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Caveolin-1 Regulates Human Immunodeficiency Virus-1 Tat-Induced Alterations of Tight Junction Protein Expression via Modulation of the Ras Signaling

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The blood–brain barrier (BBB) is the critical structure for preventing human immunodeficiency virus (HIV) trafficking into the brain. Specific HIV proteins, such as Tat protein, can contribute to the dysfunction of tight junctions at the BBB and HIV entry into the brain. Tat is released by HIV-1-infected cells and can interact with a variety of cell surface receptors activating several signal transduction pathways, including those localized in caveolae. The present study focused on the mechanisms of Tat-induced caveolae-associated Ras signaling at the level of the BBB. Treatment with Tat activated the Ras pathway in human brain microvascular endothelial cells (HBMECs). However, caveolin-1 silencing markedly attenuated these effects. Because the integrity of the brain endothelium is regulated by intercellular tight junctions, these structural elements of the BBB were also evaluated in the present study. Exposure to Tat diminished the expression of several tight junction proteins, namely, occludin, zonula occludens (ZO)-1, and ZO-2 in the caveolar fraction of HBMECs. These effects were effectively protected by pharmacological inhibition of the Ras signaling and by silencing of caveolin-1. The present data indicate the importance of caveolae-associated signaling in the disruption of tight junctions on Tat exposure. They also demonstrate that caveolin-1 may constitute an early and critical modulator that controls signaling pathways leading to the disruption of tight junction proteins. Thus, caveolin-1 may provide an effective target to protect against Tat-induced HBMEC dysfunction and the disruption of the BBB in HIV-1-infected patients.

Key words: **blood–brain barrier; HIV-1 Tat; Ras; caveolae; caveolin-1; tight junction proteins**

Introduction

Brain microvasculature is actively involved in human immunodeficiency virus-1 (HIV-1) infection of the CNS. HIV-1 crosses the blood–brain barrier (BBB) early in the course of systemic infection and resides in brain macrophages and microglia (Wu et al., 2000; González-Scarano and Martín-García, 2005; Toborek et al., 2005; Banks et al., 2006; Persidsky and Poluektova, 2006). HIV-1 can induce a variety of inflammatory mediators in a variety of cell components of the CNS, including brain endothelial cells. This is important, because HIV-1 trafficking into the brain may be mediated through a "Trojan horse" mechanism, in which HIV-infected CD4$^+$ T-lymphocyte and/or circulating monocytes enter the CNS through the BBB via interaction with inflammatory mediators (Wu et al., 2000; González-Scarano and Martín-García, 2005).

During inflammatory responses, leukocytes enter the CNS through disrupted tight junctions. Well developed tight junctions are the most prominent feature of the brain endothelium and are responsible for the integrity of the BBB. They limit paracellular flux and restrict permeability across the brain endothelium (Hawkins and Davis, 2005; Abbott et al., 2006). Transmembrane tight junction proteins, such as occludin, are responsible for sealing together brain endothelial cells (Furuse et al., 1993). Transmembrane proteins are linked to the actin cytoskeleton by tight junction accessory proteins, such as zonula occludens (ZO)-1 and ZO-2 (Hawkins and Davis, 2005; Abbott et al., 2006). Disruption of tight junctions is common in HIV-1-infected patients. For example, fragmentation and decreased immunoreactivity for occludin and ZO-1, was detected in the brains of AIDS patients (Dallasta et al., 1999; Boven et al., 2000; Persidsky et al., 2006). These changes were associated with the accumulation of HIV-1-infected macrophages, fibrinogen leakage, and apoptosis (Dallasta et al., 1999; Persidsky et al., 2006).

The present study focused on the Ras signaling cascades and its influence on tight junction protein expression. Ras proteins are small GTPases that cycle between inactive GDP-bound and active GTP-bound conformations (Ras-GDP and Ras-GTP, respectively). Several elements of the Ras signaling cascades are localized in caveolae, the dominant type of lipid rafts in endothelial cells (Dykstra et al., 2003; Pike, 2003; Cohen et al., 2004; Rao

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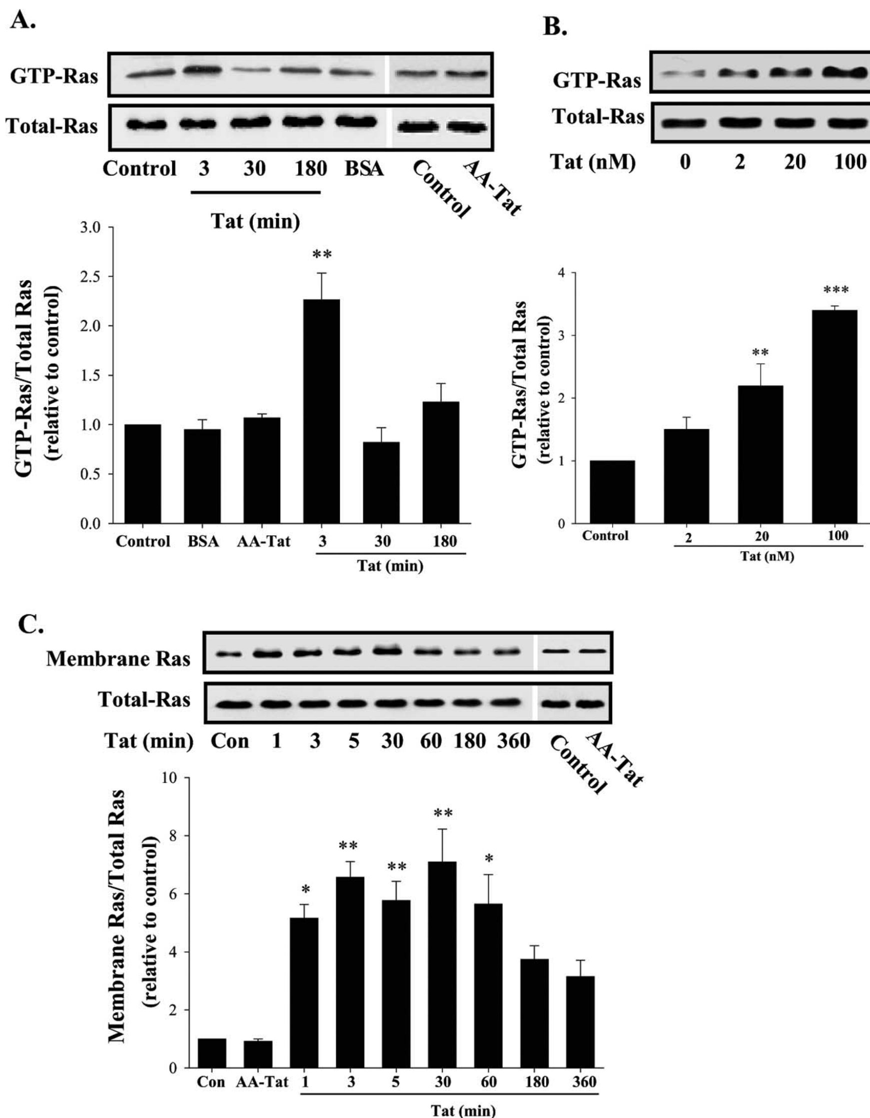

Figure 1. Tat-mediated activation of Ras in HBMECs. ***A***, ***B***, HBMECs were exposed to Tat for the indicated time points (***A***) or concentration (***B***). Active GTP-Ras was pulled down from the cell lysates with Raf-1 RBD (Ras binding domain) coupled to glutathione agarose, and Ras protein was determined by immunoblotting with monoclonal anti-Ras antibody. The bottom blots represent total Ras expression in HBMECs. ***C***, The membrane fraction was separated as described in Materials and Methods, and Ras expression was detected using monoclonal anti-Ras antibody. The whole-cell lysate was used to determine the total Ras protein. In ***A–C***, treatment with AA-Tat and/or BSA was used as negative controls. Cells were exposed to AA-Tat or BSA for 3 min. The blots reflect representative data from three different experiments and the bar graphs represent quantified results (mean ± SD) from these experiments as analyzed by densitometry. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control cells.

et al., 2004). The principal structural components of caveolae membranes are caveolins, 21–24 kDa integral membrane proteins. Caveolin-1 is the main isoform in endothelial cells and knocking it down eliminates the formation of caveolae (Cohen et al., 2004).

Several vascular effects of HIV-1 observed in the CNS can be reproduced by treatment with HIV-1 protein Tat. Tat is a strong proinflammatory agent that can recruit and induce transendothelial migration of monocytes (Weiss et al., 1999). Tat also affects endothelial cell actin microfilament dynamics, causing actin cytoskeletal rearrangements and disassembly (Wu et al., 2004). Therefore, the aim of the present study was to evaluate the role of caveolae and caveolin-1 in Tat-induced dysfunction of brain endothelial cells. Our novel observations indicate that Tat-mediated activation of the Ras signaling is regulated by caveolin-1 in brain endothelial cells. In addition, we demonstrate that inhibition of caveolin-1 and the Ras signaling can attenuate Tat-induced disruption of tight junction proteins.

Materials and Methods

Cell cultures and treatment. Immortalized human brain microvascular endothelial cells (HBMECs) (Weksler et al., 2005) were cultured in EBM-2 medium, supplemented with EGM-2 SingleQuots (Lonza Walkersville) and 5% FBS (HyClone). All cell culture dishes were coated with rat tail collagen type 1 (BD Biosciences).

Caveolin-1-deficient (cav-1 $^{-/-}$) mice were purchased from The Jackson Laboratory and bred through heterozygous mating. These mice were generated in the C57BL/6 genetic background (Razani et al., 2001). Brain microvascular cells were isolated from cav-1 $^{-/-}$ and control C57BL/6 mice as previously described (András et al., 2005). All procedures were approved by the Institutional Animal Care and Use Committee.

HIV Tat protein is released from infected cells and is found circulating in the blood of HIV-1-infected patients (Westendorp et al., 1995; Xiao et al., 2000). Evidence indicates that pathological concentrations of Tat in HIV-infected patients can reach the range of nanograms per milliliter of serum (Xiao et al., 2000). Therefore, cells were treated with Tat at levels between 2 and 100 nM in the present study. Such concentrations of Tat are consistent with literature data (Rumbaugh et al., 2006; Eugenin et al., 2007).

Tat was produced as described by Ma and Nath (1997). To determine specificity of Tat-induced effects, Tat solutions were immunoabsorbed using anti-Tat antibody conjugated to protein-A/G plus-agarose (Santa Cruz Biotechnology) and centrifuged to remove precipitated proteins. This form of Tat was called immunoabsorbed Tat (AA-Tat).

Ras GTPase pull-down assay. Ras activity was assayed by using the GTP-Ras pull-down assay according to the manufacturer's instruction (Millipore). Western blots were performed using monoclonal anti-Ras antibody (BD Biosciences Transduction Laboratories).

Caveolin-1 silencing. Caveolin-1 silencing was performed as described previously (Repetto et al., 2005) using a mixture of small interfering RNA (siRNA) corresponding to nucleotides 69–87 of the human caveolin-1 mRNA sequence, 5′-CAUCUACAAGCCCAACAAC-dTdT-3′ (cav-1 siRNA-1), and to nucleotides 223–241, 5′-CCAGAA-GGGACACACAGUU-dTdT-3′ (cav-1 siRNA-2). Control siRNA was as follows: 5′-AAAGAGCGACUUUACACAC-dTdT-3′. LipofectAMINE-plus (Invitrogen) was used to transfect HBMECs overnight with cav-1 siRNA-1 (40 nM) and with cav-1 siRNA-2 (40 nM) for an additional 5 h.

Western blotting. HMBEC membrane fractions were isolated as described by Gong et al. (1997), and total cell extracts were prepared using a standard procedure (Koon et al., 2006). Polyclonal antibodies against phospho-mitogen-activated protein kinase kinase (MEK) (Ser $^{217/221}$), MEK1/2, and extracellular signal-regulated kinase 1/2 (ERK1/2) were from Cell Signaling Technology. Caveolin-1 polyclonal antibody was from Affinity BioReagents. Antibodies against ZO-1 and occludin were from Zymed Laboratories. Antibodies against phospho-ERK1/2

and ZO-2 were from Santa Cruz Biotechnology. Pan-Ras monoclonal antibody was from BD Biosciences, and anti-actin antibody was purchased from Sigma-Aldrich. Immunoreactive bands were visualized by using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) and developed by ECL (GE Healthcare). The density of bands was calculated using the Scion Image program (Scion). Coimmunoprecipitation of caveolin-1 and Ras protein was performed according to a previously published technique (András et al., 2005) using anti-Ras antibody for immunoprecipitation and anti-caveolin-1 antibody for Western blotting.

Caveolae preparation. Cellular fractions were prepared using a detergent-free method described by Smart et al. (1995). In addition to caveolae membranes, postnuclear supernatant, plasma membranes, and intracellular membranes were also isolated during this procedure.

Immunofluorescence microscopy. Brain microvascular cells isolated from cav-1 $^{-/-}$ and control mice were grown on slides coated with a mixture of collagen type I and IV and treated with Tat. ZO-1 immunoreactivity was detected as described previously (András et al., 2003). The slides were mounted using antifade reagent containing 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen) to stain nuclear DNA. Samples were evaluated under the epifluorescence microscope.

Statistical analysis. Each experiment was repeated a minimum of three times. Data were expressed as mean ± SEM. One-way or two-way ANOVA was used to compare mean responses among the treatments. Statistical probability of $p < 0.05$ was considered significant.

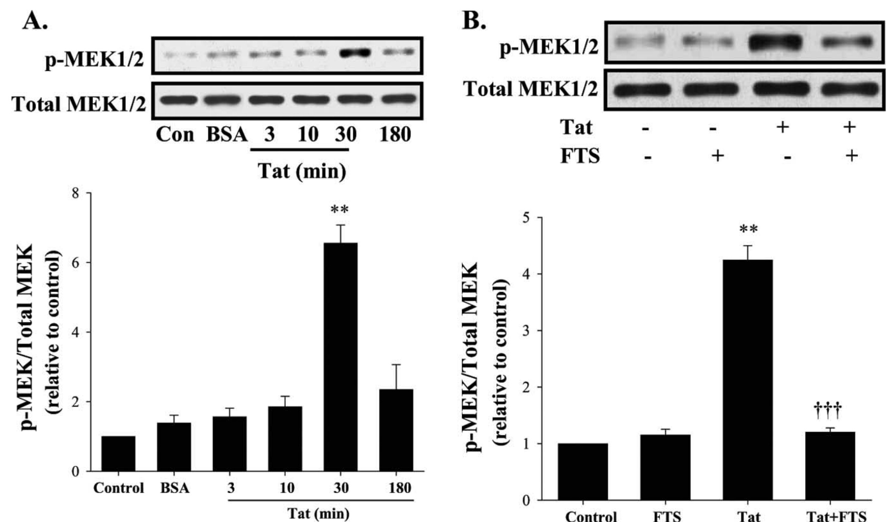

Figure 2. Tat-mediated activation of MEK1/2 in HBMECs is Ras dependent. ***A***, Time course analysis of MEK1/2 activation induced by Tat. HBMECs were treated with 100 nM Tat for the indicated time periods. Activated (phosphorylated) MEK1/2 (p-MEK1/2) was analyzed by immunoblotting with a specific phospho-MEK1/2 (Ser $^{217/221}$) antibody. In addition, total MEK1/2 was determined using a specific anti-MEK1/2 antibody. BSA was used as a negative control. ***B***, HBMECs were pretreated for 2 h with Ras inhibitor FTS (20 μM) and exposed to 100 nM Tat for 30 min. Phosphorylated and total MEK1/2 were determined as described in ***A***. In ***A*** and ***B***, the blots reflect representative data from three different experiments, and the bar graphs represent quantified results (mean ± SD) from these experiments as analyzed by densitometry. $^{**}p < 0.01$ compared with controls. †††Values in the group Tat+FTS are statistically different from those in the Tat group at $p < 0.001$.

Results

Tat stimulates Ras activation

Tat can interact with G-protein-coupled receptors, such as vascular endothelial growth factor receptor 2 (VEGFR-2) (András et al., 2005). Activation of these cell surface receptors may lead to stimulation of small GTPases, including the Ras signaling cascade. Ras activation is connected to the transformation of GDP-Ras to GTP-Ras. Therefore, we determined GTP-Ras levels in response to Tat exposure (Fig. 1*A*,*B*) using the pull-down assay. As illustrated in Figure 1*A*, exposure to 100 nM Tat resulted in a rapid and time-dependent increase in GTP-Ras. Indeed, GTP-Ras levels were elevated as the result of a 3 min treatment with Tat and returned to the control levels in cells exposed to Tat for 30 min or 3 h. The total Ras level was not affected by Tat exposure. In addition, treatment with negative controls, such as bovine serum albumin (BSA) or immunoabsorbed Tat (AA-Tat) did not alter GTP-Ras levels in HBMECs. The effects of Tat on GTP-Ras levels were dose dependent, and a marked increase was observed in HBMECs exposed to 20 nM Tat. However, a maximum activation of Ras (~2.4-fold increase over basal values) was observed in cells treated with Tat at the concentration of 100 nM (Fig. 1*B*).

Next, we evaluated the expression of Ras in the membrane fraction of Tat-treated HBMECs. Confluent cultures were exposed to 100 nM Tat for up to 6 h and membrane Ras protein was analyzed by immunoblotting. As shown in Figure 1*C*, an increase in Ras in the membrane fraction occurred as early as 1 min after Tat exposure and was preserved for up to 60 min. These effects were specific because AA-Tat did not affect membrane Ras levels. Similar to the results presented in Figure 1, *A* and *B*, total Ras was not affected by Tat exposure.

Tat activates downstream kinases of the Ras signaling cascade

To evaluate the downstream signaling effects of Tat-induced activation of Ras, we determined the levels of phosphorylated MEK1/2 and ERK1/2 in Tat-treated HBMECs. The experiments were performed using confluent cultures exposed to 100 nM Tat for up to 3 h. As shown in Figure 2*A*, exposure to Tat resulted in induction of MEK1/2 phosphorylation with a peak at 30 min (~6.5-fold increase), followed by a decline at 3 h after treatment. BSA, which was used as a negative control, did not have any effect on MEK1/2 phosphorylation. To confirm that the Ras pathway is involved in Tat-induced MEK1/2 activation, HBMECs were pretreated with farnesylthiosalicylic acid (FTS) (20 μM), a specific inhibitor of Ras, before treatment with Tat. Figure 2*B* indicates that preincubation with FTS efficiently blocked Tat-stimulated MEK1/2 phosphorylation. The total levels of MEK1/2 were not affected by Tat treatment.

Treatment with Tat significantly upregulated levels of phosphorylated ERK1/2 (~3.7-fold increase at 30 min) (Fig. 3*A*). Similar to MEK1/2 activation, these effects were also the most pronounced after 30 min of Tat exposure. Tat-induced activation of ERK1/2 was markedly blocked by the Ras inhibitor FTS (Fig. 3*B*) and MEK1/2 inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene (U0126) (0.6 μM) (Fig. 3*C*), indicating that Tat-induced activation of ERK1/2 is a downstream effect of MEK1/2 and Ras stimulation.

Tat upregulates caveolin-1 expression

We hypothesize that caveolin-1 may play a regulatory role in Tat-mediated alteration of caveolae-associated signaling and expression of tight junction proteins. Before performing the experiments that directly address this hypothesis, we determined the effects of Tat on cellular caveolin-1 levels. Treatment of confluent HBMEC cultures with Tat resulted in a dose- and time-dependent elevation of membrane caveolin-1 levels (Fig. 4*A*,*B*, respectively). The highest elevation of caveolin-1 levels were observed in cells exposed to 100 nM Tat for 3 min (~2.5-fold compared with control).

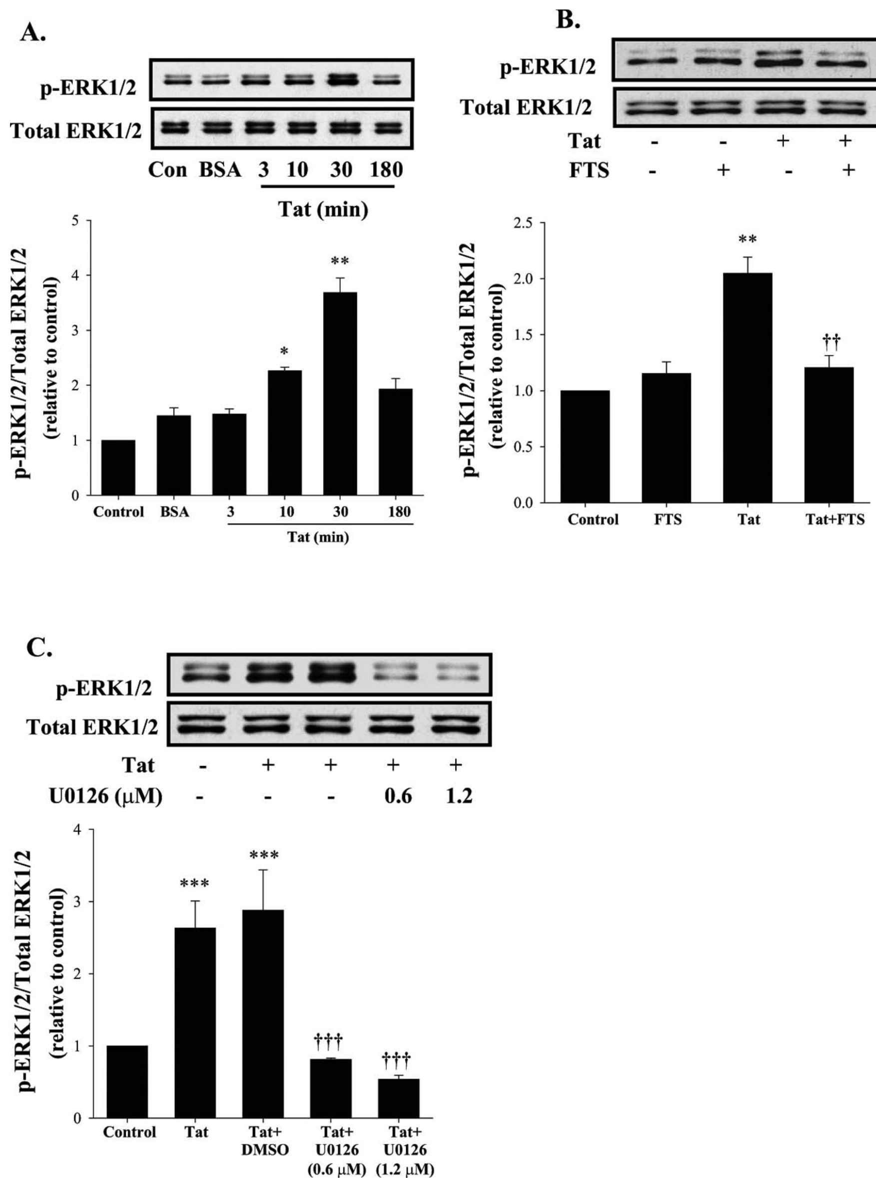

Figure 3. Tat-mediated activation of ERK1/2 in HBMECs is downstream from Ras and MEK1/2. ***A***, Time course analysis of ERK1/2 activation induced by Tat. HBMECs were treated with 100 nM Tat for the indicated time periods. Phosphorylated (p-ERK1/2) and total ERK1/2 were analyzed by immunoblotting with specific antibodies. BSA was used as a negative control. ***B***, HBMECs were pretreated for 2 h with Ras inhibitor FTS (20 μM) and exposed to 100 nM Tat for 30 min. Phosphorylated and total ERK1/2 were determined as described in ***A***. ***C***, HBMECs were pretreated with the indicated concentrations of MEK1/2 inhibitor U0126, followed by exposure to 100 nM Tat for 30 min. Phosphorylated and total ERK1/2 were analyzed as in ***A***. In ***A–C***, the blots reflect representative data from three different experiments, and the bar graphs represent quantified results (mean ± SD) from these experiments as analyzed by densitometry. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with controls; values in the group Tat+FTS or Tat+U0126 are statistically different from those in the Tat group at $^{††}p < 0.01$ or $^{†††}p < 0.001$.

However, a lower dose of Tat, such as 20 nM, also increased membrane caveolin-1 levels. The specificity of these effects was determined by treatment with AA-Tat.

In time-dependent experiments, HBMECs were exposed to 100 nM Tat for up to 6 h. Upregulation of membrane caveolin-1 was evident as early as 1 min, peaked at 3 min (approximately threefold increase), and was preserved for up to 30 min of Tat exposure. Longer treatment times with Tat did not reach statistical significance (Fig. 4*B*). The effects of Tat were limited to the membrane fraction of caveolin-1 and total caveolin-1 levels were not altered independent of the treatment dose (Fig. 4*A*) or exposure time (Fig. 4*B*).

Caveolin-1 silencing abolishes Tat-induced Ras activation

In the next series of experiments, we determined the role of caveolin-1 in Tat-mediated activation of Ras. First, we determined by coimmunoprecipitation that caveolin-1 interacts with Ras proteins. In the experiments presented in Figure 5*A*, immunoprecipitation was performed using anti-pan Ras antibody, followed by Western blotting with anti-caveolin-1 antibody. Similar results were obtained using a reverse combination of these antibodies (data not shown). Then, using a mixture of two specific caveolin-1 siRNAs, we silenced caveolin-1 protein expression by ~70% compared with mock transfected control cultures. Transfection with the same amount of control siRNA did not affect caveolin-1 expression. In addition, actin expression in the cell lysates remained unchanged in transfected cells (Fig. 5*B*).

As indicated in Figure 5*C*, silencing of caveolin-1 effectively protected against Tat-induced Ras activation. In these experiments, HBMECs were treated with 100 nM Tat for 3 min. Both Tat treatment and caveolin-1 silencing did not alter the total Ras levels in HBMECs. These results provide evidence that caveolin-1 is an upstream modulator of Ras signaling in HBMECs.

Tat alters expression of tight junction proteins in caveolae through the Ras-mediated signaling

The integrity of tight junctions is the main factor that determines the integrity and the barrier function of the brain endothelium. The functional state of tight junctions depends on phosphorylation processes; therefore, we evaluated the involvement of the Ras signaling and caveolin-1 in Tat-mediated alterations of tight junction protein expression.

Exposure to Tat (100 nM) markedly diminished the expression of several tight junction proteins, such as occludin, ZO-1, and ZO-2, in HBMECs as analyzed by Western blotting (Fig. 6*A*). These effects were induced in cells treated with Tat for 12 h and persisted 24 h after adding Tat to cell cultures. Shorter exposure times or control treatment with AA-Tat did not alter the expression of tight junction proteins. Actin was determined as a housekeeping protein and its levels were not changed in response to Tat or AA-Tat.

To further determine the relationship between tight junction proteins and caveolae, we used a detergent-free method for preparation of caveolae-rich cellular fractions (Smart et al., 1995). As indicated in Figure 6*B*, the basal expression of tight junction proteins is enriched in the caveolae fraction of HBMECs compared with other cellular components, including plasma membranes. Most importantly, Tat-mediated alterations of occludin, ZO-1, and ZO-2 expression were specifically localized to the

caveolae fraction of HBMECs. Statistical analysis of these effects is presented in Figure 6*C*. Densitometry assessment revealed that ZO-1, ZO-2, and occludin expression were reduced by 47.5, 58.6, and 49.7%, respectively, in the caveolae fraction of HBMECs treated with Tat for 15 h. Figure 6*C* also indicates that the effects of Tat on the expression of tight junction proteins are regulated by the Ras signaling. Indeed, pretreatment of HBMECs with 5 μM FTS for 3 h completely inhibited Tat-induced alterations of occludin, ZO-1, and ZO-2 expression in the caveolae fraction of HBMECs. Treatment with FTS alone did not affect expression of tight junction proteins.

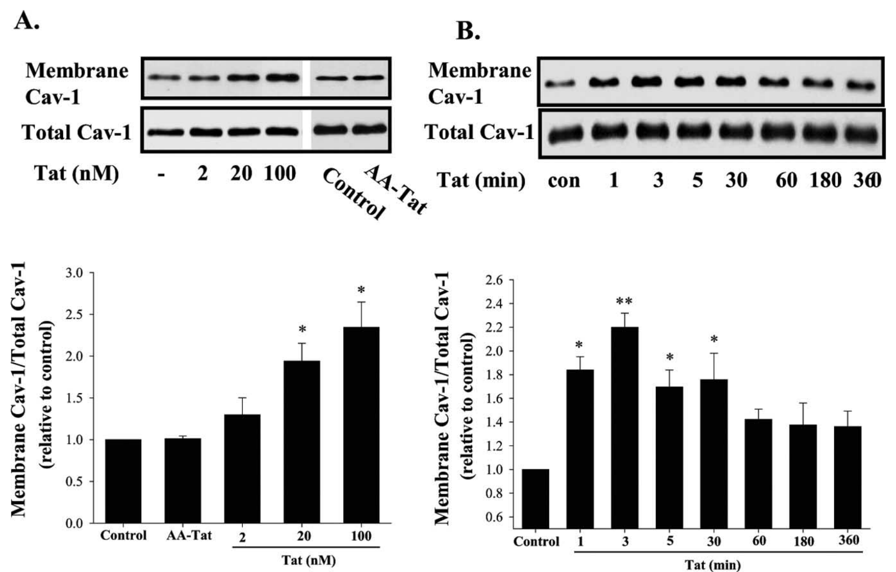

Figure 4. Exposure to Tat upregulates membrane caveolin-1 levels in HBMECs. ***A***, HBMECs were treated with indicated concentrations of Tat for 3 min. Caveolin-1 protein was analyzed in the membrane fractions and total cell extracts. Treatment with AA-Tat (100 nM) for 3 min was used as negative control. ***B***, HBMECs were exposed to 100 nM Tat for the indicated time periods and membrane and total caveolin-1 were detected as in ***A***. The blots reflect representative data from three different experiments, and the bar graphs represent quantified results (mean ± SD) from these experiments as analyzed by densitometry. $^{*}p < 0.05$; $^{**}p < 0.01$.

Lack of caveolin-1 attenuates Tat-induced alterations of tight junction protein expression and redistribution

Strong expression of tight junction proteins in the caveolae fraction of HBMECs prompted us to determine the effects of caveolin-1 silencing on Tat-induced tight junction protein expression. As indicated in Figure 7*A*, knocking down caveolin-1 expression did not affect the basal expression of tight junction proteins in untreated HBMECs. However, caveolin-1 silencing effectively protected against Tat-induced diminished the expression of occludin, ZO-1, and ZO-2. These effects were next confirmed in brain endothelial cells isolated from mice lacking caveolin-1 (cav-1 $^{-/-}$ mice) (Fig. 7*B*). Tat treatment (100 nM; 15 h) of brain microvascular endothelial cells isolated from control mice resulted in diminished and fragmented expression of ZO-1 immunoreactivity at the cell–cell borders (longer arrows) and redistribution of ZO-1 from the cell borders into the cytoplasm (shorter and open arrows). Note that the experiments were performed on confluent cultures as illustrated by uniformly distributed DAPI staining for nuclear DNA. Importantly, Tat-mediated effects on ZO-1 immunoreactivity were markedly attenuated in brain microvascular endothelial cells isolated from cav-1 $^{-/-}$ mice.

Discussion

Lipid rafts and caveolae play an important role in interactions of host cells with infectious agents, including enveloped viruses, such as HIV-1 (Suzuki and Suzuki, 2006). By increasing the spatial density of cell surface receptors, lipid rafts enhance the access of pathogens to cells and aid the cellular entry of infection agents. In addition, lipid rafts can direct pathogen trafficking to specific sites for replication and/or facilitate the assembly of the enveloped viruses during replication. It is known that lipid rafts play important roles in HIV-1 infection. It was demonstrated that HIV-1 binding to T-cells involves interactions between HIV-1 envelope proteins gp120–gp41 with raft-associated CD4 and coreceptors CCR5 and CXCR4. Disruption of lipid rafts by cholesterol depletion or by blocking sphingomyelin synthesis inhibits HIV entry into T-cells. Rafts contribute also to HIV assembly and budding. Specifically, HIV-1 proteins Gag and Env, which mediate several steps in virus assembly, are anchored into T-cell lipid rafts (Huang et al., 2007). The results of the present study provide strong evidence that caveolae play a regulatory role in activation of HBMECs and disruption of tight junction proteins in response to cellular exposure to viral proteins, such as HIV-1 Tat.

The mechanisms of Tat interaction with the cell surface, including lipid rafts and caveolar membranes, are not fully understood. It was suggested that Tat can mimic extracellular matrix proteins and bind to $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins through the arginine-glycine-aspartic domain (Toschi et al., 2006). In addition, the VEGFR-2 has been proposed to serve as a high-affinity receptor for Tat in endothelial cells (Albini et al., 1996). This notion was supported by our data that indicate that blocking VEGFR-2 can attenuate Tat-induced disruption of tight junctions (András et al., 2005). VEGFR-2 and a variety of other cell surface receptors that belong to the large family of G-protein-coupled receptors are localized to caveolae in endothelial cells. Activation of these receptors can stimulate the heterotrimeric G-proteins, which then induce the Ras signaling (Hancock, 2003; Wennerberg et al., 2005). Thus, interaction of Tat with cell surface receptors, such as VEGFR-2, can be directly responsible for an early activation of Ras signaling as observed in the present study (Fig. 1). In support of this notion, it was shown that Tat binding to cell membranes resulted in activation of the Ras/MAPK (mitogen-activated protein kinase) signaling pathway and contributed to cell progression through the G_1 phase in response to mitogen in primary and immortalized human umbilical endothelial cells (Toschi et al., 2006). Our present data also confirms that Tat-induced activation of Ras results in stimulation of the downstream kinases, such as MEK1/2 and ERK1/2 (Figs. 2, 3).

In addition to activation of the Ras cascade, treatment with Tat upregulated membrane caveolin-1 protein levels in HBMECs. These results indicate a close interaction of Tat with caveolae. The effects of Tat on caveolin-1 levels were dose dependent (Fig. 4*A*) and occurred within minutes of Tat exposure (Fig. 4*B*). To explain such an early response, it should be pointed out that caveolin proteins are present not only in caveolae but also in the *trans*-Golgi network and in caveosome, endocytic organelles

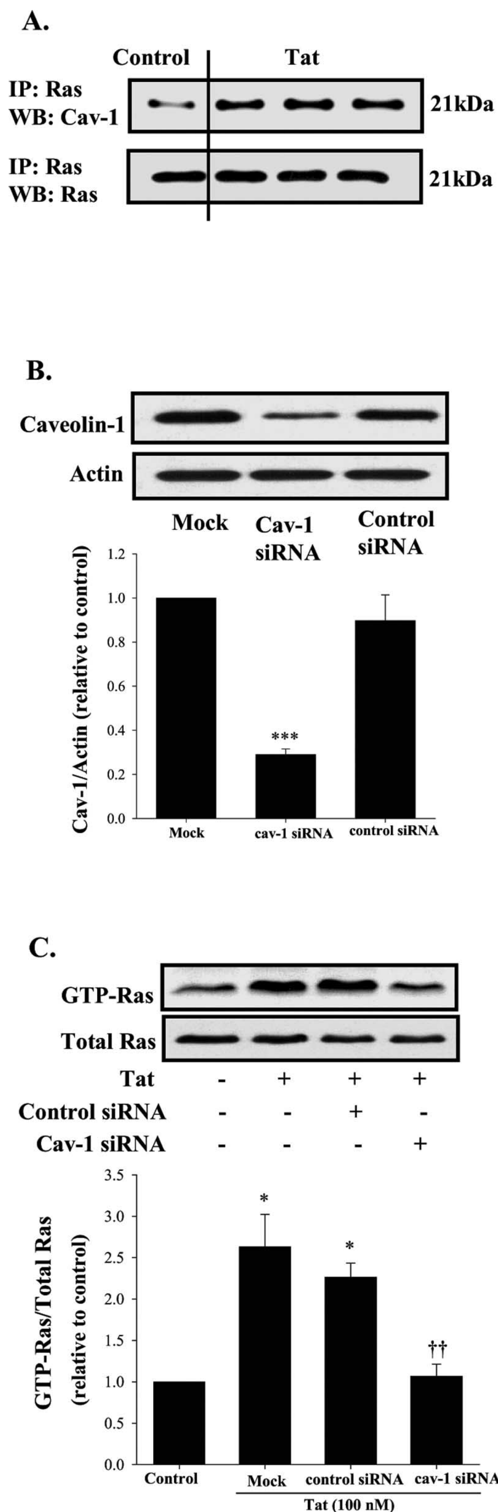

Figure 5. Tat-mediated activation of Ras is regulated by caveolin-1. ***A***, Ras coimmunoprecipitates with caveolin-1. Lysates of control and Tat-treated HBMEC cultures were immunoprecipitated using monoclonal pan-Ras antibody, followed by Western blotting with anti-caveolin-1 antibody or anti-pan-Ras antibody (control). IP, Immunoprecipitation; WB, Western blotting. ***B***, HBMECs were transfected with specific caveolin-1 siRNA or with control siRNA and caveolin-1 levels were analyzed by immunoblotting in total cell extracts. The silencing procedure resulted in ~70% decrease in cellular caveolin-1 levels. ***C***, Caveolin-1 was silenced in HBMECs as in ***A***, followed by treatment with Tat (100 nM) for 3 min. Active GTP-Ras and total Ras were determined as in Figure 1. The blots reflect representative data from four different experiments, and the bar graphs represent quantified results (mean ± SD) from these experiments as analyzed, by densitometry. $^{*}p < 0.05$, $^{***}p < 0.001$ compared with controls or mock transfection. ††Data in the Tat + cav-1 siRNA are significantly different from those in the Tat group at $p < 0.01$.

that contain multiple caveolar domains. Caveolar domains present in the Golgi complexes actively detach as vesicles distinct from the *trans*-Golgi network. It was estimated that ~100–200 caveolae can move to the plasma membrane every minute in CV-1 cells (Pelkmans et al., 2001; Pelkmans and Helenius, 2002; Tagawa et al., 2005). Because caveolae are abundant in HBMECs, membrane levels of caveolin-1 can also rapidly change in response to Tat treatment. Similarly, a dramatic enhancement of a mobile caveolae fraction was shown in response to SV40 (simian virus 40) (Tagawa et al., 2005).

Another novel finding reported in the present study is the observation that caveolin-1 can regulate Tat-induced disruption of tight junction proteins in HBMECs. These results are important because alterations of tight junctions can directly influence the integrity of the BBB and facilitate the entry of HIV-1-infected cells into the brain. In addition, the integrity of the brain microvasculature is critical to maintain brain homeostasis and viability of neurons (Hawkins and Davis, 2005; Abbott et al., 2006). In the present study, we focused on the regulation of occludin, ZO-1, and ZO-2 expression. Occludin is a 60–65 kDa transmembrane protein that is capable of linking with ZO-1. The main functions of occludin are regulation and sealing of tight junctions. In fact, vesicular removal of occludin from tight junctions correlated with loss of barrier function (Lacaz-Vieira et al., 1999; Abbott et al., 2006). ZO-1 and ZO-2 are cytoplasmic proteins that belong to the family of membrane-associated guanylate kinases. They also play a role in signal transduction mechanisms and in anchoring the transmembrane tight junction proteins to the cytoskeleton (Wolburg and Lippoldt, 2002).

Disruption of the integrity of endothelial monolayers in response to HIV-1 has been described previously (Dallasta et al., 1999; Boven et al., 2000; Persidsky et al., 2006). Clinical studies revealed the absence or fragmentation of occludin and ZO-1 in HIV-positive patients with encephalitis or HIV-1-associated dementia (Dallasta et al., 1999; Boven et al., 2000). To confirm the role of tight junctions in HIV-1-associated brain pathology, it was shown that monocyte infiltration was associated with the disruption of ZO-1 immunoreactivity in HIV-1-positive patients (Boven et al., 2000). Several HIV-1 proteins, such as gp120, Nef, and Tat were described to alter the expression of tight junction proteins *in vitro* and to enhance transendothelial migration of monocytes (Annunziata, 2003). We reported that the exposure of primary brain endothelial cells to Tat resulted in redistribution and decreased expression of several tight junction proteins, including claudin-5, ZO-1, and ZO-2 (András et al., 2003, 2005). These effects are regulated at the transcriptional levels (András et al., 2005), which may explain a temporal delay between a rapid activation of signaling processes and alterations of tight junction protein expression as observed in the present study. Indeed, we observed that Tat induced the Ras cascade and altered membrane caveolin-1 levels within minutes of cellular exposure. However, longer exposure times, such as 12 or 24 h, were required to induce tight junction changes.

The present study describes novel molecular mechanisms of Tat-induced alterations of tight junction protein expression via caveolae-associated signaling. Our immunoblotting results indicate that caveolae are enriched with tight junction proteins compared with other cellular fractions. Moreover, Tat-induced changes in occludin, ZO-1, and ZO-2 expression appear to be exclusively limited to caveolae (Fig. 6). These results are in agreement with our unpublished observations that specific tight junction proteins are associated with caveolae and can be immunoprecipitated with caveolin-1. Previous reports also indicated that

tight junction proteins are present in lipid-rich membrane microdomains with biophysical characteristics of detergent insoluble glycolipids, and the association of caveolin-1 with occludin was visualized by high-frequency imaging and morphometric analyses (Nusrat et al., 2000).

To further demonstrate the relationship between tight junctions and functional caveolae, our results demonstrate that silencing of caveolin-1 can protect against diminished tight junction protein expression in Tat-treated HBMECs (Fig. 7*A*). Caveolin-1 silencing does not result in a complete elimination of protein expression, leaving ~30% of normal caveolin-1 levels (Fig. 5*B*). Therefore, we cross-checked the effects of caveolin-1 silencing on tight junction protein expression using brain microvascular endothelial cells isolated from caveolin-1-deficient mice and from the corresponding controls. As shown in Figure 7*B*, lack of caveolin-1 markedly protected against Tat-induced disruption of ZO-1 immunoreactivity. These observations are further supported by the report that disruption of caveolae by cholesterol extraction from plasma membrane prevented both barrier dysfunction and occludin redistribution after actin depolymerization (Shen and Turner, 2005).

The protective effects of caveolin-1 silencing appear to be related to modulation of the Ras cascade. Indeed, silencing of caveolin-1 effectively prevented Tat-mediated activation of Ras (Fig. 5), the signaling pathway that regulates the expression of tight junction proteins. However, several recent reports indicated that cellular uptake of Tat may occur via caveolar-associated mechanisms (Ferrari et al., 2003; Fittipaldi et al., 2003; Jones et al., 2005; Renigunta et al., 2006). This fact may suggest that the protective effects of caveolin-1 silencing might also be related to disruption of caveolae and diminished internalization of Tat. However, pharmacological inhibition of the Ras pathway (Fig. 6) mimicked the protective effects of caveolin-1 silencing on Tat-induced alterations of tight junction protein expression (Fig. 7*A*), indicating the specificity of the observed effects. Thus, caveolin-1 may constitute an early and critical modulator that controls signaling pathways leading to the disruption of tight junction proteins.

In conclusion, the present study demonstrates that exposure to Tat results in activation of the Ras signaling, upregulation of caveolin-1, and disruption of tight junction proteins (occludin, ZO-1, and ZO-2). These events are interrelated, because inhibition of the Ras pathway protected against Tat-induced disruption of tight junction proteins. In addition, caveolin-1 silencing blocked both Tat-mediated activation of Ras and alterations of tight junction proteins. Thus, Tat-induced caveolin-1 upregulation may constitute an early event that can initiate signaling transduction pathway, leading to the disruption of tight junctions, and HIV-1 passage across the brain endothelium.

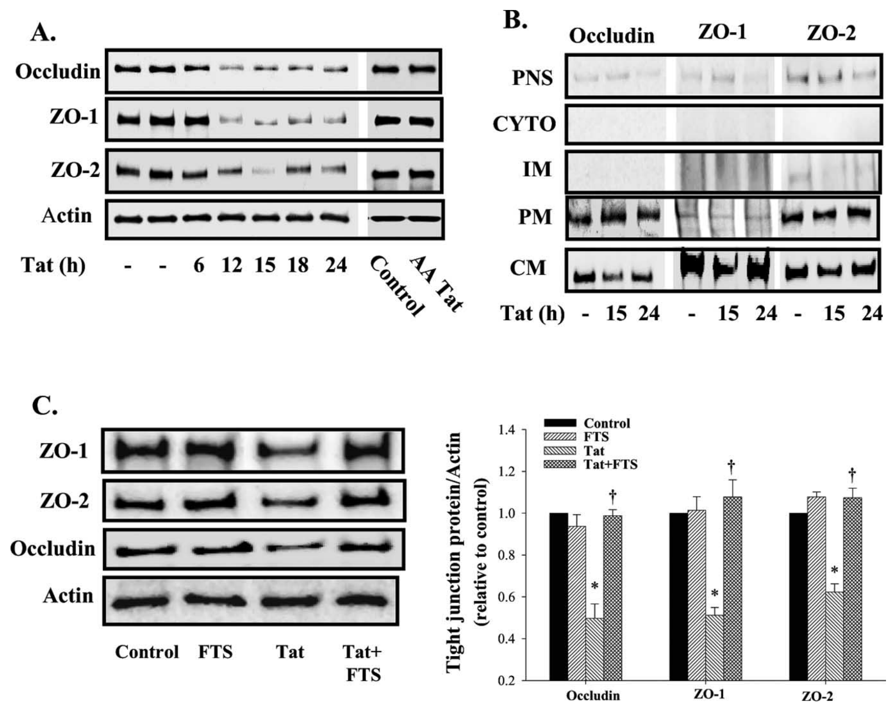

Figure 6. Exposure to Tat alters expression of tight junction proteins in caveolae fraction of HBMECs via the Ras-regulated pathway. ***A***, Confluent HBMECs were exposed to 100 nM Tat for the indicated time points. Tight junction proteins (occludin, ZO-1, and ZO-2) were detected by immunoblotting in total cell extracts. Treatment with AA-Tat (100 nM for 15 h) was used as a negative control. ***B***, Confluent HBMECs were treated with 100 nM Tat for 15 or 24 h, and cells were fractionated using a detergent-free method. Expression level of tight junction proteins was detected by immunoblotting in postnuclear soup (PNS), cytosol (CYTO), intracellular membranes (IM), plasma membrane (PM), and caveolae membranes (CM). ***C***, Confluent HBMECs were pretreated with 5 μM FTS for 3 h, and then incubated with 100 nM Tat for 15 h. FTS was retained in cell culture medium for the duration of Tat treatment. Tight junction proteins were determined in caveolae membrane fraction as in ***B***. Actin was determined as housekeeping protein and loading control. The blots reflect representative data from four different experiments, and the bar graphs represent quantified results (mean ± SD) from these experiments as analyzed by densitometry. *$p < 0.05$ compared with control (mock transfection). †Data in the Tat+FTS are significantly different from those in the Tat group at $p < 0.05$.

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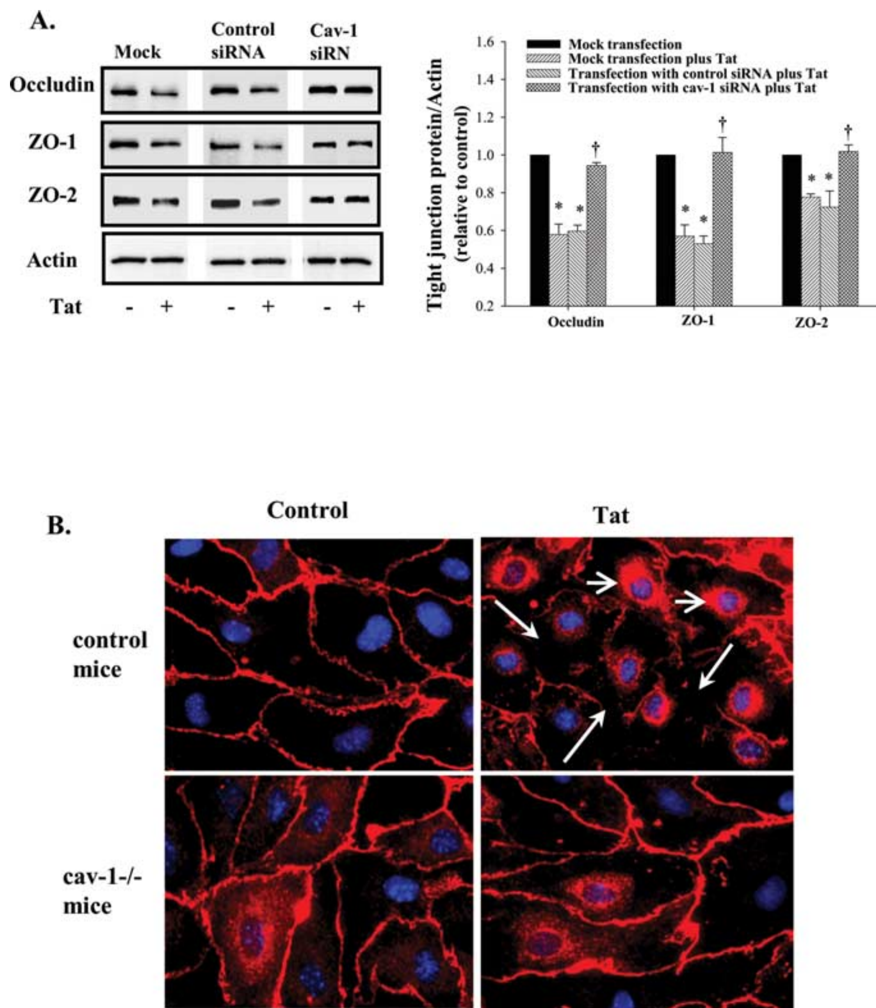

Figure 7. Caveolin-1 modulates Tat-induced alterations of tight junction protein expression. ***A***, Caveolin-1 was silenced in HBMECs as in Figure 5, followed by exposure to 100 nM Tat for 15 h. Expression of tight junction proteins (occludin, ZO-1, and ZO-2) was analyzed by immunoblotting in total cell extracts. Control experiments include mock transfection and transfection with control siRNA. The blots reflect representative data from three different experiments. *$p < 0.05$ compared with control (mock transfection). †Data in the cultures exposed to Tat and caveolin-1 siRNA are significantly different from those in the cultures exposed to Tat alone or Tat plus control siRNA at $p < 0.05$. ***B***, Brain microvascular endothelial cells were isolated from cavoelin-1-deficient (cav-1 $^{-/-}$) mice and the respective controls. Cells were cultured until confluence and treated with 100 nM Tat for 15 h. ZO-1 immunoreactivity was determined by immunofluorescence (red staining). In addition, DAPI staining was performed to visualize the nuclei (blue staining). Tat treatment resulted in diminished and fragmented expression of ZO-1 immunoreactivity at the cell–cell borders (longer arrows) and redistribution of ZO-1 from the cell borders into the cytoplasm (shorter and open arrows) in cells from control mice. These effects were markedly attenuated in cells from cav-1 $^{-/-}$ mice. The images were taken using a 60× objective and a 10× ocular lens.

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