

# TAT-mediated endocytotic delivery of the loop deletion Bcl-2 protein protects neurons against cell death

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## Abstract

Protein delivery mediated by protein transduction domains (PTD) such as the HIV-1 TAT-PTD has emerged as a promising approach for neuroprotection. The objective of this study was to generate and evaluate the neuroprotective potential of TAT fusion proteins using constructs based on Bcl-2 anti-death family proteins. A TAT-Bcl-2 construct with the loop domain deleted (TAT-Bcl-2 $\Delta$ loop) was tested for its ability to transduce neuronal cells and to promote survival. The potential mechanism of TAT-mediated protein internalization in neural cells was also investigated. The purified TAT-Bcl-2 $\Delta$ loop binds to neural cell and rat brain mitochondria, and transduces cultured neural cell lines and primary cortical neurons when used at nM concentrations. Effective internalization of TAT-Bcl-2 $\Delta$ loop

occurs at 37°C but not at 4°C, consistent with an endocytotic process. Both cell association and internalization require interaction of TAT-Bcl-2 $\Delta$ loop with cell surface heparan sulfate proteoglycans. TAT-mediated protein delivery in neuronal cells occurs through a lipid raft-dependent endocytotic process, inhibited by the cholesterol-sequestering agent nystatin. Transducible loop deleted Bcl-2 increases the survival of cortical neurons following trophic factor withdrawal and also rescues neural cell lines from staurosporine-induced death. These results support the concept of using protein transduction of Bcl-2 constructs for neuroprotection.

**Keywords:** apoptosis, Bcl-2, endocytosis, mitochondria, neuroprotection.

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The Bcl-2 family of proteins is comprised of both pro- and anti-apoptotic members. The Bcl-2 protein, the first member identified, inhibits apoptosis induced by a wide variety of stimuli, and also protects against necrotic cell death (Kane *et al.* 1995; Myers *et al.* 1995; Adams and Cory 1998). The potential of Bcl-2 for neuroprotection is demonstrated in numerous studies performed *in vitro* and *in vivo*. Increased expression of endogenous Bcl-2 protein in preconditioning paradigms, and overexpression of Bcl-2 by using viral vectors or in transgenic animals is associated with ischemic neuroprotection (Martinou *et al.* 1994; Shimazaki *et al.* 2000; Shimizu *et al.* 2001). Recently, another neuroprotective strategy was employed by using protein transduction to deliver proteins fused to protein transduction domains (PTD) into the brain (Asoh *et al.* 2002; Cao *et al.* 2002; Kilic *et al.* 2002).

A large number of short basic peptides known as protein transduction domains (PTD) or cell-penetrating peptides, derived from viral or cellular proteins, are able to cross biological membranes and, in some cases, promote the delivery of full-length proteins into cells. Various other cargoes, including plasmid DNA, oligonucleotides, liposomes and even nanoparticles were delivered using this approach (reviewed in Wadia and Dowdy 2002; Dietz and

Bahr 2004). The TAT-PTD derived from the human immunodeficiency virus (HIV)-1 transactivator of transcription (TAT) protein is one of the most used PTD and mediates protein transduction both in cultured cells and in mice (Schwarze *et al.* 1999). Two groups initially reported the ability of full-length HIV-1 TAT protein to translocate inside cells in a receptor-independent manner (Frankel and Pabo 1988; Green and Loewenstein 1988) and subsequent studies by Fawell *et al.* (1994) and Vives *et al.* (1997) led to mapping of the transduction potential to the basic domain (residues 48–60) in the HIV-1 TAT (Fawell *et al.* 1994; Vives *et al.* 1997). More recently, Dowdy's group has demonstrated

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**Abbreviations used:** Bcl, B-cell lymphoma; BH domain, homology domain; HIV, human immunodeficiency virus; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; OMM, outer mitochondrial membrane; PTD, protein transduction domain; STS, staurosporine; TAT, transactivator of transcription.

that fusion proteins with the TAT(47–57) PTD transduce efficiently in cultured cells and *in vivo* (Schwarze *et al.* 1999).

Recent studies indicate that Bcl-X<sub>L</sub>, an anti-apoptotic Bcl-2 family protein, can be transduced into cells and into the mouse brain when fused to TAT-PTD and results in post-ischemic neuroprotection (Asoh *et al.* 2002; Cao *et al.* 2002; Kilic *et al.* 2002). Delivery of Bcl-X<sub>L</sub> to pancreatic islets (Embury *et al.* 2001) or retinal ganglion cells (Dietz *et al.* 2002) and of the BH4 domains of Bcl-X<sub>L</sub> (Sugioka *et al.* 2003) and Bcl-2 (Cantara *et al.* 2004) were also reported. These studies indicate that protein transduction of Bcl-2 family proteins might be used as a neuroprotective strategy.

The mechanism of PTD-mediated protein internalization is not completely understood. Recently, the temperature-insensitive and energy-independent translocation of PTDs across cell membranes described initially (Derossi *et al.* 1996; Vives *et al.* 1997) was suggested as artifactual and evidence was provided for an endocytotic uptake (Lundberg *et al.* 2003; Richard *et al.* 2003). In non-neuronal cells, either caveolar endocytosis (Fittipaldi *et al.* 2003) or macropinocytosis (Wadia *et al.* 2004) were shown to mediate internalization of TAT-fused proteins. Even though there are now several reports of protein transduction in neurons and into the brain, the mechanisms of TAT-mediated protein delivery in neurons are not known.

Bcl-2 activity in non-neuronal cells is regulated by complex post-translational mechanisms. Phosphorylation of residues in the unstructured loop domain either inactivate or are required for Bcl-2 activity (Haldar *et al.* 1995; Ito *et al.* 1997), and caspase-cleaved Bcl-2 is pro-apoptotic (Cheng *et al.* 1997). Transduction of Bcl-2 in post-ischemic neurons might result in its inactivation, as kinases (Hara *et al.* 1990; Takagi *et al.* 2000) and caspases are activated, and endogenous Bcl-2 is cleaved during reperfusion (Krajewska *et al.* 2004). Elimination of the cleavage and phosphorylation sites through deletion of the loop domain, could improve neuroprotection by transduced Bcl-2, as shown in other cell types using transfection (Chang *et al.* 1997; Srivastava *et al.* 1999). However, as loss of anti-apoptotic activity of loop deleted Bcl-2 was also reported (Fang *et al.* 1998), the activity of this mutated Bcl-2 construct in neuronal cells and its potential for neuroprotection is unclear.

The results of this study indicate that in neuronal cells TAT-mediated protein internalization occurs through a lipid raft-dependent endocytotic process and that the ability of transducible Bcl-2 to protect neurons against at least some forms of cell death is not lost when the loop domain is deleted.

## Materials and methods

### Culture of neuronal cell lines and primary neurons

PC12*bcl-2* rat pheochromocytoma and GT1–7*bcl-2* immortalized mouse hypothalamic neurons, stably overexpressing the human

*bcl-2*, and the PC12*puro* and GT1–7*puro* cells, infected with an adenoviral vector containing only the control puromycin resistance gene were maintained in Dubelcco's modified Eagle's medium (DMEM) (Biosource, Camarillo, CA, USA) with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin (Mah *et al.* 1993). The SH-SY5Y neuroblastoma cell line was obtained from American Type Tissue Collection (ATCC; Rockville, MD, USA). The cells were cultured in 24-well plates in DMEM with 10% FBS, 1% penicillin and streptomycin. Primary rat cortical neurons were isolated from 18-day-old Sprague–Dawley rat embryo cortices as described previously (McKenna *et al.* 2001). Dissociated cortical cells were plated in 24-well plates coated with 50 µg/mL poly D-lysine (Becton Dickinson Labware, Bedford, MA, USA), at a density of  $1 \times 10^5$  cells/well, and maintained in neurobasal medium (NBM; Gibco/Life Technologies, Rockville, MD, USA) with 2% B27 serum-free supplement (Gibco). After 72 h the cells were treated with 5 µM cytosine arabinoside to inhibit proliferation of non-neuronal cells. Before use in experiments, the cells were cultured for 8–10 days when nearly pure (> 95%) neuronal cultures were obtained (McKenna *et al.* 2001).

### Expression and purification of TAT-Bcl-2 fusion proteins

For generation of the TAT fusion proteins, we constructed a new expression vector (pET-TAT) following the strategy described in (Vocero-Akbani *et al.* 2001). A double-stranded DNA oligonucleotide encoding the 11 amino acids TAT-PTD (YGRKKRRQRRR) flanked by Gly residues was ligated in frame with the 10 histidines (10 × His) tag into the *NdeI*–*XhoI* digested pET-16b(+) expression vector (Novagen, Madison, WI, USA). The cDNA encoding the Bcl-2 loop deleted construct (Bcl-2Δloop) was subcloned in the same expression vector in frame with the His-TAT sequence. Another Bcl-2 construct that lacks both the loop domain and the last 22 C-terminal hydrophobic residues (TAT-Bcl-2ΔLTM) was generated similarly. DNA sequencing by dideoxy chain termination (Biopolymer Facility, University of Maryland, Baltimore) confirmed the correct nucleotide sequence. The construct was also verified in an *in vitro* transcription/translation assay (EcoProT7, Novagen, Madison, WI, USA). The plasmid encoding the TAT-Bcl-2Δloop cDNA was transformed into the BL21(DE3)pLysS, BL21-AL, C41(DE3) and C43(DE3) *Escherichia coli* strains, and one colony was grown in 20 mL LB medium to an OD of 0.6 then inoculated into a 1-L culture. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) 100 µM to 1 mM at 30°C, in the presence of 100 µg/mL carbenicillin and 34 µg/mL chloramphenicol. Bacterial cells were harvested by centrifugation at 10 000 g, lysed using the BugBuster lysis buffer (Novagen) containing recombinant lysozyme and the benzonase nuclease (Lysonase, Novagen), then suspended in Buffer B (8 M urea, 10 mM Tris-HCl, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 20 mM imidazole, pH 8.0) containing 1% Triton X-100, and cleared bacterial lysate obtained by centrifugation. The proteins were purified by passing the cleared bacterial lysate through Ni-NTA affinity resin (Novagen), followed by washing with buffer C (8 M urea, 10 mM Tris-HCl, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 20 mM imidazole, pH 6.3) and were eluted at acidic pH in buffer D and E (8 M urea, 10 mM Tris-HCl, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, pH 5.9 and 4.5, respectively). The eluted protein fractions were dialyzed using a 5000-Da molecular weight cutoff dialysis membrane (Pierce, Rockford, IL, USA) against phosphate-buffered saline (PBS,

pH 7.4) containing 10% glycerol. The dialyzed protein was not concentrated to avoid precipitation and used immediately or aliquoted and stored at  $-70^{\circ}\text{C}$  until use. Protein purity was verified by 4–12% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie staining, and the identity confirmed by immunoblotting with an anti-Bcl-2 (mouse monoclonal, clone C2) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-His (Cell Signaling Technology, Beverly, MA, USA) antibodies.

#### ***In vitro* transduction and western blot analysis**

For transduction of cultured cells, the TAT-Bcl-2 $\Delta$ loop protein in PBS with 10% glycerol was added directly to the culture medium at the indicated concentrations. In each experiment, control cells were incubated with the vehicle alone. A carboxyl-terminal truncated Bcl-2 protein lacking the TAT-PTD (Bcl-2 $\Delta$ TM; R & D, Minneapolis, MN, USA) and containing a 6  $\times$  His tag was also used as an additional control. For cell surface binding assays, the cells were pre-cooled at  $4^{\circ}\text{C}$ , then incubated with the recombinant protein for 10 min at  $4^{\circ}\text{C}$ . The unbound protein was then washed with cold PBS and the cells lifted from the plates non-enzymatically by incubation in Tris-EDTA saline for 10 min at  $4^{\circ}\text{C}$ . To examine the intracellular uptake of the protein, the cell surface-associated protein was proteolytically removed at the end of incubation by treating the cells with trypsin (Sigma, St Louis, MO, USA; 0.5 mg/mL) for 5 min at room temperature ( $22^{\circ}\text{C}$ ), followed by trypsin inactivation and washing in PBS. The cells were lysed in radio immunoprecipitation assay (RIPA) buffer (Soane *et al.* 2001) containing a mixture of protease inhibitors (Calbiochem, La Jolla, CA, USA) and the protein concentration was measured by the bicinonic acid assay (Pierce). Equal amounts of cell lysates (25  $\mu\text{g}$ ) were separated by SDS–PAGE on 4–12% Bis-Tris gels (NuPage, Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes. The uptake and binding of TAT-Bcl-2 $\Delta$ loop was examined by immunoblotting (Soane *et al.* 1999), using an anti-Bcl-2 monoclonal antibody (Santa Cruz Biotechnology, clone C2) followed by ECL detection. A monoclonal anti-His antibody (Cell Signaling Technology) was used to detect the histidine tag (10  $\times$  His) in the purified proteins. Scan densitometric analysis was performed using the GelExpert system (NucleoTech, San Mateo, CA, USA).

#### **Role of lipid rafts and heparan sulfate proteoglycans (HSPG) in protein internalization**

Lipid raft involvement in cell surface binding and intracellular delivery was assessed by pretreating the cells for 1 h with 50  $\mu\text{g}/\text{mL}$  nystatin [Sigma; in dimethylsulfoxide (DMSO)], or with 5 mM methyl- $\beta$ -cyclodextrin (Fluka Chemicals, Buchs, Switzerland) for 30 min, followed by incubation with TAT-Bcl-2 $\Delta$ loop. Preliminary control experiments indicated that cell viability was not affected at these concentrations. The effect of heparin (from porcine intestinal mucosa; Sigma) on internalization was examined by treating the cells with TAT-Bcl-2 $\Delta$ loop and 20  $\mu\text{g}/\text{mL}$  heparin. The role of HSPG in cell binding and internalization was evaluated by enzymatic digestion with heparinase III (from *Flavobacterium heparinum*; Sigma), a glycosaminoglycan lyase specific for heparan sulfate, as in Chu *et al.* (2004). The role of glycosylphosphatidylinositol (GPI)-linked proteoglycans (glypicans) was examined as in Shen *et al.* (1995) by exposing the cells to phosphatidylinositol-specific phospholipase C (PI-PLC) (Sigma) which specifically

digests the lipid anchor of GPI-linked proteins. For these experiments, the cells were exposed to 0.05 U/mL heparinase III and 0.2 U/mL PI-PLC for 1 h at  $37^{\circ}\text{C}$  in serum-free medium, then extensively washed with PBS and incubated with TAT-Bcl-2 $\Delta$ loop.

#### **Induction of apoptosis**

SH-SY5Y cells were pretreated with TAT-Bcl-2 $\Delta$ loop (1 h) then induced to undergo apoptosis by exposure to 0.1  $\mu\text{M}$  staurosporine (STS; Sigma), a protein kinase inhibitor known to induce apoptotic cell death. Protection of primary cortical neurons by TAT-Bcl-2 $\Delta$ loop from trophic factor withdrawal-induced death, was examined by culturing the cells [days *in vitro* (DIV) 8–10] in NBM with or without the serum-free supplement B27. Cell viability was determined by a two-color fluorescence assay based on the simultaneous detection of live and dead cells (live/dead assay, Molecular Probes, Eugene, OR, USA). Cells cultured in 24-well plates ( $10^5$  cells/well) or 96-well plates ( $2 \times 10^4$  cells/well) were exposed to STS or growth-factor withdrawal, then incubated with Calcein-AM (4  $\mu\text{M}$ ) and ethidium homodimer (1  $\mu\text{M}$ ) and examined by fluorescence microscopy. The number of cells that displayed ethidium fluorescence (non-viable) to the total number of cells in three to four randomly chosen fields/sample was determined. A minimum of 500 cells was counted for each field. Viability was calculated using the formula:  $100 \times (1 - \text{ethidium-positive cells}/\text{total cell number})$ . The results are expressed as mean values  $\pm$  SEM.

#### **Isolation of mitochondria and cytochrome *c* release assay**

Isolation of mitochondria from cultured cell lines was performed essentially by the method of Moreadith and Fiskum (1984). Rat brain mitochondria were isolated according to the procedure described in Rosenthal *et al.* (1987). The protein concentration was determined using the Bradford method. Isolated mitochondria were resuspended in a KCl-based medium (mitochondrial buffer) (125 mM KCl, 2 mM  $\text{K}_2\text{HPO}_4$ , 20 mM HEPES pH 7.0) containing  $\text{MgCl}_2$  (4 mM), ATP (3 mM), respiratory substrates (5 mM malate, 5 mM glutamate) and 250  $\mu\text{M}$  EGTA. Mitochondrial binding of TAT-Bcl-2 $\Delta$ loop was examined by incubation of the protein versus the vehicle alone with isolated mitochondria (0.5 mg/mL) in 100  $\mu\text{L}$  mitochondrial buffer for 30 min at  $30^{\circ}\text{C}$ . Mitochondria were then separated from the supernatant by centrifugation at 14 000  $g$  for 10 min at  $4^{\circ}\text{C}$ . Cytochrome *c* release was assessed by immunoblotting of equal amounts of both pellet and supernatant fractions with a monoclonal anti-cytochrome *c* antibody (Pharmingen, San Diego, CA, USA) followed by ECL detection. Alamethicin (80  $\mu\text{g}/\text{mL}$ ) was used as positive control to achieve maximal release of cytochrome *c* through osmotic lysis (Andreyev and Fiskum 1999).

#### **Statistical analysis**

The data presented is representative of at least three independent experiments. The results of western blot quantification and cell survival experiments are expressed as mean  $\pm$  SEM. The statistical significance of the results was assessed either by Student's *t*-test when comparing two groups or, as indicated, by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test for multiple group comparison, using the SigmaStat 3.0 software (Systat Software Inc, Point Richmond, CA, USA), with  $p < 0.05$  considered as significant.

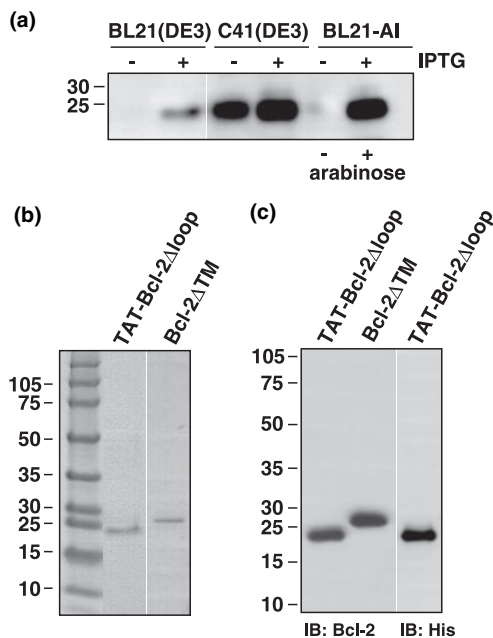
## Results

### Expression and purification of TAT-Bcl-2 $\Delta$ loop protein

In order to generate recombinant TAT-fusion proteins for transduction, we constructed a pET-TAT vector by ligation of a double-stranded oligonucleotide encoding the 11 amino acids of the TAT-PTD (47–57) in frame with the cDNA encoding the loop-deleted (amino acids 32–80) human Bcl-2 (Bcl-2 $\Delta$ loop). This Bcl-2 construct lacks both the caspase-3 cleavage site (Asp34) and several phosphorylation sites. To facilitate purification by affinity chromatography, the TAT-Bcl-2 $\Delta$ loop cDNA was ligated in frame with an N-terminal 10  $\times$  His tag. In initial studies to optimize the expression of TAT-Bcl-2 $\Delta$ loop protein, several *E. coli* strains were tested. TAT-Bcl-2 $\Delta$ loop was expressed at very low levels in the BL21(DE3) $\rho$ LysS strain, with no detectable induction band by Coomassie staining of the IPTG-induced total bacterial lysates. The C41(DE3) and C43(DE3) *E. coli* strains, selected for expression of toxic proteins, overproduce

intracellular membranes and facilitate overexpression of membrane proteins (Arechaga *et al.* 2000). Using both these strains, TAT-Bcl-2 $\Delta$ loop protein could be expressed at much higher levels than in BL21(DE3) $\rho$ LysS (Fig. 1a). The lack of  $\rho$ LysS plasmid to control basal expression from the T7 promoter in these strains results in high levels of protein expression, even in the absence of IPTG induction. Expression of TAT-Bcl-2 $\Delta$ loop in the BL21-AI *E. coli* that confers a tight control of basal transcription from the T7 promoter, also resulted in high levels of protein, similar to the C41/C43(DE3) strains (Fig. 1a).

Initial reports by Dowdy *et al.* indicated that partially denatured proteins are more efficiently transduced into the cells (Vocero-Akbani *et al.* 2001). As most of TAT-Bcl-2 $\Delta$ loop was recovered in the inclusion bodies, the protein was purified in denaturing conditions following protocols reported for purification of various TAT-fusion proteins and for purification of Bcl-2 from inclusion bodies (Anderson *et al.* 1999; Vocero-Akbani *et al.* 2001). This approach allowed us to generate significant amounts of protein (approximately 0.5 mg/L bacterial culture). The TAT-Bcl-2 $\Delta$ loop protein was purified to near homogeneity as described in the Materials and methods. By SDS-PAGE and Coomassie blue staining, a single protein band was detected at the expected molecular weight of approximately 24 kDa (Fig. 1b, lane 1), and immunoblotting with both anti-Bcl-2 and anti-His antibodies confirmed the identity of the protein (Fig. 1c, lanes 1 and 3).

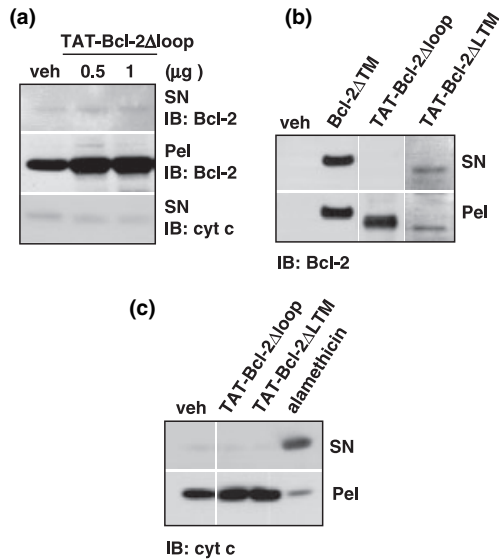


**Fig. 1** Expression of TAT-Bcl-2 $\Delta$ loop. (a) TAT-Bcl-2 $\Delta$ loop was expressed in several *E. coli* strains including BL21(DE3) $\rho$ LysS, C41/C43(DE3) and BL21-AI. Protein expression was induced with IPTG (0.5 mM) or IPTG and 0.2% L-arabinose in BL21-AI, at 30°C, and equal amounts of total bacterial lysates were analyzed by immunoblotting with an anti-Bcl-2 antibody. (b) The purity of the protein was verified by SDS-PAGE and Coomassie staining of the eluted fractions. A single protein band at the expected molecular weight was obtained for purified TAT-Bcl-2 $\Delta$ loop (lane 1). Bcl-2 $\Delta$ TM (R & D Systems) was loaded as control in lane 2. (c) The identity of the purified protein was verified by immunoblotting with both anti-Bcl-2 (lanes 1 and 2; IB: Bcl-2) and anti-His antibodies (IB: His; lane 3), using Bcl-2 $\Delta$ TM protein as positive control (lane 2).

### Effect of TAT-Bcl-2 $\Delta$ loop on isolated mitochondria

Bcl-2 protein is localized to intracellular membranes (OMM, ER membranes and nuclear envelope) (Krajewski *et al.* 1993). Recently, the ability of TAT-PTD to associate with mitochondria *in vitro* was also reported (Ross *et al.* 2004). As targeting of lipid membranes by the TAT-PTD and other cationic peptides with amphipathic  $\alpha$ -helical structure might result in non-specific membrane destabilization, we wanted to verify, using isolated mitochondria, that N-terminal fusion of TAT-PTD does not affect the normal interaction of Bcl-2 proteins with lipid membranes.

We exposed mitochondria isolated from rat brain that express very low amounts of Bcl-2, and Bax, or from the GT1-7*bcl-2* neural cell line to increasing concentrations of TAT-Bcl-2 $\Delta$ loop. The mixtures of mitochondria and proteins were incubated in a cytosol-like media containing respiratory substrates. As shown in Fig. 2(a), after a 30-min incubation at 30°C, most of the protein associated with GT1-7*bcl-2* mitochondria and was recovered in the pellet fraction. The same result was obtained with isolated rat brain mitochondria exposed to similar amounts of TAT-Bcl-2 $\Delta$ loop (Fig. 2b). In contrast, when a TAT-Bcl-2 $\Delta$ loop protein that lacks the C-terminal transmembrane domain (TAT-Bcl-2 $\Delta$ LTM; last 22 amino acids deleted) was incubated in the same conditions, only a fraction of the protein (approximately



**Fig. 2** Effect of TAT-Bcl-2 $\Delta$ loop on isolated mitochondria. (a) Mitochondrial binding of TAT-Bcl-2 $\Delta$ loop was examined by incubating the purified protein or the vehicle (veh) with mitochondria (0.5 mg/mL) isolated from GT1-7*bcl-2* cells, in mitochondrial buffer (100  $\mu$ L), for 30 min at 30°C. Mitochondria were centrifuged at 14000 *g* for 15 min and the supernatant (SN) separated from the pellet (Pel), then both fractions were analyzed by SDS-PAGE and immunoblotting with an anti-Bcl-2 antibody. TAT-Bcl-2 $\Delta$ loop was recovered in the pellet (mitochondria) and resulted in increased Bcl-2 immunoreactivity over the control. Cytochrome *c* release in the supernatant was also analyzed in the same samples by immunoblotting. (b) Mitochondrial binding was assessed as in (a), in isolated rat brain mitochondria. Recombinant Bcl-2 $\Delta$ TM (200 nm), TAT-Bcl-2 $\Delta$ loop (200 nm), and a protein lacking both the loop and TM domain (TAT-Bcl-2 $\Delta$ LTM, 50 nm), were incubated with freshly isolated mitochondria as in (a) and the SN and pellet analyzed by Bcl-2 immunoblotting. The presence of TAT-PTD did not affect mitochondrial binding as, for both TAT-Bcl-2 $\Delta$ LTM and Bcl-2 $\Delta$ TM, an equal fraction of the input protein was detected in mitochondria. (c) Potential disruption of the OMM by TAT-Bcl-2 $\Delta$ loop and TAT-Bcl-2 $\Delta$ LTM (200 nm) was examined in isolated rat brain mitochondria (0.5 mg/mL) by immunoblot analysis of cytochrome *c* release into the supernatant (SN) following exposure to the proteins (30 min, 30°C). Alamethicin (80  $\mu$ g/mL) was used as positive control for maximal cytochrome *c* release.

50%) was recovered in the mitochondrial pellet (Fig. 2b). Using another TM deleted protein (Bcl-2 $\Delta$ TM) that lacks the TAT-PTD, a similar binding to mitochondria was detected and, like for the TAT-Bcl-2 $\Delta$ LTM, only approximately 50% of the protein was associated with mitochondria (Fig. 2b).

In order to assess whether exposure of energized mitochondria to TAT-containing Bcl-2 proteins disrupts the integrity of the OMM, we measured the release of cytochrome *c* into the supernatant. No cytochrome *c* release over the background could be detected in the supernatant in conditions where most of TAT-Bcl-2 $\Delta$ loop protein was already associated with GT1-7 mitochondria (Fig. 2a), or

with the rat brain mitochondria (Fig. 2c). Additionally, we have also determined that incubation of TAT-Bcl-2 $\Delta$ loop with isolated mitochondria does not affect mitochondrial respiration as measured by O<sub>2</sub> consumption (not shown). Although the TAT-PTD itself can associate with OMM (Ross *et al.* 2004), these results indicate that N-terminal fusion of TAT-PTD does not affect interaction of Bcl-2 with mitochondria.

### *In vitro* transduction of TAT-Bcl-2 $\Delta$ loop protein in cultured cells

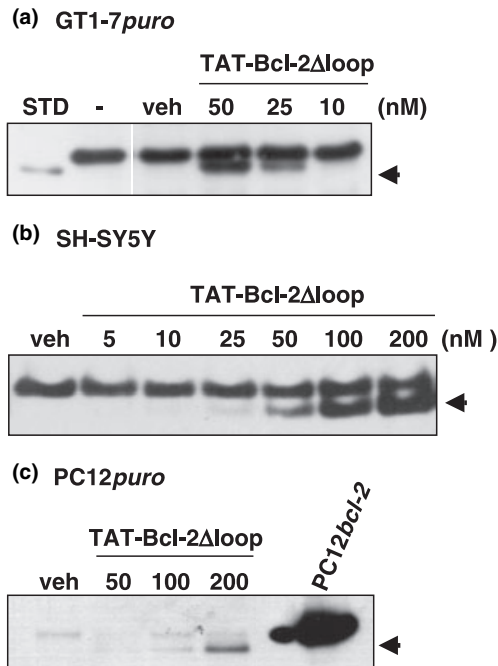
Transduction of TAT-Bcl-2 $\Delta$ loop was examined next in several neuronal cell lines. GT1-7*puro* immortalized mouse hypothalamic neuronal cells were exposed to increasing concentrations of TAT-Bcl-2 $\Delta$ loop, for 30 min at 37°C, then transduction was analyzed by immunoblotting with an anti-Bcl-2 antibody. The transduced TAT-Bcl-2 $\Delta$ loop was identified as an additional band (~24 kDa) migrating faster than endogenous Bcl-2 on SDS-PAGE. As shown in Fig. 3(a), a dose-dependent increase in the amount of transduced TAT-Bcl-2 $\Delta$ loop was detected in GT1-7*puro* cells. The transduced TAT-Bcl-2 $\Delta$ loop was detectable at 25 nM in this cell line and reached levels comparable with endogenous Bcl-2.

Similar results were also obtained for transduction of TAT-Bcl-2 $\Delta$ loop in SH-SY5Y neuroblastoma cell line (Fig. 3b), PC12*puro* cells (Fig. 3c) and primary cultures of rat cortical neurons. The identity of the transduced protein was also confirmed by immunoblotting with an anti-His antibody (Fig. 4c). In all of the cells examined, the transduced protein was readily detectable after 1 h incubation at 37°C. As for the GT1-7 cell line, the levels of transduced TAT-Bcl-2 $\Delta$ loop protein were comparable with the levels of endogenous Bcl-2, or even higher in PC12*puro* cells and in cortical neurons, that express very low levels of Bcl-2 (Figs 3c and 5d). The efficiency of protein transduction appeared to be cell-type dependent and was less effective in PC12*puro* cells where higher doses were required for detection of the transduced protein. Consistent with other studies, we have not observed toxic effects in cultured cells exposed to TAT-Bcl-2 $\Delta$ loop at doses up to 400 nM (not shown).

Protein transduction results, however, in lower levels of protein when compared with cells that overexpress Bcl-2 from a stably transfected viral vector (PC12*bcl-2*) (Fig. 3c) and suggests that it would be difficult, at least for Bcl-2, to be transduced at levels comparable with those obtained by genetic approaches.

### Mechanism of intracellular delivery of TAT-Bcl-2 $\Delta$ loop. Role of heparan sulfate proteoglycans

The experiments above suggested that intracellular delivery occur in neuronal cell lines. Several recent studies in non-neuronal cells re-evaluated the mechanism of PTD-mediated



**Fig. 3** Transduction of TAT-Bcl-2Δloop protein. (a) Transduction of TAT-Bcl-2Δloop protein into GT1-7puro neural cells was examined by treating the cells with either the vehicle (veh) or the recombinant protein at the indicated concentrations, for 30 min at 37°C. Equal amounts of cell lysates (25 μg) were separated by SDS-PAGE and analyzed by immunoblotting with an anti-Bcl-2 antibody. The blot was developed using enhanced chemiluminescence. Purified TAT-Bcl-2Δloop was loaded as a positive control (STD). The transduced protein was identified as an additional band immunoreactive with the anti-Bcl-2 antibody (arrow). A higher MW band (~26 kDa) corresponding to endogenous Bcl-2 was present in all lanes, and served as loading control. (b) TAT-Bcl-2Δloop (arrow) transduction into SH-SY5Y cells was performed as above for 1 h at 37°C. Cell lysates (25 μg) were analyzed by immunoblotting with an anti-Bcl-2 antibody. (c) The efficiency of transduction was examined by comparing the levels of TAT-Bcl-2Δloop (arrow) in PC12puro cell lysates (25 μg) after 1 h transduction at 37°C, with the levels of Bcl-2 expressed in stable transfected cells using an adenoviral expression vector (PC12bcl-2).

transduction, and indicated that cationic PTDs mediate a strong cell-surface association followed by endocytotic internalization (Lundberg *et al.* 2003; Richard *et al.* 2003). Artfactual uptake of PTDs and failure to translocate inside the cells were also reported (Falnes *et al.* 2001; Violini *et al.* 2002; Kramer and Wunderli-Allenspach 2003; Richard *et al.* 2003).

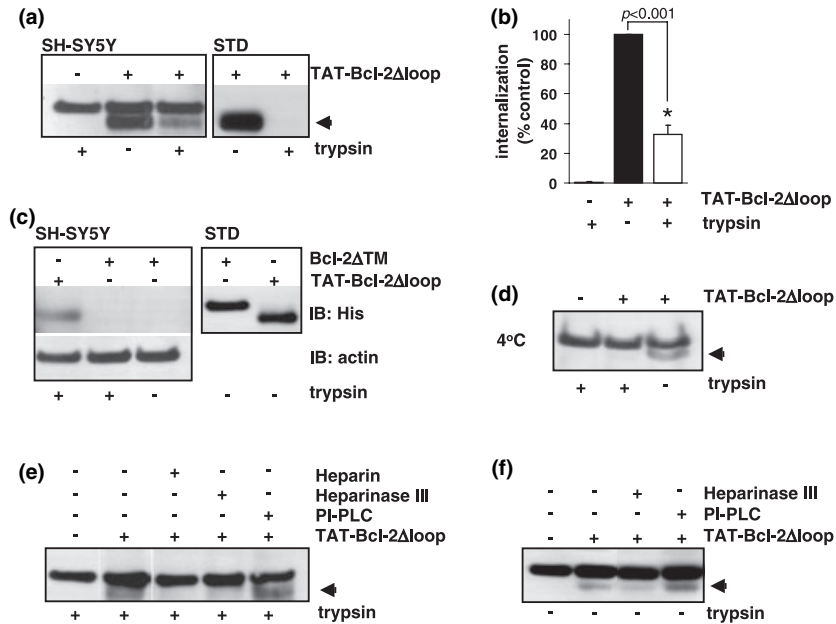
In order to verify if TAT-Bcl-2Δloop is internalized and to examine more accurately the efficiency of intracellular delivery, SH-SY5Y cells were treated with trypsin after transduction with TAT-Bcl-2Δloop, to remove the cell-surface-associated protein, then internalization was assessed by immunoblot analysis. A trypsin-resistant, Bcl-2 immunoreactive protein band is detected in cells transduced with

TAT-Bcl-2Δloop for 1 h at 37°C, demonstrating that the protein is indeed internalized (Fig. 4a). Trypsinization was effective as it resulted in complete degradation of the control TAT-Bcl-2Δloop protein, incubated in identical conditions in the absence of cells (Fig. 4a, STD). Trypsin treatment also indicated that a significant fraction of the protein remained bound to the cell surface (trypsin-sensitive). Only  $32.7 \pm 6.1\%$  ( $p < 0.001$ ) of the total cell-associated protein appeared to be delivered inside cells after 1 h at 37°C (Fig. 4b). To demonstrate the specificity of transduction, the cells were also incubated in the same conditions (1 h, 37°C) with a recombinant Bcl-2ΔTM protein, lacking the TAT-PTD. No intracellular uptake of the Bcl-2ΔTM protein was detected after trypsinization. In contrast to TAT-Bcl-2Δloop, binding of Bcl-2ΔTM to cell surface was not detected using a standard chemiluminescence technique (Fig. 4c). Taken together, these experiments confirmed that indeed intracellular delivery of Bcl-2Δloop occurs in neuronal cells in a TAT-dependent manner.

In experiments aimed at evaluating cell surface binding, when the cells were incubated with TAT-Bcl-2Δloop at 4°C for 10 min, we observed that the recovered protein was completely trypsin sensitive (Fig. 5c). Extending the incubation time at 4°C to 1 h did not result in protein internalization. As shown in Fig. 4(d) despite binding to the cell surface, the protein was completely degraded by trypsinization. Because all forms of endocytosis are inhibited at low temperature, inhibition of intracellular delivery of TAT-Bcl-2Δloop at 4°C suggested the involvement of an endocytotic pathway rather than an energy-independent translocation across plasma membrane, as previously described for various PTDs.

Heparan sulfate proteoglycans (HSPG) are known to mediate the endocytotic uptake of various endogenous ligands such as FGF, lipoproteins, or internalization of bacteria (Belting 2003). HS and other cell-surface glycosaminoglycans (GAG) were also implicated in internalization of full-length HIV-1 TAT, PTDs and TAT-fusion proteins (Tyagi *et al.* 2001; Sandgren *et al.* 2002; Suzuki *et al.* 2002; Console *et al.* 2003), although some studies failed to confirm the HS requirement (Silhol *et al.* 2002; Violini *et al.* 2002).

The role of HSPG in TAT-Bcl-2Δloop intracellular delivery was examined in SH-SY5Y cells by using internalization and cell surface binding assays. Heparin, a negatively charged structural analogue of HS, binds to cationic PTDs including the TAT-PTD (Hakansson *et al.* 2001) and polyarginine (Fuchs and Raines 2004), and was tested as competitor for binding to HS. As shown in Fig. 4(e), co-incubation of TAT-Bcl-2Δloop with heparin resulted in inhibition of protein uptake. To examine more directly the requirement of HS, we used Heparinase III that preferentially degrades HS but not other GAG such as chondroitin sulfate



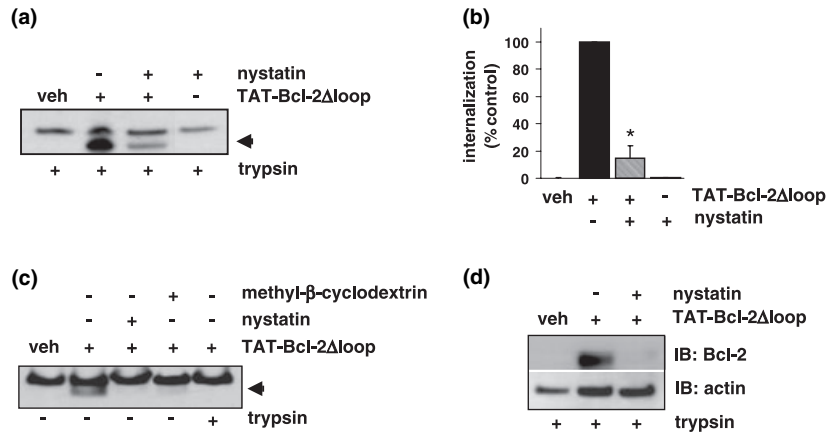
**Fig. 4** Mechanism of TAT-mediated protein internalization and role of HSPG. (a) To examine protein internalization, at the end of incubation with TAT-Bcl-2Δloop (100 nM; 1 h at 37°C), the cells were extensively washed in PBS and the cell-surface-associated protein was proteolytically removed by trypsinization. The efficiency of trypsinization was verified by digestion of TAT-Bcl-2Δloop in the same conditions, in the absence of cells (STD). Cell lysates (25 μg) were examined for internalization by immunoblotting with anti-Bcl-2. The blots were stripped and re-probed for actin as loading control. A significant fraction of TAT-Bcl-2Δloop was trypsin-resistant, demonstrating that the protein was internalized. (b) Scan densitometric analysis of TAT-Bcl-2Δloop internalization in four independent experiments performed as in (a) (1 h at 37°C). TAT-Bcl-2Δloop uptake was expressed as per cent of total cell-associated protein (without trypsinization). Results are mean ± SEM. \*Statistically significant difference ( $p < 0.001$ ) versus vehicle-treated cells (first column) by one-way ANOVA followed by Tukey post-hoc test. (c) TAT-Bcl-2Δloop (100 nM), but not Bcl-2ΔTM (100 nM) that lacks the TAT-PTD, is internalized in SH-SY5Y cells

after 1 h incubation at 37°C, indicating that internalization is TAT-dependent. (d) Internalization of TAT-Bcl-2Δloop at 4°C, was examined in SH-SY5Y cells by incubating the cells with 100 nM protein for 1 h at 4°C. TAT-Bcl-2Δloop (arrow) associates with the cells at 4°C, but is not internalized, as indicated by complete trypsin sensitivity. (e) To examine the role of HSPG in internalization, SH-SY5Y cells were treated with TAT-Bcl-2Δloop (100 nM, 1 h at 37°C) in the presence of heparin (20 μg/mL). HS and GPI-linked proteins were proteolytically removed by exposure to heparinase III (0.05 U/mL) or PI-PLC (0.2 U/mL), respectively, for 1 h at 37°C. The cells were then incubated with TAT-Bcl-2Δloop (100 nM; 1 h at 37°C). The cell-surface-associated protein was removed by trypsinization and the lysates examined by Bcl-2 immunoblotting. TAT-Bcl-2Δloop is indicated by arrow. (f) To evaluate the role of HSPG in cell-surface binding of TAT-Bcl-2Δloop, the cells were pretreated as in (e) with heparinase III or PI-PLC, then exposed to TAT-Bcl-2Δloop (100 nM) for 10 min at 4°C and lifted non-enzymatically from the plates. Cell-bound TAT-Bcl-2Δloop (arrow) was examined by anti-Bcl-2 immunoblotting.

(CS), to proteolytically remove HS from the cell surface. As shown in Fig. 4(e and f), pretreatment of SH-SY5Y cells with Heparinase III resulted in a significant decrease of cell-surface binding and almost complete inhibition of internalization of TAT-Bcl-2Δloop. This result confirmed the requirement of HS and indicated that binding of TAT-Bcl-2Δloop through electrostatic interactions to the negatively charged cell surface HS initiates subsequent internalization.

HS is ubiquitously expressed on most cells, including neurons, and is present mainly on two classes of membrane-associated proteoglycans (PG), syndecans and GPI-linked proteoglycans (glypicans) (Bandtlow and Zimmermann 2000). Although the requirement of specific GAG such as HS and CS for TAT-mediated protein delivery has been

recognized previously (Tyagi *et al.* 2001; Suzuki *et al.* 2002; Wadia *et al.* 2004) the differential requirement of GPI-linked PG (glypicans) versus syndecans has not been previously examined. In order to distinguish between the two classes of HSPG potentially involved in the first step of internalization, the cells were exposed to PI-PLC, which specifically degrades the lipid anchor of GPI-linked proteins such as glypicans. In contrast to Heparinase III treatment, PI-PLC treatment was unable to reduce cell binding or internalization of TAT-Bcl-2Δloop (Figs 4e and f). Proteolytic digestion by PI-PLC was shown previously to be effective in SH-SY5Y in conditions used here (Shen *et al.* 1995). These results indicated that, in neuronal cells, presence of glypicans is not required for internalization, and that other HSPG such as syndecans might be involved.



**Fig. 5** Effect of lipid raft disruption on TAT-Bcl-2Δloop intracellular uptake. (a) Lipid rafts were disrupted by treating SH-SY5Y cells with the cholesterol-sequestering agent nystatin (50 μg/mL) for 1 h at 37°C then the cells were incubated with TAT-Bcl-2Δloop (200 nM) for 1 h at 37°C. TAT-Bcl-2Δloop internalization (arrow) was examined by Bcl-2 immunoblotting. Disruption of lipid rafts by nystatin results in inhibition of intracellular uptake of TAT-Bcl-2Δloop. (b) Band intensities of the internalized TAT-Bcl-2Δloop from four independent experiments were analyzed densitometrically, normalized to endogenous Bcl-2 and expressed as per cent protein uptake from the amount transduced in the absence of nystatin (mean ± SEM). \*Statistically significant difference ( $p < 0.05$ ) versus TAT-Bcl-2Δloop alone by one-way ANOVA followed by Tukey post-hoc test. (c) The effect of lipid raft disruption on

TAT-Bcl-2Δloop cell-surface binding was examined in cells pretreated as above with nystatin (50 μg/mL, 1 h) or with methyl-β-cyclodextrin (5 mM, 30 min). Cell binding was assessed after 10-min incubation with TAT-Bcl-2Δloop at 4°C, by immunoblotting with anti-Bcl-2. As indicated by the complete trypsin sensitivity, TAT-Bcl-2Δloop was not internalized after 10 min at 4°C (last lane). (d) The internalization and the effect of lipid raft disruption were examined in primary rat cortical neurons exposed to TAT-Bcl-2Δloop (200 nM, 1 h at 37°C) with or without pretreatment with nystatin (50 μg/mL). Cells were trypsinized and analyzed by immunoblotting with an anti-Bcl-2 antibody as above. TAT-Bcl-2Δloop internalization is inhibited by nystatin. The blots were stripped and re-probed for actin as loading control.

### Lipid raft requirement for TAT-Bcl-2Δloop intracellular delivery

Lipid rafts are plasma membrane microdomains, rich in sphingolipids, cholesterol and GPI-linked proteins, that have important roles in neural development and signal transduction (Tsui-Pierchala *et al.* 2002). Lipid rafts presence was demonstrated in neural cells, including SH-SY5Y (Hering *et al.* 2003; Vacca *et al.* 2004). Disruption of lipid rafts by cholesterol sequestration or depletion affects both caveolar and non-caveolar endocytotic pathways (e.g. macropinocytosis) but not clathrin-mediated endocytosis (Nichols and Lippincott-Schwartz 2001).

Lipid raft dependence of TAT-PTD-mediated protein uptake in neuronal cells has not been previously examined. To test this possibility, the cells were exposed to the TAT-Bcl-2Δloop with or without pretreatment with several lipid raft-disrupting agents, including nystatin and methyl-β-cyclodextrin. The cell-surface-bound protein was removed as above by trypsinization after exposure to TAT-Bcl-2Δloop. As indicated in Fig. 5(a and b), pretreatment with nystatin (50 μg/mL) that sequesters plasma membrane cholesterol, resulted in an almost complete inhibition ( $85.5 \pm 9.05\%$ ,  $p < 0.001$ ) of internalization. Treatment with methyl-β-cyclodextrin (5 mM), a cholesterol-depleting agent, resulted in approximately 60% inhibition of TAT-Bcl-2Δloop internalization in these cells (not shown). Protein internalization

and the effect of lipid raft disruption were also examined in primary rat cortical neurons. TAT-Bcl-2Δloop is efficiently internalized in cortical neurons after 1 h at 37°C, and reaches higher levels than endogenous Bcl-2 that is expressed only at low levels (Fig. 5d). As in SH-SY5Y cells, internalization was inhibited by disruption of lipid rafts with nystatin (50 μg/mL) (Fig. 5d). These results suggest that TAT-Bcl-2Δloop internalization occurs through a lipid raft-dependent endocytotic process in neural cells.

We also examined if lipid raft disruption affects TAT-Bcl-2Δloop association with the cell surface. In these experiments, the cells were incubated with TAT-Bcl-2Δloop at 4°C for 10 min, then lifted from the plate non-enzymatically. As shown in Fig. 5(c), complete digestion of cell-associated TAT-Bcl-2Δloop by trypsin (last lane), indicated that the protein was not internalized and verified the validity of this assay for assessing surface binding of the protein. Membrane association was inhibited in cells pretreated with both nystatin and methyl-β-cyclodextrin (Fig. 5c).

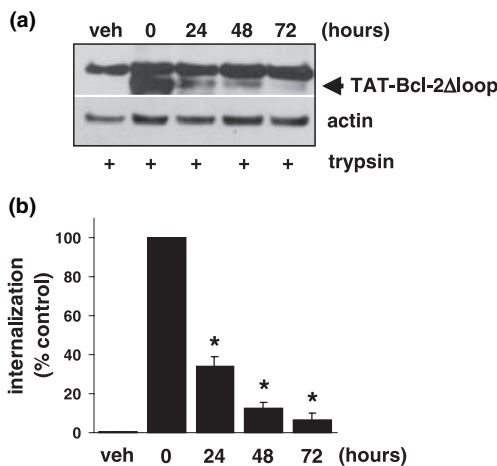
### Stability of internalized TAT-Bcl-2Δloop protein

Endocytotic internalization could result in very rapid degradation of the protein (e.g. in lysosomes). Therefore, we examined the stability of TAT-Bcl-2Δloop after delivery to SH-SY5Y cells. For these experiments, the cells were incubated with TAT-Bcl-2Δloop (200 nM) for 1 h at 37°C.

After 1 h the cells were extensively washed then further incubated in the absence of the protein for various time periods (up to 72 h). The amount of protein present intracellularly was determined by Bcl-2 immunoblotting, using trypsinization to remove the cell-surface protein. As indicated in Fig. 6, a progressive decline of intracellular levels of TAT-Bcl-2 $\Delta$ loop is observed. After 24 h,  $33.9 \pm 5.1\%$  ( $p < 0.001$ ) of the initial amount of TAT-Bcl-2 $\Delta$ loop is present intracellularly and at low levels the protein is still detectable at 72 h. This relatively slow degradation rate compared with other TAT fusion proteins, e.g. TAT-FNK with an half-life of approximately 2 h (Asoh *et al.* 2002) is consistent with that of endogenous Bcl-2, that has an estimated half-life of  $\sim 12$ –36 h (Reed 1996).

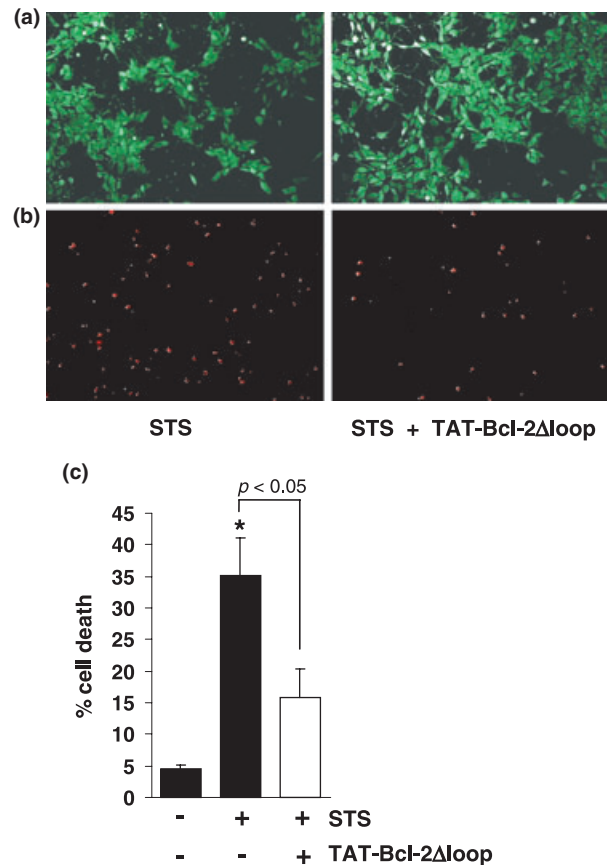
### TAT-Bcl-2 $\Delta$ loop protects neuronal cell lines from cell death

The functionality of transduced TAT-Bcl-2 $\Delta$ loop protein was examined next by evaluating its ability to protect against apoptosis induced by various stimuli. Apoptosis was induced in the SH-SY5Y cells, by treatment with  $0.1 \mu\text{M}$  staurosporine (STS) for 18 h. Pretreatment with the TAT-Bcl-



**Fig. 6** Stability of internalized TAT-Bcl-2 $\Delta$ loop protein. (a) The stability of TAT-Bcl-2 $\Delta$ loop in SH-SY5Y cells was examined after a 1 h incubation with the protein (200 nM) at  $37^\circ\text{C}$ . The cells were then extensively washed in PBS, switched to media without TAT-Bcl-2 $\Delta$ loop and further incubated for up to 72 h. Protein stability was examined by immunoblotting with an Bcl-2 antibody, by determining the amount of TAT-Bcl-2 $\Delta$ loop (arrow) present intracellularly at 24, 48 and 72 h, compared with the initial amount at the end of the incubation with the protein (0 h). The blots were stripped and re-probed for actin as loading control. Cell surface bound protein was eliminated by trypsinizing the cells. (b) TAT-Bcl-2 $\Delta$ loop bands from three independent experiments were quantified densitometrically, normalized to actin and the results expressed as per cent of protein present intracellularly from the initial amount (0 h). Error bars are SEM. A progressive decline in the level of TAT-Bcl-2  $\Delta$ loop is detected. \*Statistically significant differences ( $p < 0.001$ ) versus the initial amount at 0 h, by one-way ANOVA followed by Tukey post-hoc test.

2 $\Delta$ loop for 1 h before exposure to STS resulted in approximately 50% protection against cell death ( $p < 0.05$ ) (Fig. 7). A similar protection against STS-induced apoptosis was also obtained in PC12 cells (data not shown). These results indicated that, despite endocytotic internalization, the transduced protein was biologically active. Notably, the transduced TAT-Bcl-2 $\Delta$ loop significantly enhanced the survival of SH-SY5Y at transduced protein levels comparable (or lower) with the endogenous level of Bcl-2, but much lower than those obtained in cells overexpressing Bcl-2 (Fig. 3c), in which an increased protection is reported under similar conditions (Yuste *et al.* 2002, and our unpublished observa-



**Fig. 7** Protection of SH-SY5Y cells from STS-induced death by TAT-Bcl-2 $\Delta$ loop. SH-SY5Y cells were treated with TAT-Bcl-2 $\Delta$ loop (100 nM) or the vehicle for 1 h at  $37^\circ\text{C}$  then exposed to  $0.1 \mu\text{M}$  staurosporine (STS). Cell viability was determined after 18 h by using the live/dead assay as described in Materials and methods. Representative fluorescence microscopy images are shown in (a) for Calcein-AM ( $4 \mu\text{M}$ ) staining of viable cells and in (b) for ethidium homodimer ( $1 \mu\text{M}$ ) staining of dead cells 18 h after addition of STS, with or without pretreatment with TAT-Bcl-2 $\Delta$ loop. (c) The per cent of cell death for each condition, in four independent experiments, is expressed as mean  $\pm$  SEM. \*Statistically significant difference ( $p < 0.05$ ) versus untreated cells (first column) by one-way ANOVA followed by Tukey post-hoc test.

tions). Increased protection through deletion of the loop domain of Bcl-2 has been previously demonstrated in transfected non-neural cells, but not examined in neurons (Chang *et al.* 1997; Srivastava *et al.* 1999). Since wild-type TAT-Bcl-2 protein was more difficult to express, studies using transfected Bcl-2 constructs are underway to test the hypothesis that loop deletion also enhances Bcl-2 protection in neuronal cells.

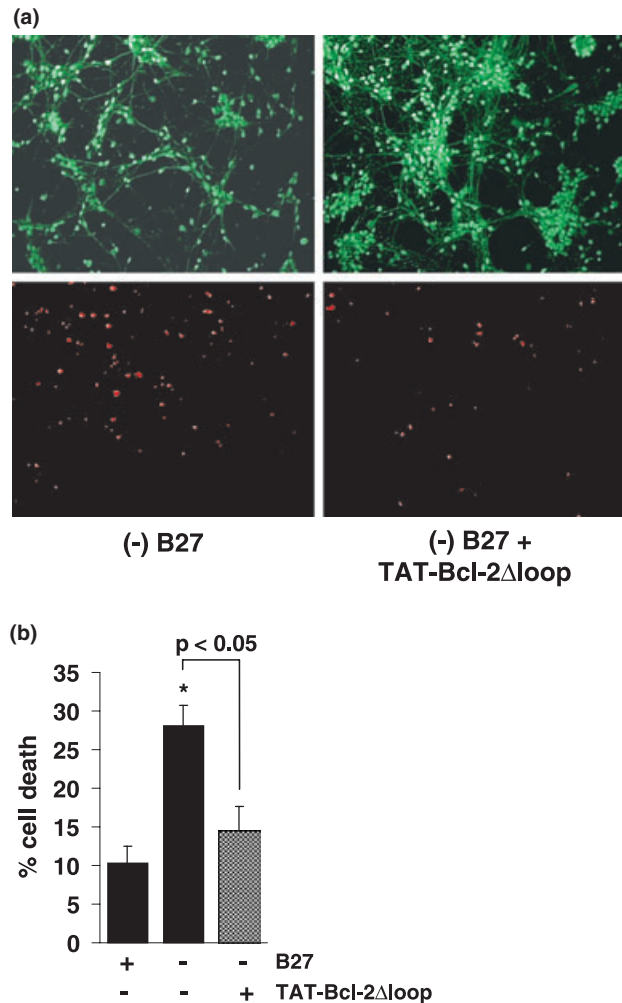
#### TAT-Bcl-2 $\Delta$ loop protects primary cultured neurons against cell death

The protective effect of TAT-Bcl-2 $\Delta$ loop was also tested using primary rat cortical neurons in a model of trophic factor withdrawal-induced death. As shown in Fig. 8, cortical neurons cultured in the absence of the serum-free supplement B27, undergo cell death. The effect of TAT-Bcl-2 $\Delta$ loop was examined without pretreatment, by adding the protein upon exposure of cells to B27-free medium. As shown in Fig. 8, transduction of cortical neurons with 100 nM TAT-Bcl-2 $\Delta$ loop resulted in significant protection against death of cortical neurons after 24 h ( $p < 0.05$ ). TAT-Bcl-2 $\Delta$ loop can therefore transduce multiple neural cell lines and primary neurons in a functional form, despite evidence for endocytotic uptake. In addition, the ability of Bcl-2 to protect neuronal cells in different cell death paradigms is not lost when the loop domain is deleted.

#### Discussion

The plasma membrane constitutes a major obstacle for intracellular delivery of macromolecules. PTD-mediated delivery of biologically active proteins in cells and in animals has therefore received increasing attention. The ability of TAT-PTD to deliver proteins into cells and across the blood-brain barrier into the brain suggests a potential strategy for delivery of therapeutic molecules as an alternative to gene-based neuroprotective approaches. The mechanisms involved in PTD-mediated internalization of protein cargoes into neurons and other brain cells are, however, not known.

We generated a TAT-fusion protein with the anti-apoptotic Bcl-2 protein using a mutated construct with the loop domain deleted, and examined the mechanisms involved in protein internalization, and the neuroprotective potential of this transducible Bcl-2. This protein and several other Bcl-2 constructs were successfully expressed and purified using the C41(DE3) and C43(DE3) *E. coli* known to facilitate expression of toxic proteins (Arechaga *et al.* 2000). We demonstrated that TAT-Bcl-2 $\Delta$ loop is capable of entering several neuronal cell lines and primary neurons. Although numerous studies report successful transduction, the actual demonstration that the proteins are internalized is not always provided. It was suggested recently that TAT-mediated protein internalization might be an artifact, as a result of strong affinity of



**Fig. 8** Protection of cortical neurons by TAT-Bcl-2 $\Delta$ loop. (a) The effect of TAT-Bcl-2 on trophic factor deprivation was examined in rat primary cortical neurons cultured in neurobasal medium (NBM) in the absence of the B27 supplement [(-) B27] for 24 h. TAT-Bcl-2 $\Delta$ loop (100 nM) was added to the culture medium immediately after withdrawal of B27. Cell survival was examined after 24 h by using the live/dead assay by incubating the cells with Calcein-AM (4  $\mu$ M; upper panel – viable cells) and ethidium homodimer (1  $\mu$ M; lower panel – dead cells). (b) TAT-Bcl-2 $\Delta$ loop protection against B27 withdrawal was expressed quantitatively by counting the ethidium and calcein positive cells and the results of three independent experiments are expressed as mean  $\pm$  SEM. \*Statistically significant difference ( $p < 0.05$ ) versus untreated cells (first column) by one-way ANOVA followed by Tukey post-hoc test.

cationic PTDs for cell surface constituents (Lundberg *et al.* 2003; Richard *et al.* 2003). The possibility that TAT-Bcl-2 $\Delta$ loop only associates with the cell surface was examined using treatment of cells with trypsin (Fig. 4). Our results clearly indicate that the TAT-Bcl-2 $\Delta$ loop protein is internalized in a TAT-PTD specific manner at 37°C, and also suggest that internalization in neuronal cells occurs through an

endocytotic pathway, as protein uptake into cells was not detected at 4°C, despite cell-surface binding.

While an endocytotic mechanism for uptake of full-length HIV-1 TAT protein was proposed in 1991 (Mann and Frankel 1991) the TAT-PTD and Antennapedia peptides were shown to 'transduce' cells even at 4°C, in an energy- and receptor-independent manner (Derossi *et al.* 1996; Vives *et al.* 1997). This mechanism was questioned recently and evidence for endocytotic uptake and endosomal sequestration was reported (Lundberg *et al.* 2003; Richard *et al.* 2003). In non-neuronal cells, involvement of a lipid raft-dependent caveolar pathway was demonstrated for internalization of the full-length HIV-1 TAT and a TAT-GFP fusion protein (Fittipaldi *et al.* 2003). However, caveolae were not identified in some cells, notably in lymphocytes and neurons (Razani *et al.* 2002). Therefore, TAT-mediated internalization might be cell specific. Indeed, in lymphocytes, Wadia *et al.* (2004) demonstrated the involvement of an alternate, lipid-raft-dependent, macropinocytotic pathway.

While internalization of full-length HIV-1 TAT in neurons occurs through a LRP receptor-dependent pathway, this possibility is unlikely for the TAT-PTD as binding of TAT to LRP involved a different domain of TAT than its PTD (Liu *et al.* 2000). Instead, we found here that TAT-PTD-mediated protein delivery in neurons occurs through a lipid raft-dependent endocytotic process. Using two different lipid raft-disrupting agents that either sequester or extract cholesterol, a marked inhibition of TAT-Bcl-2Δloop internalisation was observed in SH-SY5Y cells and cortical neurons (Fig. 5). Because lipid raft disruption does not affect clathrin-mediated endocytosis, involvement of this pathway appears unlikely. Our attempts to exclude a clathrin-dependent pathway using chlorpromazine (10–100 μM), and macropinocytosis using the specific inhibitor amiloride (100 μM–1 mM), were limited, however, by the high toxicity of these compounds. No inhibition of TAT-Bcl-2Δloop internalization was observed at doses tolerated by the cells (20 μM chlorpromazine and 1 mM amiloride; not shown) and, although further studies will be needed, these observations suggest that in neurons TAT-mediated protein internalization might occur through a raft-dependent pathway distinct from that described by Wadia *et al.* (2004) in lymphocytes.

TAT-delivered proteins and PTDs were also found to co-localize partially with markers of receptor-mediated endocytosis and, in one study, in cortical neurons, the transduced proteins were inactive and sequestered in endosomes (Sengoku *et al.* 2004). More than one pathway could mediate internalization of PTD-fused proteins, and their involvement modulated by factors such as the type and the amount of proteoglycans expressed on cells and the properties of the cargo protein. These characteristics are shared by cholera toxin, that is found in detergent-resistant domains at the plasma membrane and can be internalized by both clathrin-dependent and -independent pathways, but is active

only when endocytosed through non-clathrin raft-dependent endocytosis, leading to Golgi localization (Nichols and Lippincott-Schwartz 2001; Shogomori and Futerman 2001). A recent study indicates that the TAT-PTD share sequence similarities with bacterial toxins that use the retrograde transport system to reach the ER and Golgi (Fischer *et al.* 2004). Interestingly, four of the five arginine-rich motifs reported in that study belong to toxins or proteins known to utilize a non-clathrin-mediated and cholesterol-sensitive endocytotic pathway for internalization.

Heparan sulfate proteoglycans (HSPG) can mediate endocytotic uptake of various ligands such as basic FGF, lipoproteins and bacteria (Belting 2003), and are implicated in internalization of PTDs such as TAT-PTD and polyarginine or TAT-fused proteins (Tyagi *et al.* 2001; Suzuki *et al.* 2002; Wadia *et al.* 2004). Competitive inhibition of TAT-Bcl-2Δloop internalization by heparin, and reduction of cell-surface binding and internalization by proteolytic removal of HS from the cell surface with a HS specific GAG lyase (Heparinase III), strongly indicates that binding to HS is a required step in TAT-Bcl-2Δloop uptake. Therefore, the major TAT-mediated import pathway is apparently HSPG-dependent in neuronal cells.

HS is present on cells on two main classes of membrane-anchored PG, syndecans and glypicans. Expression of HSPG is developmentally regulated, and PG of both types are expressed on neurons (Bandtlow and Zimmermann 2000). The relative involvement of the two PG families has not been investigated, and was addressed here by selective removal of glypicans from the cell surface using PI-PLC to digest the lipid anchor of glypicans. Although glypican-1 can function as molecular carrier and mediates polyamine uptake (Belting *et al.* 2003), our results suggest that glypicans are not essential for TAT-Bcl-2Δloop transduction, as PI-PLC digestion did not significantly affect internalization or cell binding. Glypicans are enriched in lipid rafts and are therefore logical candidates for raft-dependent internalization of TAT-Bcl-2Δloop; however, syndecans also mediate raft-dependent internalization (Fuki *et al.* 2000; Chu *et al.* 2004). For TAT-Bcl-2Δloop, lipid raft disruption also reduced cell-surface binding (Fig. 5). A similar effect was observed for FGF2 binding to HSPG and explained by increased binding affinity after clustering of HSPG in lipid rafts (Tkachenko and Simons 2002). Ligand-induced clustering of syndecans was also shown to induce raft-dependent endocytosis (Fuki *et al.* 2000). Our results suggest that like FGF2, TAT fusion proteins might also induce HSPG (possibly syndecans) clustering as a prerequisite for internalization. In support of this, the TAT-PTD was, indeed, shown to induce aggregates on the cell surface in an HS-dependent manner (Ziegler *et al.* 2005).

Despite evidence of endocytosis, numerous proteins are delivered in a functional form (reviewed in Dietz and Bahr 2004), indicating that at least partial release of TAT-fused proteins from endosomes occurs. This also appears to be the

case for Bcl-2 family proteins as both Bcl-2 (this study) and Bcl-X<sub>L</sub> appear to be functional after transduction (Asoh *et al.* 2002; Cao *et al.* 2002; Kilic *et al.* 2002). PTD-induced endosome disruption (Fuchs and Raines 2004), constitutive leakage (particularly from macropinosomes) and retrograde transport to Golgi and subsequent cytosolic exit through an unknown pathway (Fischer *et al.* 2004), are potential mechanisms of cytosolic delivery. The efficacy of PTDs to promote endosomal escape of proteins appears, however, limited (Sengoku *et al.* 2004). Alternatively, the degradation products of the carrier HS might facilitate endosomal escape (Belting *et al.* 2003).

Another possible explanation for the transduction of biologically active Bcl-2 proteins is that they represent a class of proteins that can facilitate their own endosomal escape. A comparable protection against STS-induced death was reported for PTD-FNK, even when used at lower concentrations (30 pM to 3 nM) than TAT-Bcl-2 $\Delta$ loop. FNK is a mutated Bcl-X<sub>L</sub> with enhanced channel-forming properties and was also more protective than PTD-Bcl-X<sub>L</sub> (Asoh *et al.* 2002). The ability of Bcl-2 family proteins to form ion channels or pores in membranes (Schendel *et al.* 1998) suggests that TAT-Bcl-2 escape from endosomes can occur in a manner similar to that of pore forming toxins such as diphtheria toxin (DT), with which Bcl-2 proteins share structural homology. Channel formation by Bcl-2 proteins also depends on low pH, and therefore might be activated after endosomal acidification (Schendel *et al.* 1998). The mechanism of endosomal release of transduced proteins, remains a critical issue, to be addressed in future studies.

This study is the first to demonstrate that loop-deleted Bcl-2, when delivered via protein transduction, results in significant protection against cell death in both neural cell lines and primary cortical neurons. These results support the concept of using protein transduction of modified Bcl-2 constructs for neuroprotection. Our results also indicate that, in neuronal cells, a lipid raft- and HSPG-dependent endocytotic mechanism is involved in TAT-mediated protein transduction. This finding, and those of other labs, suggests that further development of protein transduction techniques by facilitating endosomal release of proteins may be essential for this technique to translate into an efficient therapeutic approach for neuroprotection.

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