Original Article

BETA2/NeuroD Protein Can Be Transduced Into Cells Due to an Arginine- and Lysine-Rich Sequence

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BETA2/NeuroD, a basic helix-loop-helix transcription factor, is a key regulator of pancreatic islet morphogenesis and insulin gene transcription. Here we report for the first time that the BETA2/NeuroD protein can permeate several cells, including pancreatic islets, due to an arginine- and lysine-rich protein transduction domain sequence in its structure. The BETA2/NeuroD protein was transduced in a dose-dependent manner up to 1 μmol/l. Transduced BETA2/NeuroD functions similarly to endogenous BETA2/NeuroD: it binds to the insulin promoter and activates its expression. We also investigated the mechanism of BETA2/NeuroD protein transduction. The BETA2/NeuroD protein penetrated cells by macropinocytosis and was released from endosomes homogeneously in cytoplasm and nuclei. These data suggest that BETA2/NeuroD protein transduction could be a safe and valuable strategy for enhancing insulin gene transcription without requiring gene transfer technology. *Diabetes* 54:2859–2866, 2005

Basic helix-loop-helix (bHLH) proteins are transcription factors involved in the specification of cell type and differentiation during development (1–3). The BETA2/NeuroD protein, a class B bHLH factor, has been cloned as both a transcriptional activator of the insulin gene (4) and a neurogenic factor in *Xenopus* embryos (2). BETA2/NeuroD is expressed transiently in a subset of neurons in the central and peripheral nervous systems at the time of their terminal differentiation (2). Moreover, this protein is important for regulating insulin gene transcription (4) and for the terminal differentiation of islet cells, including insulin- and glucagon-producing cells. In BETA2/NeuroD gene-targeting experiments, mutant mice developed severe diabetes with ketoacidosis and died perinatally (5).

Since the discovery of protein transduction domains (PTDs), which allow proteins to be translocated across the plasma membrane and into nuclei, there has been increasing interest in their potential to deliver bioactive peptides and proteins into eukaryotic cells as a valuable strategy for the transduction of therapeutic proteins into patients. The small PTDs from the TAT protein of HIV-1 (6,7), the VP22 protein of *Herpes simplex* virus (8), and the Antennapedia homeoprotein of *Drosophila* (9) have been fused to proteins, resulting in the remarkable delivery of proteins into many mammalian tissues.

Recently, we and others have shown that the important sequences in protein transduction are arginine and lysine (10–12). We have noticed that the transcription factor BETA2/NeuroD protein has an arginine- and lysine-rich sequence that is conserved across species (mice, rats, and humans) (Fig. 1*A*). The presence of a PTD in BETA2/NeuroD is intriguing, given that BETA2/NeuroD plays such an important role in islet cell differentiation and survival, islet morphogenesis, and the maintenance of normal β-cell function due to its regulation of insulin genes (4). Clinically, mutations in BETA2/NeuroD result in both type 1 and 2 diabetes, including maturity-onset diabetes of the young (13–16). BETA2/NeuroD expression and its DNA binding activity are reduced in association with decreased insulin gene expression in several diabetic models (17,18).

Here we report for the first time that the BETA2/NeuroD protein can permeate cells due to an arginine- and lysine-rich sequence in its structure and that transduced BETA2/NeuroD functions similarly to endogenous BETA2/NeuroD.

RESEARCH DESIGN AND METHODS

Construction of BETA2/NeuroD protein vectors and purification of recombinant BETA2/NeuroD proteins. Full-length BETA2/NeuroD cDNA was amplified by PCR using appropriate linker-primers and then subcloned into the *Nde* I and *Xho* I sites of pET21b(+) (Novagen, Madison, WI) using a ligation kit (TaKaRa, Tokyo, Japan). On mutant plasmids, sequences before and after each deletion were amplified by PCR using appropriate linker-primers and were then subcloned into the *Nde* I–*Hind*III and *Hind*III–*Xho* I sites of pET21b(+), respectively. BL21 (DE3) cells containing the expression plasmids were grown at 37°C to an optical density 600 of 0.8. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mmol/l, 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-nitrilotriacetic acid agarose (Invitrogen, San Diego, CA). Purified BETA2/NeuroD proteins were conjugated using a Cy5-labeling kit (Amersham Pharmacia, Piscataway, NJ).

Isolation and culture of rat pancreatic islets. Islets were isolated from the pancreases of anesthetized male SD rats (200–250 g; Taconic Farms, Germantown, NY). All animal procedures were approved by the review committee of

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bHLH, basic helix-loop-helix; dNeuroD, deletion BETA2/NeuroD; dNeuroD-6, PTD-deleted BETA2/NeuroD protein; DsRed, *Discosoma sp.* red fluorescent protein; DynDN, dominant-negative mutant of dynamin-1; EGFP, enhanced green fluorescent protein; EGFP-PTD, EGFP with BETA2/NeuroD PTD; FITC, fluorescein isothiocyanate; PDX-1, pancreatic duodenal homeobox 1; PTD, protein transduction domain; TBST, Tris-buffered saline with Tween.

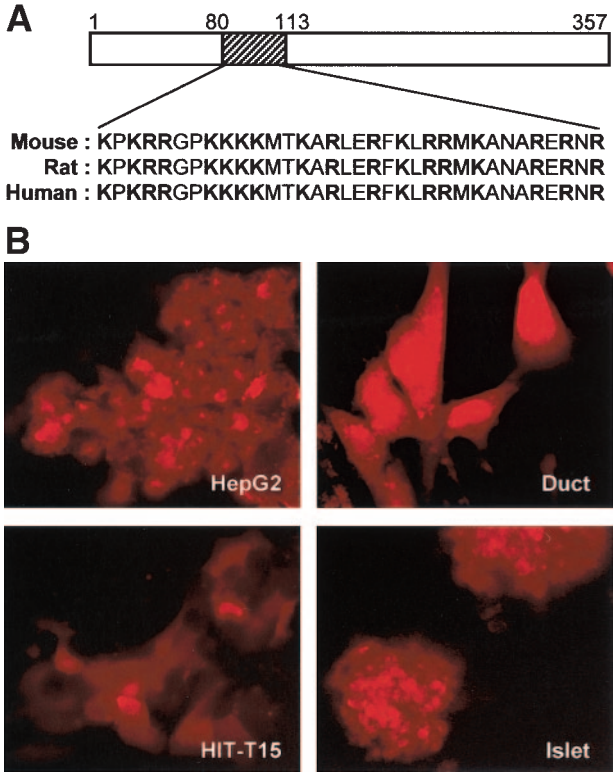


FIG. 1. Transduction of the BETA2/NeuroD protein into several cells. *A*: BETA2/NeuroD sequences in mice, rats, and humans. The arginine- and lysine-rich sequences of 34 amino acids are conserved in the BETA2/NeuroD of mice, rats, and humans. *B*: Several cells were treated with the Cy5-conjugated BETA2/NeuroD protein. BETA2/NeuroD was observed as a fluorescence signal in HepG2, primary pancreatic ductal cells, HIT-T15, and primary islets 6 h after this treatment.

Kyoto University Graduate School of Medicine. The common bile duct was cannulated and injected with 6 ml cold M199 medium containing 1.5 mg/ml collagenase (Roche Boehringer Mannheim, Indianapolis, IN). The islets were separated on Histopaque 1077 (Sigma, St. Louis, MO) density gradient, hand picked under a dissecting microscope to ensure a pure islet preparation, and used immediately afterward. In these experiments, islets were cultured in RPMI medium with 10% FCS.

Western blotting. Western blotting was conducted in accordance with the method described previously (19). In brief, after being washed three times with PBS and high salt buffer, cells were scraped from the dish and sonicated. Then 10 μg of cell extracts were fractionated by 10% SDS-PAGE and transferred to polyvinylidine fluoride membranes (Immun-Blot PVDF Membrane; Bio-RAD, Hercules, CA) using a transfer buffer containing 20% methanol, 25 mmol/l Tris base, and 192 mmol/l glycine (300 mA, 2 h). After being blocked at room temperature for 1 h in 50 mmol/l Tris-HCl, 150 mmol/l NaCl, and 0.1% Tween-20 (TBST) with 5% nonfat dry milk, the membranes were incubated overnight at 4°C in TBST using 5% nonfat dry milk containing goat anti−BETA2/NeuroD antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-actin antibody (1:1,000; Sigma), or horseradish peroxidase−conjugated anti−6 histidine antibody (1:5,000; Invitrogen). For anti−BETA2/NeuroD antibody or anti-actin antibody, the membranes were then incubated for 1 h at room temperature in TBST with 5% nonfat dry milk containing anti-goat or anti-mouse IgG antibody coupled to horseradish peroxidase (1:1,000; Bio-Rad).

Gel-mobility shift assay. For this assay, 2 μg nuclear extract were incubated with 2 μg poly (dI-dC), 25 mmol/l HEPES (pH 7.8), 0.2 mmol/l EDTA, 150 mmol/l KCl, 5 mmol/l dithiothreitol, and 9% glycerol 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 BETA2/NeuroD−binding region (E-box) and surrounding sequences (ACGTTCTGGC*CATCTG*CTGAT CCTACGT; E-box in italics) or E-box−mutated sequences (ACGTTCTGGC*TTTCCC*CTGAT CCTACGT) (Sigma Genosys, Woodlands, TX) was used as a binding probe. In some binding assays, anti−BETA2/NeuroD, anti−6 histidine antibody, and preimmune antisera were added to the reaction mixture 1 h before DNA probes were added.

Gene transfection and luciferase assay. A rat insulin II promoter-reporter (luciferase) plasmid (1.0 μg) containing 238-bp 5′-flanking sequences of the rat insulin II promoter region (20) or E-box−mutated insulin II promoter-reporter plasmid (1.0 μg) (18) was transfected into cells with LipofectAMINE (Invitrogen) using the conditions recommended by the manufacturer. Then 48 h after the transfection, the cells were harvested and assayed (Promega, Madison, WI).

Fluorescein isothiocyanate−conjugated BETA2/NeuroD PTD. Fluorescein isothiocyanate (FITC)−conjugated BETA2/NeuroD PTD peptide was synthesized by Sigma Genosys-Japan. The peptide was purified by preparative reversed-phase high-performance liquid chromatography, was confirmed as >95% pure as analyzed by high-performance liquid chromatography, and had the expected amino acid composition and mass spectra.

Treatment with FITC−BETA2/NeuroD PTD and FM4–64. Cervix-derived HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS and penicillin-streptomycin. Cells were treated with 1 μmol/l BETA2/NeuroD PTD for the specified time and then washed with fresh medium. To evaluate endocytosis, cells were incubated with 1 μmol/l FITC−BETA2/NeuroD PTD and 10 μmol/l FM 4–64 (Molecular Probes, Eugene, OR), a marker of endocytosis. Treated living cells were analyzed by conventional fluorescence microscopy and confocal microscopy.

Treatment with a plasmid expressing a dominant-negative mutant of dynamin. HeLa cells were transfected with a plasmid expressing a dominant-negative mutant of dynamin-1 (DynDN), *Discosoma sp.* red fluorescent protein (DsRed), as previously described (21). After 24 h, cells were washed and treated with 1 μmol/l FITC−BETA2/NeuroD PTD in complete medium. After cells were incubated overnight, the FITC and DsRed signals were examined.

Treatment with amiloride and cytochalasin D. To measure the mechanism of BETA2/NeuroD internalization, HeLa cells were plated and cultured in Dulbecco's modified Eagle's medium with 10% FBS. Cells were then pretreated for 10 or 30 min in serum-free medium with 5 mmol/l amiloride (Sigma) or 10 μmol/l cytochalasin D (Sigma), respectively. After the BETA2/NeuroD PTD was added, cells were maintained for 1 h in the presence of inhibitors, washed several times, and incubated for 1 h in complete medium.

RESULTS

Transduction of the BETA2/NeuroD protein into cells. To test whether the purified BETA2/NeuroD protein can be transduced into cells, cervix-derived HeLa, liver-derived HepG2, β-cell−derived HIT-T15 cells, and primary rat islets and duct cells were treated with Cy5-conjugated BETA2/NeuroD protein. Then 6 h after this treatment, BETA2/NeuroD was observed as a fluorescence signal in primary islets, duct cells, HIT-T15, HepG2 (Fig. 1*B*), and HeLa cell lines (Fig. 2*C1*). Although it has previously been shown that some homeoprotein transcription factors can enter a large number of cell types (9,20–22), our results showed that the BETA2/NeuroD protein, a bHLH transcription factor, is delivered into cells without requiring any additional sequences.

Protein transduction domain of BETA2/NeuroD protein. To clarify the location of the PTD of BETA2/NeuroD, we made several Cy5-conjugated deletion proteins (Fig. 2*A* and *B*). Full-length BETA2/NeuroD protein and deletion BETA2/NeuroD (dNeuroD)-2, -4, -7, -8, and -9, but not the other dNeuroDs, could be transduced into HeLa cells (Fig. 2*C* and *D*). Both dNeuroD-8 and -9 proteins could be transduced into cells, but the Cy5 signals were weaker than that with full-length protein treatment (Fig. 2*C*). These data show that the 34 amino acids of the putative BETA2/NeuroD PTD are necessary for the BETA2/NeuroD protein to be efficiently transduced into cells (Fig. 3*A*). As we and others have recently shown, the sequences of BETA2/NeuroD protein are rich in arginine and lysine (10–12).

Transduction efficiency of the BETA2/NeuroD PTD. To test the transduction efficiency of the PTD of BETA2/NeuroD, we made enhanced green fluorescent protein (EGFP)−fused PTDs. EGFP with 11 arginine (11R) PTD,

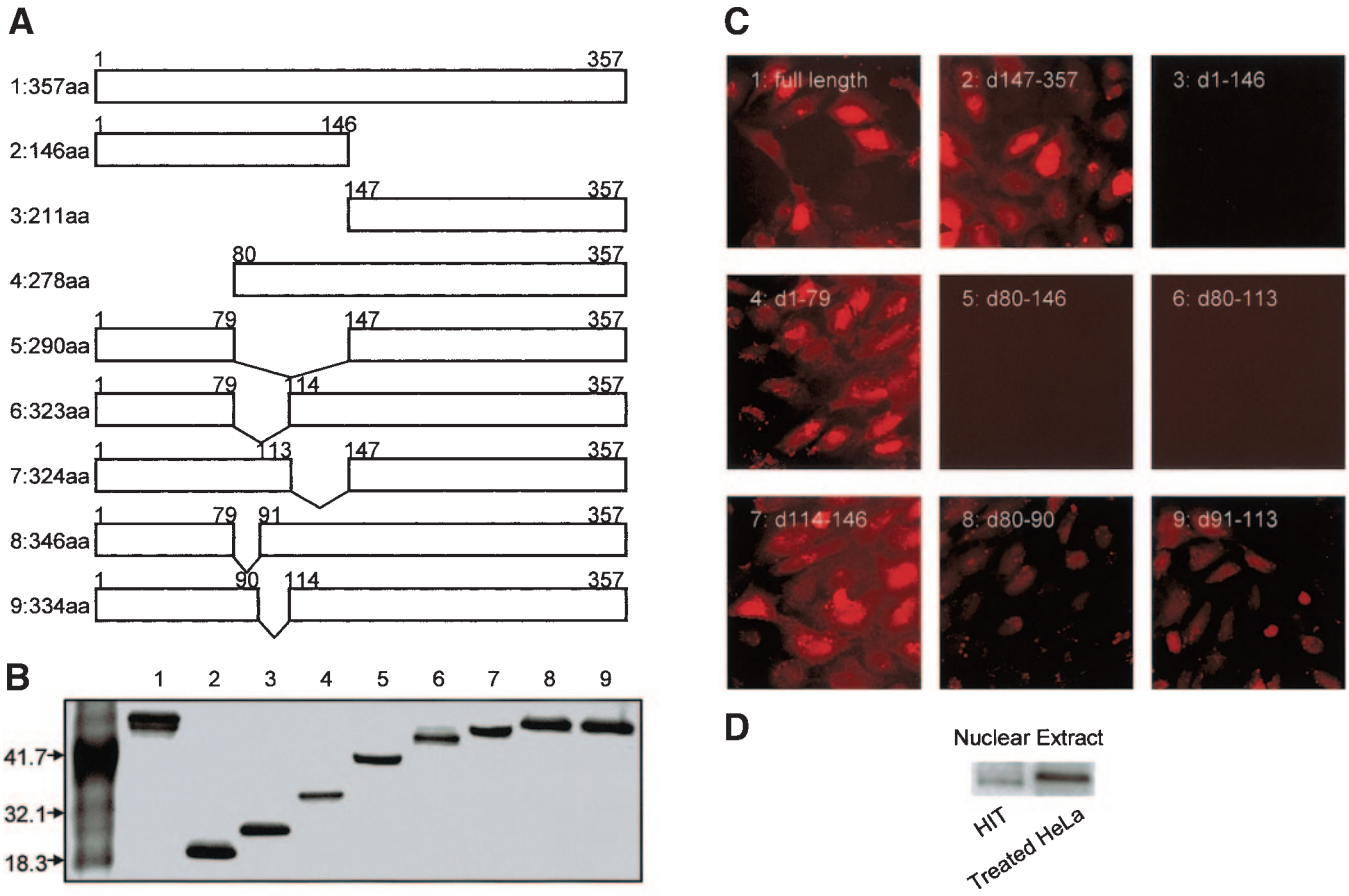


FIG. 2. Transduction of full-length and mutant BETA2/NeuroD proteins. *A*: **We made Cy5-conjugated full-length BETA2/NeuroD and mutant BETA2/NeuroD proteins with several deleted sequences (*A1*–*A9*).** *B*: **Purified full-length and mutant BETA2/NeuroD proteins were confirmed on Coomassie staining.** *C*: **HeLa cells were treated with Cy5-conjugated full-length (*A1*) and mutant (dNeuroD; *A2*–*A9*) BETA2/NeuroD proteins. The full-length BETA2/NeuroD protein (*A1*) and dNeuroD-2, -4, -7, -8, and -9 (*A2*, *A4*, *A7*, *A8*, and *A9*) can be transduced into HeLa cells 6 h after this treatment but not dNeuroD-3, -5, or -6 (*A3*, *A5*, or *A6*).** *D*: **Western blotting using anti-BETA2/NeuroD antibody on nuclear extracts of HeLa cells treated with BETA2/NeuroD protein and nuclear extracts of HIT cells.**

the most effective protein transduction domain (10,11), and EGFP without PTD were used as positive and negative controls, respectively (Figs. 3*A* and *B*). HeLa cells were treated with these EGFP proteins for 6 h and analyzed by Western blotting (Fig. 3*C*) and confocal microscopy (Fig. 3*D*). The transduction efficiency of the full-length BETA2/NeuroD PTD was similar to that of the 11R PTD, whereas the efficiency of the partial sequence of the PTD was about half that of the 11R PTD (Figs. 3*C* and *D*). These data show that the transduction efficiency of BETA2/NeuroD PTD is as strong as that of 11R PTD.

Dose-dependent transduction of BETA2/NeuroD and the stability of transduced BETA2/NeuroD protein. To test the effectiveness of transduced BETA2/NeuroD, HeLa cells were treated with the BETA2/NeuroD protein at several concentrations for 2 h, washed with PBS, incubated with conventional medium, and analyzed by Western blotting (Fig. 4*A*). The BETA2/NeuroD protein was transduced in a dose-dependent manner up to 1 μmol/l. To determine the stability of the BETA2/NeuroD protein, HeLa cells were treated with 1 μmol/l BETA2/NeuroD for 2 h, washed with PBS, incubated with conventional medium for 1–46 h, and analyzed by Western blotting. The half-life of BETA2/NeuroD was ~5 h (Fig. 4*B*). In addition, the transduced BETA2/NeuroD could be detected using an antibody to the histidine tag included in this synthesized protein.

Effects of transduced BETA2/NeuroD on clonal cells. The insulin-enhancer element E-box is the binding site for BETA2/NeuroD and plays an important role in the regulation of insulin gene transcription (4,17,18). We therefore used a gel shift assay to test whether the transduced BETA2/NeuroD protein binds to the insulin-enhancer element. HeLa cells were treated with 1 μmol/l BETA2/NeuroD protein, dNeuroD-6 (PTD-deleted BETA2/NeuroD protein), and EGFP with BETA2/NeuroD PTD (EGFP-PTD) for 24 h. The nuclear extract of HIT-T15 cells was used as a positive control. The E-box binding complex, but not the mutated E-box, was observed in nuclear extracts from treated HeLa cells. The specificity of the band was confirmed by its supershift with BETA2/NeuroD antibody and anti–6 histidine antibody but not by preimmune serum (Fig. 5*A*).

To test whether transduced BETA2/NeuroD protein can activate insulin gene transcription, we examined its effect on insulin promoter activity using a luciferase assay. HeLa cells were transfected with an insulin promoter–luciferase plasmid and allowed to grow for 24 h; they were then treated with 1 μmol/l BETA2/NeuroD protein, dNeuroD-6, and EGFP-PTD for an additional 24 h, after which a luciferase assay was performed. Insulin promoter activity was increased by treatment with BETA2/NeuroD protein in HeLa cells (Fig. 5*B*) but not by treatment with dNeuroD-6 or EGFP-PTD. Mutated insulin promoter activity was not

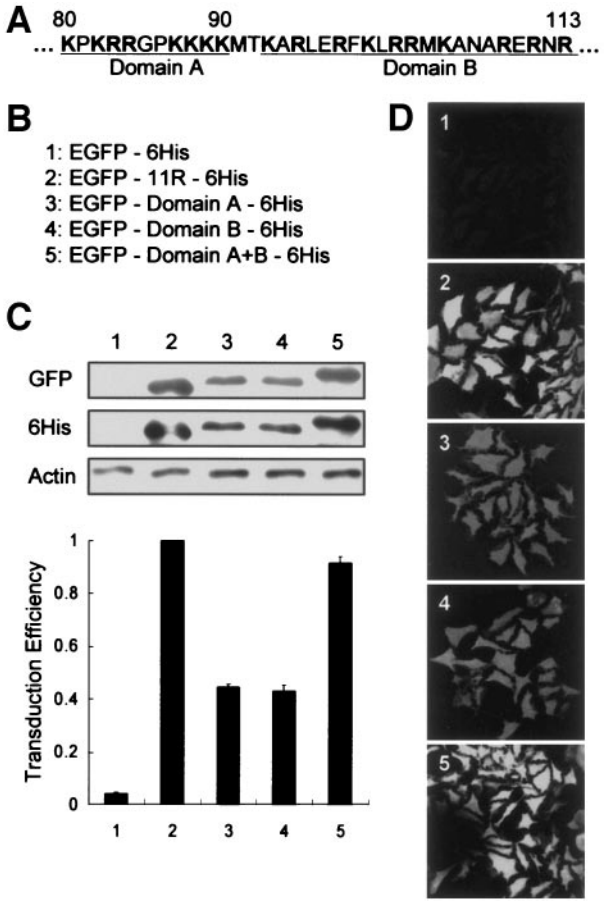


FIG. 3. Transduction efficiency of BETA2/NeuroD PTD. *A*: Protein transduction domain of BETA2/NeuroD, domain A, and domain B. *B*: Different EGFP-fused PTDs were generated as proteins. *C*: Western blotting on extracts of HeLa cells treated with EGFP-fused PTD proteins. The EGFP signal was observed in HeLa cells only when EGFP with PTDs were used. Actin expression was used as a loading control. This gel is representative of three independent experiments. *D*: HeLa cells were treated with 1 μmol/l EGFP-fused PTD proteins for 6 h. 6His, 6 histidine.

significantly changed by any treatment. Taken together, these results suggest that exogenous BETA2/NeuroD protein can be transduced into cells and their nuclei, bind to the E-box, and activate the insulin promoter.

Transduction of BETA2/NeuroD protein by endocytosis. To investigate the mechanism of BETA2/NeuroD protein transduction, HeLa cells were treated with FITC-conjugated BETA2/NeuroD PTD and FM 4–64, a marker of endocytosis, for 30 min and then washed with fresh media. The PTD fluorescence signal appeared as punctate within living, unfixed HeLa cells. This punctate PTD mainly co-localized with FM 4–64 in living cells (Fig. 6*A*). After an additional 90 min, the PTD was homogeneously distributed in the cytosol and nuclei of some cells, whereas the FM dye remained in the endosome (Fig. 6*A*). These data show that the cellular uptake of BETA2/NeuroD PTD occurs through electrostatic interaction with the plasma membrane, subsequent endocytosis, and escape from the endosome with eventual homogeneous distribution in the cytoplasm.

BETA2/NeuroD protein transduction is independent of phagocytosis or clathrin- or caveolar-mediated endocytosis. It was then determined whether the cellular uptake of BETA2/NeuroD occurs through a specific endocytic pathway. Multiple forms of endocytosis, including phagocytosis and clathrin- and caveolar-mediated endocytosis, require dynamin GTPase activity for vesicle formation at the cell surface. The expression of DynDN effectively blocks these endocytic pathways (21,23). We transfected cells with the DynDN-DsRed plasmid, treated the cells with FITC-conjugated BETA2/NeuroD PTD 24 h after transfection, and analyzed FITC signals at 48 h. The expression of DynDN did not block BETA2/NeuroD PTD uptake and recombination (Fig. 6*B*). These observations show that the BETA2/NeuroD internalization process does not involve caveolar- or clathrin-mediated endocytosis.

BETA2/NeuroD protein enters cells by lipid raft macropinocytosis. Recently, it was reported that PTD-mediated cellular entry occurs by macropinocytosis (7,21). Macropinocytosis is a rapid, lipid raft–dependent, and receptor-independent form of endocytosis. It requires actin membrane protrusions that envelope vesicles, termed macropinosomes (23). To examine the involvement of macropinocytosis in transduction, cells were pretreated with amiloride, a specific inhibitor of the Na^+/H^+ exchange required for macropinocytosis (24), or cytochalasin D, an F-actin elongation inhibitor (25). Pretreated cells were then incubated with BETA2/NeuroD PTD for 1 h and washed with complete medium. Cell treatment with either

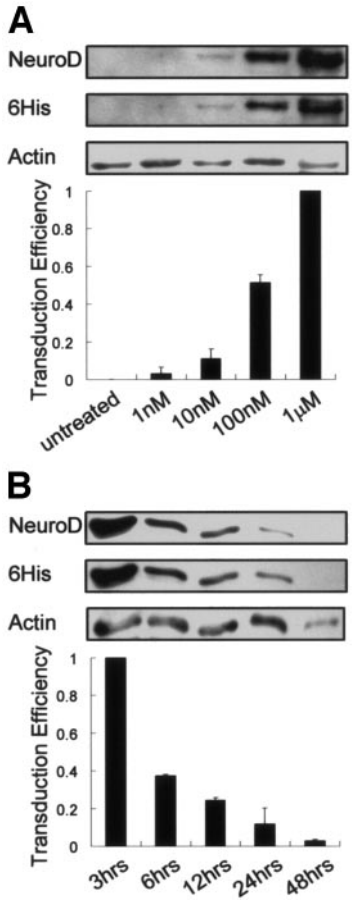
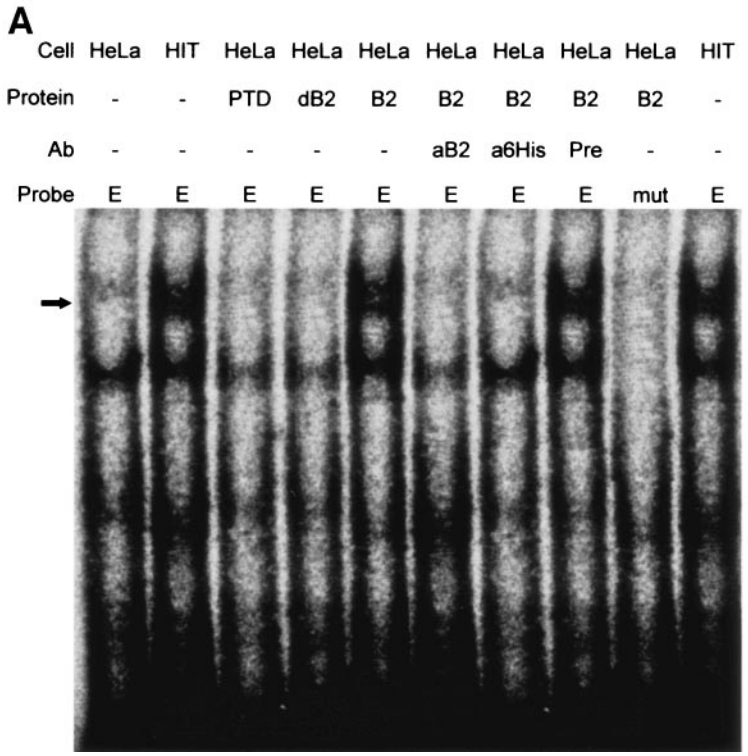


FIG. 4. Dose-dependent transduction of BETA2/NeuroD protein and stability of transduced BETA2/NeuroD protein. *A*: Western blotting using anti–BETA2/NeuroD antibody and anti–6 histidine (6His) antibodies on extracts of HeLa cells treated with BETA2/NeuroD protein at several concentrations. *B*: Using cells treated with 1 μmol/l BETA2/NeuroD for 2 h, washed with PBS, and incubated with conventional medium for 1–46 h, the protein is seen to be stable for only a few hours. These gels are representative of three independent experiments. Actin expression was used as a loading control. The same membranes were used after stripping for different antibodies.



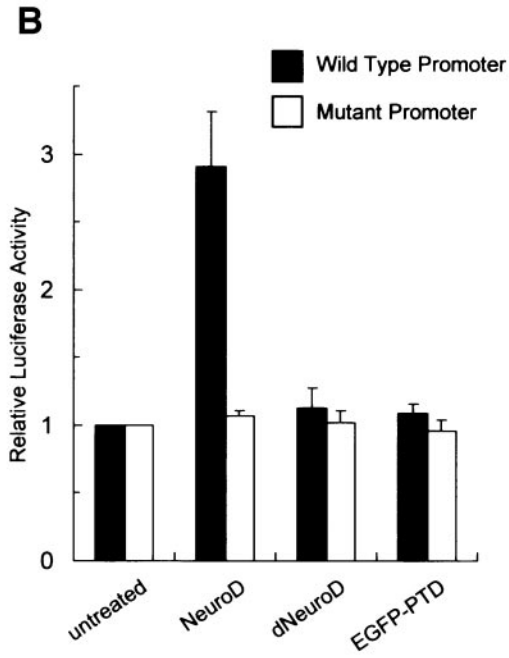


FIG. 5. Effects of transduced BETA2/NeuroD on clonal cells. *A*: Increase of BETA2/NeuroD–binding activity by transduced BETA2/NeuroD protein. After being treated with 1 μmol/l BETA2/NeuroD protein (B2) for 24 h, nuclear extracts from HeLa cells were used in a gel shift assay. An increased intensity of the E-box (*E*) binding complex (*arrow*) was observed with nuclear extracts from treated HeLa cells. Anti–BETA2/NeuroD antiserum (aB2), anti–6 histidine antibody (a6His), and preimmune serum (Pre) were used to show specificity. The nuclear extract from HIT-T15 (HIT) cells was used as a positive control. The nuclear extracts from untreated (−), EGFP-PTD (PTD)–treated (Fig. 3*B5*), and dNeuroD-6 (dB2)–treated (Fig. 2*A6*) HeLa cells and the mutated probe (mut) were used as negative controls. This gel is representative of three independent experiments. *B*: Insulin promoter activity using a luciferase assay. HeLa cells were transfected with an insulin promoter–luciferase plasmid and allowed to grow for 24 h; they were then treated with 1 μmol/l BETA2/NeuroD protein, dNeuroD-6 (Fig. 2*A6*), and EGFP-PTD (Fig. 3*B5*) for an additional 24 h, after which a luciferase assay was performed. Insulin promoter activity was significantly increased by treatment with the BETA2/NeuroD protein in HeLa cells compared with treatment with dNeuroD-6 or EGFP-PTD ($P < 0.01$). Data are means ± SE, with the basal insulin promoter activity being arbitrarily set at 1 ($n = 4$).

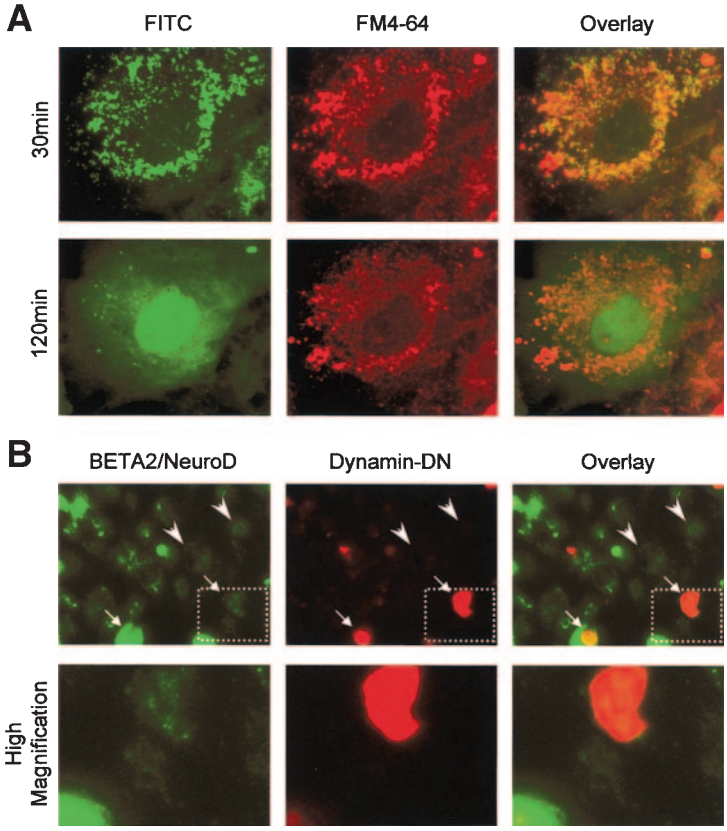


FIG. 6. BETA2/NeuroD protein transduction by endocytosis. *A*: HeLa cells were treated with FITC-conjugated BETA2/NeuroD PTD and FM 4–64, a marker of endocytosis, for 30 min and then washed with fresh medium. The PTD fluorescence signal appeared as punctate within living, unfixed HeLa cells. This punctate PTD mainly co-localized with FM 4–64 in the living cells (30 min). After an additional 90 min, the PTD was homogeneously distributed in the cytosol and nuclei of some cells, whereas the FM dye remained in the endosome (120 min). *B*: BETA2/NeuroD protein transduction is independent of phagocytosis or clathrin- or caveolar-mediated endocytosis. Cells were transfected with the DynDN-DsRed plasmid and then treated with FITC-conjugated BETA2/NeuroD PTD 24 h after transfection. The FITC signals were analyzed at 48 h. FITC signals were observed in both DsRed-positive and -negative cells. The expression of DynDN (DsRed positive) did not block BETA2/NeuroD PTD uptake and recombination. *Arrow*, DsRed-positive cells; *arrow head*, DsRed-negative cells.

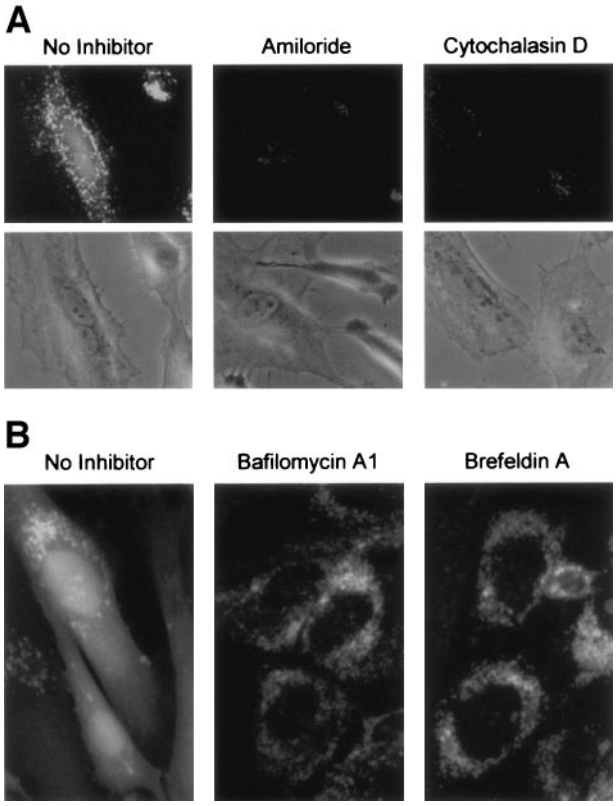


FIG. 7. Mechanism of BETA2/NeuroD protein transduction. *A*: BETA2/NeuroD protein enters cells by lipid raft macropinocytosis. Cells were pretreated with macropinosome inhibitors, amiloride, or cytochalasin D, incubated with BETA2/NeuroD PTD for 1 h, and washed with complete medium. Cell treatment with both inhibitors resulted in the reduction in BETA2/NeuroD PTD transduction into vesicles, with no cytotoxicity. *B*: The impact of bafilomycin A1 or brefeldin A on the intracellular distribution of BETA2/NeuroD PTD was tested: 30 min before the addition of the PTD peptide, cells were washed once and 300 nmol/l bafilomycin or 20 μmol/l brefeldin A was added to the medium. After a 2-h incubation with PTDs, no cytoplasmic fluorescence was present and only vesicular staining was observed.

macropinosome inhibitor resulted in the reduction of BETA2/NeuroD PTD transduction into vesicles with no cytotoxicity (Fig. 7*A*). Taken together, these observations showing rapid transduction into cells, dynamin-1 independence, and uptake inhibition by amiloride and cytochalasin D, indicate that BETA2/NeuroD PTD−mediated cellular entry occurs by lipid raft−mediated macropinocytosis.

Impact of bafilomycin A1 or brefeldin A on the cellular distribution of BETA2/NeuroD PTD. Bafilomycin A1 is a highly potent and selective inhibitor of vacuolar H^+ ATPases and inhibits endosomal acidification (26). The effect of bafilomycin A1 on the intracellular localization of the peptide was tested: 30 min before the PTD peptide was added, cells were washed once and then 300 nmol/l bafilomycin was added to the medium. After a 2-h incubation with BETA2/NeuroD PTD, no cytoplasmic fluorescence was present and only vesicular staining was observed (Fig. 7*B*). These data show that the release of 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 BETA2/NeuroD PTD. Brefeldin A interferes with the integrity of the Golgi and trans-Golgi network and affects the uptake of the TAT fusion protein (27). In this study, cells were washed once and 20 μmol/l brefeldin A was added 30 min before the peptide was introduced. After a 2-h incubation with the peptide, the cellular fluorescence of BETA2/NeuroD PTD was reduced (Fig. 7*B*), similar to what was seen with bafilomycin A1. Taken together, these data suggest that the BETA2/NeuroD protein penetrated cells by macropinocytosis and entered the cytoplasm by means of retrograde transport (Fig. 8).

DISCUSSION

PTDs are a series of small domains that have been shown to cross biological membranes efficiently, independently of transporters or specific receptors, and promote the delivery of full-length proteins in a rapid and concentration-dependent fashion into a large number of cells. These PTDs are arginine- and lysine-rich sequences, especially the TAT PTD. We and others have shown that proteins with fused polyarginine or polylysine sequences can permeate several cell types even more efficiently (10–12,28). It has also been shown that >40% of lysine- and arginine-rich peptides within 11–16 amino acids can permeate cells (29). BETA2/NeuroD has arginine- and lysine-rich se-

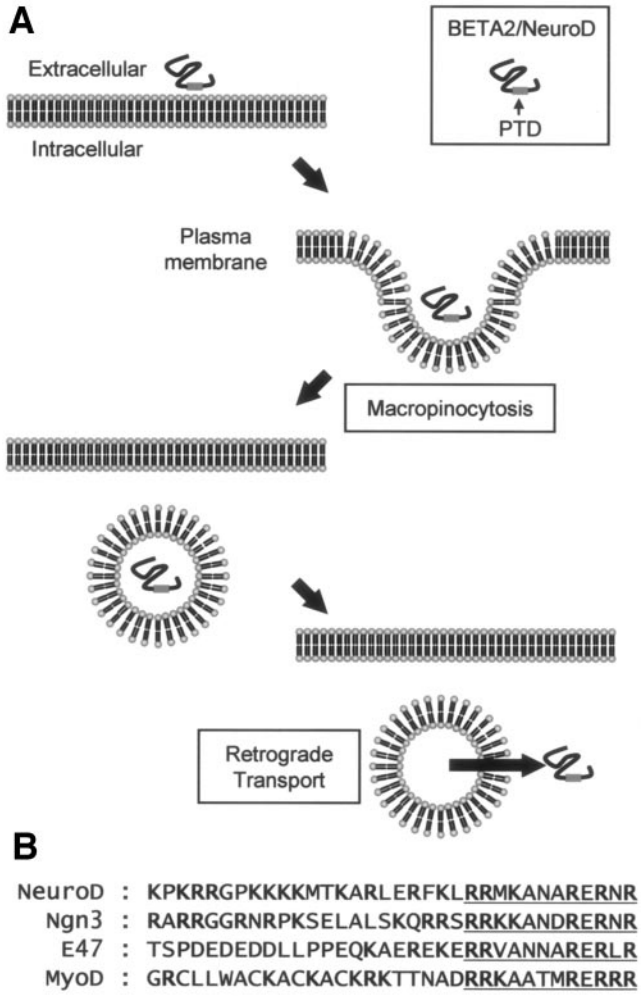


FIG. 8. Schema of BETA2/NeuroD protein transduction. *A*: Main mechanism of BETA2/NeuroD protein transduction was electrostatic interaction with the plasma membrane, penetration into cells by macropinocytosis, and a release from endosome by retrograde transport. *B*: BETA2/NeuroD protein transduction domain and other bHLH proteins. Ngn3, neurogenin 3; MyoD, myogenic determination factor.

quences in its own sequence and the arginine and lysine content is 53% over 34 amino acids.

BETA2/NeuroD is selectively expressed in the developing endocrine pancreas, the small intestine, and the nervous system (2) and plays an important role in maintaining the differentiation of endocrine cells, proper islet morphogenesis, and normal cell function, especially β-cell function, by regulating the insulin gene (4,5). Mice with an inactivated *BETA2/NeuroD* gene die of severe diabetes caused by a major reduction in the number of β-cells and a lack of proper islet formation (4). BETA2/NeuroD strongly stimulates DNA-binding capacity by heterodimerization with class A bHLH proteins, such as the ubiquitous E47. The PTD of BETA2/NeuroD includes the DNA-binding region of the bHLH factor. Thus the 34 amino acids of BETA2/NeuroD PTD are extremely important in several other aspects of its function.

Because the DNA-binding domain is conserved between different bHLH proteins (Fig. 8*B*, *underlined*), most, but not all, bHLH proteins could be transduced into cells. However, transduction efficiency is extremely different between each bHLH protein because sequences before the DNA binding domain are quite different. Neurogenin 3 and myogenic determination factor could be transduced into cells, but the efficiency is less than that of the BETA2/NeuroD protein (H.N., M.M., unpublished observations). E47 is hardly transduced into cells.

Our study showed that BETA2/NeuroD protein has a native PTD and, without modification, can permeate several cell types and enhance insulin promoter activity in these cells. In the insulin promoter, E-boxes, corresponding to the consensus CANNTG, are the binding site of heterodimer E47/BETA2 and are juxtaposed to AT-rich elements (A-box) that bind pancreatic duodenal homeobox 1 (PDX-1). The synergistic activation of *cis*-elements is observed between BETA2 and PDX-1 (30,31). Moreover, these two proteins are important in pancreatic development (5,32). We have reported that the PDX-1 protein can transduce cells because of its own Antennapedia-like protein and that PDX-1 protein induces insulin expression in pancreatic ductal cells, thought to be islet progenitor cells (20). The exogenous BETA2/NeuroD protein also induces insulin expression in pancreatic ductal progenitor cells (H.N., S.M., unpublished observations). The *BETA2/NeuroD* gene has E-boxes, and the BETA2/NeuroD protein could stimulate its own transcription (33). Furthermore, the *pdx-1* gene has A-boxes, and the PDX-1 protein could positively autoregulate its expression (34). Once the BETA2/NeuroD and PDX-1 proteins are transduced into progenitor cells, endogenous *BETA2/NeuroD* and *pdx-1* gene transcription are amplified by these proteins, stimulate insulin transcription, and may facilitate their differentiation into insulin-producing cells. Our finding of a functional PTD in transcription factor BETA2/NeuroD suggests that exogenous BETA2/NeuroD could be a novel approach for generating surrogate β-cells without requiring gene transfer technology.

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REFERENCES

1. Bang AG, Goulding MD: Regulation of vertebrate neural cell fate by transcription factors. *Curr Opin Neurobiol* 6:25–32, 1996
2. Lee JE, Hollenberg SM, Snider L, Turner DL, Lipnick N, Weintraub H: Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science* 268:836–844, 1995
3. Olson EN, Klein WH: bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. *Genes Dev* 8:1–8, 1994
4. Naya FJ, Stellrecht CM, Tsai MJ: Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. *Genes Dev* 9:1009–1019, 1995
5. Naya FJ, Huang HP, Qiu Y, Mutoh H, DeMayo FJ, Leiter AB, Tsai MJ: Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. *Genes Dev* 11:2323–2334, 1997
6. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF: In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* 285:1569–1572, 1999
7. Wadia JS, Stan RV, Dowdy SF: Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med* 10:310–315, 2004
8. Elliott G, O'Hare P: Intercellular trafficking and protein delivery by a herpes virus structural protein. *Cell* 88:223–233, 1997
9. Joliot A, Prochiantz A: Transduction peptides: from technology to physiology. *Nat Cell Biol* 6:189–196, 2004
10. Matsushita M, Tomizawa K, Moriwaki A, Li ST, Terada H, Matsui H: A high-efficiency protein transduction system demonstrating the role of PKA in long-lasting long-term potentiation. *J Neurosci* 21:6000–6007, 2001
11. Matsui H, Tomizawa K, Lu YF, Matsushita M: Protein therapy: in vivo protein transduction by polyarginine (11R) PTD and subcellular targeting delivery. *Curr Protein Pept Sci* 4:151–157, 2003
12. Noguchi H, Matsushita M, Okitsu T, Moriwaki A, Tomizawa K, Kang S, Li ST, Kobayashi N, Matsumoto S, Tanaka K, Tanaka N, Matsui H: A new cell-permeable peptide allows successful allogeneic islet transplantation in mice. *Nat Med* 10:305–309, 2004
13. Malecki MT, Jhala US, Antonellis A, Fields L, Doria A, Orban T, Saad M, Warram JH, Montminy M, Krolewski AS: Mutations in NEUROD1 are associated with the development of type 2 diabetes mellitus. *Nat Genet* 23:323–328, 1999
14. Kristinsson SY, Thorolfsdottir ET, Talseth B, Steingrimsson E, Thorsson AV, Helgason T, Hreidarsson AB, Arngrimsson R: MODY in Iceland is associated with mutations in HNF-1alpha and a novel mutation in NeuroD1. *Diabetologia* 44:2098–2103, 2001
15. Iwata I, Nagafuchi S, Nakashima H, Kondo S, Koga T, Yokogawa Y, Akashi T, Shibuya T, Umeno Y, Okeda T, Shibata S, Kono S, Yasunami M, Ohkubo H, Niho Y: Association of polymorphism in the NeuroD/BETA2 gene with type 1 diabetes in the Japanese. *Diabetes* 48:416–419, 1999
16. Hansen L, Jensen JN, Urioste S, Petersen HV, Pociot F, Eiberg H, Kristiansen OP, Hansen T, Serup P, Nerup J, Pedersen O: NeuroD/BETA2 gene variability and diabetes: no associations to late-onset type 2 diabetes but an A45 allele may represent a susceptibility marker for type 1 diabetes among Danes: Danish Study Group of Diabetes in Childhood and the Danish IDDM Epidemiology and Genetics Group. *Diabetes* 49:876–878, 2000
17. Jonas JC, Sharma A, Hasenkamp W, Ilkova H, Patane G, Laybutt R, Bonner-Weir S, Weir GC: Chronic hyperglycemia triggers loss of pancreatic beta cell differentiation in an animal model of diabetes. *J Biol Chem* 274:14112–14121, 1999
18. Kaneto H, Sharma A, Suzuma K, Laybutt DR, Xu G, Bonner-Weir S, Weir GC: Induction of c-Myc expression suppresses insulin gene transcription by inhibiting NeuroD/BETA2-mediated transcriptional activation. *J Biol Chem* 277:12998–13006, 2002
19. Noguchi H, Kobayashi N, Westerman KA, Sakaguchi M, Okitsu T, Totsugawa T, Watanabe T, Matsumura T, Fujiwara T, Ueda T, Miyazaki M, Tanaka N, Leboulch P: Controlled expansion of human endothelial cell populations by Cre-loxP-based reversible immortalization. *Hum Gene Ther* 13:321–334, 2002
20. Noguchi H, Kaneto H, Weir GC, Bonner-Weir S: PDX-1 protein containing its own antennapedia-like protein transduction domain can transduce pancreatic duct and islet cells. *Diabetes* 52:1732–1737, 2003
21. Noguchi H, Matsumoto S, Okitsu T, Iwanaga Y, Yonekawa Y, Nagata H, Matsushita M, Wei FY, Matsui H, Minami K, Seino S, Masui Y, Futaki S, Tanaka K: PDX-1 protein is internalized by lipid raft-dependent macropinocytosis. *Cell Transplant.* In press
22. Noguchi H, Matsushita M, Matsumoto S, Lu YF, Matsui H, Bonner-Weir S:

Mechanism of PDX-1 protein transduction. *Biochem Biophys Res Commun* 332:68–74, 2005
23. Conner SD, Schmid SL: Regulated portals of entry into the cell. *Nature* 422:37–44, 2003
24. West MA, Bretscher MS, Watts C: Distinct endocytotic pathways in epidermal growth factor-stimulated human carcinoma A431 cells. *J Cell Biol* 109:2731–2739, 1989
25. Sampath P, Pollard TD: Effects of cytochalasin, phalloidin, and pH on the elongation of actin filaments. *Biochemistry* 30:1973–1980, 1991
26. Bowman EJ, Siebers A, Altendorf K: Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc Natl Acad Sci U S A* 85:7972–7976, 1988
27. Drecktrah D, de Figueiredo P, Mason RM, Brown WJ: Retrograde trafficking of both Golgi complex and TGN markers to the ER induced by nordihydroguaiaretic acid and cyclofenil diphenol. *J Cell Sci* 111:951–965, 1998
28. Noguchi H, Nakai Y, Matsumoto S, Kawaguchi M, Ueda M, Okitsu T, Iwanaga Y, Yonekawa Y, Nagata H, Minami K, Masui Y, Futaki S, Tanaka K: Cell permeable peptide of JNK inhibitor prevents islet apoptosis immediately after isolation and improves islet graft function. *Am J Transplant* 5:1848–1855, 2005
29. Mi Z, Mai J, Lu X, Robbins PD: Characterization of a class of cationic peptides able to facilitate efficient protein transduction in vitro and in vivo. *Mol Ther* 2:339–347, 2000
30. Glick E, Leshkowitz D, Walker MD: Transcription factor BETA2 acts cooperatively with E2A and PDX1 to activate the insulin gene promoter. *J Biol Chem* 275:2199–2204, 2000
31. Ohneda K, Mirmira RG, Wang J, Johnson JD, German MS: The homeodomain of PDX-1 mediates multiple protein-protein interactions in the formation of a transcriptional activation complex on the insulin promoter. *Mol Cell Biol* 20:900–911, 2000
32. Jonsson J, Carlsson L, Edlund T, Edlund H: Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* 371:606–609, 1994
33. Miyachi T, Maruyama H, Kitamura T, Nakamura S, Kawakami H: Structure and regulation of the human NeuroD (BETA2/BHF1) gene. *Brain Res Mol Brain Res* 69:223–231, 1999
34. Marshak S, Benshushan E, Shoshkes M, Havin L, Cerasi E, Melloul D: Functional conservation of regulatory elements in the pdx-1 gene: PDX-1 and hepatocyte nuclear factor 3β transcription factors mediate β-cell–specific expression. *Mol Cell Biol* 20:7583–7590, 2000