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The vimentin-tubulin binding site peptide (Vim-TBS.58-81) crosses the plasma membrane and enters the nuclei of human glioma cells

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ABSTRACT

Cell-penetrating peptides (CPPs) can translocate through the plasma membrane and localize in different cell compartments providing a promising delivery system for peptides, proteins, nucleic acids, and other products. Here we describe features of a novel cell-penetrating peptide derived from the intermediate filament protein vimentin, called Vim-TBS.58-81. We show that it enters cells from a glioblastoma line via endocytosis where it distributes throughout the cytoplasm and nucleus. Moreover, when coupled to the pro-apoptogenic peptide P10, it localizes to the nucleus inhibiting cell proliferation. Thus, the Vim-TBS.58-81 peptide represents an effective vector for delivery of peptides and potentially a broad range of cargos to the nucleus.

1. Introduction

The first cell-penetrating peptide (CPP) was described more than 20 years ago launching the concept of protein transduction domains. Frankel and Pabo (1988) showed that the transactivating Tat protein of HIV-1 can cross the plasma membrane and activate a viral promoter. Thereafter, the group of Lebleu identified Tat amino acids 48–60 as the minimal sequence with capacity to enter cells (Vives et al., 1997). The Tat.48-60 peptide was shown later to translocate through the plasma membrane and localize to the nuclei of glioma cells like U251, T98G (glioblastoma, grade IV) and U373 (astrocytoma, grade III) (Baker et al., 2007; Darbinian et al., 2001; Lo and Wang, 2008).

Peptides able to enter neurons and localize to nuclei also have been described including penetratin, corresponding to the third helix of Antennapedia homeodomain (Derossi et al., 1994). To deliver oligonucleotides to the nucleus, Morris et al. (1997) used the artificial CPP, MPG that contains a hydrophobic domain derived from the fusion sequence of HIV gp41 and a hydrophilic domain derived from the nuclear localization sequence of SV40 T-antigen. Futaki et al. (2001) demonstrated that chimeric cationic peptides, composed of several arginines (called polyArg: 8 or 9 Arg residues) also were able to deliver different molecules into cells. Such CPPs have been used to transport a variety of cargos including oligonucleotides (Astriab-Fisher et al., 2002), liposomes (Torchilin et al., 2003), complete proteins (Snyder and Dowdy, 2005) and protein fragments (Niesner et al., 2002) into different cellular compartments.

We demonstrated previously that intermediate filaments have the capacity to bind unpolymerized tubulin at discrete *tubulin-binding sites* (TBSs). Peptides corresponding to some of these TBS, like the NFL derived NFL-TBS.40-63 peptide, are able to enter glioma cells where they disrupt the microtubule network and induce apoptosis (Bocquet et al., 2009). In this report we describe the properties of another TBS peptide, Vim-TBS.58-81, derived from the intermediate filament, vimentin. Vim-TBS.58-81 enters T98G human glioblastoma cells and distributes to both the cytoplasmic and the nuclear compartments. However, it does not affect the microtubule network. Using well-established cell biology techniques (Richard et al., 2003), we show that Vim-TBS.58-81 enters cells by an energy dependant process. Additionally, we ligated

Abbreviations: ATP, adenosine tri-phosphate; BSA, bovine serum albumin; CF, carboxyfluorescein; CPP, cell penetrating peptides; Ctx-B, cholera toxin subunit B; DAPI, 4′,6′-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagles medium; DMSO, dimethylsulfoxide; FACS, fluorescence activated cell sorting; HIV, human immunodeficiency virus; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 tetrazolium; MβCD, methyl-β-cyclodextrin; NFL, neurofilament light subunit; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; PFA, paraformaldehyde; PI, propidium iodide; PTD, protein transduction domain; TAT, trans-activator of transcription; TBS, tubulin-binding site.

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the Vim-TBS.58-81 peptide to the P10 peptide, a pro-apoptogenic fragment of p21/WAF1 protein (Baker et al., 2007; Mutoh et al., 1999; Warbrick et al., 1995), and show that it crosses the plasma membrane and localizes to the nucleus where P10 maintains its pro-apoptogenic activity. In contrast, when the P10 peptide was ligated to the Tat.48-60 peptide, entry to the nucleus was strongly reduced and its pro-apoptogenic activity was attenuated. These results demonstrate that Vim-TBS.58-81 can be a useful vector capable of maintaining the desirable properties of certain molecular cargos.

2. Materials and methods

2.1. Materials

Labeled peptides were synthesized by Millegen (Toulouse). Human glioma T98G cells were obtained from ATCC (Manassa, VA, USA). Cells were cultured in 60 mm dishes at 37 °C under 5% CO_2 in DMEM (Lonza, France) supplemented with 10% fetal calf serum, 5% L-glutamine (Lonza, France) and 5% penicillin/streptomycin (Lonza, France) and passaged every 2–3 days.

2.2. Immunocytochemistry and fluorescence microscopy

Cells were plated on coverslips one night before experiment, and cultured in medium containing biotinylated peptides for 6 h. After washing in PBS, the cells were fixed for 10 min in 4% paraformaldehyde (PFA in PBS), and washed three times in PBS. The cells were then incubated for 10 min in a 0.5% Triton X-100 permeabilization solution, and washed three times in PBS. Cells were incubated in a blocking solution of 5% BSA in PBS for 15 min followed by mouse anti-β-tubulin antibody (Sigma) at 1/200 overnight. Tubulin and biotinylated peptides were localized using respectively Alexa 568 nm anti-mouse antibody or streptavidin Alexa 488 nm (Molecular Probes) at 1/200 for 1 h, followed by washing in PBS. The preparations were counterstained with 3 μM 4′,6′-diamidino-2-phenylindole (DAPI; Sigma) for 5 min, washed twice with PBS and coverslips were mounted with an antifading solution. The cell staining was visualized using a Leica DMI6000 inverted microscope and images were acquired with a CoolSNAP HQ2 camera and analyzed with Metamorph 7.1.7.0. software. A minimum of 200 cells were

Table 1
Name and sequence of synthesized biotin and carboxyfluorescein-peptides.

Peptide	Sequence
Vim-TBS.58-81	Biot-GGAYVTRSSAVRLRSSVPGVRLLQ-$CONH_2$
CF-Vim-TBS.58-81	CF-GGAYVTRSSAVRLRSSVPGVRLLQ-$CONH_2$
Tat.48-60	Biot-GRKKRRQRRRPPQ-$CONH_2$
CF-Tat.48-60	CF-GRKKRRQRRRPPQ-$CONH_2$
P10	Biot-RQTSMTDFYHSKRRLIFS-$CONH_2$
Vim-TBS.58-81-P10	Biot-GGAYVTRSSAVRLRSSVPGVRLLQRQTSMTDFYHSKRRLIFS-$CONH_2$
Tat.48-60-P10	Biot-GRKKRRQRRRPPQRQTSMTDFYHSKRRLIFS-$CONH_2$

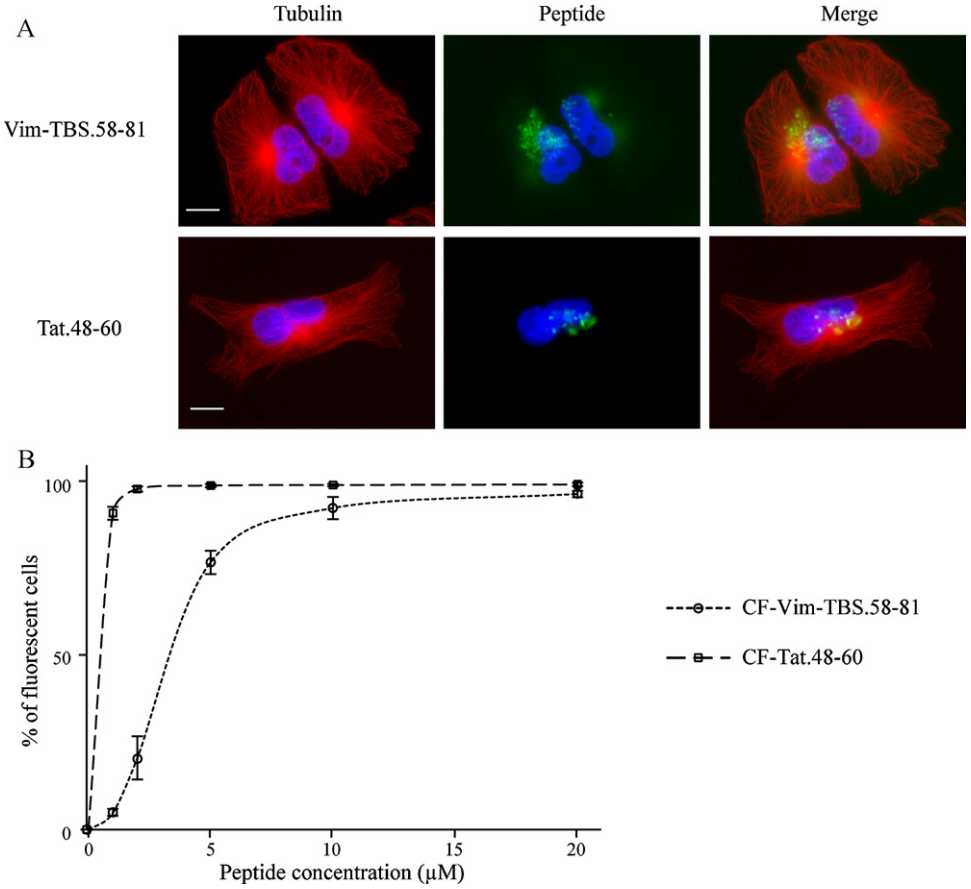

Fig. 1. Cytoplasmic and nuclear localization of Vim-TBS.58-81 and Tat.48-60 peptides in T98G cells. (A) Fluorescent microscopy of T98G cells incubated for 6 h at 37 °C in the presence of biotinylated Vim-TBS.58-81 (50 μM) or Tat.48-60 (20 μM). Localization of biotinylated peptides was revealed with streptavidin Alexa 488 (green fluorescence). The microtubule network was revealed using anti-tubulin immunocytochemistry (red fluorescence). Nuclei were stained with DAPI (blue fluorescence). Images were obtained using a Leica DMR 6000 inverted microscope and analyzed using MetaMorph software. Scale bar = 20 μm. (B) T98G cells were incubated for 1 h with different concentrations of carboxyfluorescein-tagged Vim-TBS.58-81 or Tat.48-60 peptides and fluorescence measured using a FACSCalibur flow cytometer (Becton Dickinson). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

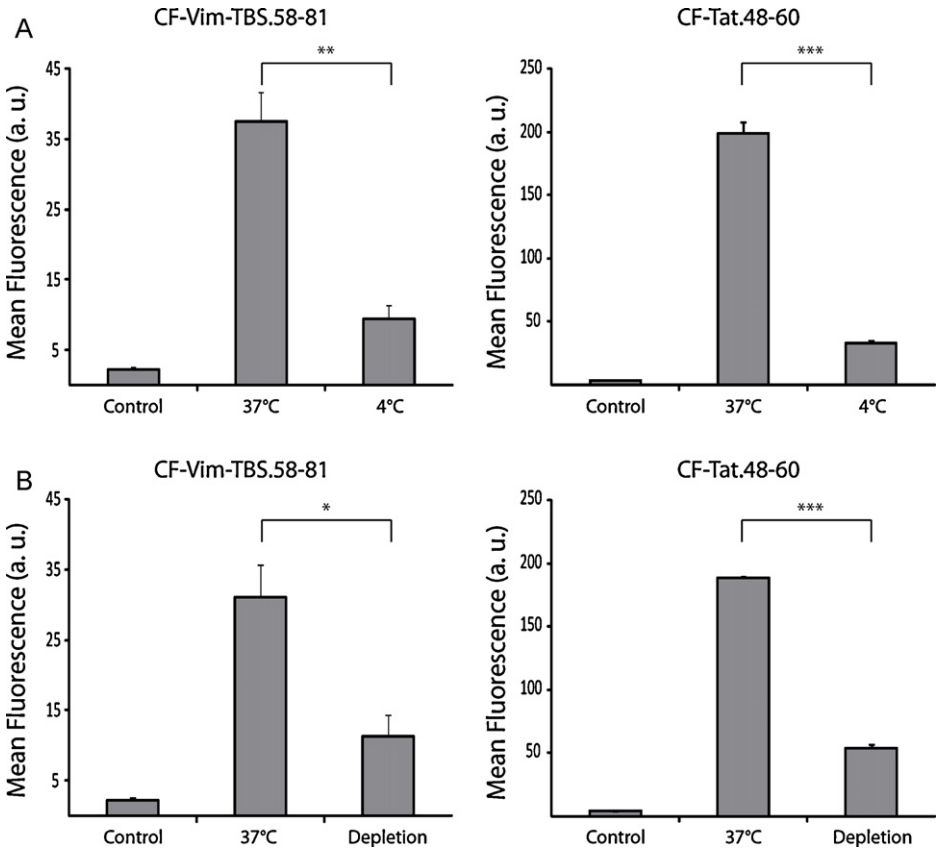

Fig. 2. Uptake of both Vim-TBS.58-81 and Tat.48-60 peptides by T98G cells is temperature and energy-dependant. (A) Incorporation of carboxyfluorescein-tagged Vim-TBS.58-81 (50 μM) or carboxyfluorescein-tagged Tat.48-60 (10 μM) following incubation for 1 h at 4 °C or 37 °C. The mean fluorescence and standard deviations are the results of three independent experiments. (B) Depletion of cellular ATP inhibits uptake of Vim-TBS.58-81 and Tat.48-60. Flow cytometric analysis of cells incubated with CF-Vim-TBS.58-81 (50 μM) or CF-Tat.48-60 (10 μM) for 30 min at 37 °C, with or without ATP depletion via a 30 min pre-incubation in 10 mM sodium azide and 6 mM deoxyglucose. Average fluorescence intensities and standard deviations are the results of three independent experiments. Statistically significant differences were evaluated by Student's *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

scored for peptide incorporation, and experiments were repeated three times.

2.3. Peptide uptake analysis by flow cytometry

Cells were plated in 6 well plates (4–5 × 10^5 cells/well), and after 24 h treated with carboxyfluorescein-peptides at different concentrations for 1 h at 37 °C. Subsequently, cells were washed two times with PBS. To dissociate cells and to release peptide attached to membrane, they were then treated with Trypsin-EDTA 1× (Sigma) for 15 min. After two washes and centrifugation for 5 min at 2000 × *g*, cells were re-suspended in 350 μL of PBS containing 5 μL propidium iodide (1 mg/mL, Sigma) and subjected to FACS analysis (FACSCalibur, Becton Dickinson). Twenty thousand events per sample were analyzed and experiments were repeated three times. Cells stained with propidium iodide were excluded from analysis.

To investigate the entry mechanism, cells were depleted of intracellular ATP by incubating for 30 min with 6 mM 2-deoxy-D-glucose and 10 mM sodium azide for 30 min prior to incubation with the peptide. In another set of experiments, we attempted to alter plasma membrane rigidity and reduce endocytosis by incubating cells at 4 °C starting 1 h prior to incubation with the peptide.

2.4. Study of the colocalization of Vim-TBS.58-81 with cholera toxin or transferrin by confocal microscopy

Cholera toxin subunit B conjugated to AlexaFluor 555 nm (Ctx-AlexaFluor555; Molecular Probes) (10 μg/mL) associates with lipid rafts and is internalized by caveolae. Transferrin from human serum conjugated to AlexaFluor 568 nm (Transferrin-AlexaFluor568; Molecular Probes) (25 μg/mL) associates with transferrin receptor and is internalized from clathrin-coated invaginations. To determine if Vim-TBS.58-81 colocalizes with the cholera toxin subunit B or transferrin during cell entry, cells were seeded on coverslips and co-incubated in media for 1 h with CF-Vim-TBS.58-81 peptide (50 μM) and Ctx-AlexaFluor555 (10 μg/mL), or with CF-Vim-TBS.58-81 peptide (50 μM) and Transferrin-AlexaFluor568 (25 μg/mL). After washing in PBS, the cells were fixed for 10 min in 2% paraformaldehyde (PFA in PBS), and washed three times in PBS before mounting with an antifading solution. The stained cells were visualized using a Zeiss LSM 700 confocal microscope, and images were analyzed with the Zen 2009 software.

For specific caveolar endocytosis inhibition experiment, methyl-β-cyclodextrin (MβCD) is used to disrupt lipid rafts by removing cholesterol from membranes. Cells were pretreated 30 min with MβCD (10 μg/mL in serum free medium) before treatment with the CF-Vim-TBS.58-81 peptide and Ctx-AlexaFluor555.

2.5. Analysis of cell proliferation

The effects of the P10 peptide fused or not to the Vim-TBS.58-81 peptide (Vim-TBS.58-81-P10) or to the Tat.48-60 peptide (Tat.48-60-P10) on the growth rate of T98G glioma cells were determined by using the MTS proliferation assay (Promega). 500 cells were seeded in 96-well plates, incubated at 37 °C for 24 h, and treated with either vehicle (PBS) or the indicated concentrations of the compounds for 72 h. Media and compounds were replaced daily.

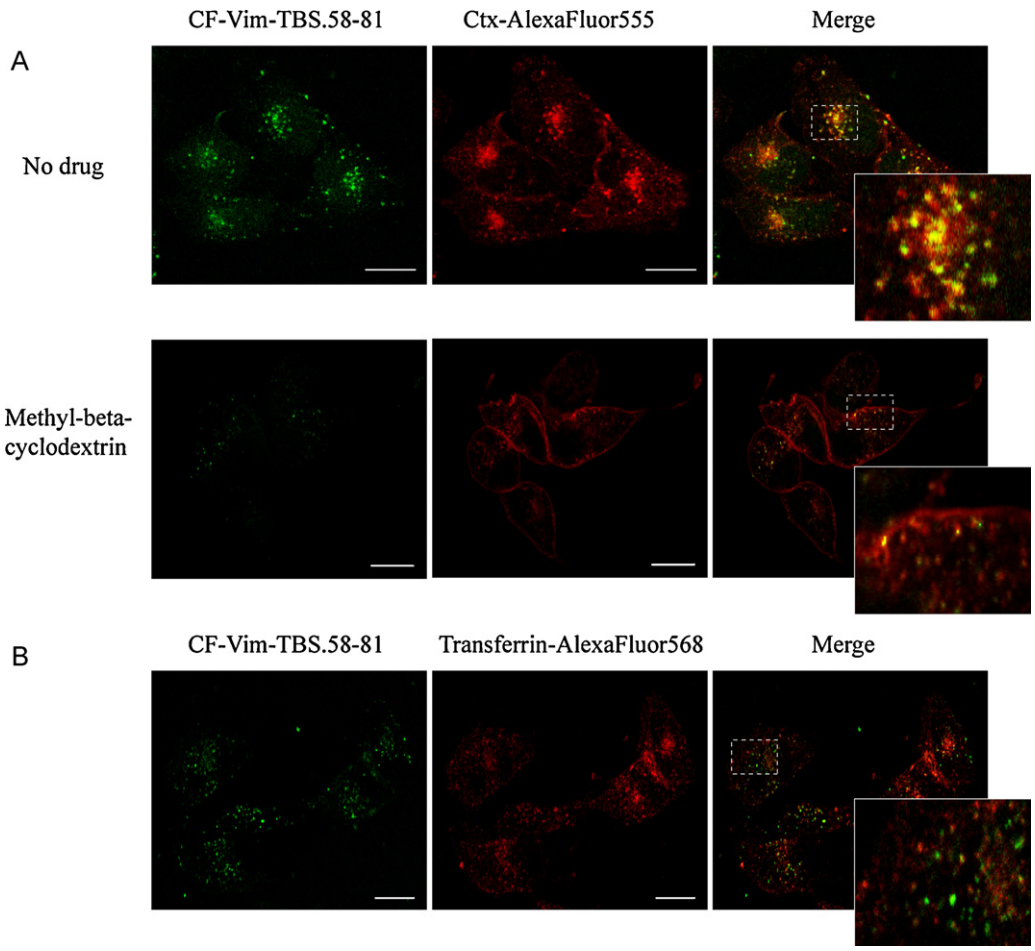

Fig. 3. Internalization of Vim-TBS.58-81 occurs by lipid raft-dependant endocytosis. (A) Confocal microscopy of T98G cells incubated with CF-Vim-TBS.58-81 (50 μM) and AlexaFluor 555-labeled cholera toxin B (Ctx-AlexaFluor555, 10 μg/mL) for 1 h, with or without methyl-beta-cyclodextrin (MβCD, 10 mg/mL). (B) Confocal microscopy of T98G cells incubated with CF-Vim-TBS.58-81 (50 μM) and AlexaFluor 568-labeled transferrin (Transferrin-AlexaFluor586, 25 μg/mL) for 1 h. Cells were fixed and analyzed by Zeiss LSM700 confocal microscope, using Zen 2009 software. Scale bar = 20 μm.

Peptides were prepared in DMEM, and Paclitaxel was dissolved in DMSO at a concentration of 2 mM and further diluted into DMEM. MTS produce color change upon bioreduction with accumulation measured at 490 nm proportional to the number of living cells in culture (Barltrop et al., 1991; Cory et al., 1991). Each condition was repeated three times and the results averaged.

The peptides used in this study are shown in Table 1. Biotinylated-peptides were used for fluorescence microscopy and MTS/PMS assays while carboxyfluorescein-labeled peptides were used for FACS analysis and confocal microscopy.

3. Results

3.1. Vim-TBS.58-81 and Tat.48-60 peptides distribute to the cytoplasm and nuclei of T98G cells

We demonstrated previously that the NFL derived NFL-TBS.40-63 peptide is internalized by T98G cells where it remains in the cytoplasm and disrupts the microtubule cytoskeleton (Bocquet et al., 2009). Here, to determine if a tubulin binding peptide derived from another intermediate filament has the same properties, we evaluated Vim-TBS.58-81. Both NFL-TBS.40-63 and Vim-TBS.58-81 were readily detectable in T98G cells by fluorescence microscopy, following a 6 h incubation. In contrast to the cytoplasmic distribution and microtubule disruption caused by NFL-TBS.40-63 internalization, Vim-TBS.58-81 was found both in the cytoplasm and the nucleus and had no obvious effect on the microtubule network (Fig. 1A).

To evaluate the dynamics of internalization and intracellular distribution, we compared Vim-TBS.58-81 with Tat.48-60, a peptide known to enter T98G cells and to localize to nuclei (Darbinian et al., 2001; Lo and Wang, 2008). A FACS based dose-dependant uptake study revealed that both Vim-TBS.58-81 and Tat. 48-60 uptakes followed a sigmoidal curve. Similarly, at 10 μm peptide concentration, more than 92% of the cells incorporated the Vim peptide while 99% incorporated Tat.48-60 (Fig. 1B).

3.2. The uptake of Vim-TBS.58-81 and Tat.48-60 peptides is temperature and energy-dependent

To investigate the mechanism of peptide entry, the uptake of labeled peptides under different temperature and energy conditions was assessed using flow cytometry. At reduced temperature (4 °C vs. 37 °C), where numerous properties of the plasma membrane are affected, the proportion of cells labeled following a 1 h incubation with carboxyfluorescein-labeled Vim-TBS.58-81 or Tat.48-60 was reduced to 25% and 16% respectively (Fig. 2A).

As the temperature dependent decrease could result from changes in membrane properties, reduced availability of energy or a combination of both, we next evaluated the consequences of depleting the cellular ATP pool in cells maintained at 37 °C. Cells were treated for 30 min with sodium azide and deoxyglucose prior

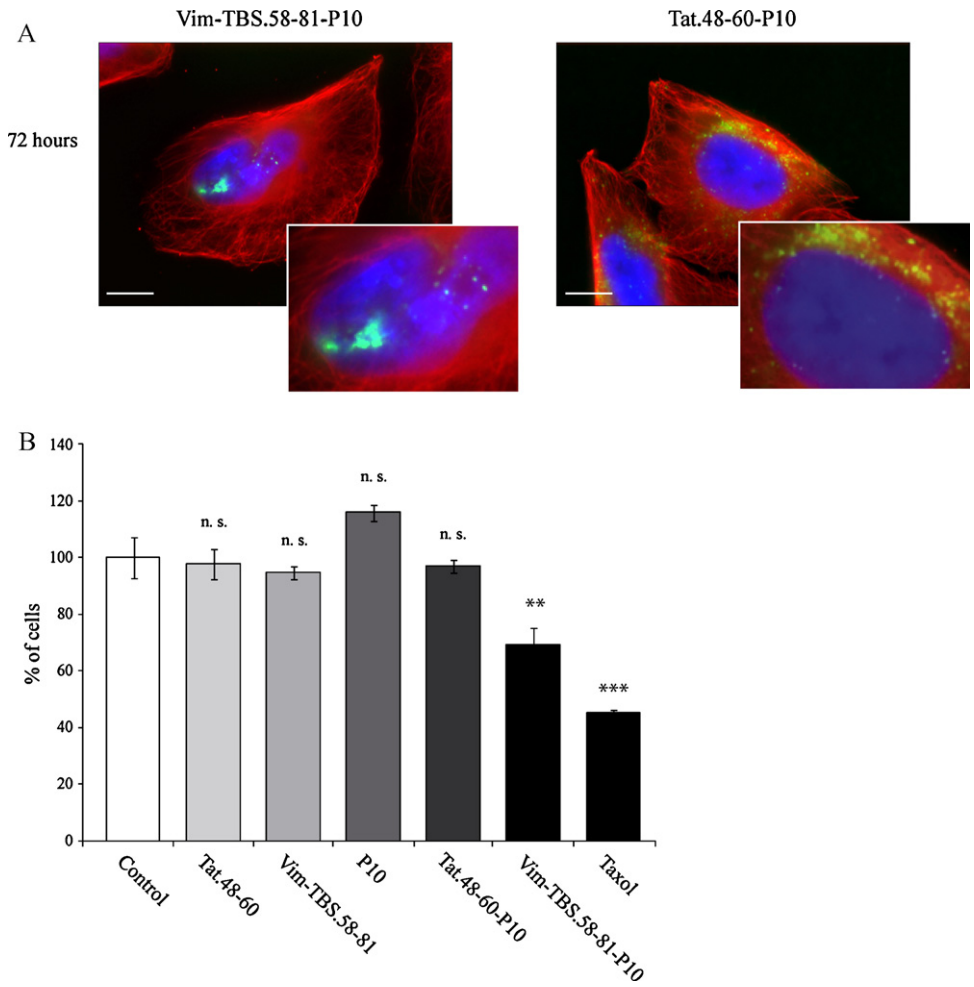

Fig. 4. In vitro effects of Vim-TBS.58-81-P10 and Tat.48-60-P10 peptides on the T98G population present at 72 h. (A) Fluorescent confocal microscopy of fixed T98G cells incubated with Vim-TBS.58-81-P10 or Tat.48-60-P10 (100 μM) for 72 h at 37 °C. Scale bar = 20 μm. (B) Effects of peptides on viability of T98G cells. Cells were treated with different peptides (100 μM) or Taxol (40 nM) for 72 h. Taxol was used as positive control. Statistically significant differences compared to control were evaluated by Student's *t*-test (n. s.: not significant; $^{**}p < 0.01$; $^{***}p < 0.001$).

to peptide exposure (Fig. 2B). Such ATP depletion reduced peptide uptake to 37% and 28% for Vim-TBS.58-81 and Tat.48-60 respectively consistent with an uptake process that is heavily energy dependent.

3.3. Vim-TBS.58-81 internalization occurs through the caveolar endocytic pathway

The Tat.48-60 peptide was shown previously to associate with lipid rafts and to be internalized through the caveolar endocytic pathway in numerous cell lines including HeLa, Cos-1 and CHO (Ferrari et al., 2003; Fittipaldi et al., 2003). This strategy of internalization can be inhibited by cholesterol depletion using methyl-β-cyclodextrin, a drug that extracts cholesterol from cell membranes (Yancey et al., 1996). Moreover, cholera toxin B interacts with the ganglioside GM1 present in plasma membrane and can be used as a marker of lipid rafts and caveoli (Heyningen, 1974). Transferrin is internalized from clathrin-coated invaginations that can detach to form clathrin-coated vesicles and endosomes (Hanover et al., 1984; Marsh et al., 1995). Here, using immunocytochemistry and confocal microscopy, we show that CF-Vim-TBS.58-81 co-localizes with Ctx-AlexaFluor555 in treated T98G cells (Fig. 3A), and not with transferrin (Fig. 3B). Moreover, uptake of Vim-TBS.58-81 was strongly inhibited by treatment with MβCD (Fig. 3A). Both results are consistent with a lipid raft based endocytotic pathway supporting Vim-TBS.58-81 entry into T98G cells.

3.4. Vim-TBS.58-81-P10 prevents normal expansion of T98G cell population

p21/WAF1 is a cyclin-dependant kinase inhibitor. P10 is a peptide mimetic that can bind to PCNA and also inhibit kinase activity (Mutoh et al., 1999; Warbrick et al., 1995). When U251 human glioblastoma cells are exposed to a fusion peptide consisting of Tat.48-60 and P10, they internalize the peptide where it localizes to the nucleus and exerts cytotoxic effects (Baker et al., 2007). In contrast, unlike the predominantly nuclear accumulation of Tat.48-60-P10 in U251 cells, the fusion peptide accumulates primarily in cytoplasm of the T98G cells.

To explore the capacity of Vim-TBS.58-81 to transport the P10 peptide to the nucleus, we evaluated the capacity of T98G cells to accumulate and compartmentalize Vim-TBS.58-81-P10. Following incubation with Vim-TBS.58-81-P10 or Tat.48-60-P10 peptides for 72 h, internalization of both fusion peptides was observed and consistent with the distribution of Vim-TBS.58-81 alone Vim-TBS.58-81-P10 accumulated preferentially in the nuclei of T98G cells (Fig. 4A).

Following the demonstration that both Tat.48-60-P10 and Vim-TBS.58-81-P10 are internalized by T98G cells, we next evaluated

the expansion of peptide treated T98G cell populations using the MTS/PMS cell proliferation assay. Evaluation was performed following incubation in media containing peptides for 72 h. Exposure of T98G cells to 100 μM of Vim-TBS.58-81, Tat.48-60, P10 or Tat.48-60-P10 had no effect on the population size after 72 h of incubation (Fig. 4B). In contrast, exposure to 100 μM of the Vim-TBS.58-81-P10 fusion peptide induced a significant failure of the cell population to expand. As a positive control for the assay, other cultures were treated with 40 nM Taxol, a treatment known to inhibit cell proliferation.

4. Discussion

In this study, we investigated the features of the tubulin binding peptide (Vim-TBS.58-81) derived from the intermediate filament protein vimentin (Bocquet et al., 2009). Like other TBS peptides derived from the diverse family of intermediate filament proteins, it is internalized by T98G glioblastoma cells. Once internalized, it translocates to the nuclear compartment though an unknown mechanism and its interactions, if any, with endogenous nuclear components remain to be illuminated. However, both neurofilaments and vimentin contact nuclear pores (Metuzals and Mushynsk, 1974; Traub et al., 1983, 1985) and have been shown to interact with RNA and DNA (Traub et al., 1983, 1985). The amino acids 1–95 sequence of vimentin, that overlap the Vim-TBS.58-81 peptide, has been shown to bind DNA through electrostatic interactions or a stacking/intercalation mechanism (Shoeman et al., 1999). Regardless of the mechanism used, the nuclear localizing capacity of Vim-TBS.58-81 suggests that it may have utility as a novel vector capable of delivering a variety of cargo molecules into the nuclei of diverse cell types.

The mechanisms through which CPPs are internalized remain somewhat controversial and many parameters including both the composition and secondary structure of the peptide may be involved (Heitz et al., 2009).

Consistent with its global positive charge, Vim-TBS.58-81 initially associates with the plasma membrane (data not shown). As a strong decrease in its intra-cellular accumulation was observed when cells were incubated at 4 °C or when their ATP pool was depleted, an energy-dependant mechanism of internalization is suggested. Further, confocal microscopy revealed that cholera toxin B, a well-known marker of lipid rafts that also tracks caveolar endocytosis, co-localized with Vim-TBS.58-81 during internalization. Thus, our collective observations suggest that a major route of Vim-TBS.58-81 entry is through the energy dependent but clathrin independent endocytic pathway.

Finally, we show that a fusion between Vim-TBS.58-81 and the pro-apoptogenic P10 peptide accumulates and distributes in glioblastoma cells on the schedule predicted by internalization of Vim-TBS.58-81 alone. Moreover, internalization of Vim-TBS.58-81-P10 prevents normal proliferation of T98G cells. Interaction between P10 and PCNA interfere with DNA polymerase delta by competition for the same binding site, thus inhibiting DNA replication (Flores-Rozas et al., 1994; Maga and Hubscher, 2003). It is noteworthy that the Tat.48-60-P10 fusion peptide remains largely in the cytoplasm of T98G cells and has minimal or no effect on their proliferation. The different consequences of the 2 fusion peptides could result from the differences observed in their sub-cellular localizations or from difference in fusion peptide properties caused by the significantly different charge properties of Vim-TBS.58-81 and Tat.48-60. Regardless of the underlying mechanism, the results of this investigation suggest that Vim-TBS.58-81 may provide an effective vector for peptide delivery into cells that are resistant to the Tat peptide delivery system.

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