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Intracellular Delivery of Proteins into Mammalian Living Cells by Polyethylenimine-Cationization

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In the post-genomic era, there is pressing need for development of protein manipulation methodology to analyze functions of proteins in living cells. For this purpose, techniques to deliver functional proteins into living cells are currently being evaluated as alternative approaches to the introduction of transcriptionally active DNA. Here, we describe a novel method for efficient protein transduction into living cells in which a protein is simply cationized with polyethylenimine (PEI) by limited chemical conjugation. PEI-cationized proteins appear to adhere to the cell surface by ionic charge interaction and then internalize into cells in a receptor- and transporter-independent fashion. Since PEI is an organic macromolecule with a high cationic-charge density, limited coupling with PEI results in endowment of sufficient cationic charge to proteins without causing serious decline in their fundamental functions. A number of PEI-cationized proteins, such as ribonuclease (RNase), green fluorescent protein (GFP) and immunoglobulin (IgG), efficiently entered cells and functioned in the cytosol. Our results suggest that protein cationization techniques using PEI will be useful for the development of protein transduction technology.

[**Key words:** protein transduction, polyethylenimine (PEI), cationization, chemical modification, endocytosis]

Protein transduction, an efficient method for delivery of proteins into living cells, has many potential applications that range from basic study of protein function to development of novel therapeutics. Recent progress in protein transduction technology is promising for such purposes. Small cationic regions of proteins, called cell-penetrating peptides or protein transduction domains (PTD) such as human immunodeficiency virus TAT-(48-60), Antenapedia-(43-58) or Arg-rich peptides are among the most well-known peptides for these purposes (1–6). By attaching these carrier peptides, efficient protein transduction of $p16P^{INK4}$ (7), $p27P^{Kip1}$ (8), p53 (9), an HIV protease-activated caspase-3 (10) and Cre recombinase (11) *in vitro* and/or *in vivo* has been demonstrated to enable modulation of cellular events.

Although the mechanism remains controversial, the efficiency of PTD-mediated protein transduction is probably controlled by cell surface adsorption by ionic charge interaction between anionic cellular surface and cationic region of proteins (12–14), and that interaction appears to be followed by internalization through a caveolar endocytotic pathway (15). Likewise, highly cationic proteins, such as human eosinophile cationic protein (pI=11.9) (16) and artificially cationized proteins (17–26), have also shown efficient intracellular delivery. It was proposed more than a decade ago protein cationization is applicable to intracellular protein delivery by adsorption-mediated endocytosis (17, 18). This protein cationization method is accomplished by extensive amidation of carboxyl groups with various diamines (*e.g.*, ethylenediamine, Fig. 1A). Recently, we have demonstrated that non-toxic secretory RNases are converted to highly cytotoxic ones by cationization with ethylenediamine (25). Although these cationized RNases show markedly decreased enzymatic activity because of extensive modification of carboxyl groups, acquirement of a new function to pass through the cell membrane contributed to their cyto-

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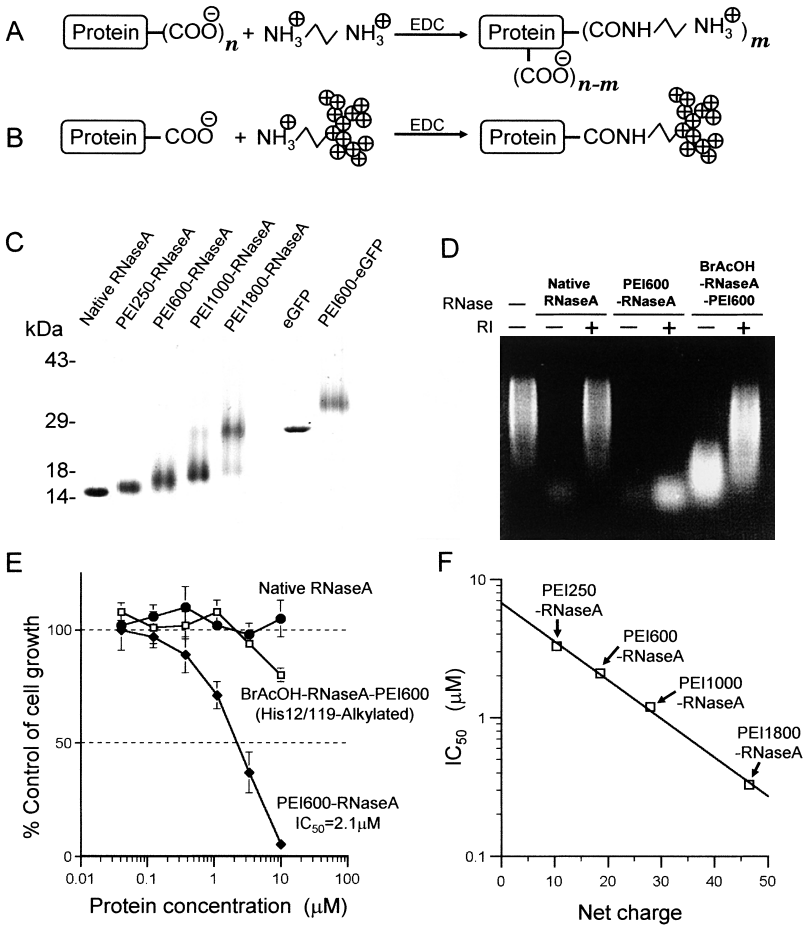

FIG. 1. Preparation and characterization of PEI-cationized proteins. Schema of protein cationization by amidation of the carboxyl group with ethylenediamine (A) and PEI (B) by a carbodiimide reaction. (C) SDS–PAGE analysis under reducing conditions using 15% polyacrylamide gel. (D) Agarose gel-based assay of enzymatic activity and RNase inhibitor resistance of RNases. *E. coli* ribosomal RNA was treated with 10 ng of RNases in the presence or absence of 10 molar excess RNase inhibitor for 15 min at 37°C, and then analyzed. (E) Cytotoxicity of RNases against Swiss 3T3-SV40 cells determined by an MTT assay after 3-d incubation. (F) Correlation between cytotoxic activity and net-charge of PEI-cationized RNaseA's.

toxicity. Thus, cationization of proteins with diamines is considered to be a powerful strategy to promote their internalization. However, unfavorable effects on protein function and stability due to the required extensive modification of carboxyl groups with diamines may limit its application (26).

In this report, we describe a new protein transduction method, cationization with polyethylenimine (PEI). PEI allows a protein to be more efficiently cationized than diamine and to maintain protein function as much as possible by a limited chemical conjugation (Fig. 1B). PEI, a polycationic polyamine, is a polymer with a branched backbone of two carbons followed by one potentially protonated nitrogen atom in every molecular mass unit of 43. PEIs with various molecular masses, ranging on average from 250 to 70,000, are available. Since PEI is toxicologically safe, it has been used as a food additive. Recently, PEI-mediated gene transfection by formation of a PEI/DNA noncovalent complex has been developed as a possible alternative to viral and liposomal routes of gene delivery (reviewed in Refs. 27–29).

Our experiments showed that a number of PEI-cationized proteins were efficiently delivered into living cells both *in vitro* and *in vivo*. Our results suggest that in addition to current PTD-fusion methodologies, the covalent conjugation of PEI to proteins is also a possible approach in protein transduction methodology for analyzing protein function in living cells or for specific manipulation of cellular events.

MATERIALS AND METHODS

Materials PEIs with various molecular masses (Epomin™ SP series: manufactured by Nippon Shokubai, Osaka) were donated by Nippon Shokubai or purchased from Wako Chemical (Osaka). The average molecular masses of PEIs (250, 600, 1000 and 1800) were used for their designations such as PEI600 in the text. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) was purchased from Pierce (Rockford, IL, USA). Bovine RNaseA (Type XII-A), deoxyribonuclease (DN-25), holotransferrin and Rhodamine B isothiocyanate (RITC) were obtained from Sigma (St. Louis, MO, USA). Bafilomycin A1, Fluorescein isothiocyanate (FITC) and recombinant human RNase inhibitor were purchased from Wako Chemical. Preparation of rabbit antibody against human S100C was described previously (30).

Preparation of recombinant proteins Complementary