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BTat, a *trans*-acting regulatory protein, contributes to bovine immunodeficiency virus-induced apoptosis

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Summary

Bovine immunodeficiency virus (BIV) is a member of the lentivirus subfamily of retroviruses highly related to human immunodeficiency virus in morphologic, antigenic and genomic features. BIV is known to induce chronic pathological changes in infected hosts, which are often associated with the development of immune-mediated lesions. However, the molecular events underlying the cytopathic effect of BIV remain poorly understood. In this study, BIV was found to induce apoptotic cell death, and a small *trans*-acting regulatory protein encoded by BIV, BTat, was found to participate in the pro-apoptotic action of BIV. Introduction of exogenous BTat to cells triggered apoptosis dramatically, as revealed by assays such as terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling, nuclear morphology analysis, flow cytometry, and cleavages of caspases and poly(ADP-ribose)polymerase. Interestingly, the pro-apoptotic effect of BTat was found to be mediated through its interaction with cellular microtubules and its interference with microtubule dynamics. These results provide the first evidence that induction of apoptosis may contribute to the cytopathic effect of BIV. In addition, these results uncover a novel role for BTat in regulating microtubule dynamics in addition to its conventional role in regulating gene transcription.

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Introduction

Apoptosis is an active process regulated by a series of programmed signal cascades and activated by various extracellular or intracellular stimuli. Apoptosis plays an essential role in a number of normal physiological processes (Arends and Wyllie, 1991), and participates in the pathogenesis of many diseases, such as cancer and acquired immunodeficiency syndrome (AIDS) (Thompson, 1995). Apoptosis has been recognized as an essential defence mechanism against bacterial and viral infections during innate and adaptive immunity (Williams, 1994). Many viruses adopt strategies to interfere with host apoptotic pathways; they have the ability to inhibit or promote apoptosis of host cells by targeting various points in the apoptotic pathway. Viral proteins such as LMP1 from Epstein-Barr virus, K13 from Kaposi's sarcoma herpesvirus, and CrmA from cowpox virus have been shown to inhibit host apoptosis (Dbaibo and Hannun, 1998; Young *et al*., 1999; Low *et al*., 2001; Vockerodt *et al*., 2001). On the other hand, a variety of viruses and their proteins have been found to induce apoptosis. For example, the prototypic alphavirus, Sindbis virus, is able to replicate lytically in many different mammalian cell lines due to the induction of apoptosis (Levine *et al*., 1993). In addition, chicken anaemia virus triggers efficient apoptosis through the viral protein VP3 (Noteborn *et al*., 1994; Danen-Van Oorschot *et al*., 1997).

Human immunodeficiency virus (HIV)-induced apoptosis has been regarded as one of the important mechanisms for the depletion of CD4+ T cells, and some viral structural and regulatory proteins have been implicated in this process (Roshal *et al*., 2001), among which HTat, a small *trans*-acting regulatory protein encoded by the *tat* gene of HIV, has been studied most extensively. Several different mechanisms which HTat employs to induce apoptosis have been proposed, for example, up- or downregulation of various cytokines, cell survival factors, superoxide dismutase, and p53 (Flores *et al*., 1993; Westendorp *et al*., 1994, 1995; Li *et al*., 1995a; Zauli *et al*., 1995; Sastry *et al*., 1996; Wang *et al*., 1999), activation of cyclin-dependent kinases (Li *et al*., 1995b), and interaction with cytoskeletal proteins (Chen *et al*., 2002; Epie *et al*., 2005).

Bovine immunodeficiency virus (BIV) is a lentivirus initially isolated from the leucocytes of a cow with a

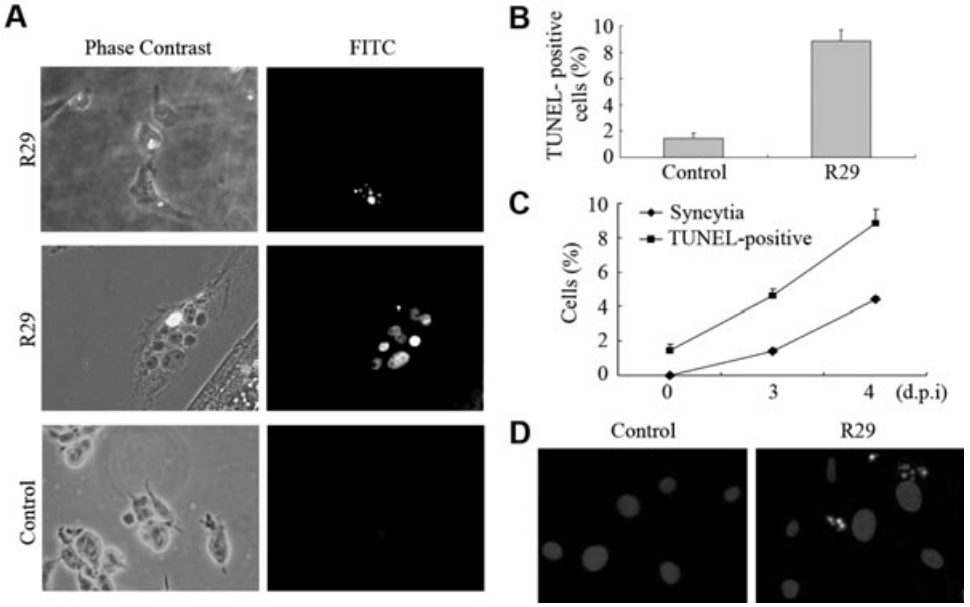

Fig. 1. BIV infection triggers apoptosis in FBL cells. The data shown in the graphs represent means and standard deviations of three independent experiments.
A. FBL cells untreated or treated with BIV (R29 strain) were stained with the TUNEL (FITC) reagents as described in *Experimental procedures*. Cells were observed under a fluorescence microscope with phase contrast. TUNEL-positive (apoptotic) cells stain bright green.
B. FBL cells untreated or treated with R29 were stained with the TUNEL reagents, and the percentage of TUNEL-positive cells was quantified by flow cytometry.
C. FBL cells were infected with R29 for 0, 3 or 4 days and stained with the TUNEL reagents, and the percentage of TUNEL-positive cells was then analysed by flow cytometry. The percentage of syncytia was counted by microscopy. d.p.i., days post infection.
D. FBL cells treated or untreated with R29 were stained with DAPI and observed under a fluorescence microscope. Apoptotic cells exhibit condensed and fragmented nuclei.

wasting syndrome. Although most BIV infections occur with no evident clinical disease in cattle, it does replicate in monocyte/macrophage cells, with a possible dysfunction of the immune system (Carpenter *et al*., 1992; Onuma *et al*., 1992; Zhang *et al*., 1997). Like HIV and other lentiviruses, BIV genome contains structural genes *gag*, *pol*, *env* and several accessory genes, including *vif*, *tat*, *rev*, *vpw*, *vpy* and *tmx*, which encode important regulatory proteins. BTat is encoded by the *tat* gene of BIV containing 103 amino acids in size and shares sequence homology with HTat. Similar to HTat, BTat contains five structural domains, i.e. acidic amino terminal region (amino acids 1–37), cysteine-rich region (38–51), core region (52–64), basic region (65–73) and carboxyl terminal region (74–103) (St-Louis *et al*., 2004). The cysteine-rich regions of HTat and BTat share 50% sequence homology, and the core regions share 62% homology. BTat binds to the transactivation-responsive region to promote transcription elongation, and as a transcriptional activator it plays an essential role in viral replication and viral life cycle (Barboric *et al*., 2000). In the present study, our data demonstrate for the first time that BIV infection causes apoptosis and BTat-mediated disruption of microtubule dynamics and induction of apoptosis may contribute to this action. These findings may furnish novel insights not only into BIV-related disease but also into HIV/AIDS.

Results

Bovine immunodeficiency virus triggers prominent apoptosis in FBL cells

The BIV strain R29 was used in this study to investigate the cytopathic effect of BIV. BIV infection is known to induce in cell cultures the formation of syncytia (large multinucleated cells), a dramatic event that is associated with the cytopathic effect of BIV (St-Louis *et al*., 2004). By terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay, which detects DNA breaks during the process of apoptosis, we found that BIV infection resulted in prominent apoptosis in FBL cells. As shown in Fig. 1A, both the individual infected cells and the infection-induced syncytia exhibited robust TUNEL-positive staining. In contrast, TUNEL signal was hardly detectable in the control cells. We did not find other significant cytopathic changes in BIV-infected cells apart from apoptosis and multinuclear syncytia. Flow cytometric analysis revealed that 8.8% of BIV-infected FBL cells were TUNEL-positive (Fig. 1B). In addition, there was a

correlation between the percentage of syncytia and the percentage of TUNEL-positive cells upon BIV infection (Fig. 1C). BIV-induced apoptosis was also observed by nuclear morphology analysis after staining cells with 4′-6-diamidino-2-phenylindole (DAPI), a DNA dye. As shown in Fig. 1D, a subset of BIV-infected FBL cells displayed typical apoptotic nuclear morphology (condensed and fragmented nuclei), while the control cells contained normal nuclei. Together, these results reveal that BIV infection induces apoptosis and this action may play an important role in the cytopathic effect of BIV.

BTat induces typical apoptotic characteristics

Because BTat is one of the factors involved in BIV/host interactions and is homologous to HTat, which is known to trigger apoptosis, we hypothesized that BTat might play a role in BIV-induced apoptosis. To test this hypothesis, we treated FBL cells with the cell-permeable, bacterially purified BTat and then examined cells by TUNEL assay. Our result revealed that the introduction of exogenous BTat was able to trigger apoptosis dramatically in FBL cells (Fig. 2A). Flow cytometric analysis revealed that 8.2% of BTat-treated FBL cells were TUNEL-positive (Fig. 2B). We also found that BTat could induce apoptosis in Jurkat cells (Fig. 2B). In addition, transfection of FBL and Jurkat cells with a plasmid expressing BTat was able to trigger apoptosis (Fig. 2C and D).

The ability of BTat to induce apoptosis in Jurkat cells was also investigated by nuclear morphology analysis. As shown in Fig. 2E, BTat treatment induced condensed and fragmented nuclei quite rapidly in Jurkat cells.

Flow cytometric analysis of cellular DNA content was then performed to further examine BTat-induced apoptosis. Cells treated with BTat were stained with the DNA dye propidium iodide and then analysed by a flow cytometer. The percentage of sub-G1 (apoptotic) cells, which have less than 2N DNA content, gradually increased over the time of BTat treatment (Fig. 3A), and reached 26% and 30%, respectively, after treatment with 1 μM of BTat for 4 and 8 h (Fig. 3B). In addition, BTat increased the percentage of sub-G1 cells in a dose-dependent manner (Fig. 3C). BTat-induced DNA fragmentation was confirmed by electrophoresis of total DNA extracted from BTat-treated cells (Fig. 3D), and was in a dose-dependent manner (Fig. 3E). We also found that low concentrations of BTat could induce apoptosis in Jurkat cells upon treatment for a longer period (e.g. treatment with 10 nM of BTat for 48 h) (data not shown).

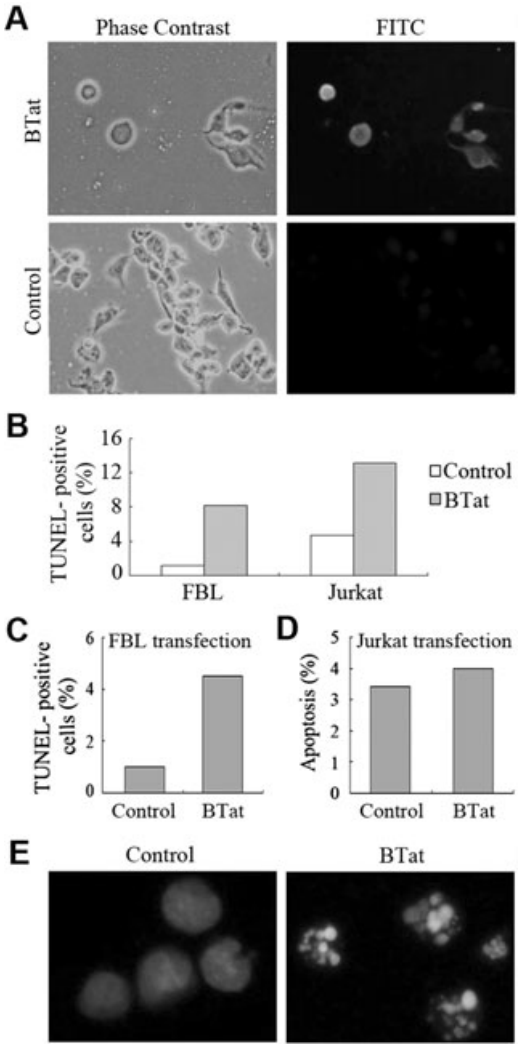

Fig. 2. BTat induces apoptosis in FBL and Jurkat cells.
A. FBL cells treated with 0.2 μM of purified BTat for 2 h were stained with the TUNEL (FITC) reagents, and observed under a fluorescence microscope with phase contrast.
B. FBL and Jurkat cells treated with 0.2 μM of purified BTat for 2 h were stained with the TUNEL reagents, and the percentage of TUNEL-positive cells was quantified by flow cytometry.
C. FBL cells were transfected with a plasmid expressing Myc-tagged BTat or an empty vector for 72 h and stained with the TUNEL reagents, and the percentage of TUNEL-positive cells was analysed by flow cytometry.
D. Jurkat cells were transfected with the Myc-BTat plasmid or empty vector for 72 h and stained with propidium iodide, and the percentage of apoptotic cells (sub-G1 cells that have less than 2N DNA content) was then quantified by flow cytometry.
E. Jurkat cells were treated with 1 μM of BTat for 4 h, stained with DAPI, and then observed under a fluorescence microscope.

Involvement of caspases in BTat-induced apoptosis

Numerous cellular factors have been reported to participate in the apoptotic process, among which the cysteine proteases caspases have been studied most extensively. Upon extracellular or intracellular death stimuli, caspases can be cleaved (activated) by upstream proteases, which in turn leads to the cleavage of various substrates. For example, poly(ADP-ribose)polymerase (PARP), a 116 kDa nuclear protein involved in DNA mainten-

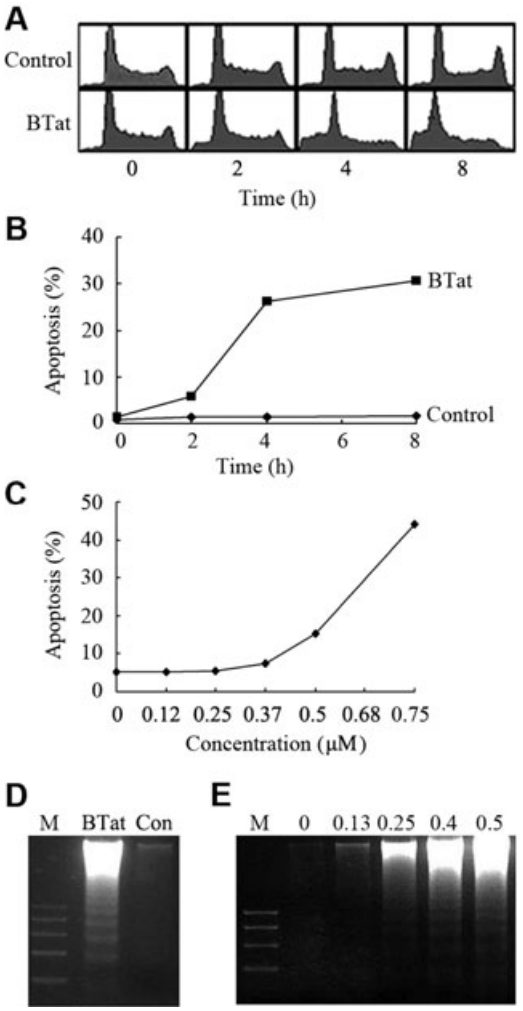

Fig. 3. BTat-induced apoptosis examined by flow cytometry and DNA electrophoresis.
A. Jurkat cells were untreated or treated with 1 μM of BTat for indicated times, stained with propidium iodide, and flow cytometry was then performed as described in *Experimental procedures*.
B. Experiments were performed as in (A), and the percentage of cells with less than 2N DNA content (sub-G1 cells) was quantified.
C. Jurkat cells were treated for 4 h with different concentrations of BTat, and the percentage of sub-G1 cells was determined by flow cytometry.
D. DNA electrophoresis pattern in Jurkat cells untreated or treated with 1 μM of BTat for 4 h. M, marker.
E. DNA electrophoresis pattern in Jurkat cells treated with indicated concentrations of BTat for 4 h. M, marker.

ance and repair, is a well-characterized substrate for caspase-3, and can be cleaved by activated caspase-3 into 89 kDa and 24 kDa fragments upon death stimuli.

To investigate whether caspase-3 was functionally activated by BTat, we examined the integrity of PARP by Western blotting. As shown in Fig. 4A, the cleavage of PARP in Jurkat cells was detected as early as 2 h after treatment with 1 μM of BTat, and the cleavage of PARP was more evident for longer treatment. In addition, we found that BTat-induced PARP cleavage was in a concentration-dependent manner (Fig. 4B). The cleavage of PARP was also detected in FBL cells after BTat treatment (Fig. 4C). These results indicate the functional activation of caspase-3 by BTat treatment. We also used an antibody specifically against the cleaved form of caspase-3 to further examine its activation. Strikingly, BTat resulted in the cleavage of caspase-3 at a concentration as low as 0.13 μM (Fig. 4D). Similarly, using an antibody against cleaved caspase-9, we found that BTat treatment resulted in the cleavage of caspase-9 in Jurkat cells (Fig. 4E). These data thus indicate that activation of caspase-3 and -9 is involved in BTat-induced apoptosis.

BTat interacts with tubulin and microtubules

After we established that BTat triggered apoptosis, we investigated the molecular mechanisms underlying the pro-apoptotic action of BTat. HTat has been recently implicated in microtubule polymerization (Chen *et al.*, 2002). Given the high similarity of BTat to HTat, it is possible that BTat also affects microtubule polymerization eventually

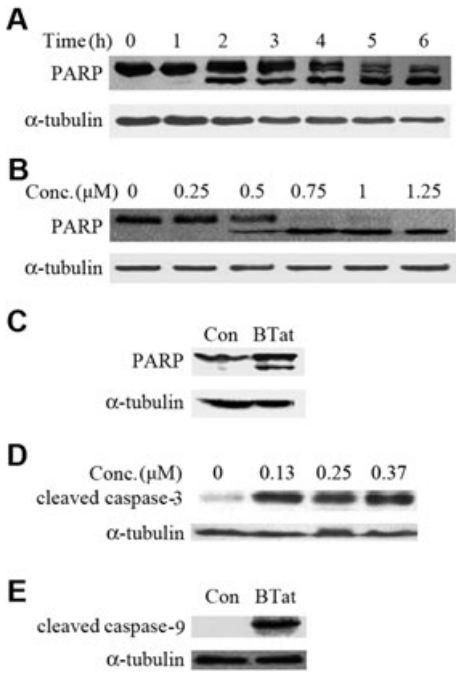

Fig. 4. BTat induces the activation of caspase-3 and caspase-9.
A. Western blot analysis of PARP in Jurkat cells treated with 1 μM of BTat for 0, 1, 2, 3, 4, 5 or 6 h.
B. Western blot analysis of PARP in Jurkat cells treated for 4 h with the indicated concentrations of BTat.
C. Western blot analysis of PARP in FBL cells treated with 0.5 μM of BTat for 2 h.
D. Jurkat cells were treated with different concentrations of BTat for 4 h, and the activation of caspase-3 was then determined by Western blotting using an antibody against cleaved caspase-3.
E. Western blot analysis of cleaved caspase-9 in Jurkat cells untreated or treated with 1 μM of BTat for 4 h.

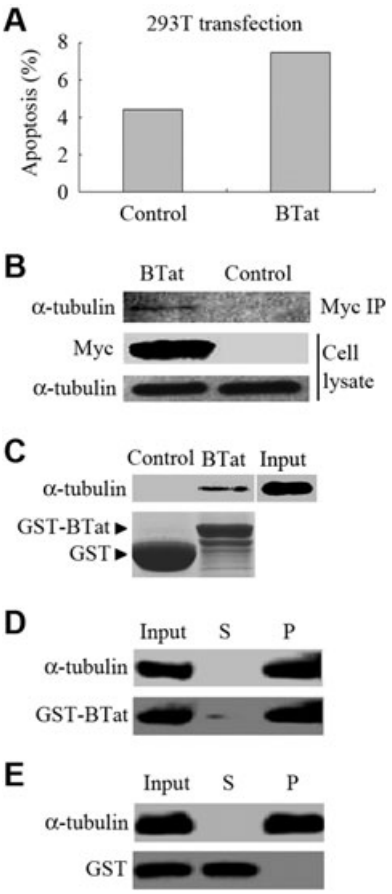

Fig. 5. Interaction of BTat with tubulin and microtubules.
A. 293T cells were transfected with a Myc-BTat expression construct or an empty vector for 72 h and stained with propidium iodide, and the percentage of apoptotic (sub-G1) cells was quantified by flow cytometry.
B. 293T cells were transfected with the Myc-BTat plasmid or empty vector, and the interaction between BTat and tubulin was analysed by immunoprecipitation (IP) using an anti-Myc antibody and Western blotting of the precipitate with an anti-α-tubulin antibody.
C. Purified tubulin was incubated with GST-BTat or GST immobilized on sepharose beads. GST-pulldown was then performed, and the presence of tubulin in the pulldown preparation was detected by Western blotting using an anti-α-tubulin antibody. Purified GST-BTat and GST used in the pulldown assay were detected by Coomassie blue staining.
D. Purified GST-BTat was incubated with preformed microtubules, and the mixture was then centrifuged. Proteins present in the pellet (P) and supernatant (S) fractions were detected by Western blotting.
E. Experiments were done as (D), except that GST was used instead of GST-BTat.

leading to apoptotic death. We tested this possibility first by investigating whether BTat interacts with microtubules/tubulin. A plasmid expressing Myc-tagged BTat or the empty vector was transfected into 293T cells, which undergo modest apoptosis upon BTat transfection (Fig. 5A) and are known to have high transfection efficiency. The lysate of the transfected cells was immunoprecipitated with an anti-Myc antibody, and Western blotting was then performed. Using an anti-α-tubulin antibody, we found the presence of α-tubulin in the BTat precipitate (Fig. 5B), indicating the interaction of BTat with tubulin. *In vitro* GST-pulldown assay was then performed to investigate whether the interaction of BTat with tubulin is direct. Purified GST-BTat or GST was immobilized on glutathione sepharose beads and incubated with purified tubulin. By this assay, BTat was found to bind directly to tubulin (Fig. 5C).

We next studied whether BTat also binds microtubules, the polymeric form of tubulin. Purified GST-BTat or GST was incubated with microtubules, and the mixture was then centrifuged to collect the pellet (P) and supernatant (S) fractions. GST-BTat was found mostly in the pellet fraction, which contained microtubules (Fig. 5D). In contrast, GST was present only in the supernatant fraction (Fig. 5E). These results thus reveal that BTat interacts with microtubules in addition to tubulin.

BTat increases microtubule polymerization

To examine the effect of BTat on microtubule polymerization, we performed *in vitro* tubulin assembly assay and measured the absorbance at 350 nm of wavelength, resulting from the turbidity produced upon tubulin assembly into microtubules. BTat clearly enhanced microtubule polymerization at a concentration of 1.25 μM, and the enhancive effect was more significant when 2.5 μM of BTat was used (Fig. 6A).

We then examined whether BTat increases microtubule polymerization in cells. BTat-induced increase of microtubule polymerization was reflected in the organization of cellular microtubules. As shown in Fig. 6B, BTat treatment resulted in the bundling of microtubules, especially in the cell periphery. Cell extracts containing free, soluble, dimeric tubulin (S) and polymerized tubulin (microtubules, P) were then prepared from Jurkat cells treated with 0.5 μM of BTat and analysed by Western blotting. We found that BTat significantly altered the balance of tubulin between dimer and polymer (Fig. 6C). In control cells 28% of tubulin was in the soluble form, whereas in BTat-treated cells soluble tubulin was hardly detectable (Fig. 6C).

BTat increases microtubule stability

The dramatic effect of BTat on microtubule polymerization suggests that it might alter microtubule stability. α-Tubulin is known to be acetylated at lysine-40, and this modification is a well-established marker of microtubule stability because it is predominantly associated with stable microtubules (Palazzo *et al.*, 2003). We thus investigated the effect of BTat on microtubule stability by examining the level of tubulin acetylation. We found that treatment of

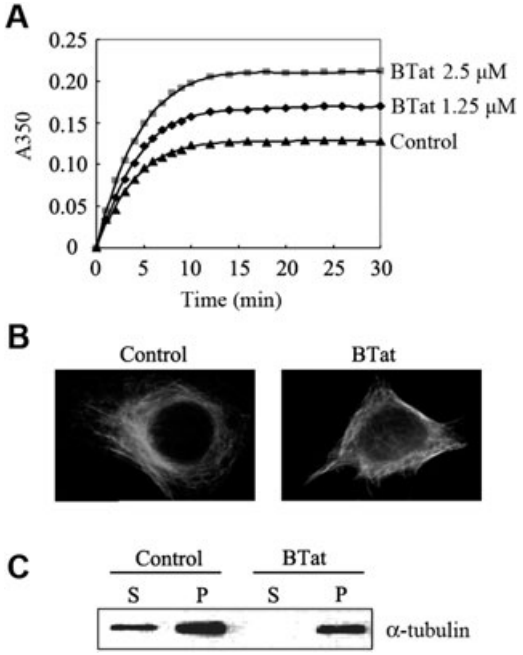

Fig. 6. BTat promotes microtubule polymerization *in vitro* and in cells.
A. Effects of different concentrations of BTat on tubulin polymerization were measured by light scattering, reflected as the absorbance at 350 nm wavelength at 37°C.
B. HeLa cells grown on glass coverslips were untreated or treated with 0.5 μM of BTat for 4 h, and immunofluorescence microscopy were then performed using an anti-α-tubulin antibody.
C. Jurkat cells were treated with 1 μM of BTat for 4 h. The lysates that contain polymeric (P) and soluble, dimeric (S) tubulin were collected, respectively, and detected by Western blotting with an anti-α-tubulin antibody.

cells with 0.5 μM of BTat for 4 h significantly enhanced the extent of tubulin acetylation (Fig. 7A), and this effect of BTat was dose-dependent (Fig. 7B), indicating the ability of BTat to increase microtubule stability.

It has been reported that microtubules in HeLa cells are cold-sensitive; most microtubules disassemble after cold exposure (Lieuvin *et al*., 1994). We thus used this approach to further examine the effect of BTat on microtubule stability. BTat-treated HeLa cells or control cells were incubated at 4°C for 1 h, and the morphology of cellular microtubules was then examined by immunofluorescence microscopy. As shown in Fig. 7C, in control cells most microtubules disassembled, whereas in BTat-treated cells, microtubules were rather resistant to cold exposure. These results thus suggest that BTat could significantly increase the stability of microtubules.

Discussion

Virus–host interaction is a dynamic process that has been extensively studied in the past decades. The immune system of host cells attempts to suppress and eliminate virus infection. After being infected, many cells undergo apoptosis to protect themselves, whereas viruses often employ the existing cellular machinery to promote or inhibit the onset of apoptosis. Elucidation of the molecular mechanisms by which viruses achieve the promotion or inhibition of host apoptosis is one of the most important subjects on virus–host interactions (Roshal *et al*., 2001; Coffey *et al*., 2006; Moon and Yang, 2006).

Bovine immunodeficiency virus has attracted much attention in the past years because of its high similarity to HIV on morphology, genome structure and antigenic reactivity and because of the urgent need for developing animal models for AIDS (Gonda *et al*., 1987). In this study, our data reveal for the first time that BIV infection induces apoptosis in cells. It has been reported that for some viruses the apoptotic death of a host cell results in the formation of small membrane-bound structures known as apoptotic bodies, which can pinch off from the dying cell and consumed by the phagocytic action of neighbouring cells. This effect is thought to provide a means for virus spread without initiating a concomitant host response, followed by the release of the progeny into neighbouring cells (Teodoro and Branton, 1997). Similarly, the pro-apoptotic action exerted by BIV infection may play a critical role in viral dissemination, and BIV-induced apoptosis may contribute to its cytopathic effect in the infected animals.

Bovine immunodeficiency virus has a complex genomic structure characterized by the presence of several regu-

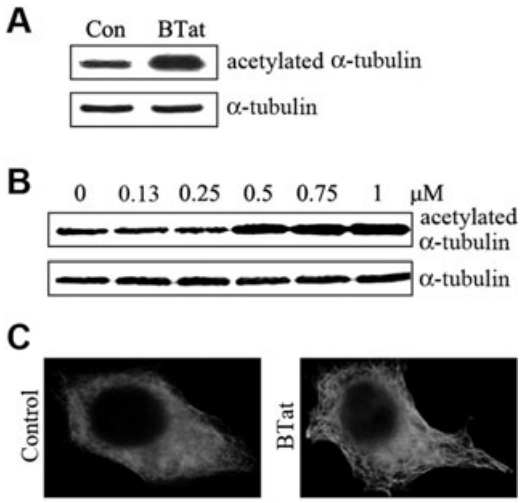

Fig. 7. BTat increases microtubule stability in cells.
A. Western blot analysis of acetylated α-tubulin and total α-tubulin in Jurkat cells untreated or treated with 1 μM of BTat for 4 h.
B. Western blot analysis of acetylated α-tubulin and total α-tubulin in A549 cells treated with different concentrations of BTat for 4 h.
C. HeLa cells grown on glass coverslips were untreated or treated with 0.5 μM of BTat for 4 h, then incubated at 4°C for 1 h, and immunofluorescence microscopy was then performed to detect cellular microtubules.

latory genes that encode proteins involved in the regulation of viral gene expression and virus–host interaction. Cellular effects elicited by lentiviral infection involves a complex interaction between the virus and the host immune response, as well as an interplay between viral and cellular regulatory factors (Fultz, 1991; Novembre *et al*., 1993). BTat is an early regulatory protein that is required for the replication of BIV genome and plays an essential role in viral life cycle. As BTat is highly similar to HTat on protein structure as well as transcription elongation function and HTat is known to induce robust apoptosis, we have investigated in this study whether BTat can induce apoptosis. Using assays such as TUNEL, nuclear morphology analysis, flow cytometry, and cleavages of caspases and PARP, we have demonstrated that BTat is able to trigger apoptosis. This is consistent with the view that like HTat, BTat may affect cellular signalling pathways in addition to regulating the replication of viral genome (Peruzzi, 2006).

Lentiviral cytopathogenicity is a complex phenomenon involving many viral and host proteins. The data presented in this study suggest that BTat may contribute to BIV-induced apoptosis. To achieve a better understanding of the relationship between BTat and BIV-induced apoptosis, we have tried to treat cells with BIV in the presence of BTat antibody to block the entry of extracellular BTat. We find that addition of BTat antibody to the culture medium does not cause a significant reduction of apoptosis in BIV-infected cells (data not shown). However, this result cannot undervalue the potential contribution of BTat to BIV-induced apoptosis, because it is uncertain at present whether adding BTat antibody to the culture medium is capable of blocking the entry of extracellular BTat, in addition to that both exogenous and endogenous BTat can induce apoptosis. Given the complexity of lentiviral cytopathogenicity, it is conceivable that many other viral or host proteins, apart from BTat, may be identified in the future to participate in BIV-induced apoptosis.

Low concentrations of HTat are known to promote cell proliferation in some cell types (Gibellini *et al*., 2005; Pugliese *et al*., 2005). For example, HTat has been reported to increase the proliferation of cells derived from Kaposi's sarcoma lesions of AIDS patients (Ensoli *et al*., 1990). In addition, increased cell proliferation has been shown in HTat-treated cell lines in association with slightly increased apoptosis (Pugliese *et al*., 2005). However, in our preliminary experiment, low concentrations of BTat do not obviously affect cell proliferation despite its homology to HTat (data not shown).

The microtubule cytoskeleton plays an important role in a variety of cellular processes, such as cell shaping, cell trafficking and cell division. Microtubules are intrinsically dynamic, and the dynamic property is critical for many functions of microtubules (Mollinedo and Gajate, 2003). Our data have shown that BTat interacts with both tubulin and microtubules. In addition, our recent experiments reveal that BTat and HTat interact with tubulin to similar degrees and the cysteine-rich region may be important for the interaction (data not shown). In this study, we find that BTat promotes microtubule polymerization and increases microtubule stability. It is conceivable that BTat-induced interference of microtubule dynamics may underlie its pro-apoptotic action, given that disruption of microtubule dynamics by chemical agents such as taxol and vinca alkaloids causes apoptosis (Mollinedo and Gajate, 2003). On the other hand, microtubule-interfering agents have been reported to cause the release of Bim from microtubules, which translocates to mitochondria to either bind Bcl-2 or Bcl-x_L to neutralize their anti-apoptotic activity or to induce conformational change in Bax to promote apoptosis (Puthalakath *et al*., 1999; Marani *et al*., 2002). It is possible that BTat may adopt a similar strategy to transduce signals from the disruption of microtubule dynamics to apoptosis.

Experimental procedures

Materials

Trypan blue, DAPI, propidium iodide, and antibodies against α-tubulin, acetylated α-tubulin, and β-actin were purchased from Sigma-Aldrich. Antibodies against PARP, cleaved caspase-3, and cleaved caspase-9 were from Cell Signaling. Antibodies against GST and Myc were from Santa Cruz Biotechnology. The fluorescein-conjugated anti-mouse secondary antibody was purchased from Jackson ImmunoResearch Laboratories, and horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were from Santa Cruz Biotechnology.

Cells, viruses, plasmids and proteins

HeLa, 293T and FBL cells were maintained in Dulbecco's modified Eagle's medium as monolayers in tissue culture plates, whereas Jurkat and A549 were maintained in RPMI 1640 medium. Both media were supplemented with 10% fetal calf serum, 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin at a 5% CO_2, 95% air atmosphere. The R29 strain of BIV was provided by Dr Charles Wood (University of Nebraska Lincoln). The bacterial expression plasmid for GST-tagged BTat was constructed by insertion of BTat cDNA in frame into the pGEX6P1 vector. The mammalian expression plasmid Myc-BTat was provided by Dr Matjaz Barboric (University of California, San Francisco, CA, USA). The BL21 (DE3) strain of *E. coli* was used to express the GST-BTat fusion protein or GST alone, and proteins were purified in the presence of 500 units of Benzonase nuclease (Sigma-Aldrich), using glutathione sepharose 4B beads according to the manufacturer's instruction (Promega). Tubulin (from bovine brain, >99% pure) was from Cytoskeleton Inc.

TUNEL assay

TUNEL assay was performed by using the *in situ* cell detection kit (FITC) (Roche Molecular Biochemicals) as described (Zhou

et al., 2002a). In brief, cells grown on glass coverslips or cell suspensions were fixed by a freshly prepared paraformaldehyde solution (4% in phosphate-buffered saline, PBS) for 1 h at room temperature. Cells were then washed with PBS and incubated in permeabilization solution (0.1% Triton X-100, 0.1% NaAc) for 2 min on ice. Then, 50 μl of TUNEL reaction mixture was added and incubated in a humidified chamber for 1 h at 37°C in the dark. Finally, cells were mounted and examined under a fluorescence microscope or directly analysed by flow cytometry as described (Aneja *et al.*, 2006).

DNA electrophoresis

Cells were harvested by centrifugation at 2000 r.p.m. and then washed three times with PBS. The pellets were treated with cell lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, 1% SDS, pH 7.5) for 10 s. After centrifugation for 10 min at 12 000 r.p.m., the supernatants were collected and treated for 2 h with RNaseA (final concentration 3 μg ml^{-1}) at 37°C followed by digestion with proteinase K (final concentration 2.5 μg ml^{-1}) at 50°C. After addition of 1/10 volume 3 M NaAc and 2 volume ethanol, DNA was precipitated at –20°C for 4 h and collected by centrifugation at 12 000 r.p.m. for 10 min. The pellet was dissolved in the TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.5) and separated by electrophoresis on 2% agarose gel.

Flow cytometry

Flow cytometric analysis of cell cycle was performed as described previously (Liu *et al.*, 2006). Briefly, 10^6 cells were harvested by centrifugation at 2000 r.p.m. and washed three times with ice-cold PBS. Cells were fixed in cold 70% ethanol for 24 h at –20°C, centrifuged at 2000 r.p.m. for 10 min, and then washed in PBS for three times. Cells were digested with RNaseA (final concentration 20 μg ml^{-1}) and stained with propidium iodide (final concentration 20 μg ml^{-1}). The samples were then analysed on a flow cytometer (BD FACSCalibur).

Western blotting

Cell extracts were subjected to SDS-PAGE and blotted onto the polyvinylidene difluoride membrane for 1 h at 100 V. After blocking with PBS containing 0.1% tween 20 (PBST) and 5% dry milk for 45 min at room temperature, the membrane was incubated overnight at 4°C with the primary antibody in PBST. The membrane was washed for three times with PBST and incubated with the horseradish peroxidase-conjugated second antibody for 45 min at room temperature. The membrane was washed for five times with PBST, and protein bands were visualized with enhanced chemiluminescence detection reagent (Pierce Biotechnology).

GST-pulldown and immunoprecipitation

For GST-pulldown *in vitro*, GST or GST-BTat fusion protein immobilized on glutathione sepharose beads was incubated with purified tubulin at 4°C for 2 h in 20 mM Tris, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM EDTA and 500 units of Benzonase nuclease (Sigma-Aldrich). Benzonase nuclease was used in the GST-pulldown experiments to avoid unspecific bridging by nucleic acids, given the potent nucleic acid-binding activity of Tat family proteins. The beads were washed extensively and boiled in the SDS loading buffer. The proteins were then analysed by SDS-PAGE and Western blotting. For immunoprecipitation, Myc-BTat expression construct or vector was transfected into 293T cells. The cell lysate was incubated with an anti-Myc antibody for 2 h at 4°C, and Protein A agarose beads were then added to incubate for another 3 h. The beads were washed extensively, and the precipitated proteins were detected by Western blotting.

Preparation of polymeric and dimeric tubulin

The polymeric (cytoskeletal) tubulin and free, dimeric (soluble) tubulin were prepared as described (Zhou *et al.*, 2002b). Essentially, cells were washed for three times with PBS, and soluble proteins were then extracted under conditions that prevent microtubule depolymerization (0.1% Triton X-100, 0.1 M MES, pH 6.75, 1 mM $MgSO_4$, 2 mM EGTA, 4 M glycerol). The remaining cytoskeletal fraction was dissolved in 0.5% SDS in 25 mM Tris (pH 6.8). Equivalent amounts for each treatment group were loaded on the gel, and detected by Western blotting with an anti-α-tubulin antibody.

Tubulin assembly into microtubules in vitro

Spectrophotometer cuvettes (0.4 cm path length) held a solution consisting of microtubule assembly buffer (100 mM Pipes, 2 mM EGTA, 1 mM $MgCl_2$, 1 mM GTP, pH 6.8) and BTat. The cuvettes were kept at room temperature before the addition of 10 μM purified tubulin and shifted to 37°C in a temperature controlled spectrophotometer (Amersham Biosciences). The assembly was monitored by measuring the changes in absorbance (350 nm) at 0.5 min intervals as described previously (Zhou *et al.*, 2003).

Microtubule co-sedimentation assay

Purified GST-BTat or GST was incubated at 30°C for 30 min with paclitaxel-stabilized microtubules in PEMG buffer (100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, 1 mM GTP, pH 6.8). Microtubules were pelleted by centrifugation through a 60% glycerol/PEMG cushion at 60 000 r.p.m. for 20 min at 37°C. The pellet and supernatant fractions were collected individually, and proteins present in each fraction were examined by Western blotting.

Immunofluorescence microscopy

Cells grown on glass coverslips were fixed with cold (–20°C) methanol for 5 min and then washed with PBS for 5 min. Cells were blocked by incubating with 100 μl of 2% bovine serum albumin (BSA) in PBS at 37°C for 15 min. A mouse monoclonal antibody against α-tubulin (DM1A, Sigma) was diluted 1:500 and incubated (50 μl) with the coverslips at 37°C for 1 h. Cells were then washed with 2% BSA/PBS for 10 min at room temperature before incubating with a 1:200 dilution of a FITC-labelled donkey anti-mouse IgG antibody (Jackson ImmunoResearch) at 37°C for 30 min. Coverslips were then rinsed with 2% BSA/PBS for 10 min and cells were examined with an Olympus fluorescence microscope.

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References

Aneja, R., Zhou, J., Vangapandu, S.N., Zhou, B., Chandra, R., and Joshi, H.C. (2006) Drug-resistant T-lymphoid tumors undergo apoptosis selectively in response to an antimicrotubule agent, EM011. *Blood* **107:** 2486–2492.

Arends, M.J., and Wyllie, A.H. (1991) Apoptosis: mechanisms and roles in pathology. *Int Rev Exp Pathol* **32:** 223–254.

Barboric, M., Taube, R., Nekrep, N., Fujinaga, K., and Peterlin, B.M. (2000) Binding of Tat to TAR and recruitment of positive transcription elongation factor b occur independently in bovine immunodeficiency virus. *J Virol* **74:** 6039–6044.

Carpenter, S., Miller, L.D., Alexandersen, S., Whetstone, C.A., VanDerMaaten, M.J., Viuff, B., *et al.* (1992) Characterization of early pathogenic effects after experimental infection of calves with bovine immunodeficiency-like virus. *J Virol* **66:** 1074–1083.

Chen, D., Wang, M., Zhou, S., and Zhou, Q. (2002) HIV-1 Tat targets microtubules to induce apoptosis, a process promoted by the pro-apoptotic Bcl-2 relative Bim. *EMBO J* **21:** 6801–6810.

Coffey, C.M., Sheh, A., Kim, I.S., Chandran, K., Nibert, M.L., and Parker, J.S. (2006) Reovirus outer capsid protein micro1 induces apoptosis and associates with lipid droplets, endoplasmic reticulum, and mitochondria. *J Virol* **80:** 8422–8438.

Danen-Van Oorschot, A.A., Fischer, D.F., Grimbergen, J.M., Klein, B., Zhuang, S., Falkenburg, J.H., *et al.* (1997) Apoptin induces apoptosis in human transformed and malignant cells but not in normal cells. *Proc Natl Acad Sci USA* **94:** 5843–5847.

Dbaibo, G.S., and Hannun, Y.A. (1998) Cytokine response modifier A (CrmA): a strategically deployed viral weapon. *Clin Immunol Immunopathol* **86:** 134–140.

Ensoli, B., Barillari, G., Salahuddin, S.Z., Gallo, R.C., and Wong-Staal, F. (1990) Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. *Nature* **345:** 84–86.

Epie, N., Ammosova, T., Sapir, T., Voloshin, Y., Lane, W.S., Turner, W., *et al.* (2005) HIV-1 Tat interacts with LIS1 protein. *Retrovirology* **2:** 6.

Flores, S.C., Marecki, J.C., Harper, K.P., Bose, S.K., Nelson, S.K., and McCord, J.M. (1993) Tat protein of human immunodeficiency virus type 1 represses expression of manganese superoxide dismutase in HeLa cells. *Proc Natl Acad Sci USA* **90:** 7632–7636.

Fultz, P.N. (1991) Replication of an acutely lethal simian immunodeficiency virus activates and induces proliferation of lymphocytes. *J Virol* **65:** 4902–4909.

Gibellini, D., Vitone, F., Schiavone, P., and Re, M.C. (2005) HIV-1 tat protein and cell proliferation and survival: a brief review. *New Microbiol* **28:** 95–109.

Gonda, M.A., Braun, M.J., Carter, S.G., Kost, T.A., Bess, J.W., Jr, Arthur, L.O., and Van der Maaten, M.J. (1987) Characterization and molecular cloning of a bovine lentivirus related to human immunodeficiency virus. *Nature* **330:** 388–391.

Levine, B., Huang, Q., Isaacs, J.T., Reed, J.C., Griffin, D.E., and Hardwick, J.M. (1993) Conversion of lytic to persistent alphavirus infection by the bcl-2 cellular oncogene. *Nature* **361:** 739–742.

Li, C.J., Wang, C., Friedman, D.J., and Pardee, A.B. (1995a) Reciprocal modulations between p53 and Tat of human immunodeficiency virus type 1. *Proc Natl Acad Sci USA* **92:** 5461–5464.

Li, C.J., Friedman, D.J., Wang, C., Metelev, V., and Pardee, A.B. (1995b) Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein. *Science* **268:** 429–431.

Lieuvin, A., Labbe, J.C., Doree, M., and Job, D. (1994) Intrinsic microtubule stability in interphase cells. *J Cell Biol* **124:** 985–996.

Liu, M., Aneja, R., Liu, C., Sun, L., Gao, J., Wang, H., *et al.* (2006) Inhibition of the mitotic kinesin Eg5 up-regulates Hsp70 through the phosphatidylinositol 3-kinase/Akt pathway in multiple myeloma cells. *J Biol Chem* **281:** 18090–18097.

Low, W., Harries, M., Ye, H., Du, M.Q., Boshoff, C., and Collins, M. (2001) Internal ribosome entry site regulates translation of Kaposi's sarcoma-associated herpesvirus FLICE inhibitory protein. *J Virol* **75:** 2938–2945.

Marani, M., Tenev, T., Hancock, D., Downward, J., and Lemoine, N.R. (2002) Identification of novel isoforms of the BH3 domain protein Bim which directly activate Bax to trigger apoptosis. *Mol Cell Biol* **22:** 3577–3589.

Mollinedo, F., and Gajate, C. (2003) Microtubules, microtubule-interfering agents and apoptosis. *Apoptosis* **8:** 413–450.

Moon, H.S., and Yang, J.S. (2006) Role of HIV Vpr as a regulator of apoptosis and an effector on bystander cells. *Mol Cells* **21:** 7–20.

Noteborn, M.H., Todd, D., Verschueren, C.A., de Gauw, H.W., Curran, W.L., Veldkamp, S., *et al.* (1994) A single chicken anemia virus protein induces apoptosis. *J Virol* **68:** 346–351.

Novembre, F.J., Johnson, P.R., Lewis, M.G., Anderson, D.C., Klumpp, S., McClure, H.M., and Hirsch, V.M. (1993) Multiple viral determinants contribute to pathogenicity of the acutely lethal simian immunodeficiency virus SIVsmmPBj variant. *J Virol* **67:** 2466–2474.

Onuma, M., Koomoto, E., Furuyama, H., Yasutomi, Y., Taniyama, H., Iwai, H., and Kawakami, Y. (1992) Infection and dysfunction of monocytes induced by experimental inoculation of calves with bovine immunodeficiency-like virus. *J Acquir Immune Defic Syndr* **5:** 1009–1015.

Palazzo, A., Ackerman, B., and Gundersen, G.G. (2003) Cell biology: tubulin acetylation and cell motility. *Nature* **421:** 230.

Peruzzi, F. (2006) The multiple functions of HIV-1 Tat: proliferation versus apoptosis. *Front Biosci* **11:** 708–717.

Pugliese, A., Vidotto, V., Beltramo, T., Petrini, S., and Torre,

D. (2005) A review of HIV-1 Tat protein biological effects. *Cell Biochem Funct* **23:** 223–227.

Puthalakath, H., Huang, D.C., O'Reilly, L.A., King, S.M., and Strasser, A. (1999) The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell* **3:** 287–296.

Roshal, M., Zhu, Y., and Planelles, V. (2001) Apoptosis in AIDS. *Apoptosis* **6:** 103–116.

Sastry, K.J., Marin, M.C., Nehete, P.N., McConnell, K., el-Naggar, A.K., and McDonnell, T.J. (1996) Expression of human immunodeficiency virus type I tat results in down-regulation of bcl-2 and induction of apoptosis in hematopoietic cells. *Oncogene* **13:** 487–493.

St-Louis, M.C., Cojocariu, M., and Archambault, D. (2004) The molecular biology of bovine immunodeficiency virus: a comparison with other lentiviruses. *Anim Health Res Rev* **5:** 125–143.

Teodoro, J.G., and Branton, P.E. (1997) Regulation of apoptosis by viral gene products. *J Virol* **71:** 1739–1746.

Thompson, C.B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* **267:** 1456–1462.

Vockerodt, M., Haier, B., Buttgereit, P., Tesch, H., and Kube, D. (2001) The Epstein-Barr virus latent membrane protein 1 induces interleukin-10 in Burkitt's lymphoma cells but not in Hodgkin's cells involving the p38/SAPK2 pathway. *Virology* **280:** 183–198.

Wang, Z., Morris, G.F., Reed, J.C., Kelly, G.D., and Morris, C.B. (1999) Activation of Bcl-2 promoter-directed gene expression by the human immunodeficiency virus type-1 Tat protein. *Virology* **257:** 502–510.

Westendorp, M.O., Li-Weber, M., Frank, R.W., and Krammer, P.H. (1994) Human immunodeficiency virus type 1 Tat upregulates interleukin-2 secretion in activated T cells. *J Virol* **68:** 4177–4185.

Westendorp, M.O., Frank, R., Ochsenbauer, C., Stricker, K., Dhein, J., Walczak, H., *et al.* (1995) Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. *Nature* **375:** 497–500.

Williams, G.T. (1994) Programmed cell death: a fundamental protective response to pathogens. *Trends Microbiol* **2:** 463–464.

Young, L.S., Dawson, C.W., and Eliopoulos, A.G. (1999) Epstein-Barr virus and apoptosis: viral mimicry of cellular pathways. *Biochem Soc Trans* **27:** 807–812.

Zauli, G., Gibellini, D., Caputo, A., Bassini, A., Negrini, M., Monne, M., *et al.* (1995) The human immunodeficiency virus type-1 Tat protein upregulates Bcl-2 gene expression in Jurkat T-cell lines and primary peripheral blood mononuclear cells. *Blood* **86:** 3823–3834.

Zhang, S., Wood, C., Xue, W., Krukenberg, S.M., Chen, Q., and Minocha, H.C. (1997) Immune suppression in calves with bovine immunodeficiency virus. *Clin Diagn Lab Immunol* **4:** 232–235.

Zhou, J., Gupta, K., Aggarwal, S., Aneja, R., Chandra, R., Panda, D., and Joshi, H.C. (2003) Brominated derivatives of noscapine are potent microtubule-interfering agents that perturb mitosis and inhibit cell proliferation. *Mol Pharmacol* **63:** 799–807.

Zhou, J., Gupta, K., Yao, J., Ye, K., Panda, D., Giannakakou, P., and Joshi, H.C. (2002a) Paclitaxel-resistant human ovarian cancer cells undergo c-Jun NH2-terminal kinase-mediated apoptosis in response to noscapine. *J Biol Chem* **277:** 39777–39785.

Zhou, J., Panda, D., Landen, J.W., Wilson, L., and Joshi, H.C. (2002b) Minor alteration of microtubule dynamics causes loss of tension across kinetochore pairs and activates the spindle checkpoint. *J Biol Chem* **277:** 17200–17208.

Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Effect of BTat antibody on BIV-induced apoptosis. FBL cells untreated or treated with BIV (R29 strain) in the absence or presence of BTat antibody were stained with the TUNEL reagents, and the percentage of TUNEL-positive cells was quantified by flow cytometry.

Fig. S2. Effect of BTat on the proliferation of Jurkat cells. Jurkat cells untreated or treated with 1 nM of BTat for 1, 3, 4, 5, 6 or 7 days, and the number of cells was then counted by a haemocytometer.

Fig. S3. Induction of apoptosis in Jurkat cells by low concentration of BTat. Jurkat cells untreated or treated with 10 nM of BTat for 48 h and stained with propidium iodide, and the percentage of apoptotic (sub-G1) cells was then examined by flow cytometry.

Fig. S4. BTat and HTat interact with tubulin to similar degrees. Purified tubulin was incubated with GST-BTat, GST-HTat or GST immobilized on sepharose beads. GST-pulldown was then performed, and the presence of tubulin in the pulldown preparation was detected by Western blotting using an anti-α-tubulin antibody. Purified GST-BTat, GST-HTat and GST used in the pulldown assay were detected by Coomassie blue staining.

Fig. S5. The cystine-rich region is important for the interaction of tubulin with JTat, a protein highly homologous to BTat. Cells were transfected with a plasmid expressing GST or the indicated truncated forms of JTat tagged with GST. GST-pulldown and Western blotting were then performed to examine their interaction with α-tubulin.

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