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Mechanism of PDX-1 protein transduction

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Abstract

PDX-1 plays a central role in differentiation of insulin-producing cells. We previously reported that exogenous PDX-1 protein can permeate cells and induce insulin gene expression in progenitor cells. These data suggest a strategy for facilitating differentiation into insulin-producing cells. Here we show the mechanism of PDX-1 protein transduction. Initially, a punctate cytoplasmic distribution of PDX-1 protein transduction domain (PTD), which co-localized with an endosomal marker, was observed in treated cells. However, homogeneous distribution of PDX-1-PTD was observed in some cells, indicating that PDX-1 is transduced by endocytosis and then released. The experiments using inhibitors suggested that the PDX-1 is transported through the Golgi complex and to the endoplasmic reticulum. Moreover, we observed in real-time PDX-1-PTD release from endosomes. These data suggest that mechanism of transduction of PDX-1 protein is by endocytosis and subsequent release from the endosome homogeneously in cytoplasm and nuclei, and that PDX-1 protein transduction could be a valuable strategy for facilitating differentiation of progenitor cells into insulin-producing cells.

Keywords: Islet transplantation; Regeneration; PDX-1; Protein transduction domain; Endocytosis; Endosomal release

Islet transplantation is a promising therapeutic option for the treatment of type 1 diabetes [1–3]. However, the shortage of suitable donor tissues remains a major obstacle. Pancreatic stem cells residing within the ductal epithelium have been used to generate human islet like clusters [4], but there is less efficient strategy for facilitating differentiation of progenitor cells into insulin-producing cells. Pancreatic and duodenal homeobox factor-1 (PDX-1), a homeodomain-containing transcription factor, plays a central role in regulating pancreatic development and insulin gene transcription [5–8]. Even in adults, PDX-1 is associated with islet neogenesis and differentiation of insulin-producing cells from progenitor cells [4,9]. We previously have reported that purified PDX-1 protein can be transduced into cells and that the 16 amino acids of PDX-1 truly form a protein transduction domain (PTD) [10,11]. The transduced PDX-1 stimulated the gene expression of insulin, PDX-1 itself, and several other β-cell genes in pancreatic duct cell cultures. These data suggest that PDX-1 protein transduction could be a safe and valuable strategy for facilitating differentiation of progenitor cells into insulin-producing cells without requiring gene transfer

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technology. The presence of a PTD in PDX-1 is intriguing since PDX-1 plays such a crucial role in pancreatic development [5,8], β-cell differentiation [4,9], and maintenance of normal β-cell function [12–14].

Moreover, the demonstration that the full-length homeoproteins can be internalized led us to consider that, in some circumstances, the homeoproteins may have paracrine activities, in that they are released by one group of cells and then internalized by other cells. It has been reported that engrailed homeoproteins can be released into surrounding medium, internalized by neighboring cells, and conveyed intact to the cytoplasm and nuclei [15–20]. Similar observations have been made for Hoxa-5 [20], Hoxc-8, and Hoxb4 [15,18]. These findings suggest that PTDs in such homeobox proteins convey important *intercellular* signaling properties [15].

However, the mechanism of internalization of PDX-1 is not understood. Recent studies on cell-surface binding properties of TAT-PTD from HIV-1 [21,22] show that the initial binding of PTD to the cell surface involves an ionic interaction. Some studies demonstrated that the cellular internalization of TAT-PTD involved endocytosis [23,24]. Moreover, it is reported that TAT-PTD first interacts with cell membrane lipid rafts in a receptor-independent manner, stimulating a rapid internalization by macropinocytosis [22].

Here we present the first report of protein transduction in real time. PDX-1 protein penetrates into cells by endocytosis and is released from the endosome homogeneously into cytoplasm and nuclei, thus providing the mechanism of PDX-1 protein transduction and its intercellular trafficking.

Materials and methods

Construction of vectors and purification of recombinant PDX-1 proteins. Full-length PDX-1 cDNA was amplified by PCR using appropriate linker-primers and then subcloned into the *Nde*I and *Xho*I sites of pET21a (+) (Novagen, Madison, WI) using a ligation kit (TaKaRa, Tokyo, Japan). For deletion of the PTD, sequences before and after the PTD of PDX-1 cDNA were amplified by PCR using appropriate linker-primers and then subcloned into the *Nde*I–*Bam*HI and *Bam*HI–*Xho*I sites of pET21a (+), respectively. For EGFP-11 arginine (11R), full-length enhanced green fluorescent protein cDNA was amplified by PCR using appropriate linker-primers, and 11R sequence was synthesized by Sigma Genosys (The Woodlands, TX) and then subcloned into the *Nde*I–*Bam*HI and *Not*I–*Xho*I sites of pET21a (+). BL21 (DE3) cells containing the expression plasmids were grown at 37 °C to an OD600 of 0.8. Isopropyl-D-thiogalactopyranoside was added to a final concentration of 0.1 mM, and the cells were then incubated for 12 h at 24 °C. Cells were sonicated, and the supernatants were recovered and applied to a column of Ni–NTA agarose (Invitrogen, San Diego, CA). Purified PDX-1 proteins were conjugated using a fluorescein isothiocyanate (FITC)-labeling kit (American Qualex Antibodies, San Clemente, CA).

FITC-conjugated PDX-1-PTD. FITC-conjugated peptides were synthesized by Sigma Genosys, USA (PDX-1-PTD) and Sigma Genosys, Japan (TAT-PTD, 11R-PTD) using the following peptide sequences (PDX-1-PTD, RHIKIWFQNRRMKWKK; TAT-PTD, YGRKKRRQRRR; and 11R-PTD, RRRRRRRRRRR). Each peptide was purified by preparative reversed-phase HPLC, with >95% purity as analyzed by HPLC, and had the expected amino acid composition and mass spectra.

Gel-mobility shift assay. Nuclear extract (2 μg) was incubated with 2 μg poly(dI–dC), 10 mM Hepes (pH 7.8), 0.1 mM EDTA, 75 mM KCl, 2.5 mM $MgCl_2$, and 1 mM dithiothreitol, 3% Ficoll, at room temperature. The binding reaction was initiated by adding ^{32}P-labeled double-stranded oligonucleotide probes. A double-stranded oligonucleotide reproducing the rat insulin gene II PDX-1 binding region and surrounding sequences (ACGTCCTCTTAAGACTCTAATTACCCT ACGT) (Sigma Genosys) was used as a binding probe. In some of the binding assays, anti-PDX-1, anti-6 His, and preimmune antisera [10] were added to the reaction mixture 1 h before addition of the DNA probes.

Semiquantitative radioactive multiplex PCR. PCRs were performed in a Perkin-Elmer 9700 Thermocycler with 3 μl cDNA (20 ng RNA equivalents), 160 μmol/L cold dNTPs, 2.5 μCi [α-^{32}P]dCTP (3000 Ci/mmol), 10 pmol appropriate oligonucleotide primers, 1.5 mmol/L $MgCl_2$, and 5 U AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT). The oligonucleotide primers and cycle number used for semiquantitative radioactive multiplex PCR were insulin (forward) TCTTCTACACACCCATGTCCC, (reverse) GGTGCAGCACTGA TCCAC, 15 cycles for islets, 28 cycles for duct cells; cyclophilin: (forward) AACCCCACCGTGTTCTTC, (reverse) TGCCTTCTTTC ACCTTCCC, 28 cycles. The thermal cycle profile used a 10-min denaturing step at 94 °C followed by amplification cycles (1 min denaturation at 94 °C, 1 min annealing at 55 °C, and 1 min extension at 72 °C) and an extension step of 10 min at 72 °C. The steps taken to validate these measurements were previously reported [10].

Treatment with FITC-PTDs and FM 4-64. Cervix-derived HeLa cells were maintained in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS) and penicillin–streptomycin. Cells were treated with 10 μM PDX-1-PTD for specified times and then washed with the fresh medium. To evaluate endocytosis, cells were incubated with 10 μM FITC-PDX-1-PTD and with 10 μM FM 4-64 (Molecular Probes, OR, USA), a marker of endocytosis. Treated living cells were analyzed by conventional fluorescence microscopy and confocal microscopy. In some cases, cells were fixed with 4% paraformaldehyde in PBS buffer for 20 min or with 99% methanol for 10 min.

Treatment with bafilomycin A1 or brefeldin A. Cells were cultured in DMEM with 10% FCS and washed once with serum-free medium before addition of inhibitors or peptides. Thirty minutes before addition of peptides, 300 nM bafilomycin A1 or 20 μM brefeldin A was added to the medium. After 2 h incubation with peptides, images were acquired immediately at room temperature with excess peptide in the medium.

Results

Transduction of PDX-1 protein into cells and effect of transduced PDX-1

As we have previously reported [10], FITC-conjugated full-length PDX-1, and the PTD peptide (PDX-1-PTD) can be transduced into cells. Full-length PDX-1 protein and the peptide of the 16 amino acids similar to Antp PTD transduced MIN6 and HeLa cells, but PTD-deleted PDX-1 protein did not penetrate the cells (Fig. 1A,B). The transduced PDX-1 protein binds to the A-box binding complex of the insulin enhancer in gel shift assays (Fig. 1C). Moreover, the PDX-1

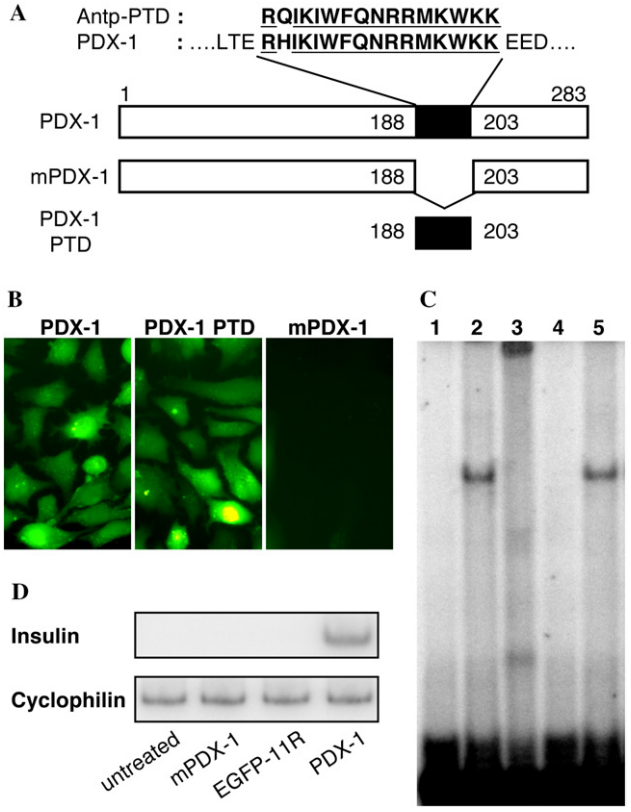

Fig. 1. Transduction of PDX-1 protein into cells and protein transduction domain. (A) Full-length PDX-1 protein, mutant PDX-1 protein with deletion of 16 amino acids similar to Antennapedia-PTD, and the PTD peptide of the 16 amino acids are shown. (B) HeLa cells showed a nuclear and cytoplasmic fluorescence signal after 6 h treatment with 10 μM FITC-conjugated PDX-1 or PDX-1 PTD peptide but not with FITC-conjugated mutant protein with deleted PTD. (C) PDX-1 binding activity by transduced PDX-1 protein. After 10 μM PDX-1 treatment for 6 h, nuclear extracts from HeLa cells were used in gel shift assay. Lane 1, nuclear extracts from untreated cells. Lanes 2–5, nuclear extract from PDX-1 treated cells. Anti-PDX-1 (lane 3), anti-6His (lane 4), and preimmune serum (lane 5) were used to show specificity. (D) Induction of insulin gene expression in duct cells treated with PDX-1 protein. Cultured duct cells were treated with 100 nM PDX-1 protein three times in a week (days 0, 3, and 6); 24 h after final treatment, RNA was extracted for examination of insulin mRNA levels by RT-PCR.

protein transduced into pancreatic ducts, thought to be islet progenitor cells [4,10], induces insulin gene expression (Fig. 1D). Thus, full-length PDX-1 can be transduced into cells and their nuclei, bind to the A-box, facilitate differentiation of progenitor cells into insulin-producing cells, and the 16 amino acids of PDX-1 form a functional PTD.

Cellular uptake of several PTDs into living and fixed cells

Although FITC-PDX-1 protein can be transduced into cells, the efficiency of transduction is strongly reduced because FITC binds to cationic amino acids, which are important in the PDX-1 protein transduction domain. Therefore, the PDX-1-PTD was used in the following experiments. To investigate the cellular uptake of PDX-1-PTD into both living and fixed cells, HeLa cells were treated with FITC-conjugated PDX-1-PTD (Fig. 2). Two hours after treatment, the PTD was observed as a fluorescent signal in living unfixed HeLa cells (Fig. 2B, left panel). A punctate cytoplasmic distribution of the fluorescence was observed in most cells, a pattern which is similar to that of the endosome as shown by recent studies [23,24]. In some living (unfixed) cells, FITC-PTD was homogeneous by distributed in the cytosol and nucleus. The distribution of the PTD changed dramatically after methanol fixation (Fig. 2B, middle panels), confirming that shown in recent studies [25]. Unlike the diffuse cytoplasmic and nuclear localization observed in methanol-fixed cells, in paraformaldehyde-fixed cells (Fig. 2B, right panels) PDX-1-PTD had a similar localization as in living cells, as has been recently reported for other PTDs [26,27]. Thus, PDX-1-PTD is transduced into living unfixed cells with a punctate distribution but can be found diffusely in the

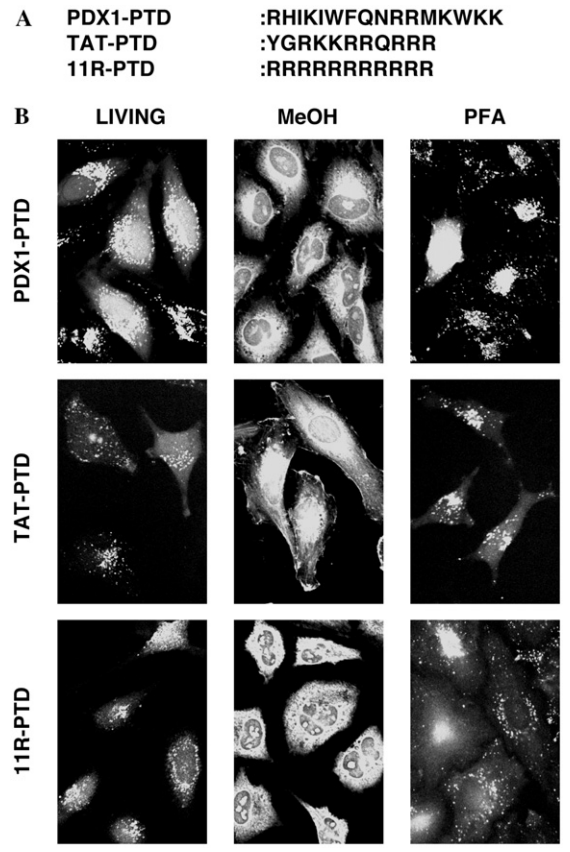

Fig. 2. Cellular uptake of several PTDs into living cells and after fixation. (A) The sequence of the three PTDs synthesized for this study. (B) Transduction of these PTDs into HeLa cells. HeLa cells were treated with 1–10 μM of FITC-conjugated PTDs for 2 h, then washed with fresh media, and photographed by confocal microscopy either as living (unfixed) (left panels) or after fixation with methanol (middle panels) or 4% (para)formaldehyde (PFA) (right panels).

cytoplasm and nuclei of some cells. Two other PTDs, TAT-PTD and 11R-PTD, gave similar patterns as PDX-1-PTD (Fig. 2B).

Transduction of PTDs by endocytosis and release from endosome

To investigate whether the punctate cytoplasmic distribution resulted from endocytosis, HeLa cells were treated with both FITC-conjugated PDX-1-PTD and FM 4-64, a fluorescent (red) molecule that binds to the membrane and thus is used to mark endocytosis, and then observed in real time by confocal microscopy. As shown in Fig. 3, initially the FITC signal was outside the cells (in the medium) and FM dye signal on the surface of cells. Ten minutes after treatment, the FITC signal appeared within unfixed living HeLa cells in a punctate pattern, mainly co-localized with FM 4-64 as indicated by the resultant yellow color (Fig. 3A, 10 min). Since in our previous study [10] we found both PDX-1-PTD and PDX-1 itself within the nuclei by 6 h of culture, a time course was performed. HeLa cells were incubated with both FITC-conjugated PDX-1-PTD and FM 4-64 for 30 min and then washed with fresh media (Fig. 4A, 30 min, as well as Fig. 3A). After an additional 60 min, the PTD was homogeneously distributed in cytosol and nuclei of some cells, while the FM-dye remained endosomal (Fig. 4A, 90 min). These data suggest that endocytosis plays an important role in the bulk uptake of the PDX-1-PTD and that a partial intracellular release from endosomes occurs in some cells. Similar data were found for TAT-PTD (Fig. 4B) and 11R-PTD (Figs. 3B and 4C).

Cellular uptake of PDX-1-PTD at 4 °C

To further test the role of endocytosis, FITC PDX-1-PTD entry was examined in HeLa cells at 4 °C. After a change to cold media, the cells were cultured for 30 min at 4 °C, and then for another hour at 4 °C in fresh

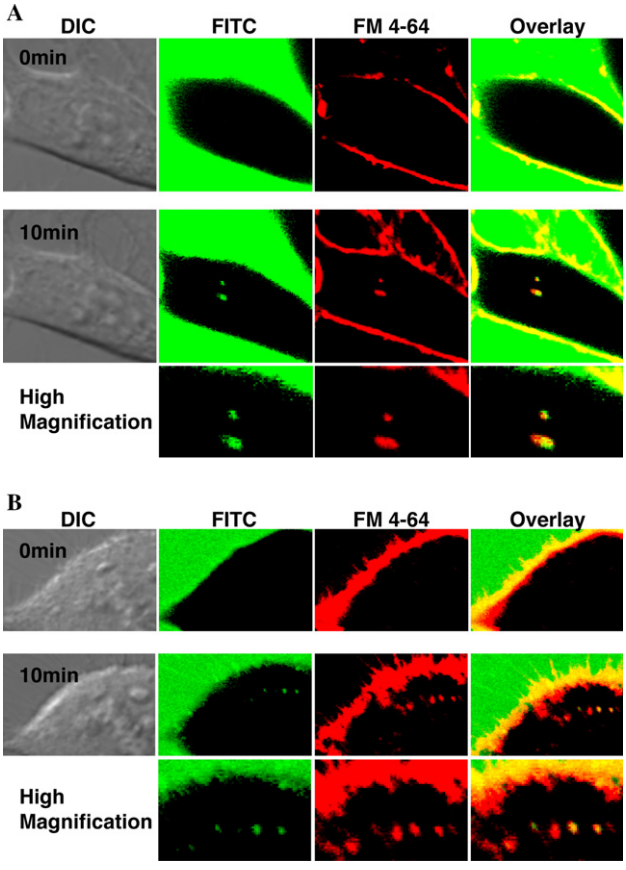

Fig. 3. Transduction of PTDs by endocytosis. HeLa cells were treated with FM 4-64, a marker of endocytosis, and either FITC PDX-1-PTD (A) or FITC 11R-PTD (B) and analyzed by confocal microscopy immediately after addition of fluorochromes. Initially, the FITC signal was outside the cells (in the medium) and FM dye signal on the membrane of the cells. Ten minutes after treatment, the FITC signal appeared as a punctate pattern within unfixed living HeLa cells, mainly co-localized with FM 4-64 (red) in the endosome as indicated by the resultant yellow color.

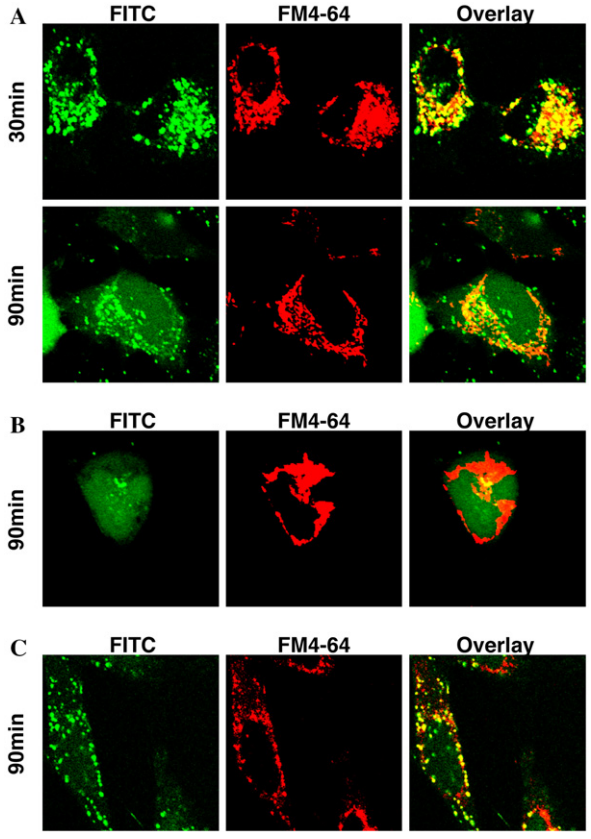

Fig. 4. Release of PTDs from endosome. HeLa cells were incubated with PDX-1-PTD (A), TAT-PTD (B), and 11R-PTD (C) and with FM 4-64 for 30 min and then washed with fresh media. Treated living cells were analyzed by confocal microscopy after an additional 30 or 90 min. PTDs were initially in endocytic vesicles/endosome and become diffuse throughout the cytoplasm and nucleus, but the FM4-64 remains in the endosome.

medium with PDX-1-PTD and FM 4-64. The cells were then washed with 4 °C medium and immediately observed. Little to no signal of either FITC or FM dye was observed within the HeLa cells (data not shown). These data showing that PDX-1-PTD is transduced into cells in a temperature-dependent manner suggest mainly an endocytotic uptake.

Impact of bafilomycin A1 or brefeldin A on the cellular distribution of PDX-1-PTD

Balfomycin A1 is a highly potent and selective inhibitor of vacuolar H^+-ATPases and inhibits endosomal acidification [28]. The effect of bafilomycin A1 on the intracellular localization of the peptide was tested: 30 min before addition of the PTD peptide, cells were washed once and 300 nM bafilomycin was added in the medium. After 2 h incubation with PTDs, no cytoplasmic fluorescence was present and only vesicular staining was observed (Fig. 5B). These data show that the release of PDX-1-PTD into the cytosol occurs by a mechanism dependent on endosomal acidification and preserved endosomal integrity. We next addressed the potential involvement of the Golgi complex in the cellular trafficking of PDX-1-PTD. Brefeldin A interferes both with the integrity of the Golgi and *trans*-Golgi network (TGN) and affects the uptake of TAT fusion protein [29]. Thirty minutes before addition of the peptide cells were washed once and 20 μM brefeldin A was added. After 2 h incubation with the peptide, the cellular fluorescence of PDX-1-PTD was reduced (Fig. 5B), similar to what was seen with bafilomycin A1. Together these data suggest that PDX-1-PTD enters the cytoplasm by means of retrograde transport.

Real-time observation of PDX-1-PTD release from endosomes in living cells

Although other studies have suggested that PTDs enter into cells by endocytosis [23,24], endosomal release of these PTDs in real time has not been previously reported. However, we observed the real-time release

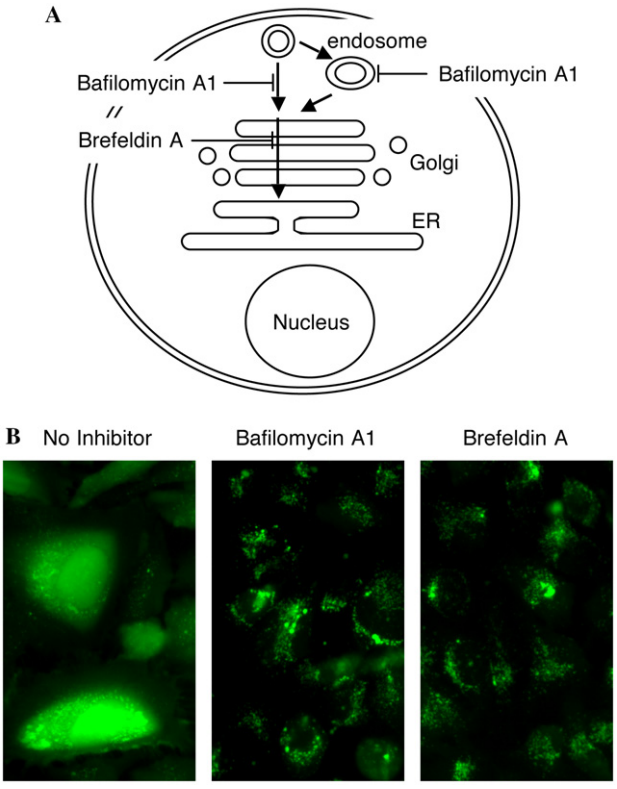

Fig. 5. Impact of bafilomycin A1 or brefeldin A on the cellular distribution of PDX-1-PTD. (A) Potential PTD-trafficking along the endosomal/retrograde pathway and the effect of small molecule inhibitors bafilomycin A1 and brefeldin A on this trafficking. (B) Cells were cultured in DMEM with 10% FBS and washed once with serum-free medium before addition of inhibitors or PTD peptide. Thirty minutes before addition of PTD peptide, 300 nM bafilomycin A1 (middle) or 20 μM brefeldin A (right) was added in the medium. After 2 h incubation with peptide, images were acquired immediately at room temperature with excess peptide in the medium.

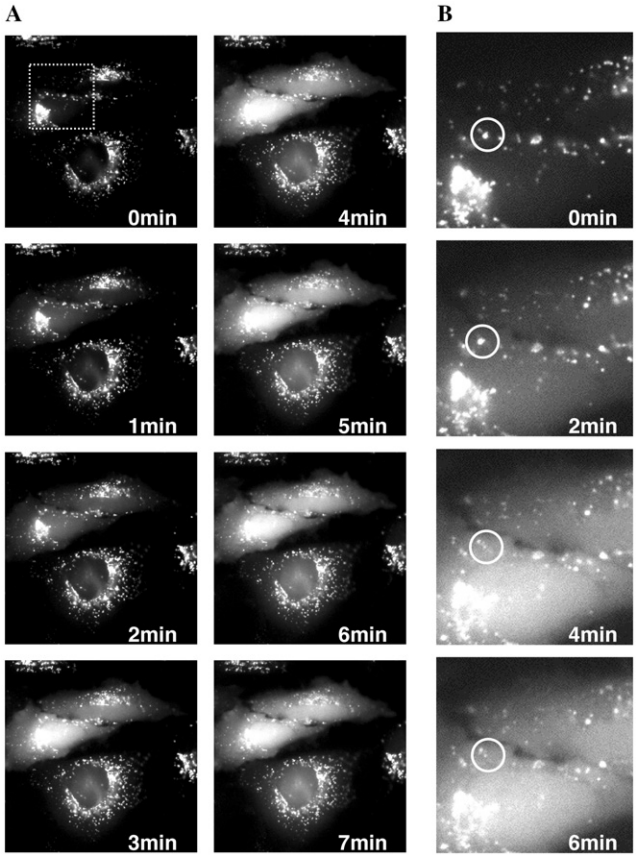

Fig. 6. Real-time observation of PDX-1-PTD release from endosome in cells. HeLa cells were treated with 10 μM of FITC-conjugated PDX-1-PTD for 2 h, washed with fresh medium, and photographed every minute under conventional fluorescence microscopy. In (B), a high magnification of the box in (A) shows that the PDX-1-PTD is released from endosome (circle).

of PDX-1-PTD from endosomes in HeLa cells treated with 10 μM PDX-1-PTD for 2 h (Fig. 6). After washing well, fluorescence is seen as large particles or vesicles within the cells (Fig. 6, 0 min). With observation time, the particulate nature of the fluorescent signal disappears; there was a time-dependent increase in the number of cells with homogeneous distribution (Figs. 6A and B). These data support the concept that cellular uptake of PDX-1-PTD occurs through electrostatic interaction with plasma membrane, subsequent endocytosis, and eventual release from endosome with resultant homogeneous, diffuse distribution in cytoplasm and nucleus.

Discussion

A series of small domains, called protein transduction domains, have been shown to cross biological membranes efficiently and to promote the delivery of peptides and proteins into cells. The homeodomain of Antennapedia, a *Drosophila* transcription factor, and related homeodomains are internalized by cells in culture, and this homeodomain, or its third α-helix, has delivered many peptides, phosphopeptides, and oligonucleotides into cells [30,31]. TAT protein from HIV-1 [21,32,33] and VP22 protein from *Herpes simplex* virus (HSV) [34] are also able to deliver biologically active proteins into cells in vitro and in vivo. The synthetic poly-R (11R) delivers peptides and proteins even more efficiently [35–37]. Recent studies clearly show that the homeoproteins can be transferred from cell to cell and have direct non-cell autonomous activities, the physiological role of the protein transduction system [15–20]. This intercellular transfer has not only important biotechnological consequences but also the physiological and developmental implications.

Early studies on the internalization mechanism of PTDs and PTD-fused proteins showed that internalization was (1) not inhibited by incubation at 4 °C, depletion of the cellular ATP pool, nor by inhibitors of endocytosis and (2) did not depend on a specific primary sequence for transporter or receptor recognition [20,30]. Therefore, it was commonly accepted that PTD internalization did not involve classical endocytosis or specific protein transporters. Investigation of the delivery of small peptide cargos by Antp-PTD implied that membrane shuttling occurred through the formation of unilamellar phospholipid bubbles or inverted micelles [31]. However, inverted micelle formation would limit cargo size and require the presence of hydrophobic amino acids, which are absent in TAT-PTD and poly-arginine-PTDs; thus, such a mechanism is unlikely to be a common PTD delivery mechanism. Recent studies demonstrated that the cellular internalization of PTDs involved endocytosis [23,24]. We found the majority of PTD uptake results from endocytosis followed by endosomal release. However, one cannot completely eliminate the possibility of partial uptake across the plasma membrane via inverted micelle and/or direct penetration.

Our data suggest that the main mechanism of PDX-1 protein transduction is an electrostatic interaction with the plasma membrane, penetration into cells by endocytosis, and a release to cytoplasm and nuclei. Recently, the role of endosomal acidification and retrograde transport for the uptake of the Antp-PTD, TAT-PTD, and 9R-PTD was reported [38]. A number of well-characterized toxins reach the cytosol of eukaryotic cells after binding to the cell surface, undergoing endocytosis and retrograde transport to the Golgi apparatus and endoplasmic reticulum [39,40]. The arginine-rich motif of 8–10 amino acids in the A subunits of these toxins, reported to be transported by means of retrograde transport, is similar to TAT-PTD, Antp-PTD [38], and PDX-1-PTD. Our data suggest that the mechanism of PDX-1 protein transduction is similar to those of other PTDs and may have important applications in diabetes research.

Acknowledgments

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