Photo-acceleration of protein release from endosome in the protein transduction system

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Abstract The protein transduction system has been employed for delivery of bioactive proteins into cells via an endocytotic mechanism. However, trapping of endocytosed proteins in the endosome may significantly attenuate biological actions in cells. The present investigation demonstrated that endosomal release of transduced protein could be artificially accelerated by exposure to fluorescent light. Exposure to light at 480 nm stimulated endosomal release of transduced FITC-11 arginine-protein transduction domain (11R-PTD), TAT-PTD and Antennapedia-PTD. Moreover, FITC-11R-p53 protein was released from endosomes following stimulation with light. These data suggest that photo-acceleration is a more efficient strategy in terms of the protein transduction system.

Keywords: Photo; FITC; Endosomal release; Protein transduction; Protein transduction domain

1. Introduction

Peptidic drugs and/or therapeutic proteins typically are molecules characterized by small size consequent to limitations associated with cell entry [1]. However, protein transduction systems have been employed for delivery of full-length proteins in rapid, concentration-dependent fashion into many cell types [2–15]. A series of small domains, referred to as protein transduction domains (PTDs, also known as cell-penetrating peptides; CPPs), have been shown to cross biological membranes efficiently and to promote the delivery of peptides and proteins into cells. The Antennapedia homeodomain, a Drosophila transcription factor, or its third helix, can deliver many peptides, phosphopeptides and oligonucleotides into cells [2,3]. Similarly, TAT protein from human immunodeficiency virus (HIV-1) is able to deliver biologically active proteins in vitro and in vivo [4,5]; furthermore, VP22 protein from Herpes simplex virus (HSV) also promotes delivery of covalently linked peptides or proteins into cells [6,7]. Poly-arginine and poly-lysine exhibit even greater efficiency in terms of delivery of several peptides and proteins [8–12]. Studies by our group as well as by others have demonstrated that these protein transduction systems are effective experimental and protein therapy strategies [2–15].

Early investigations regarding the mechanism of internalization by PTDs and PTD-fused proteins revealed that internalization was not attenuated significantly by incubation at 4 °C, by depletion of the cellular ATP pool or by inhibitors of endocytosis, which indicated that internalization of PTD did not involve endocytosis or specific protein transporters [16–18]. However, recent studies suggested that import and nuclear localization of protein involving PTDs occurred during fixation and not in living cells [19,20]. Other investigations indicated that the majority of PTD uptake in living cells resulted from initial rapid electrostatic interaction with the plasma membrane followed by endocytotic uptake [21,22]. A recent study clearly demonstrated that TAT-PTD was internalized by lipid raft-dependent macropinocytosis [23]. However, a large number of published reports documented PTD-mediated delivery of biologically active cargo, including proteins, peptides and oligonucleotides. These studies suggested the occurrence of partial endosomal release of these conjugates.

To improve endosomal escape, photochemical reactions are initiated by photosensitization of compounds localized in endocytic vesicles, inducing rupture of these vesicles upon light exposure [24]. The present investigation documented the artificial acceleration of endosomal release of transduced fluorescence peptides and proteins by photo-excitation with respect to the protein transduction system. This technique could provide a more efficient protein transduction strategy.

2. Results

2.1. Transduction of PTDs by endocytosis

In order to examine the mechanism of internalization of PTDs into living cells, cervix-derived HeLa cells were treated with FITC-conjugated 11 arginine-PTD (11R-PTD), TAT-PTD and/or Antennapedia-PTD (Antp-PTD) (green fluorescence) with FM 4–64, an endocytotic marker (red fluorescence).

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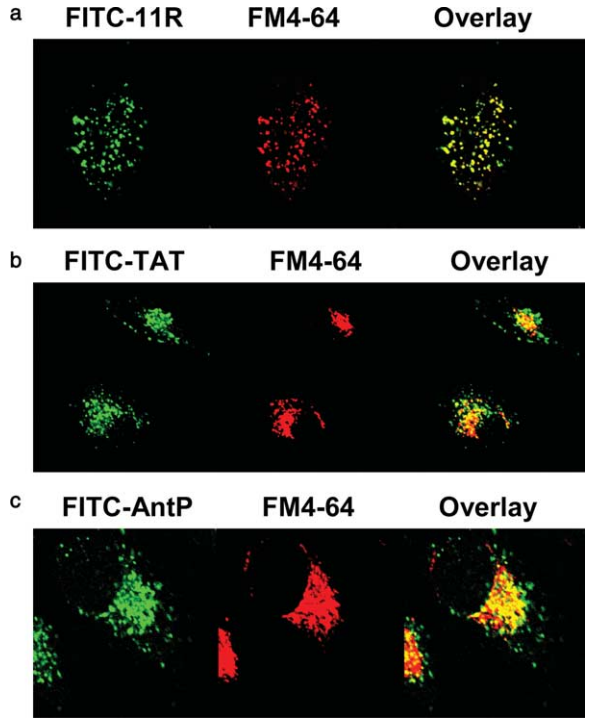

Fig. 1. Transduction of PTDs by endocytosis. HeLa cells were treated with 1–10 μM FITC-conjugated 11R-PTD (a), TAT-PTD (b) and Antp-PTD (c) (green fluorescence; left panels) with FM 4–64, an endocytotic marker, (red fluorescence; middle panels) for 30 min. The right panels are overlays of these figures, i.e., the merger of both red and green channels. Treated living cells were analyzed via confocal microscopy.

Thirty minutes after treatment, punctuated cytoplasmic distribution of the peptides was observed as a fluorescent signal in living unfixed HeLa cells (Fig. 1, left panels). PTDs predominantly co-localized with FM 4–64 in living cells as indicated by the resultant yellow coloration of most punctuated vesicles (Fig. 1, overlay). These data suggest that endocytosis plays a major role in the uptake of PTDs.

2.2. Photo-acceleration of peptide release from endosomes

During these experiments, transduced peptide was artificially released from endosomes upon stimulation with light at 480 nm. Thirty minutes after treatment with PTDs, punctuated cytoplasmic distribution of 11R-PTD (Fig. 2), TAT-PTD and Antp-PTD (data not shown) was observed in living cells. However, subsequent exposure of these cells to light at 480 nm for 30 s, followed by an additional 5-min incubation, led to dramatic alteration of the distribution of PTDs. PTDs were released from endosomes; however, homogeneous distribution throughout the cells was evident exclusively in areas exposed to light (Fig. 2(a) and (b)). These data suggest that endosomal release of PTDs is accelerated by photo-activation. To examine the cell toxicity of this method, FITC-11R treated cells were exposed to light at 480 nm for 30 s. These cells were observed after 8 h in the presence of propidium iodide (PI) (Fig. 2(c)). The cells did not show the red fluorescence. Taken together, these data showed that photo acceleration of PTDs could be use without cell toxicity.

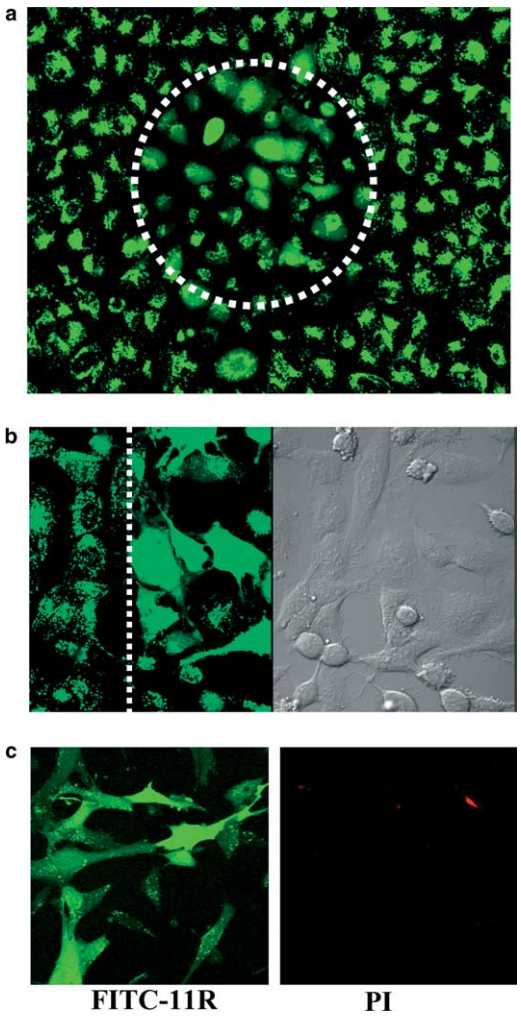

Fig. 2. Photo-acceleration of release of transduced peptide from endosomes. HeLa cells were treated with 1 μM FITC-conjugated 11R-PTD for 30 min. Treated living cells were analyzed via confocal microscopy. These cells were exposed to light at 480 nm for 30 s exclusively in circled areas, followed by an additional 5-min incubation (a). High-magnification with a DIC image of the same field (b). Cells within the circle exposed to light exhibited homogeneous fluorescence following the release of transduced peptide from endosomes.

2.3. Real-time observation of peptide release

Peptide release from endosomes was observed in real-time. HeLa cells were treated with 1–10 μM 11R-PTD, TAT-PTD and Antp-PTD for 30 min. PTDs translocated across the membrane due to endocytosis (Fig. 3(a)–(c), 0 min). Following exposure to light at 480 nm, the fluorescent peptide extended markedly from endosomes; moreover, fluorescence became more evenly distributed in nuclei and cytoplasm (Fig. 3(a)–(c), 10 min and graphs). This enhanced release was not induced by light at either 340 or 540 nm (Fig. 3(d)). Released FITC-peptides in the cytosol exhibited much greater fluorescent intensity than those in endosomes. This enhancement of fluorescent intensity may reflect impaired fluorescence of endosomally trapped FITC-peptides as a consequence of the low pH environment present within endosomes. In the primary neural cells, punctuated appearance of transduced peptides was observed initially, followed by alteration to a more homogeneous

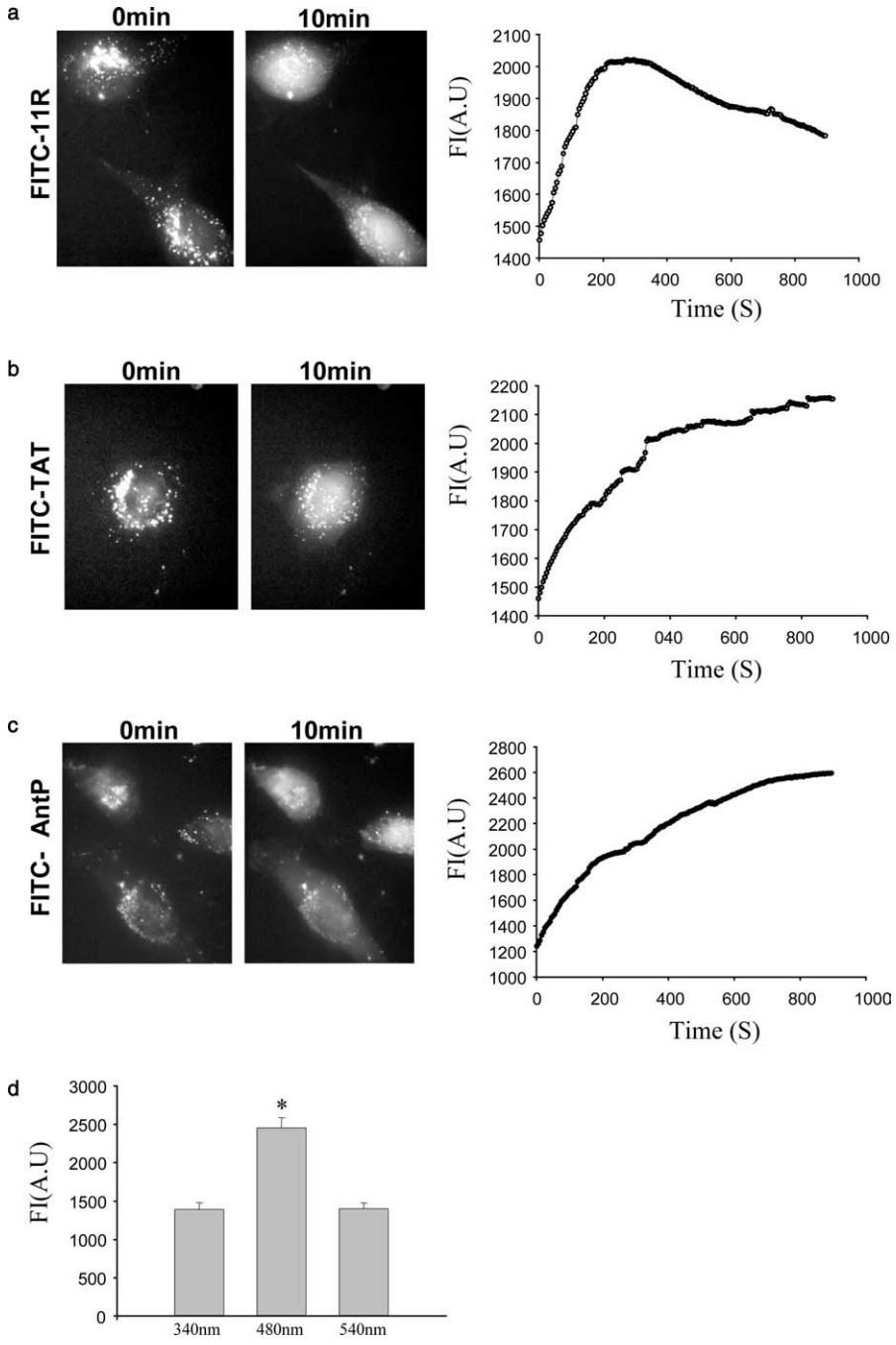

Fig. 3. Real-time observation of endosomal peptide release in HeLa. HeLa cells were treated with 1–10 μM FITC-conjugated 11R-PTD (a), TAT-PTD (b) and Antp-PTD (c) for 30 min; subsequently, cells were exposed continuously to light at 480 nm. Treated cells were analyzed every 5 s via conventional fluorescence microscopy. Fluorescent intensity (FI), which was measured with AquaCosmos software (Hamamatsu Photonics), is presented for each field. Effect of several wavelengths on endosomal release (d). HeLa cells were treated with 1 μM FITC-conjugated 11R-PTD for 30 min and exposed to light at 340, 480 or 540 nm for 30 s. Treated cells were analyzed in real-time as noted above. Cells exhibiting normal morphology were considered with respect to acquisition of fluorescent intensity per cell; furthermore, analysis was performed utilizing the entire soma of individual cells as the region of interest (ROI). The fluorescent intensity of 10 Hela cells was counted; five sets of experiments were conducted. Background fluorescent intensity was subtracted from all experiments. $^{*}P < 0.01$.

distribution upon stimulation with light at 480 nm (Fig. 4(a) arrow, c and d). Uptake of PTDs was evident in the same areas of the neuron (Fig. 4(c) and (d)). These data suggest the presence of hot spots in terms of uptake of PTDs. The fluorescence in the whole cell body including the nucleus increased constantly following release from the endosome (Fig. 4(b)). These findings suggest that light at 480 nm stimulates the accelerated endosomal release of PTDs; furthermore, released PTDs assume a homogeneous distribution in the cytoplasm and the nucleus.

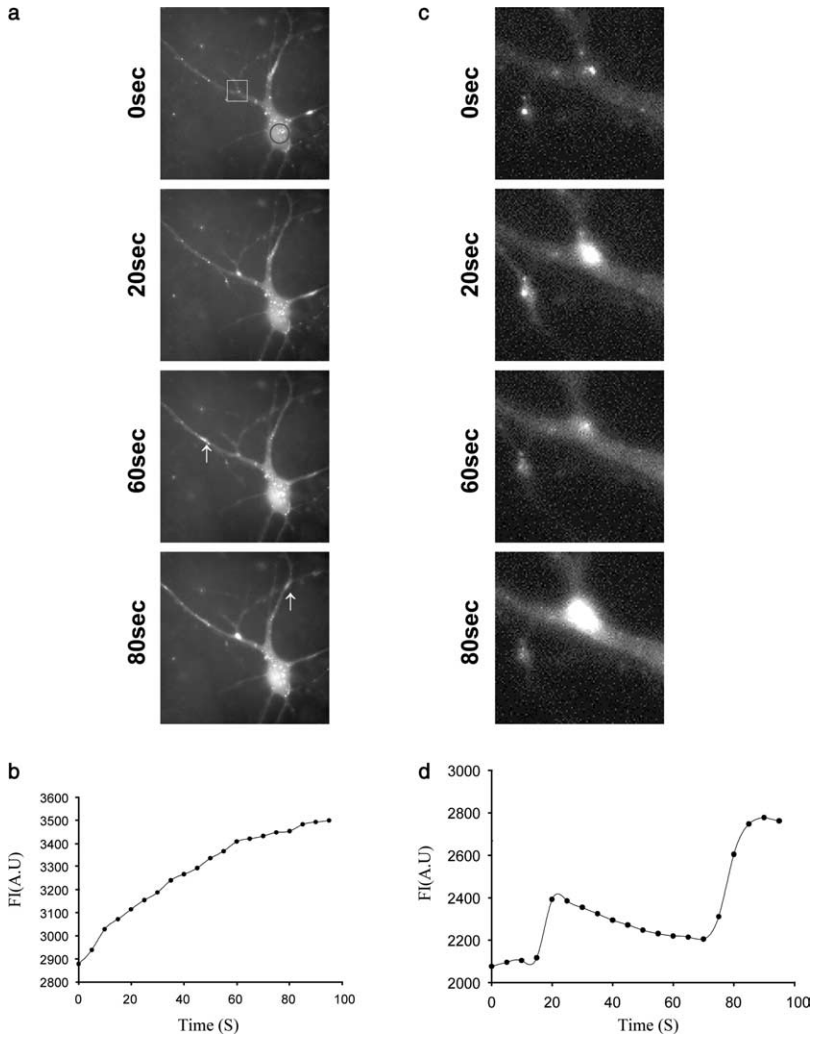

Fig. 4. Real-time observation of 11R-PTD release from endosomes in primary neural cells. Primary neural cells were treated with 1 μM FITC-conjugated 11R-PTD for 30 min; subsequently, cells were exposed continuously to light at 480 nm. Treated cells were analyzed every 5 s via conventional fluorescence microscopy. Arrows indicate the occurrence of endocytosis of PTD; the PTD is released immediately (a). Nuclear fluorescent intensity (FI) (red circles) revealed a continuous increase in fluorescence with time (b). High-magnification of the square in figure (a) is shown in (c). Fluorescent intensity of the square area is presented in (d).

2.4. The effect of 480-nm light stimulation on PTD-fused functional peptides and protein

In order to determine whether peptides/proteins released from endosomes operate as functional peptides, a nuclear localization signal was fused to the FITC-conjugated 11R (11R-NLS). HeLa cells were treated with FITC-11R-NLS for 30 min, followed by exposure to light at 480 nm for 30 s. Photo-exposed cells displayed nuclear localization of peptides relative to the number observed prior to photo-stimulation (Fig. 5(a); A and B). At higher magnification, fluorescence is clearly localized in nuclei as well as in endosomal vesicles (Fig. 5(a); C). These data suggest that transduced peptides released from endosomes are not degraded; rather, these peptides remain functional.

We next determined whether trapped protein release occurred as a result of photo-exposure. Glioma cells were treated with FITC-labeled 11R-p53 protein for 2 h, followed by exposure to light at 480 nm for 30 s. The punctuated appearance of transduced 11R-p53 was observed initially (Fig. 5(b); A), followed by alteration to a more homogeneous distribution upon stimulation with light at 480 nm (Fig. 5(b); B). At high magnification, predominant localization of p53 in the nucleus was apparent in some cells (Fig. 5(b); C). Cells did not exhibit red fluorescence typical of propidium PI (Fig. 5(b); D). These observations indicate that photo-acceleration of the protein transduction system was suitable for proteins.

3. Discussion

A number of studies, which employed this system for reporter proteins as well as for functional peptides and proteins with PTDs, appear in the literature [2–5,7–15]. It is commonly accepted that internalization of PTD, which occurs in a rapid, ATP- and temperature-independent manner, does not involve endocytosis or specific protein transporters [16–18]. Investigation of the delivery of small peptide cargos by Antp-PTD indicated that membrane shuttling occurred via the formation of unilamellar phospholipid bubbles or inverted micelles [2]. However, inverted micelle formation requires the presence of

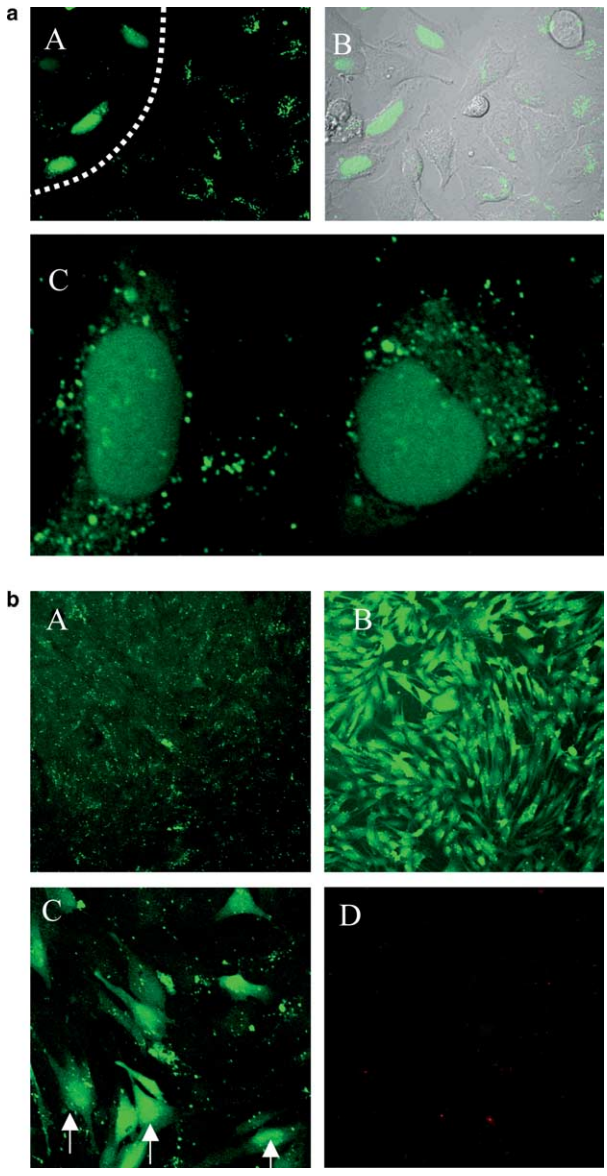

Fig. 5. (a) Effect of light at 480 nm on PTD-fused functional peptides. (A) HeLa cells were treated with 1 μM FITC-conjugated 11R-NLS peptides for 30 min. The left side of the white line was exposed to light at 480 nm for 30 s. Cells were visualized by confocal laser microscopy and as a merger of fluorescence and DIC (B). High-magnification of light-exposed cells demonstrated fluorescence in the nuclei following peptide release from endosomes (C). (b) Glioma cells were treated with 1 μM FITC-conjugated 11R-p53 for 2 h; subsequently, cells were washed with conventional fresh medium and visualized by confocal laser microscopy (A). Then, cells were exposed to light at 480 nm for 30 s. Cells were visualized by confocal laser microscopy. After 2 h, light-exposed cells demonstrated homogeneous fluorescence in the cytosol and nuclei following protein release from endosomes (B). At higher magnification, the white arrow indicates nuclear localization of p53 (C). These cells did not display PI staining (D).

hydrophobic amino acids, which are absent in TAT-PTD and 11R-PTD. Additionally, cargo size is limited. These findings suggest that this PTD delivery mechanism is improbable. Recent studies demonstrated the involvement of cellular internalization of PTDs in endocytosis [21–23]. Therefore, it is likely that the major mechanism of protein transduction involves in endocytotic uptake and subsequent release from endosomes. The present data support the revised version of the transduction mechanism.

Additionally, this investigation revealed that endosomal release can be accelerated under specific conditions, i.e., photoactivation. The current study demonstrated accelerated release of transduced FITC-PTDs from endosomes by stimulation with light at 480 nm. These findings established a novel method for delivery of a large variety of macromolecules and small molecules, which are unable to enter the cytosol without assistance.

4. Materials and methods

4.1. Peptide synthesis

Peptides (11R-PTD; RRRRRRRRRRR, TAT-PTD; YGRKKRRQRRR, Antp-PTD; RQIKIWFQNRRMKWKK, and 11R-NLS; RRRRRRRRRRR-PKKKRKV) and fluorescein-isothiocyanate- (FITC) conjugated peptides were synthesized by Sigma Genosys, Japan. The peptides, which were purified by preparative reverse-phase HPLC, displayed >95% purity by HPLC. All peptides exhibited the expected amino acid composition and mass spectra.

4.2. Protein labeling with FITC

11R-p53 was purified by Ni-NTA chromatography as previously described [12]. The purified protein was labeled by coupling to FITC (Molecular Probes).

4.3. Cell line and primary neural culture from rat brain

Cervix-derived HeLa cells and KR158 glioma cells were maintained in DMEM (Gibco) supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin (50 IU/ml and 50 μg/ml). The cells were plated onto glass coverslips in 35-mm culture dishes. Dissociated neuronal culture was prepared and maintained by a modified procedure as previously described [8]. Cultures could be maintained in a healthy condition for up to four weeks without medium change. Cultures at 9–14 days after plating were utilized in this study.

4.4. Treatment of FITC-PTDs and/or FM 4–64

Cells were incubated with 1–10 μM FITC-11R-PTD, TAT-PTD and Antp-PTD and 10 μM FM 4–64 (Molecular Probes); subsequently, cells were washed with the conventional medium. Treated cells were analyzed with a confocal microscope in either the conventional or confocal mode. Nuclear fluorescent intensity was measured via Fluoview (Olympus).

4.5. Statistical analysis

All values are expressed as means + S.E. Significance levels between groups were calculated with Scheffe's test following the ANOVA-test. A P-value <0.05 was considered significant.

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