Intracellular Internalization Mechanism of Protein Transfection Reagents

Makoto Oba* and Masakazu Tanaka

Graduate School of Biomedical Sciences, Nagasaki University; 1–14 Bunkyo-machi, Nagasaki 852–8521, Japan.
Received December 27, 2011; accepted March 25, 2012

A protein transfection reagent is a powerful tool for elucidating a protein function in a cell, and plays an important role in the fields of cell biology and drug discovery. Many researchers have developed protein delivery systems and several systems are commercially available. In this study, we focus on the biological functions of three commercially available protein transfection reagents, Pro-DeliverIN, Xfect, and TurboFect, especially in their internalization routes by HeLa cells. A cellular uptake study using specific endocytosis inhibitors and confocal laser scanning microscope observation revealed that each reagent was internalized into HeLa cells by different mechanism. It is our hope that the results presented here will help in the choice and use of protein transfection reagents for experiments.

Key words protein transfection reagent; intracellular internalization; endocytosis; protein delivery system

Proteins are fundamental biomacromolecules and are generally produced in all living cells. A number of studies have attempted to clarify protein functions and to use proteins for therapeutic approaches. Obviously, a major key to success in such studies is the development of protein delivery systems that have high delivery efficiency without any cytotoxicity. Much effort has been therefore devoted to developing the excellent delivery systems,[1–4] and there are already several commercially-available protein transfection reagents; lipid-based, polymer-based, or peptide-based formulations.[5,6] One of the important factors for the protein delivery into cells is where the proteins are delivered. Depending on the purpose of the studies, proteins may be delivered to targeted organelles such as nuclei, cytoplasm, lysosomes, mitochondria, and so on. Therefore, we need to know the properties of the protein transfection reagents used in our experiments in order to choose appropriate reagents according to the experiments.

The aim of this study was to gain insights into the biological properties of commercially-available protein transfection reagents. We used three types of reagents: a lipid-based Pro-DeliverIN, a cell penetrating peptide-based Xfect, and a cationic polymer-based TurboFect, and evaluated their complexes with bovine serum albumin (BSA). These products may be described as follows: (1) Pro-DeliverIN is a lipid-based formulation, forms non-covalent complexes with proteins, and can be used under conditions both with and without serum; (2) Xfect is a cell penetrating peptide and used in serum-free medium; (3) TurboFect is a cationic polymer and can be used in the presence or absence of serum, but transfectin in serum-free medium is recommended for best results in the manufacturer's protocol. A cellular uptake study using specific inhibitors of endocytosis and confocal laser scanning microscope (CLSM) observation clarified the internalization routes and the final intracellular localization of each complex with BSA. The results obtained in this study may be helpful in choosing and using protein transfection reagents according to the type of experiments.

MATERIALS AND METHODS

Materials Pro-DeliverIN was purchased from OZ Biosciences (Marseille, France). Xfect was obtained from Clontech Laboratories, Inc. (Palo Alto, CA, U.S.A.). TurboFect was purchased from Fermentas (Glen Burnie, MD, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), BSA, fluorescein isothiocyanate conjugate BSA (FITC-BSA), filipin III from *Streptomyces filipinensis*, and amiloride hydrochloride were purchased from Sigma-Aldrich Co. (ST. Louis, MO, U.S.A.). Heparin, Cell lysis buffer M, and sucrose were products of Wako Pure Chem. Co., Ltd. (Osaka, Japan). Cell counting kit-8 was purchased from Dojindo Laboratories (Kumamoto, Japan). Lyso Tracker Red was purchased from Molecular Probes (Eugene, OR, U.S.A.).

Preparation of Protein Transfection Reagent/BSA Complexes Each protein transfection reagent/BSA complex was prepared according to the manufacturer's protocols. Briefly, 1.0 μL of Pro-DeliverIN reagent, 3.0 μL of 1X Xfect protein transfection reagent stock solution, and 0.8 μL of TurboFect protein transfection reagent were used for preparation of complexes containing 1.0 μg of BSA, respectively.

Cell Viability HeLa cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and the cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. HeLa cells were seeded onto 96-well culture plates (10000 cells/well) and incubated overnight in 100 μL of DMEM containing 10% FBS. After the medium was replaced by fresh medium with or without 10% FBS, protein transfection reagent/BSA complex solution was applied to each well (0.25 μg BSA/well). After 3 h incubation, the Cell counting kit-8 was used according to the manufacturer's protocol. Cell viability was evaluated on the basis of the absorbance of formazan from each well, where 100% cell viability was calculated from the wells without the complexes. The results are presented as the mean and standard deviation obtained from 5 samples (Fig. 1).

Cellular Uptake Each protein transfection reagent FITC-BSA complex was used for these experiments. HeLa cells were seeded onto 96-well culture plates (10000 cells/well) and incubated overnight in 100 μL of DMEM containing

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: moba@nagasaki-u.ac.jp

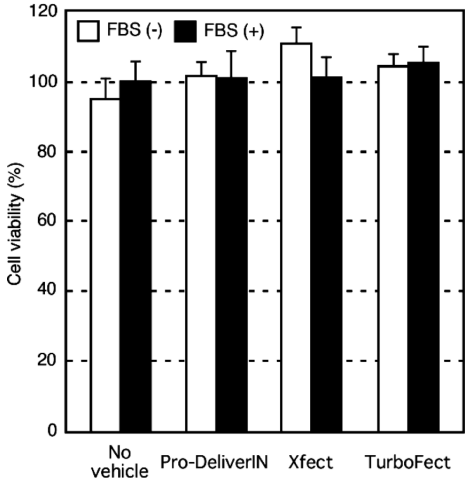

Fig. 1. Cytotoxicity of BSA Complexes with No Vehicle, Pro-DeliverIN, Xfect, and TurboFect against Hela Cells

Experiments were carried out in culture medium without (open bars) or with (closed bars) 10% FBS. Error bars in the graph represent standard deviation, $n=5$.

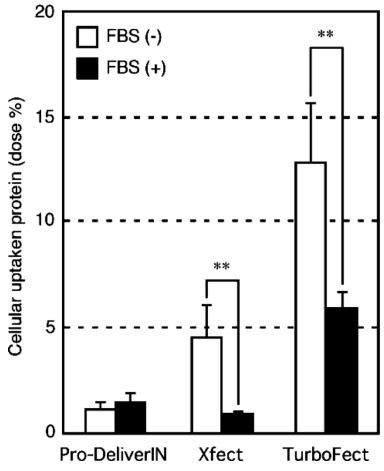

Fig. 2. Cellular Uptake of FITC-BSA Complexes with Pro-DeliverIN, Xfect, and TurboFect

Experiments were carried out in culture medium without (open bars) or with (closed bars) 10% FBS. Error bars in the graph represent standard deviation, $n=6$. $^{**}p<0.01$.

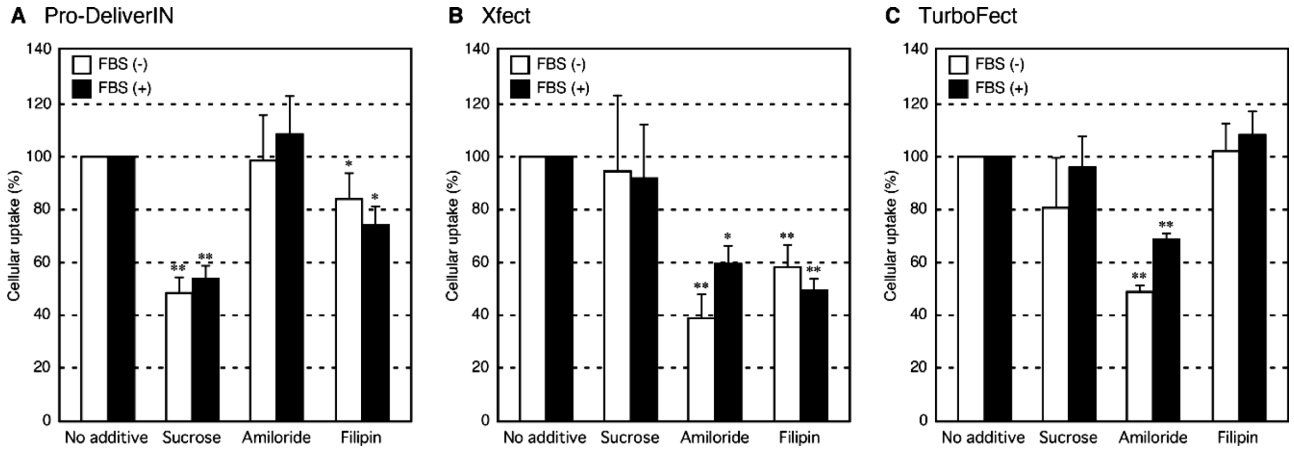

Fig. 3. Effects of Inhibitors on Internalization of FITC-BSA Complexes with Pro-DeliverIN (A), Xfect (B), and TurboFect (C)

Experiments were carried out in culture medium without (open bars) or with (closed bars) 10% FBS. Error bars in the graph represent standard deviation, $n=5$. $^{*}p<0.05$ and $^{**}p<0.01$.

10% FBS. The medium was replaced by fresh medium with or without 10% FBS and then protein transfection reagent/FITC-BSA complex solution was applied to each well (0.25 μg FITC-BSA/well). After 3 h incubation, the medium was removed, and the cells were washed 3 times with ice-cold PBS supplemented with heparin (20 units/mL) and treated with Cell lysis buffer M. The fluorescence intensity of lysate was measured by a spectrofluorometer (ND-3300, NanoDrop, Wilmington, DE, U.S.A.) and cellular uptaken protein (% dose) was calculated using a calibration curve. The results are presented as the mean and standard deviation obtained from 6 samples (Fig. 2).

Inhibition of Endocytosis HeLa cells were seeded onto 96-well culture plates (10000 cells/well) and incubated overnight in 100 μL of DMEM containing 10% FBS. After replacement with fresh medium (containing 10% FBS or not) in the absence or presence of sucrose (0.4 M), or amiloride (5 mM), or filipin (5 μg/mL), cells were pre-incubated at 37°C for 30 min. Each protein transfection reagent/FITC-BSA complex solution was then applied to each well (0.25 μg FITC-BSA/well) and incubated for 1 h (in the case of amiloride and filipin) or 3 h (in the case of sucrose). The experimental conditions were as reported previously.[7–9] After each incubation time, the medium was removed and the cells were washed 3 times with ice-cold PBS supplemented with heparin (20 units/mL) and treated with Cell lysis buffer M. The fluorescence intensity of lysate was measured by a spectrofluorometer (ND-3300, NanoDrop). The results are presented as the mean and standard deviation obtained from 5 samples (Fig. 3).

CLSM Observation HeLa cells were seeded onto 8-well chambered coverglasses (Iwaki, Tokyo, Japan) (20000 cells/well) and incubated overnight in 200 μL of DMEM containing 10% FBS. The medium was replaced by fresh medium with or without 10% FBS and each protein tranfection reagent/FITC-BSA complex solution was then applied to each well (0.5 μg FITC-BSA/well). After 3 h incubation, the medium was removed and the cells were washed three times with ice-cold PBS supplemented with heparin (20 units/mL). The intracellular distribution of the complexes was observed by CLSM after staining acidic late endosomes/lysosomes with Lyso

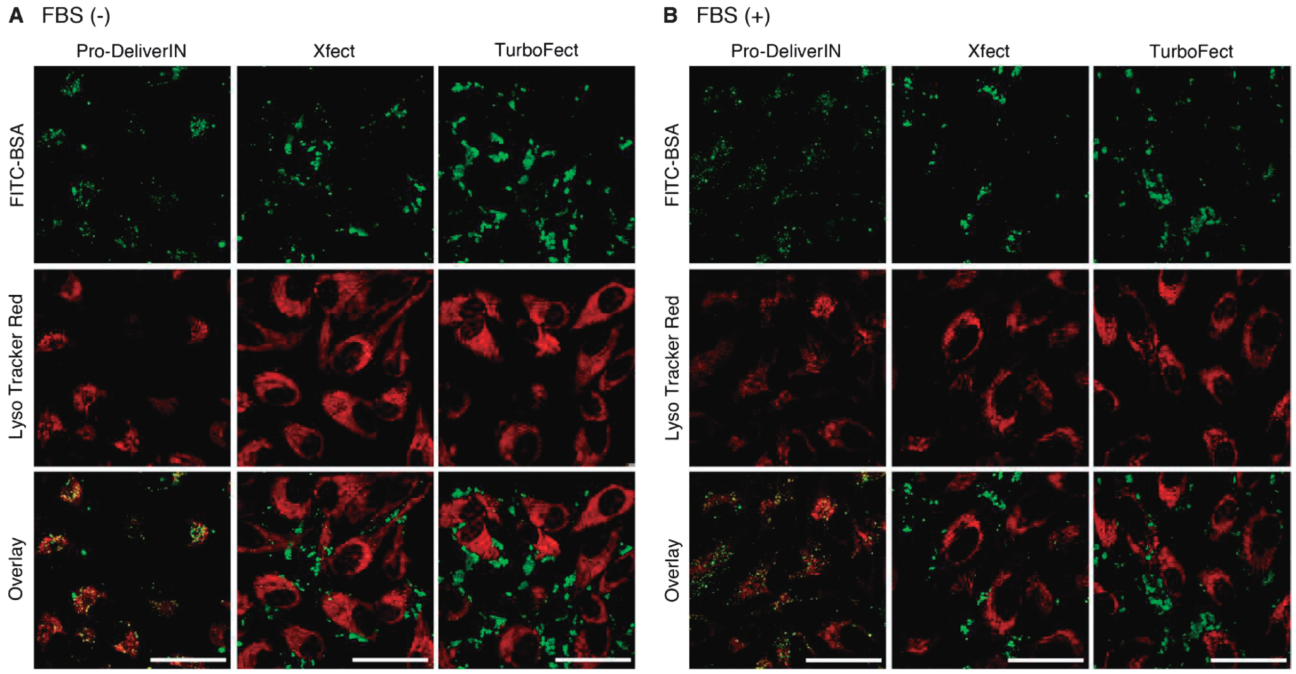

Fig. 4. Intracellular Distribution of FITC-BSA (Green) Complexes with Pro-DeliverIN, Xfect, and TurboFect

The acidic late endosomes/lysosomes were stained with Lyso Tracker Red (red). Experiments were carried out in culture medium without (A) or with (B) 10% FBS. The scale bars represent 50 μm.

Tracker Red. The CLSM observation was performed using an LSM 710 (Carl Zeiss, Oberlochen, Germany) equipped with a 63X objective (Plan-Apochromat, Carl Zeiss) at excitation wavelengths of 488 nm (Ar laser) for FITC-BSA and 543 nm (He–Ne laser) for Lyso Tracker Red (Fig. 4). The rate of co-localization of FITC-BSA complexes with Lyso Tracker Red was quantified.[10] Colocalization was quantified as follows:

$$\text{colocalization ratio} = \text{FITC-BSA pixels}_{\text{colocalization}} / \text{FITC-BSA pixels}_{\text{total}}$$

where FITC-BSA $\text{pixels}_{\text{colocalization}}$ represents the number of FITC-BSA pixels colocalizing with Lyso Tracker Red in the cell, and FITC-BSA $\text{pixels}_{\text{total}}$ represents the number of all the FITC-BSA pixels in the cell. The results are presented as the mean and standard deviation obtained from 16 cells (Fig. 5).

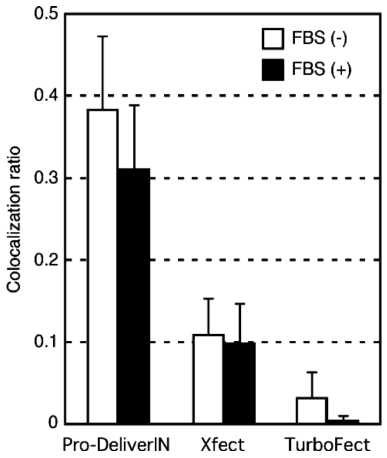

Fig. 5. Quantification of FITC-BSA Complexes Colocalization with Lyso Tracker Red in the HeLa Cells

Experiments were carried out in culture medium without (open bars) or with (closed bars) 10% FBS. Error bars represent standard deviation, n=16.

RESULTS

Cytotoxicity of Protein Transfection Reagent/BSA Complexes Figure 1 shows the results of cytotoxicity analysis treated with BSA only (no vehicle) or each protein transfection reagent/BSA complex for 3 h. None of the complexes showed significant cytotoxicity either with or without FBS.

Cellular Uptake of BSA Complexes The cellular uptake of the complexes into HeLa cells was evaluated using FITC-conjugate BSA-incorporated complexes (Fig. 2). Regardless of FBS presence, the uptake of FITC-BSA was higher for TurboFect than for Pro-DeliverIN and Xfect. Note that cell lysate treated with FITC-BSA only (no vehicle) showed no fluorescence in this experiment (data not shown). In the case of Xfect and TurboFect, the cells treated with the complexes in medium with FBS tended to be taken up less efficiently those in medium without FBS. On the other hand, there was no significant decrease in the uptake of Pro-DeliverIN complexes even by adding FBS. These results indicate that Pro-DeliverIN is compatible with serum-containing media.

Effect of Endocytosis Inhibitors on the Internalization of BSA Complexes To clarify the mechanism of internalization of each complex into HeLa cells, inhibitory experiments of cellular uptake were carried out using specific endocytosis inhibitors. The effects of the following endocytosis inhibitors on internalization of the complexes were examined: sucrose (a specific inhibitor of clarthrin-mediated endocytosis); amiloride (a specific inhibitor of macropinocytosis); and filipin (a specific inhibitor of caveolae-mediated endocytosis).[11] Figure 3A shows the internalization of Pro-DeliverIN complexes in the presence of inhibitors. Treatment of the cells with amiloride had no significant effect on the protein delivery by

Pro-DeliverIN. In contrast, sucrose treatment caused an approximately 50% decrease in the amount of cellular uptake. Also, filipin significantly decreased the internalization of Pro-DeliverIN complexes. These results implied that Pro-DeliverIN complexes were internalized into the HeLa cells mainly *via* clathrin-mediated endocytosis but with the addition of caveolae-mediated endocytosis. As shown in Fig. 3B, the cellular uptake of the Xfect complexes treated with amiloride and filipin was diminished by approximately 50% relative to control values, while sucrose had no significant effect. The internalization of Xfect complexes into HeLa cells may occur *via* macropinocytosis and caveolae-mediated endocytosis. Figure 3C shows data on the internalization of the TurboFect complexes by HeLa cells. The incubation in the presence of amiloride significantly affected the internalization of TuboFect complexes, while that in the presence of sucrose and filipin did not. These results indicate that the TuboFect complexes were internalized preferentially through macropinocytosis by HeLa cells.

Intracellular Distribution of Complexes The intracellular distribution of the complexes was investigated by CLSM using FITC-BSA (green) incorporated complexes (Fig. 4). Lyso Tracker Red (red) was used to label late endosomes/lysosomes. Intracellular distribution of Pro-DeliverIN complexes was observed as small green spots. Meanwhile, the CLSM images of the cells transfected by TurboFect complexes showed spreading green regions in the cells. Interestingly, Xfect complexes displayed both small green spots and spreading green regions, as shown in the cells treated with Pro-DeliverIN and TurboFect, respectively. Note that distribution of FITC-BSA in nuclei was not observed in the HeLa cells treated with all three complexes. Colocalization of FITC-BSA with the late endosomes/lysosomes was quantified and is shown in Fig. 5. More than 30% of FITC-BSA in Pro-DeliverIN complexes was localized in the late endosomes/lysosomes, while only 10% and less than 5% of those in Xfect and TurboFect complexes, respectively, were localized there. Adding FBS to the culture medium changed the colocalization ratio of TurboFect complexes, but not those of Pro-DeliverIN and Xfect complexes. Thus, it is reasonable to assume that there may be distinct routes of internalization for Pro-DeliverIN, Xfect, and TurboFect complexes, and therefore their final destinations and their shapes observed in the cells may be different.

DISCUSSION

There are several commercially-available protein transfection reagents. They are very useful for determining the functions of a protein in a cell, and have been widely used in the fields of cell biology and drug discovery. Nevertheless, those studies focused primarily on the functions of the delivered proteins and demanded high transfection efficiency and no cytotoxicity for protein transfection reagents, and less attention has been paid to the intracellular trafficking of the proteins.[12,13] In this study, we evaluated three protein transfection reagents, Pro-DeliverIN, Xfect, and TurboFect, that deliver the proteins into HeLa cells, and especially the intracellular internalization routes of their complexes with BSA.

Comparison among ProDeliverIN, Xfect, and TurboFect/BSA complexes revealed that each reagent has different properties for protein delivery into HeLa cells. TurboFect showed the highest protein transfection efficiency of all (Fig. 2). As shown in the manufacturer's protocols, adding serum to the cell culture medium significantly decreased the amount of cellular protein uptake by Xfect and TurboFect to less than 50%. Pro-DeliverIN showed no such decrease regardless of serum in the medium, although the protein transfection efficiency of TurboFect was higher than that of Pro-DeliverIN. Note that no cytotoxicity against HeLa cells was observed under all experimental conditions (Fig. 1), indicating low cellular toxicity of each reagent.

To elucidate the intracellular internalization routes of each complex, inhibitory experiments using specific inhibitors of endocytosis (Fig. 3) and CLSM observation with late endosomes/lysosomes staining (Figs. 4, 5) were carried out. Sucrose, amiloride, and filipin are specific inhibitors of clathrin-mediated endocytosis, macropinocytosis, and caveolae-mediated endocytosis, respectively.[11] Cellular uptake of Pro-DeliverIN complexes was decreased by adding sucrose and filipin to the medium with and without FBS, indicating that Pro-DeliverIN complexes were preferentially internalized into the HeLa cells *via* clathrin-mediated endocytosis and, in addition, caveolae-mediated endocytosis (Fig. 3A). The CLSM observation of HeLa cells challenged with Pro-DeliverIN complexes revealed that Pro-DeliverIN complexes were distributed in the cells as punctate spots and localized in late endosomes/lysosomes at over 30% (Figs. 4, 5). Clathrin-mediated endocytosis was reported to deliver uptaken substances to the acidic compartment of a lysosome,[14] which agrees with our results derived from Pro-DeliverIN complexes. The cellular uptake of TurboFect complexes treated with amiloride was diminished by about 50%, while treatment with sucrose and filipin had no significant affect (Fig. 3C). TurboFect complexes seemed to be internalized into the HeLa cells by macropinocytosis, which is consistent with the result that the colocalization ratio of FITC-BSA with late endosomes/lysosomes was less than 5%. Macropinosomes are thought not to fuse into lysosomes and to be inherently leaky vesicles compared with other types of endosomes.[11,14,15] Thus, almost TuboFect complexes internalized through macropinocytosis might escape from macropinosomes to the cytoplasm (Fig. 5), leading us to see spreading distribution of TurboFect complexes in the HeLa cells (Fig. 4). Note that some parts of complexes might still remain in the macropinosomes or early endosomes because Lyso Tracker Red cannot stain them. In the case of Xfect, amiloride and filipin reduced the cellular uptake of complexes by more than 40%, but sucrose did not cause any decrease (Fig. 3B). Xfect complexes were internalized into the HeLa cells using macropinocytosis and caveolae-mediated endocytosis, and thus both spreading and punctate green regions might be observed in the cells (Fig. 4). Caveolae-mediated endocytosis is not associated with a pH decrease and dose not deliver uptaken substances to lysosomes, which is one of the reasons why the colocalization ratio of Xfect complexes with late endosomes/lysosome was low[11,14,16] (Fig. 5).

CONCLUSION

In conclusion, we evaluated three commercially-available protein transfection reagents, Pro-DeliverIN, Xfect, and TurboFect, and revealed that these reagents are internalized into HeLa cells preferentially *via* different routes. We should

be aware of the internalization mechanisms of foreign substances, and these findings may be helpful in choosing and using protein transfection reagents for experiments.

Acknowledgement This work was financially supported by Grant-in-Aid for Young Scientists (A) (No. 20689024 to M.O.) from the Japan Society for the Promotion of Science (JSPS).

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