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Human immunodeficiency virus type 1 gp120–mediated disruption of tight junction proteins by induction of proteasome-mediated degradation of zonula occludens-1 and -2 in human brain microvascular endothelial cells

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The infiltration of human immunodeficiency virus (HIV)-1, such as by HIV-infected leukocytes, across an injured blood-brain barrier (BBB) is a characteristic pathologic manifestation of HIV-1–associated dementia. HIV-1 gp120 has been implicated as a cause of breakdown of tight junctions between endothelial cells of the BBB, though the disrupting molecular mechanisms are unexplained. This study offers a new explanation for the increased BBB microvascular permeability, due to the degradation of tight junction proteins by the proteasome induced by gp120, and the negative regulation of this process by the scaffold protein, 14-3-3τ. gp120 reduced the amount of zonula occludens (ZO)-1 and ZO-2 in human brain microvascular endothelial cells (HBMECs). The treatment of HBMECs with the proteasome inhibitor, lactacystin, blocked the degradation of ZO-1 and ZO-2, suggesting that these proteins were targeted by gp120 for degradation by the proteasome. gp120 also specifically increased the expression of 14-3-3τ in HBMECs, and its down-regulation by RNAi facilitated the breakdown of tight junction proteins induced by gp120. Our results demonstrate the novel molecular mechanisms of the BBB breakdown by gp120. *Journal of NeuroVirology* (2008) **14,** 186–195.

Keywords: 14-3-3 protein; HIV-1 gp120; HIV-1–associated dementia; proteasome; tight junction protein

Introduction

Infection of the central nervous system (CNS) by the human immunodeficiency virus type 1 (HIV-1) causes its degeneration in patients with acquired immunodeficiency syndrome (AIDS), who develop cognitive and motor dysfunctions known as HIV-1–associated dementia (HAD) (Adle-Biassette *et al*, 1995; Petito and Robert, 1995; An *et al*, 1996). HAD is characterized by neuropathological changes, including brain atrophy, microglial activation, astroglial cell reaction, and loss of neurons (Budka, 1991; Everall *et al*, 1991; Wiley *et al*, 1991; Price and Perry, 1994). Because these adverse manifestations are caused by HIV-1 infiltration, toxic virus products and blood-derived, activated macrophages across the blood-brain barrier (BBB), the loss of BBB integrity is a highly relevant adverse pathogenetic event in HAD (Nottet *et al*, 1996; Persidsky *et al*, 1997). Indeed, a disruption of the BBB is more regularly observed in

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AIDS patients with dementia than in patients who do not present with dementia (Power *et al*, 1993). Brain microvascular endothelial cells (BMECs), a major cellular component of the BBB, are the first neural cells to be exposed to blood-borne viral products or HIV-infected cells, and the tight junction, composed of specific junctional proteins, including zonula occludens proteins (ZO)-1, ZO-2, claudin, and occludin between BMECs, plays a crucial role in the protection against entry of the virus into the brain (Dallasta *et al*, 1999; Luabeya *et al*, 2000; Boven *et al*, 2000). There is now growing evidence that neurotoxic viral proteins, such as HIV-1 gp120 and Tat, increase the endothelial permeability to facilitate the passage of HIV-1 across the BBB (Annunziata *et al*, 1998; Banks *et al*, 1998; Oshima *et al*, 2000; Arese *et al*, 2000; Kim *et al*, 2003). The decreased expression of tight junction proteins by Tat, and changes in their distribution via a signaling pathway in brain endothelial cells (Pu *et al*, 2005) support the theory that HIV proteins injure the endothelium. It has recently been reported that gp120 also down-regulates the tight junction proteins (Kanmogne *et al*, 2005), consequently causing the dysfunction of the BBB, such as increased monocyte migration (Kanmogne *et al*, 2007), though the precise mechanisms behind these effects remain undetermined.

The 14-3-3 protein is a highly conserved and ubiquitously expressed protein family, which is involved in the regulation of a wide range of cellular processes, including signal transduction, cell cycle, and apoptosis (Fu *et al*, 1994; Reuther *et al*, 1994; Conklin *et al*, 1995; Zha *et al*, 1996). The stabilization of various target proteins by 14-3-3 proteins is based on their functional characteristics as scaffold proteins or molecular chaperones (Obsil *et al*, 2001; Yano *et al*, 2006). On the other hand, the high tissue concentrations of 14-3-3 proteins in the brain, representing approximately 1% of the total amount of soluble proteins (Boston *et al*, 1982), have made their role in neuronal function a worthy target of investigation. They often aggregate in disease-specific lesions in the brain of patients with various neurological disorders (Berg *et al*, 2003), and their presence in the cerebrospinal fluid (CSF) has been identified as a highly sensitive diagnostic marker of progressive dementia, such as Creutzfeld-Jakob disease (Hsich *et al*, 1996; Zerr *et al*, 2000). The importance of 14-3-3 proteins has also been evoked in the context of HAD. We have shown previously that 14-3-3 proteins are present in the CSF of patients presenting with HAD, in whom they might be a reliable real-time marker of the rate and amount of neural cell destruction (Wakabayashi *et al*, 2001). Another study has also found a close relationship between CSF concentrations of 14-3-3 and encephalitis in the simian immunodeficiency virus (SIV)/macaque model of HIV CNS disease (Helke *et al*, 2005). Although the presence of 14-3-3 proteins in the CSF of patients with HAD or other diseases of the CNS can be attributed to leakage from destroyed neurons, their neuropathological role in HAD has recently become clearer after we observed that a 14-3-3 protein, induced by HIV-1 gp120 stimulation, negatively regulates gp120/CXCR4-mediated cell death in vitro (Yano *et al*, 2007). Furthermore, in the CNS, 14-3-3 proteins are often induced in response to stress, such as nerve injury and oxidation, perhaps as a cell survival mechanism (Namikawa *et al*, 1998; Satoh *et al*, 2006).

With a view to deepen our biological understanding of BBB injury in HAD, we have focused on the role of 14-3-3 proteins and proteasome in the gp120-mediated disruption of tight junction proteins in human brain microvascular endothelial cells (HBMECs). In this study, we found that the treatment of HBMECs with gp120 induced the degradation of tight junction proteins, ZO-1 and ZO-2, but not occludin or claudin, by the proteasome. Inhibition of the proteasome caused their accumulation in the cytosol. Furthermore, among the various 14-3-3 isoforms present, 14-3-3τ was specifically up-regulated in response to treatment with gp120. Down-regulation of 14-3-3τ by RNAi accelerated the gp120-dependent degradation of tight junction proteins, thus promoting their elimination mediated by gp120. These observations demonstrate that 14-3-3τ preserves the integrity of the cell junction system, and that it might play a protective role against the disruption of the BBB caused by HIV-1 infection.

Results

HIV-1 gp120 decreases the expression of ZO-1 and ZO-2, but not occludin and claudin-1, in HBMECs

It has recently been shown that the exposure of primary HBMECs to HIV-1 proteins, such as gp120 and Tat, alters the expression and distribution of tight junction proteins. To study the effects of gp120 on the expression of tight junction proteins, their levels were determined by immunoblot analysis in HBMECs exposed to recombinant gp120 for 24 h. After treatment with gp120, the expression of ZO-1 and ZO-2, the accessory proteins necessary to form the structural support of tight junctions, was decreased in a concentration-dependent manner, though the expression of the integral transmembrane proteins, occludin and claudin-1, did not change (Figure 1A). It is noteworthy that the reverse transcriptase–polymerase cahin reaction (RT-PCR) analysis of the tight junction proteins showed no decrease in RNA levels following gp120 treatment (Figure 1B), suggesting that it does not inhibit the synthesis of ZO-1 and ZO-2, but causes their intracellular degradation. Immunofluorescence microscopy was used to further examine the effect of gp120 on the expression of ZO-1, ZO-2, and claudin-1 in HBMECs. In contrast to what was observed in untreated cells, gp120 disrupted the continuous, linear arrangement of ZO-1 and ZO-2 among HBMECs (Figure 1C). The

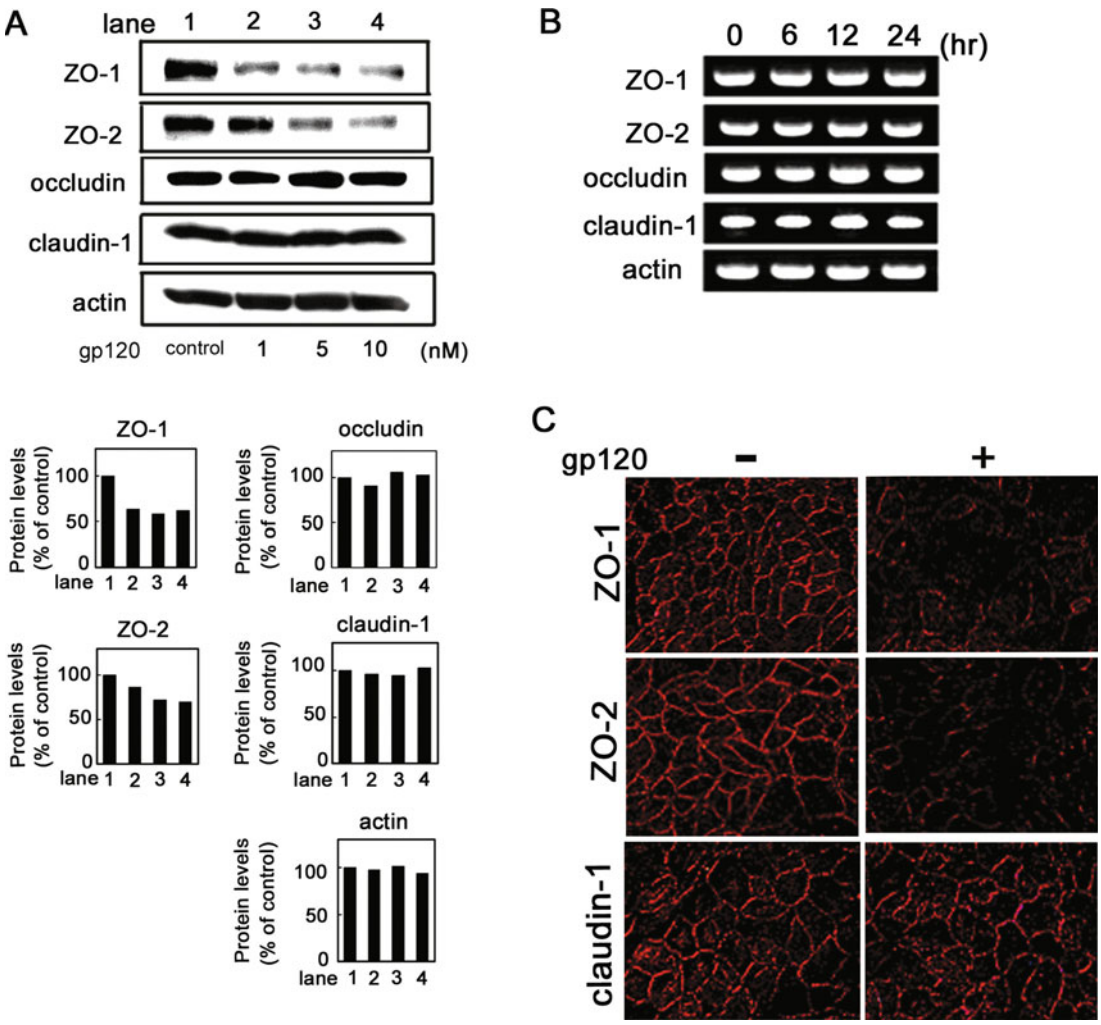

Figure 1 Selective decrease in the expression of ZO-1 and ZO-2 proteins by HIV-1 gp120 in HBMECs. (**A**) Expression of tight junction proteins after exposure of HBMECs to gp120. Cell extracts from HBMECs, treated for 24 h with the indicated concentrations of gp120, or heat-inactivated gp120 (control), were separated by SDS-PAGE and immunoblotted with each tight junction protein–specific antibody. The expression of actin was monitored as internal control. The protein levels were measured by densitometric analysis, using Scion Image (Scion, Frederick, MD). The results were expressed as percentages of each protein level with heat-inactivated gp120, set at 100%. (**B**) Time course of the mRNA expression levels of tight junction proteins in HBMECs after treatment with gp120. (**C**) Representative example (from among three separate experiments) of immunofluorescence showing a decrease in ZO-1 and ZO-2 but not claudin-1 expression in gp120-treated HBMECs. Cells were cultured to confluence, then incubated for 24 h in absence or presence of 10 nM of gp120.

immunoreactivity of claudin-1 in control cells and in gp120-exposed cultures was apparent as a continuous linear staining pattern.

Gp120 disrupts the endothelial barrier tightness

There is growing evidence indicating that changes in the organization of the tight junction complex, including breakdown of ZO-1, increase the endothelial permeability. The increase in HBMEC monolayer permeability by gp120 was measured by quantifying the passage of dextran conjugated to flourescein isothiocyanate (FITC) through cocultures. The exposure of HBMECs to 10 nM of gp120 for 24 h increased the HBMEC monolayer permeability 1.7-fold, as compared with the clearance of a control culture incubated without gp120 (Figure 2A). The endothelial barrier disruption was assessed by measurements of the transendothelial electrical resistance (TEER) in HBMECs treated for 0 to 24 h with 10 nM of gp120. Treatment of the cells with gp120 caused a significant time-dependent decrease in TEER (Figure 2B). These functional observations were corroborated by immunoblotting and immunofluorescence studies, which showed that gp120 decreases the expression of ZO-1 and ZO-2.

gp120 specifically up-regulates the expression of 14-3-3τ in HBMECs, and its down-regulation accelerates the gp120-dependent disruption of ZO-1 and ZO-2

We observed, in a previous study, that 14-3-3τ, a protein that, under normal conditions, is barely

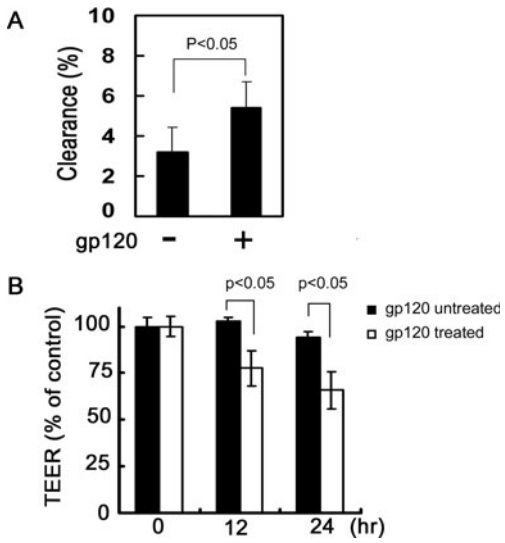

Figure 2 Effects of HIV-1 gp120 on the permeability and TEER of HBMEC monolayers. (**A**) Exposure to gp120 increased the permeability of HBMEC monolayers to FITC-dextran. Confluent HBMEC monolayers were cultured with or without 10 nM gp120 at 37°C for 24 h, and the clearance of FITC-dextran measured fluorometrically. The fluorescence of the lower well was expressed as a percentage of the total fluorescence (lower + upper well). *Columns*, mean of four independent experiments; *bars*, SD. (**B**) Decrease in TEER by gp120. The TEER of confluent HBMEC monolayers, untreated or treated with gp120 for the indicated times, was measured as described in Materials and Methods. The measurements are expressed as percentages of the values with or without gp120 at 0 h, set at 100%. *Columns*, mean of three independent experiments; *bars*, SD.

detectable in human umbilical vein endothelial cells (HUVECs), is up-regulated by treatment with gp120 and protects against cell injury caused by gp120 (Yano *et al*, 2007). To examine whether 14-3-3 proteins might serve as one of the host cell factors that regulate the elimination of tight junction proteins induced by gp120 in HBMECs, we first ascertained the expression patterns of 14-3-3 proteins in HBMECs. We identified 14-3-3β, ζ, ε, γ, and η to be the main isoforms in HBMECs, whereas we observed a very low expression of 14-3-3τ (Figure 3A). However, after gp120 treatment, immunoblotting with a 14-3-3τ antibody revealed considerable up-regulation of the expression of 14-3-3τ, which was increased approximately 2.5-fold in comparison with untreated cells, without apparent increase in the expression of the other isoforms. To determine the functional importance of 14-3-3τ in the disruption of tight junction proteins mediated by gp120, we lowered the amounts of endogenous 14-3-3τ present in HBMECs with RNAi, and studied its effect on the expression of tight junction proteins. Treatment of HBMECs with 14-3-3τ dsRNA accentuated the decrease in ZO-1 and ZO-2 levels mediated by gp120 compared with that measured in cells untreated with dsRNA or in cells treated with 14-3-3ε RNAi (Figure 3B). On the other hand, 14-3-3τ RNAi had no effect on the expressions of occludin and claudin-1, which were unchanged by treatment with gp120. These observations clearly indicate an inhibitory role played by 14-3-3τ in the destruction of tight junction proteins caused by gp120 in HBMECs.

Inhibition of the proteasome blocks the gp120-induced degradation of ZO-1 and causes its accumulation in the cytoplasm

The mechanisms by which gp120 decreases the expression of tight junction proteins, such as ZO-1 and ZO-2, are poorly known. The absence of decrease in ZO-1 and ZO-2 RNA levels following gp120 treatment suggests that gp120 causes their degradation in HBMECs. It is noteworthy that several cytoplasmic junctional components can serve as proteasome substrates (Laing and Beyer, 1995; Aberle *et al*, 1997), and that they are stabilized by inhibition of the proteasome (Tsukamoto and Nigam, 1999; Traweger *et al*, 2002). To study the machinery that turns over ZO-1 and ZO-2, we pretreated HBMECs with the proteasome inhibitor, lactacystin, for 30 min before exposure to gp120. The total amounts of ZO-1 and ZO-2 were nearly the same in absence of gp120, regardless of whether the cells had been exposed to lactacystin (Figure 4A). Lactacystin prominently blocked the gp120-dependent decrease in expression of ZO-1 and ZO-2 in HBMECs (Figure 4B). This inhibitory effect became obvious in the cells treated with 14-3-3τ dsRNA. These observations indicate that gp120 induces the degradation of ZO-1 and ZO-2 by the proteasome. To examine the gp120-coupled cellular events underlying the proteasome-mediated degradation of tight junction proteins, we next examined whether gp120 causes the aberrant intracellular localization of ZO-1. After treatment with gp120, immunofluorescence studies showed an abnormal distribution of ZO-1 protein in HBMECs treated with lactacystin. Under control conditions, without gp120, the distribution pattern of ZO-1 was continuous, localized along the plasma membrane at the cell-to-cell interface (Figure 4C, top). In contrast, exposure to gp120 caused a decrease in ZO-1, with discontinuous staining at the cell borders, and an apparently more diffuse distribution inside the cytoplasm (Figure 4C, bottom). These data suggest that gp120 promotes the proteasome-mediated degradation of ZO-1 in HBMECs.

Effect of 14-3-3τ in the distribution of tight junction proteins in gp120-treated HBMECs

To clarify the role played by 14-3-3τ protein in the abnormal distribution of tight junction proteins mediated by gp120, we examined their localization pattern by fractional analysis, using sequential centrifugation (Figure 5). In HBMECs untreated with lactacystin, ZO-1, ZO-2, and occludin were found in the pellet after low-speed centrifugation of cells lysed in hypotonic buffer, consistent with a localization in the membrane. gp120 decreased significantly the levels of ZO-1 and ZO-2, but not occludin, in the membrane

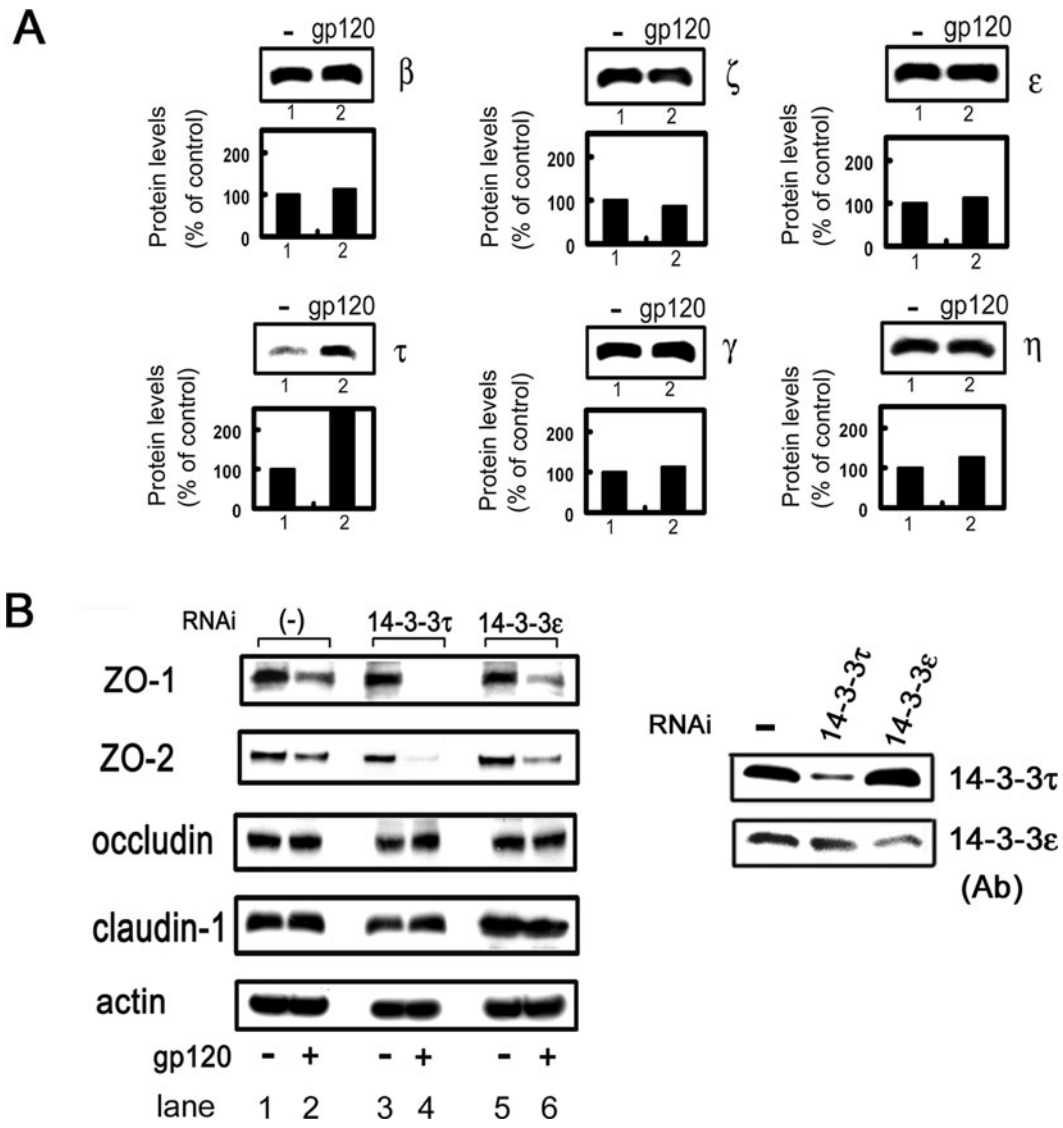

Figure 3 Induction of 14-3-3τ expression in HIV-1 gp120–treated HBMECs and effect of its down-regulation on the expression of tight junction proteins. (**A**) Up-regulation of the expression of 14-3-3τ by gp120. Extracts prepared from HBMECs, untreated or treated for 24 h with 10 nM of gp120, were separated by SDS-PAGE, and the expressions of 14-3-3 proteins were measured by immunoblotting with each specific anti-14-3-3 antibody. The quantity of 14-3-3 protein was estimated by densitometric analysis using Scion Image. The results are expressed as percentages of each protein level without gp120, set at 100%. (**B**) A decrease in 14-3-3τ by RNAi promoted the gp120-induced disruption of ZO-1 and ZO-2, but not occludin or claudin-1. HBMECs, untreated or treated with dsRNA for 14-3-3τ or ε, were exposed to gp120 for 24 h, and cell extracts were prepared as described in Materials and Methods. Each tight junction protein was examined by immunoblotting, using appropriate antibodies (*left panel*). The silencing efficiency of 14-3-3τ or ε was determined by immunoblotting with the appropriate antibodies (*right panel*).

fraction without displacement to the cytosol fraction. However, after lactacystin treatment, ZO-1 and ZO-2, but not occludin, were predominantly found in the cytosol of HBMECs exposed to gp120, whereas only small amounts of ZO-1 and ZO-2 remained in the membrane. When we attenuated the expression of 14-3-3τ by RNAi in absence of lactacystin, gp120 completely eliminated ZO-1 and ZO-2 in HBMECs. On the other hand, treatment with lactacystin promoted the changes in ZO-1 and ZO-2 distributions induced by gp120, displacing them away from the membrane into the cytoplasmic compartment. In cells treated with 14-3-3τ double-stranded RNA (dsRNA), the expression and distribution of occludin during lactacystin treatment in presence of gp120 remained unchanged. These observations indicate an inhibitory role played by 14-3-3τ in the gp120-mediated disruption of the tight junction.

Discussion

In the present study, we showed that HIV-1 gp120 decreased the expression of ZO-1 and ZO-2 by inducing

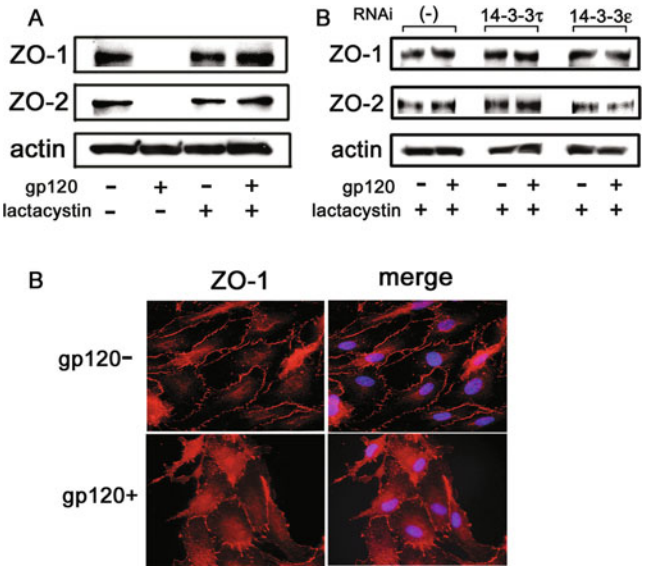

Figure 4 Prevention of decrease in ZO-1 expression and changes in its intracellular localization by the proteasome inhibitor, lactacystin, in gp120-treated HBMECs. (**A**) Effects of lactacystin on the expression of ZO-1 and ZO-2. Cell extracts from HBMECs, untreated or treated for 30 min with 10 μM of lactacystin before exposure to gp120, were separated by SDS-PAGE and immunoblotted with each tight junction protein-specific antibody. The expression of actin was monitored and served as internal control. (**B**) HBMECs untreated or treated with dsRNA for 14-3-3τ were incubated with lactacystin before the addition of gp120. Extracts prepared from the cells, with or without 10 nM of gp120 for 24 h, were then subjected to SDS-PAGE. The expressions of ZO-1 and ZO-2 were measured by immunoblotting with each specific antibody. (**C**) The effects of lactacystin on the intracellular distribution of ZO-1 in gp120-treated HBMECs. Lactacystin-treated HBMECs grown to confluence on glass chamber slides were incubated with or without 10 nM of gp120 for 24 h and stained for ZO-1. The cells were visualized by immunofluorescence microscopy. Nuclei were stained with Hoechst 33342. Merge of both staining procedures is shown in the right-hand preparations.

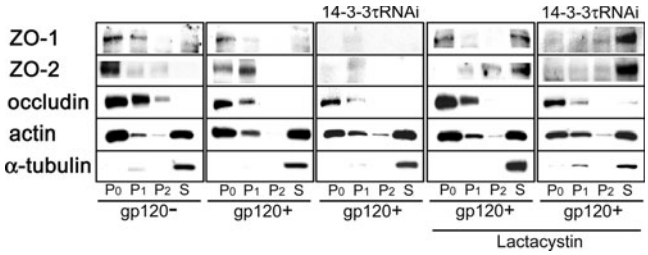

Figure 5 Effect of gp120 and lactacystin on the distribution of tight junction proteins in HBMECs. In HBMECs exposed to gp120, lactacystin treatment displaced ZO-1 and ZO-2, but not occludin or claudin-1, from the membrane to the cytosol. HBMECs untreated or treated with dsRNA for 14-3-3τ were incubated in presence or absence of 10 μM of lactacystin for 30 min before exposure to gp120, then homogenized in a buffer without detergent and fractionated by successive centrifugation as described in Materials and Methods. Each fraction was separated by SDS-PAGE, and the expressions of tight junction proteins were measured by immunoblotting with each specific antibody.

a proteasome-mediated degradation, disrupting the brain microvascular endothelial cell junctions. To link this gp120-dependent event mechanistically, we showed that the scaffold protein, 14-3-3, negatively regulated the breakdown of tight junction proteins by the proteasome. This is the first clarification of the regulatory steps involved in the gp120-mediated disruption of the tight junction. Several studies have shown that the dysfunction of BMECs, a key component of the BBB, is central to HIV infection of the CNS (Nottet *et al*, 1996; Persidsky *et al*, 1997; Power *et al*, 1993). The paracellular passage across endothelial cell monolayers is generally regulated by specialized intercellular tight junctional structures, consisting of integral (claudin-1 and occludin) and membrane-associated (ZO-1 and ZO-2) proteins. There is now strong evidence that the cytotoxic HIV-1 products, such as gp120 and Tat, are involved in the opening of brain microvascular inter–endothelial cell tight junctions (Annunziata *et al*, 1998; Banks *et al*, 1998; Oshima *et al*, 2000; Arese *et al*, 2000; Kim *et al*, 2003). Although the cellular and molecular mechanisms underlying the disruption of tight junction mediated by HIV-1 remain unknown, it is possible that HIV-1 products either down-regulate the synthesis or increase the degradation of tight junction proteins. The administration of Tat to mice brain, for example, causes a significant decrease in mRNA and protein levels of ZO-1, disrupting the integrity of the BBB. Pu *et al*, in that case, proposed that the extracellular signal-regulated kinase 1/2, activated by Tat-induced oxidative stress, decreases the expression of ZO-1 at the transcriptional level (Pu *et al*, 2005), whereas it might also cause the activation of matrix metalloproteinase-9, which degrades ZO-1.

A previous study has shown that HIV-1 gp120 decreases the plasma membrane–associated expressions of ZO-1, ZO-2, and occludin in HBMECs (Kanmogne *et al*, 2005). In fact, gp120 enhances monocyte migration and permeability of BBB *in vitro* via protein kinase C (PKC) activation and intracellular calcium release (Kanmogne *et al*, 2007). Our study explored the mechanisms by which gp120 decreases the expression of tight junction proteins. We first focused our investigations on the role of 14-3-3 protein because 14-3-3τ, which was barely detectable in HBMECs as well as in HUVECs under normal conditions, was up-regulated after treatment with gp120. As in our previous study showing the protective role of 14-3-3τ in gp120-induced cell injury in HUVECs (Yano *et al*, 2007), the down-regulation of 14-3-3τ by RNAi promoted the disruption of ZO-1 and ZO-2 mediated by gp120 in HBMECs, suggesting that 14-3-3τ plays an important role in the organization of tight junction proteins as scaffold proteins. Among the six isoforms examined, 14-3-3τ responded selectively to gp120, suggesting that different isoforms are regulated differently and fulfill specific functions. In our study, gp120 decreased the expression of ZO-1 and

ZO-2 without changing their expressions at the transcriptional level, such that the decreased expressions might have been due to degradation of the proteins. The functional importance of the degradation of junctional architecture has been suggested by an increase in the half-life of junctional components, such as β-catenin (Laing and Beyer, 1995), connexin (Aberle *et al*, 1997), and occludin (Traweger *et al*, 2002) by proteasome inhibition. Therefore, we next hypothesized a role played by the proteasome in the down-regulation of the tight junction proteins in HBMECs mediated by gp120. This hypothesis was supported by the prevention, by proteasome inhibition, of the decrease in the expressions of ZO-1 and ZO-2 by gp120, and the observation of their disappearance from the plasma membrane and relocation to the cytosol.

Because of its role in connecting transmembrane proteins to the actin cytoskeleton (Fanning *et al*, 1998), ZO-1 is considered a key element of the proper organization of tight junctions (Tsukita *et al*, 1999). In fact, the abnormal expression or distribution of ZO-1 has been implicated in an increase in the paracellular permeability of the cerebral microvascular endothelium (Mark and Davis, 2002; Fischer *et al*, 2002), and of the seminiferous (Celine *et al*, 2004; Fink *et al*, 2006), glomerular (Rincon-Choles *et al*, 2006), and intestinal epithelium (Youakim *et al*, 1999) under pathological conditions. Although the loss of ZO-1 from the plasma membrane was a common occurrence under all pathophysiological conditions, its content and localization pattern within the cells has been variable. For example, in porcine cerebral microvascular endothelial cells, hypoxia decreased the expression of ZO-1, which was displaced to the cytosol (Fischer *et al*, 2002). A similarly decreased expression of ZO-1, associated with aberrant intracellular localization, has been observed in glomerular epithelial cells exposed to high glucose (Rincon-Choles *et al*, 2006), or in Sertoli cells in presence of carcinoma *in situ* (Fink *et al*, 2006). On the other hand, inflammatory factors, such as histamine and interferon-γ, down-regulate ZO-1 without a detectable increase in other cellular compartments, including the cytoplasm (Youakim *et al*, 1999; Gardner *et al*, 1996). These observations indicate that there are several alternate mechanisms behind the decreased ZO-1 expression induced by various agents. In the present study, we found that ZO-1 and ZO-2 are targets of gp120 for their degradation by the proteasome, a new mechanism of increased microvascular permeability. The accelerated redistribution of ZO-1 and ZO-2 from membrane to cytoplasm by 14-3-3τ RNAi in presence of lactacystin, versus their enhanced down-regulation in absence of lactacystin, also suggests that these two events, loss and translocation of ZO-1 and ZO-2, are closely related intracellular events. In addition, our observations identified 14-3-3τ protein as a key molecular switch candidate, which regulates the destruction of ZO-1 and ZO-2 by the proteasome in response to gp120 in HBMECs. Because the mechanisms that modulate the expression and localization of the junctional protein ZO-1 have not been well characterized, the role of the proteasome in the regulation of tight junction proteins is noteworthy. The specific mechanisms behind the degradation of zonula occludens proteins mediated by the proteasome remain unknown. The lysine residues in the amino termini of ZO-1 and ZO-2 are putative sites of modification by ubiquitination, whereas claudin-1, integral tight junction protein, has no lysine residue in the NH_2-terminal region. Furthermore, it remains to be determined whether the gp120-mediated displacement of ZO-1 and ZO-2 to the cytosol prompted by lactacystin is an early event of proteasomal degradation or an intracellular movement of nondegraded proteins. Studies of direct or indirect actions of 14-3-3τ toward the degradation of ZO-1 and ZO-2 evoked by gp120 are in progress, which should explain the disruption of tight junction by gp120 in HBMECs, and make important contributions to pathogenetic studies of HAD.

Materials and methods

Biochemicals

Recombinant HIV-1 gp120 protein isolated from the T cell–tropic IIIB and MN strains of HIV-1 was obtained from ImmunoDiagnostics (Woburn, MA). The rabbit antibodies against 14-3-3 total (K-19), isoform-specific antibodies against 14-3-3τ and ζ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and β, ε, γ, and η from IBL (Gunma, Japan). The rabbit anti-ZO-1, -ZO-2, -occludin, and -claudin-1 antibodies were obtained from Zymed Laboratories (San Francisco, CA). The mouse monoclonal antibody (MAb) against Actin was acquired from Chemicon International (Temecula, CA), Hoechst 33342 from Wako (Osaka, Japan), and the mouse anti-α-tublin antibody, lactacystin, and all other reagents from Sigma (St. Louis, MO).

Cell cultures

Primary HBMECs were purchased from Applied Cell Biology Research (Kirkland, WA), and were grown according to the manufacturer's protocol in Endothelial Cell Medium (Cell Systems, Kirkland, WA) containing an endothelial cell growth supplement. The culture medium was changed twice weekly and the cells, kept at 37°C in 5% CO_2, were treated or untreated with gp120, at 10 nM, for 24 h. The cells treated with heat-inactivated gp120 served as controls. Lactacystin, 10 μM, was added to the HBMECs for 30 min before exposure to gp120.

Reverse transcriptase–polymerase chain reaction

A RNeasy Mini Kit (QIAGEN, Valencia, CA) was used to isolate total RNA from HBMECs. Reverse

transcriptase–polymerase chain reaction (RT-PCR) was carried out, using the following primer pairs to amplify the *ZO-1* GenPeptide accession no. NM003257: forward primer (F) 5′TAGAGAGAGGAG CTTGTCCCC3′, reverse primer (R) 5′GCGAGTGCCT GGAATGAG3′; *ZO-2*, NM201629, (F) 5′CAGGACTAC GAGCGAGCCTAT3′, (R) 5′TGATGACGTCTCTCCTC TGG3′; *Occludin*, NM002538, (F) 5′TGGAACTTC-CCTTTTAGGAGG3′, (R) 5′GCAAAAGCCACAATAA TCATG3′; *Claudin-1*, NM021101, (F) 5′TTCATTCTC GCCTTCCTGG3′, (R) 5′TTTTTCGGGGACAGGAAC-A3′; *Actin*, NM001101, (F) 5′AGAGGCATCCTCACC-CTGA3′, (R) 5′CATCTCTTGCTCGAAGTCCA3′. The products were examined by agarose gel electrophoresis after 21 cycles.

RNA interference
The sequences of the sense strands used to generate specific siRNA were obtained as follows: 14-3-3τ, GenPeptide accession no. X56468.1, 5′-AAG-TTGCAGCTGATTAAGGAC-3′; 14-3-3ε, U28936, 5′-AACCACATCCATCCCTGCTAC-3′. The siRNAs were synthesized using the Silencer siRNA construction kit (Ambion, Austin, TX) according to the manufacturer's instructions. HBMECs were transfected with each siRNA, 10 nM, using the HiPerFect Transfection Reagent (Qiagen), and grown for 72 h to allow a sufficient decrease in the expression of the respective target molecules.

Permeability assay
HBMECs were grown to confluence on 6-well tissue culture plates with Falcon Cell Culture Inserts (3.0 μm), and exposed to gp120 for 24 h. To detect changes in the monolayer permeability, 2.0 ml of assay medium were added to each lower well, and 1.0 ml of assay medium containing 1 mg/ml FITC-dextran (molecular weight 10,000) was added to each insert. These volumes equalized the hydrostatic pressure on both sides of the HBMEC monolayer. After incubation at 37°C for 1 h, the fluorescence intensity of the medium was measured in each lower well (2.0 ml) at 490 nm excitation and 520 nm emission, using a fluorescence microplate reader (Spectra Max; Geminiem, Molecular devices, Sunnyvale, CA). Activation of the HBMEC monolayer permeability was quantified as clearance of FITC-dextran from the upper well/insert to lower well, after subtracting the background fluorescence from all values.

Transendothelial electrical resistance
The electrical resistance across monolayers of endothelial cells was measured using Millicell-ERS (Millipore, Billerica, MA) as previously described (Kanmogne *et al*, 2007). HBMECs were plated in the upper chamber of 6-well tissue culture inserts (0.4 μm pore size). After 10 days, the cells, grown to confluence, were either untreated or treated with 10 nM gp120, for 0 to 24 h, and TEER was measured. The electrical resistance of blank inserts containing medium only was subtracted from the TEER measurements made from inserts containing confluent endothelial cell monolayers.

Immunoblotting
HBMECs treated or untreated with HIV-1 gp120 were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 1% NP 40, 0.5% deoxycholate, 0.4 mM EDTA, 0.5 mM sodium orthovanadate) for 30 min at 4°C. The cell lysates were resolved in Laemmli sample buffer. After sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), the samples were transferred to a polyvinylidene difluoride membrane, reacted with the respective antibodies, and detected with an enhanced chemiluminescence detection kit.

Immunofluorescence
The immunofluorescence of gp120-treated HBMECs grown to confluence on glass chamber slides was analyzed. The cells were fixed in 2% paraformaldehyde at 37°C, and permeabilized with 0.05% Triton X-100 for 5 min at room temperature. The cells were stained with an anti-ZO-1 (1 μg/ml), ZO-2 (4 μg/ml), or claudin-1 (7 μg/ml) antibody, diluted in blocking buffer for 45 min, and incubated for 1 h with Alexa Fluor 594 (Red)–conjugated goat secondary antibody against mouse immunoglobulin G (IgG) (Invitrogen, Carlsbad, CA). The stained cells were examined on a confocal microscope.

Cell fractionation
Subcellular fractionation was performed as described previously (Suzuki *et al*, 2004). Confluent HBMECs were lysed in a hypotonic buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 4 mM EGTA, 25 mM sodium fluoride, 1 mM sodium orthovanadate) containing a cocktail of protease inhibitors (EMB Bioscience, La Jolla, CA). The cells were homogenized by repeated passages through a 27-gauge needle, and homogenates were centrifuged at 500 $\times$ *g* for 5 min. The pellets, solubilized in Laemmli sample buffer, were collected as P0 fraction. The supernatants were further fractionated by subsequent centrifugation in P1 (pellet of 20,000 $\times$ *g* for 20 min), and P2 (pellet of 100,000 $\times$ *g* for 45 min). The resulting supernatants were collected as S fraction. Each fraction dissolved in sample buffer was analyzed by SDS-PAGE and immunoblotting.

Statistics
A Student's *t* test was used. A value of $P < .05$ was considered statistically significant.

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