Louis, MO), anti-V5 epitope, anti-mouse horseradish peroxidase (HRP), anti-rabbit HRP (Cell Signaling Technology, Danvers, MA), and anti-gp1 (kindly provided by the NIH AIDS Research and Reference Reagent Program).

Luciferase assay. 293T cells were seeded at a density of 2 × 10^5 cells/well and cotransfected with NL4-3 R E' luc and pC-Luc-NL4-3-Env. SupT1 cells were added along with 8 μg/ml of Cav-1 or control peptide 3 days posttransfection. SupT1 cells were harvested after 3 days of coculture, and cell lysates were subjected to a luciferase assay. Luciferase activity from cell lysates was determined as described by the manufacturer (Promega, Madison, WI).

Apoptosis assays. CHO or THP-1 cells were seeded into 24-well plates at 2 × 10^5 cells/well. THP-1 cells were transfected with pc-Cav-1 and the HIV Env constructs or vector alone. After 24 h, SupT1 cells or primary CD4+ T cells were added at 1 × 10^6 cells per well. Cells were cocultured for 24 h. After that, nonadherent cells were collected and stained with annexin V (BD Biosciences, San Jose, CA) according to the manufacturer’s instructions and subjected to flow cytometry analysis by using an LSR II apparatus with FACSDiva software, v6.3. Jurkat cells were transfected with the HIV Env expression construct or control vector alone (10 μg DNA). After 2 days, SupT1 cells were added in a ratio of 1:5 (Jurkat to SupT1 cells) along with the Cav-1 or control peptide (2 μg/ml). Cells were collected 24 h later, and SupT1 cells were isolated by using phycoerythrin (PE)-labeled anti-CD8 (BD Biosciences) and magnetic beads (Miltenyi Biotech, Auburn, CA). CD8+ SupT1 cells were stained with annexin V and analyzed by flow cytometry. Apoptosis induced by the effector cells, CHOWT, Jurkat, or THP-1 cells, were assigned a value of 100%, with the other groups’ results being expressed as apoptosis relative to that of these groups. Caspase-3 activities in SupT1 cell lysates were determined by using the caspase-3 fluorogenic substrate Ac-DEVD-AFC (BD Biosciences, San Jose, CA) and an Aminco Bowman series 2 spectrophotofluorometer (440-nm excitation wavelength and 500-nm emission wavelength) according to the manufacturer’s instructions (Thermo Fisher Scientific Inc., Waltham, MA). The activity was expressed in arbitrary fluorescence units.

Cav-1 and gp41

Cav-1 interaction with Env in 293T, HeLa, and CHO cells was studied by using transfected pCZ-cav-1 into 293T cells. A Cav-1 interaction with gp41 was detected by coimmunoprecipitation and immunoblotting analysis. As shown in Fig. 1, the association of Cav-1 and gp41 was evident in both 293T (Fig. 1A) and HeLa (Fig. 1B) cells. In addition, we examined the endogenous Cav-1 interaction with Env in CHO cells stably expressing the entire HIV Env protein (CHOWT cells). Similar to the results with 293T and HeLa cells, endogenous Cav-1 and Env binding was demonstrated by coimmunoprecipitation and immunoblotting analysis (Fig. 1C). The interaction was specific, as the transfaction of the Cav-1 gene into CHOWT cells significantly increased binding. Furthermore, such an association of Cav-1 and Env was lacking in control CHOEE cells, where the cells contained an expression vector devoid of the Env gene.

Both the Cav-1 and HIV-1 Env proteins have been shown to localize in lipid rafts (12). However, it is not known whether Cav-1 interacts with HIV-1 Env in these lipid rafts. In order to establish whether Cav-1 binds to gp41 of HIV Env in lipid rafts, we first examined the localization of Cav-1 and Env gp41. As shown in Fig. 1D, immunoblotting analysis showed that a portion of gp41 was found in fraction 4, which is regarded as a caveolar fraction, as evidenced by the presence of Cav-1 and flotillin-1. Coimmunoprecipitation and immunoblotting analysis revealed an association of Cav-1 and gp41 in fraction 4 (Fig. 1E). Taken as a whole, these studies, for the first time, provide evidence of an interaction between Cav-1 and HIV Env within lipid rafts.

Cav-1 modulates HIV-1 envelope-induced bystander apoptosis.

Three stable cell lines overexpressing Cav-1 (CHOWT/ cav-1/1, CHOWT/cav-1/2, and CHOWT/cav-1/3) were established by parental Env-expressing CHOWT cells in an effort to investigate whether Cav-1 binding to HIV Env gp41 can influence Env-induced bystander apoptosis. Furthermore, three additional stable cell lines expressing the vector alone were derived from CHOWT cells (CHOWT/vec-1, CHOWT/vec-2, and CHOWT/vec/3). Each of these effector cell lines was cocultured with SupT1 cells as targets for 24 h, which was followed by the collection of the nonadherent SupT1 cells and analysis of apoptosis with phosphatidylserine exposure by annexin V staining. As shown in Fig. 2A, CHOWT cells transfected with vector alone caused apoptosis in cocultured SupT1 cells ranging from 18.9% to 21.3%. The CHOWT/Cav-1 clones, which overexpressed Cav-1, markedly reduced apoptosis in the range of 8.4% to 9.5% in the target cells (Fig. 2A). The overexpression of Cav-1 in CHOEE cells (CHOEE/cav-1/1-3), where Env was lacking, did not affect apoptosis in cocultured SupT1 cells (Fig. 2B). To further validate the findings, we transfected an siRNA targeting the Cav-1 mRNA sequence into CHOWT cells to knock down endogenous Cav-1 expression and determine whether the apoptosis of SupT1 cells can be enhanced. At 48 h posttransfection, endogenous Cav-1 protein levels were significantly decreased compared to those of the control, as evaluated by immunoblotting analysis (Fig. 2C). In agreement with our observations that the overexpression of Cav-1 suppressed HIV-1 Env-induced bystander apoptosis, the knockdown of Cav-1 in CHOWT cells exhibited a significant

RESULTS

Interaction of Cav-1 and gp41.

In an effort to test whether Cav-1 modulates the function of HIV Env in the induction of apoptosis in bystander cells, we first examined the interaction of Cav-1 and HIV-1 Env in 293T, HeLa, and CHO cells to select an appropriate effector cell line. The coding sequence of the gp41 subunit of HIV-1 Env was subcloned from the CXXCR4-tropic (NL4-3) or CCR5-tropic (AD8) virus and tagged with V5 for expression (pcDNA3.1/V5-HisA-NL4-3-gp41 or pcDNA3.1/V5-HisA-AD8-gp41). These were then cotransfected with plasmid pCZ-cav-1 into 293T cells. A Cav-1 interaction with gp41 was detected by coimmunoprecipitation and immunoblotting analysis. As shown in Fig. 1, the association of Cav-1 and gp41 was evident in both 293T (Fig. 1A) and HeLa (Fig. 1B) cells. In addition, we examined the endogenous Cav-1 interaction with Env in CHO cells stably expressing the entire HIV Env protein (CHOWT cells). Similar to the results with 293T and HeLa cells, endogenous Cav-1 and Env binding was demonstrated by coimmunoprecipitation and immunoblotting analysis (Fig. 1C). The interaction was specific, as the transfaction of the Cav-1 gene into CHOWT cells significantly increased binding. Furthermore, such an association of Cav-1 and Env was lacking in control CHOEE cells, where the cells contained an expression vector devoid of the Env gene.

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augmentation in SupT1 bystander apoptosis (27.5%) compared to that of control siRNA (18.7%) (Fig. 2D).

To demonstrate the physiological relevance of our results, we used macrophages (THP-1 macrophages) as effector cells instead of CHO cells to determine if Cav-1 modulates bystander apoptosis in SupT1 cells. The HIV Env expression plasmid was transfected alone or in combination with pCZ-cav-1 into THP-1 cells and allowed to differentiate into macrophages. Target SupT1 cells were then cocultured with these macrophages and harvested, with the level of apoptosis being measured by staining with annexin V and flow cytometry. When THP-1 macrophages overexpressing Cav-1 were used, the level of apoptosis of target SupT1 cells was decreased significantly (by 32%) compared to that of cells expressing endogenous Cav-1 only (Fig. 2E). These results establish the role of Cav-1 in cells physiologically relevant to HIV infection.

To preclude the possibility that the modulation of apoptosis was specific only to SupT1 cells, we examined whether Cav-1 modulates bystander apoptosis in primary CD4 T cells. Target CD4 T cells were isolated from peripheral blood of healthy humans with a purity level of 98% (Fig. 3A). As shown in Fig. 3B, the cells underwent significant bystander apoptosis when cocultured with CHOWT/vector clones. In contrast, Cav-1 overexpression in CHOWT/Cav-1 cells markedly suppressed (4-fold) CD4 T-cell bystander apoptosis, thus confirming the results observed for SupT1 cells.

Mutation of the Cav-1 binding site of gp41 influences the inhibition of innocent-bystander apoptosis. We generated a mutant gp41 in which an alanine was substituted for the consensus tryptophan in the Cav-1 binding motif (W111A). This mutation did not affect the relative expression levels of Cav-1 and gp41 (Fig. 4A). When 293T cells were cotransfected with mutant gp41 and Cav-1 expression constructs, the interaction between the two was completely abolished (Fig. 4A). The gp41/Cav-1 interaction upon downstream bystander apoptosis was evaluated for CHO cells stably transfected with Cav-1 and either the HIV-1 NL4-3 envelope (wild-type Env [WTEnv]) or the HIV-1 NL4-3 envelope containing mutant gp41 (MTEnv). These cells were cocultured with SupT1 cells, and the induction of apoptosis on the target cells was measured. As shown in Fig. 4B, Cav-1 overexpression significantly suppressed WTEnv-induced bystander apoptosis, whereas Cav-1 has no impact on the induction of apoptosis of SupT1 cells by MTEnv. No significant differences were observed when the level of apoptosis by MTEnv was compared to that of WTEnv in the absence of Cav-1 overexpression. First, these studies suggest that the mu-
Migration introduced into gp41 of HIV Env has only a minor effect on the ability of Env to induce apoptosis on bystander cells. Second, the blockage of bystander apoptosis by the association of Cav-1 with WTEnv was shown to be specific due to the inability of Cav-1 to bind MTEnv, thus allowing bystander apoptosis.

Cav-1 modulates gp41-induced membrane hemifusion. The Cav-1 binding motif lies in the heptad repeat 2 (HR-2) region (Fig. 5A) of the gp41 domain of HIV-1 Env (30). This region is critical for cell membrane fusion and hemifusion. The fusion inhibitors C34 and T20 (enfuvirtide) target this region of gp41 (71). Neutralizing antibodies 2F5 and 4E10 bind to the nearby membrane-proximal ectodomain region (MPER) (24, 60). Previous studies demonstrated that membrane hemifusion is essential for the induction of apoptosis in bystander cells (31). To determine whether the inhibition of apoptosis in target cells was a consequence of the blockage of gp41-mediated membrane hemifusion due to a Cav-1/Env interaction, a cell fusion assay was performed. CHOWT/Cav-1 and CHOWT/vector cells were cocultured with SupT1 cells for 24 h. The suspended cells were harvested and subjected to annexin V FITC staining and flow cytometry analysis. Representative clones are shown. Cav-1 expression was determined by immunoblotting analysis of the six individual clones. Coomassie blue stain served as the loading control. (B) Overexpression of Cav-1 does not affect apoptosis in CHOEE cells. SupT1 cells were cocultured with CHOEE cells stably transfected with the pCZ-cav-1 or pCZ vector for 24 h. The experiments were performed as described above (A). (C) siRNA targeting Cav-1 or control siRNA was transfected into CHOWT cells. After 48 h, the cells were harvested and subjected to immunoblotting to determine Cav-1 expression levels. Coomassie blue stain served as the loading control. CTL, control. (D) CHOWT cells transfected with Cav-1 or control siRNA were cocultured with SupT1 cells for 24 h. The suspended cells were harvested and subjected to annexin V FITC staining and flow cytometry analysis. Apoptosis is expressed as annexin V staining relative to that of the control siRNA transfection group. (E) THP-1 macrophages cotransfected with Env-expressing plasmid along with pCZ-cav-1 or pCZ and used as effector cells with SupT1 as targets. Apoptosis is expressed as annexin V staining relative to that of cells receiving vector alone (pCZ). Results are expressed as the means ± standard deviations (SD) from three determinations. * P < 0.05.
contrast, no cytoplasmic dye (CMTMR) was detected in the target cells, confirming that Cav-1 blocks gp41 membrane-mediated hemifusion. Furthermore, Cav-1 overexpression failed to block membrane dye transfer when MTEv was used (Fig. 5E). This therefore establishes the requirement of a specific interaction of Cav-1 and gp41 for the Cav-1 modulation of the gp41-induced hemifusion of effector and target cells.

Cav-1 modulates apoptotic signal pathways. Caspases are crucial mediators of apoptosis, and caspase-3 was shown to be involved in HIV-1 Env-induced bystander apoptosis (29). To explore the influence of a Cav-1/gp41 interaction on the subsequent underlying signal apoptosis pathways in a target cell, we first measured caspase-3 activity in target SupT1 cells using CHOWT/Cav-1 and CHOWT/vector cell lines as effector cells. As shown in Fig. 6A, caspase-3 activation in target SupT1 cells was markedly increased in SupT1 cells when CHOWT/Cav-1 were used as an effector compared to that of CHOWT/vector cells (Fig. 6B). To determine whether the level of Hsp70 affects caspase-3 activation, we transfected SupT1 cells with siRNA targeting Hsp70. As shown in Fig. 6D, the downregulation of Hsp70 was associated with enhanced caspase-3 activation. These results suggest a potential link between the level of Hsp70 and caspase-3 activity in the Cav-1-mediated inhibition of bystander apoptosis.

Cav-1 peptide modulates HIV envelope-induced bystander apoptosis. Since the Cav-1 scaffold domain is essential for the interaction of caveolin with gp41, we designed a peptide containing the Cav-1 scaffold domain and tested its effects on bystander apoptosis. As shown in Fig. 7A, the Cav-1-based peptide significantly suppressed HIV-1 envelope-induced bystander apoptosis compared to the control peptide, in a dose-dependent manner, reaching 3-fold inhibition at an 8-µg/ml concentration. To determine whether the peptide can block cell fusion similarly to the Cav-1 protein, we examined gp41-induced membrane hemifusion. The Cav-1 peptide consistently showed, similarly to Cav-1, an inhibition of hemifusions (Fig. 7B). The Cav-1 peptide also decreased envelope-induced caspase-3 activation (Fig. 7C) and increased Hsp70 expression levels in a dose-dependent manner (Fig. 7D). Furthermore, the Cav-1 peptide markedly suppressed primary CD4+ T-cell bystander apoptosis (Fig. 7E). The peptide therefore exerts an efficient inhibition of cell fusion and bystander apoptosis in a manner similar to that of the Cav-1 protein and therefore shows promising potential for HIV therapy applications.

The efficacy of the peptide was further tested by using T cells as effectors to establish the physiological relevance for an HIV infection. We used Jurkat cells, which express CD4 but lack CD8, as an effector cell line. These cells were transfected with the HIV Env-expressing plasmid or vector alone and then cocultured with target T cells (SupT1) in the presence of Cav-1 or control peptide. Since SupT1 cells express both CD4 and CD8, the SupT1 cells were isolated by using magnetic beads followed by annexin V staining and flow cytometry analysis. As shown in Fig. 6A, caspase-3 activation in target SupT1 cells was significantly decreased (3-fold) when the CHOWT/Cav-1 cell line was used compared to CHOWT/vector cells, thus corroborating the apoptosis results observed with annexin V. HIV infection causes an increase in oxidative stress, and ROS production was shown to be enhanced in bystander apoptotic cells (57). Hence, we analyzed ROS production in SupT1 cells cocultured with CHOWT/Cav-1 or CHOWT/vector cells. Surprisingly, we did not detect any difference between CHOWT/Cav-1 and CHOWT/vector cells (Fig. 6B). In addition, Hsp70 was reported previously to be upregulated in bystander apoptotic cells, as demonstrated by immunoblotting and proteomic analyses (57). We found consistent phenomena in target SupT1 cells when we used CHOWT effector cells. Interestingly, the level of Hsp70 expression was markedly increased in SupT1 cells when CHOWT/Cav-1 cells were used as an effector compared to that of CHOWT/vector cells (Fig. 6C). To determine whether the level of Hsp70 affects caspase-3 activation, we transfected SupT1 cells with siRNA targeting Hsp70. As shown in Fig. 6D, the downregulation of Hsp70 was associated with enhanced caspase-3 activation. These results suggest a potential link between the level of Hsp70 and caspase-3 activity in the Cav-1-mediated inhibition of bystander apoptosis.

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were used to measure luciferase activity. As shown in Fig. 8, luciferase activity was markedly reduced when the Cav-1 peptide was used compared to that when the control peptide was used. Therefore, the peptide shows efficacy in the relevant cell type that is prone to HIV infection.

**DISCUSSION**

Cav-1 was previously demonstrated to interact with gp41 of HIV Env, and the scaffold domain of Cav-1 is essential for this association between the two proteins (42, 43, 52). The role of this interaction of Cav-1 and Env in HIV pathogenesis remains to be determined. In this report, we show the Cav-1 inhibition of Env-mediated bystander T-cell killing. In addition, the interaction between Cav-1 and Env reduces the hemifusion of effector and target T cells, which subsequently results in the blocking of bystander cell killing. The introduction of mutations into the Cav-1 binding domain of gp41 of Env leads to the formation of hemifusions/fusions and bystander apoptosis in the presence of Cav-1, confirming that the block of bystander apoptosis is due to a specific interaction of Cav-1 and the gp41 region of Env. Furthermore, a peptide derived from the scaffold domain of Cav-1 exerts the same effect as full-length Cav-1, thus effectively blocking cell fusion and inhibiting bystander apoptosis. These results show a potential role for Cav-1 in HIV pathogenesis and establish a basis for further investigations of Cav-1 expression and HIV replication.

The depletion of uninfected CD4+ T cells is common with HIV infection and is a major contributor to pathogenesis in infected individuals. The HIV envelope is believed to be a major player in the HIV-mediated killing of uninfected CD4 T cells. Envelope-induced apoptosis occurs as a result of an infected cell’s interaction with an uninfected cell CD4 receptor (1, 23, 40) and/or the CXCR4/CCR5 coreceptor (11, 13, 14, 45). Infected macrophages can also contribute to the depletion of uninfected T lymphocytes by inducing apoptosis in both CD4+ and CD8+ cells (6, 8). Cav-1 is known to be expressed in macrophage and dendritic cells, yet it is not known whether such an expression influences infected-macrophage-induced bystander apoptosis of T cells. This report shows the Cav-1 modulation of HIV envelope-induced bystander apoptosis of T cells through a Cav-1/gp41 interaction in which Cav-1 suppresses envelope-induced caspase-3 activation and downstream apoptosis using CHO cell lines as effectors. Furthermore, our results demonstrate that THP-1 macrophages expressing HIV Env can induce apoptosis in cocultured SupT1 cells and that the apoptosis of these cells can be inhibited by Cav-1. This finding leads to the possibility that cells infected by HIV that express Cav-1 could influence bystander cell killing. Since there is an abundance of macrophages and dendritic cells in lymph nodes, Cav-1 may play a role in regulating bystander T-cell apoptosis in HIV pathogenesis. The significant increase in levels of bystander cell killing when endogenous Cav-1 expression is blocked by targeting with siRNA strengthens the notion that HIV-infected cells with the ability to express Cav-1 can modulate Env-induced bystander apoptosis by interacting with gp41. Innocent-bystander cell killing involves the binding of gp120 to CD4 and CXCR4/CCR5, which is followed by hemifusion or fusion mediated by gp41 (30). The blocking of gp41-mediated fusion inhibits apoptosis in target cells (31). Similarly, our results show that Cav-1 binding to gp41 blocks the Env-mediated hemifusion of effector and T cells, which is
a prerequisite for bystander apoptosis. Since the fusogenic potential of HIV Env is proven to correlate with the pathogenesis of both CXCR4- and CCR5-tropic viruses, the blocking of this process of HIV infection with Cav-1 may have important implications for HIV therapy.

Llano et al. previously discovered that the cotransfection of human Cav-1 and HIV-1 into 293T cells blocked virion production, even at low ratios of Cav-1 to HIV-1 DNA (50). Cav-1 also blocked HIV-2 virion production (50). Such a suppression of HIV replication may have to do with a strong interaction between Cav-1 and the HIV envelope protein gp41 (42, 43, 52). First indentified by phage display, the Cav-1 scaffolding domain (amino acids 61 to 101) demonstrated a strong binding to the gp41 core N36(L8)C34, a six-helix bundle motif seen in a physiological situation (43). Interestingly, a synthetic peptide corresponding to the consensus Cav-1 binding motif in gp41 is capable of eliciting antibodies in rabbits. These antibodies were shown to inhibit the infection of primary CD4+ T lymphocytes by several primary HIV-1 isolates (42). Based on these findings, it was hypothesized that the interaction of Cav-1 and gp41 might be essential for the formation of fusion pores or the endocytosis of HIV-1 (43). However, further evidence to prove such a hypothesis is still lacking. Our discovery of the association of Cav-1 with HIV Env in lipid rafts along with the strikingly diminished bystander apoptosis in SupT1 cells and CD4+ T cells isolated from peripheral blood sheds new light onto a potential role of Cav-1 in HIV pathogenesis.

Hsp70 is a molecular chaperone involved in protection against environmental stresses. Its expression has been shown to be upregulated in umbilical cord blood mononuclear cells and A2.01/CD4.403 T cells cocultured with effector HEK cells expressing the HIV-1 envelope (57). Hsp70 coimmunoprecipitated with HIV-1 viral protein R (Vpr), and the overexpression of Hsp70 reduced Vpr-dependent G1 arrest and apoptosis in T cells (44). Our studies show that Cav-1 markedly enhances Env-induced Hsp70 expression, while the downregulation of...
Hsp70 increases caspase-3 activation in bystander cells. This finding suggests a possible link between Hsp70 and the Cav-1-mediated inhibition of Env-induced bystander apoptosis. Furthermore, this finding supports the observation by others (44) that the overexpression of Hsp70 reduces apoptosis in T cells.

The level of production of ROS is increased in both HIV-1-infected cells (59) and bystander cells (57). Several HIV-1 virus proteins, including Tat, Nef, Vpr, and gp120, have been implicated in the initiation and intensification of oxidative stress (59). Fas, tumor necrosis factor (TNF), and T-cell receptors are involved in the ROS-regulated apoptotic signaling pathway (59). Consistent with those reports, we found that HIV Env increased ROS production in bystander SupT1 cells. However, the overexpression of Cav-1 did not affect ROS production, suggesting that ROS may not be involved in Cav-1-modulated bystander apoptosis.

Fusion inhibitors confer a strong inhibition of both bystander apoptosis and virus infection. However, their clinical use can be limited because of the rapid emergence of resistant viruses (17). A new strategy for the blocking of fusion/hemifusion is necessary to overcome this clinical dilemma. The Cav-1 binding motif lies in the HR-2 region, near the MPER of gp41 (Fig. 5A). Those regions have been highly investigated and believed to be critical for the function of gp41-mediated cell membrane fusion and hemifusion (30). The HR regions have been targets for the development of fusion inhibitors such as C34 and T20 (enfuvirtide) (71). Two broadly neutralizing antibodies, 2F5 and 4E10, bind to the MPER and inhibit Env-mediated fusion and viral infection (24, 60). Therefore, Cav-1 binding to the HR-2 region could function as a fusion inhibitor. Our findings establish evidence that both Cav-1 and a peptide based on the Cav-1 sequence of the gp41 binding domain demonstrate a strong inhibition of HIV-1 Env-induced membrane hemifusion. This evidence suggests that Cav-1 or a peptide derived from Cav-1 could serve as a candidate for a new fusion inhibitor. The effectiveness of fusion inhibitors in blocking cell-to-cell transmission via synapsis formation, which is believed to be a major route of HIV dissemination in vivo (reviewed in reference 54), remains inconclusive. The Cav-1/Env association that we identified in the lipid raft and Cav-1’s ability to inhibit effector/target cell hemifusion suggest that Cav-1 could be a strong candidate for blocking HIV cell-to-cell transmission.

Cav-1 has been implicated in the regulation of apoptosis in a variety of cells under differing circumstances. Whether Cav-1 displays proapoptotic or antiapoptotic characteristics may depend on cellular specificity and particular conditions (67). The mechanism of HIV-1-induced apoptosis in vivo is complicated and multifactorial. Besides the envelope, many viral proteins, including Tat, Nef, and Vpr, are capable of eliciting apoptosis.

**FIG. 6.** Cav-1 modulation of apoptotic signal pathways. SupT1 cells were cocultured with CHOWT cells, stably transfected with the pCZ-cav-1 or pCZ vector, for 24 h. (A and B) The suspended cells were collected and subjected first to a caspase-3 activity assay (A) and second to an ROS production assay (B). Results are expressed as the means ± SD from three determinations. *, P < 0.05. SupT1 cell lysates served as the control (CTL). (C) SupT1 cells under the same conditions as those described above (A and B) were also harvested and subjected to immunoblot analysis with Hsp70. β-Actin served as the loading control. (D) CHOWT cells transfected with Hsp70 siRNA or control siRNA for 48 h. These cells were cocultured with SupT1 cells for 24 h. The suspended cells were collected and subjected to a caspase-3 activity assay. Results are expressed as the means ± SD from three determinations. *, P < 0.05.
in CD4+ T lymphocytes (58). It remains to be determined whether Cav-1 can modulate other viral proteins involved in apoptosis during HIV infection. Infected macrophages are relatively resistant to cytopathic effects and consequently play an essential role in viral dissemination into host tissues and organs (18). Furthermore, in this viral reservoir, HIV infection appears to be associated not with apoptosis but with a chronic, productively infected phenotype (37, 63). Cav-1 serves as a protein that is protective against cellular stress and inflammation (10, 15, 32, 55) and is involved in supporting multiple antiapoptotic and survival pathways (16, 72). Investigation of the interaction of Cav-1 and HIV Env in macrophages will be critical to establishing whether the binding of Cav-1 modulates the ability of Env to induce apoptosis in macrophages. Our observation of the inhibition of bystander apoptosis by Cav-1 using Env-expressing THP-1 macrophages as an effector cell line supports such a notion. Furthermore, Cav-1 expression in macrophages coupled first with our observation that it inhibits

FIG. 7. The Cav-1 peptide modulates HIV-1 Env-induced bystander apoptosis. SupT1 cells were cocultured with CHOWT cells for 24 h in the presence of control and Cav-1 peptides at a concentration of 0.5 μg/ml, 2 μg/ml, or 8 μg/ml. (A to D) The suspended cells were harvested and subjected to annexin V-APC staining and flow cytometry analysis (A), a fusion assay (B), a caspase activity assay (C), and Hsp70 immunoblotting (D). (E) CD4+ T cells isolated from peripheral blood were cocultured with CHOWT cells transfected with vector or the Cav-1 gene for 24 h. The suspended cells were collected and subjected to annexin V-APC staining and flow cytometry analysis. Apoptosis is expressed as annexin V staining relative to that of the vector group. (F) Jurkat cells were transfected with an Env expression or vector-alone plasmid construct and were used as effector cells. These transfected cells were cocultured with SupT1 cells for 24 h in the presence of the control or Cav-1 peptide at a concentration of 0.8 μg/ml. SupT1 cells were isolated by using magnetic beads and anti-CD8 antibody. Apoptosis is expressed as annexin V staining relative to that of the vector group. Results are expressed as the means ± SD from three determinations. *, *P < 0.05.

FIG. 8. The Cav-1 peptide inhibits HIV production. The efficacy of the Cav-1 peptide was tested in a limiting single-round infection using NL4-3 R′ E′ luc HIV. NL4-3 R′ E′ luc and pCI-NL4-3-Env (Env-expressing construct) were cotransfected into 293T cells. Supernatants harvested from transfected 293T cells were used to infect SupT1 cells. To determine infection levels, lysates from infected cells were used to measure luciferase activity. The level of luciferase activity is expressed relative to that of the control peptide (Ctl). Results are expressed as the means ± SD from three determinations. *, *P < 0.05.
hemifusion and bystander apoptosis and second with the findings reported by Llano et al. that Cav-1 restricts virus replication (50) suggest that Cav-1 may play a role in the persistent infection of macrophages, thus warranting further investigation of a Cav-1/HIV Env interaction within macrophages.

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