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Induction by NeuroD of the components required for regulated exocytosis

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Abstract

NeuroD is a transcriptional factor critical in differentiation of neuronal cells, enteroendocrine cells, and pancreatic endocrine cells. However, little is known of its roles in cellular functions. We show here that introduction of NeuroD into human fetal epithelial cell line Intestine 407 cells induces neuron-like morphology. In addition, multiple genes associated with vesicular trafficking and exocytotic machinery, including Sec24D, carboxypeptidase E, myosin Va, SNAP25, syntaxin 1A, Rab, Rims, Munc18-1, and adenylyl cyclase, were up-regulated by NeuroD gene transfer. Moreover, low osmotic pressure-induced exocytosis monitored by FM1-43 was enhanced by overexpression of NeuroD. These results suggest that NeuroD plays an important role in regulated exocytosis by inducing expressions of various components required in the process.

Keywords: NeuroD; SNAREs; Rabs; Exocytosis

NeuroD (also known as BETA2) is a basic helix–loop–helix (bHLH) transcription factor expressed in pancreatic endocrine, enteroendocrine, and neuronal cells [1,2]. Lack of NeuroD expression in mice results in a reduction of mature pancreatic islets and decreased numbers of secretin- and cholecystokinin-producing enteroendocrine cells, leading to perinatal death [3]. When NeuroD expression is rescued by a transgene under the insulin promoter, the mice survive but exhibit severe loss of inner sensory neurons and granule cells in cerebellum and hippocampus [4,5]. In addition, NeuroD has been implicated in cell fate determination, differentiation, and cell survival in neurons [6,7]. These findings indicate that NeuroD is essential in normal development of pancreatic endocrine, enteroendocrine, and neuronal cells.

NeuroD is known to directly regulate the expression of genes in differentiated cells. NeuroD binds and transactivates the insulin gene upon dimerization with ubiquitous bHLH transcription factor E47 [8]. Sulfonylurea receptor 1 (SUR1), the regulatory subunit of the ATP-sensitive K^+ channel in pancreatic β-cells, is up-regulated by NeuroD [9]. Expression of glucokinase, the rate-limiting enzyme of glucose metabolism in pancreatic β-cells, also is activated by NeuroD [10]. In addition, NeuroD activates promoters of the islet-specific glucose-6-phosphatase catalytic subunit-related protein, secretin, and Pax6 [11–13]. However, the roles of NeuroD in the regulation of cellular functions are poorly understood.

The human fetal epithelial cell line Intestine 407 possesses undifferentiated enteric cell properties [14] without expression of NeuroD. IEC6, a cell line derived from rat immature intestinal epithelium, has been shown to differentiate into insulin-secreting cells by introduction of transcription factors important in pancreatic β-cell development [15,16]. These findings indicate that undifferentiated

Abbreviations: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SNAP25, synaptosome-associated protein of 25 kDa; cAMP, cyclic AMP.

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intestinal cells possess potency to differentiate into cells with endocrine phenotype. In the present study, we have used Intestine 407 cells to clarify the roles of NeuroD in the regulation of gene expression and exocytosis including vesicle trafficking and membrane fusion.

Materials and methods

Cell culture. Intestine 407 (Dainippon Sumitomo Pharma, Osaka, Japan) human fetal intestinal epithelial cells were cultured in DMEM (Sigma, St. Louis, MO) containing 10% fetal calf serum under humidified condition of 5% CO_2/95% air at 37 °C.

Adenoviruses. Human NeuroD recombinant adenovirus (Ad-NeuroD) was constructed under cytomegalovirus (CMV) promoter, using an adenovirus expression vector kit (Takara, Ohtsu, Japan) according to the manufacturer's instruction. Adenovirus expressing β-galactosidase (Ad-LacZ) was used as a negative control.

Immunoblotting of NeuroD. Cells were washed with PBS and lysed in a buffer containing 150 mM NaCl, 20 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF, and protein inhibitor cocktail (Nacalai Tesque, Kyoto Japan). The total cell lysates (20 μg of protein) were subjected to SDS–PAGE and separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were probed with anti-NeuroD antibody (N-19) (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-actin antibody (I-19) (Santa Cruz Biotechnology). The secondary antibody used was horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology). Signals were visualized using ECL plus Western Blotting Detection System (GE Healthcare Bio-Sciences, Piscataway, NJ). Blocking peptide (N-19P) (Santa Cruz Biotechnology) for anti-NeuroD antibody was used to confirm specificity of the reaction.

Reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated using the RNeasy Mini kit (Qiagen, Venlo, The Netherlands). After treatment with DNaseI (Qiagen), 1 μg of the RNA was reverse-transcribed by RevaTraAce (Toyobo, Osaka, Japan) with random priming. PCR primers were designed such that the amplified regions spanned introns in the gene. β-Actin was amplified to confirm equivalence between samples. The PCR conditions were as follows: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s. The cycle number was 35, 33, and 23 for glucokinase, SUR1, and β-actin, respectively.

For real-time RT-PCR analysis, TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) were used. All reactions and analyses were performed using ABI PRISM 7300 Sequence Detection System (Applied Biosystems). Gene expression levels were calculated using comparative threshold cycle methods according to ABI PRISM 7700 Sequence Detection System User Bulletin #2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The GAPDH expression level was unchanged even when NeuroD was overexpressed. Experiments were repeated four to seven times, each of which included duplicate measurements.

GeneChip analysis. Intestine 407 cells were infected with Ad-LacZ or Ad-NeuroD at 25 multiplicity of infection (moi). Two days after infection, total RNA isolated as described was subjected to GeneChip analysis. Labeled cRNA was synthesized and hybridized by Human Genome Focus Array (Affymetrix, Santa Clara, CA), which represents over 8500 human genes, according to the manufacturer's instruction. The probe arrays were washed and stained. The signals were scanned using GeneChip Scanner 3000 (Affymetrix). The scanning data were analyzed using Microarray Suite 5.1 (Affymetrix).

Measurement of exocytosis with FM1-43. Intestine 407 cells were seeded on a 96-well plate the day before infection. Cells were infected with Ad-LacZ or Ad-NeuroD (100 moi) and cultured for 72 h. After washing with Opti-MEM (Invitrogen), 2 μM FM1-43 (Molecular Probes, Eugene,

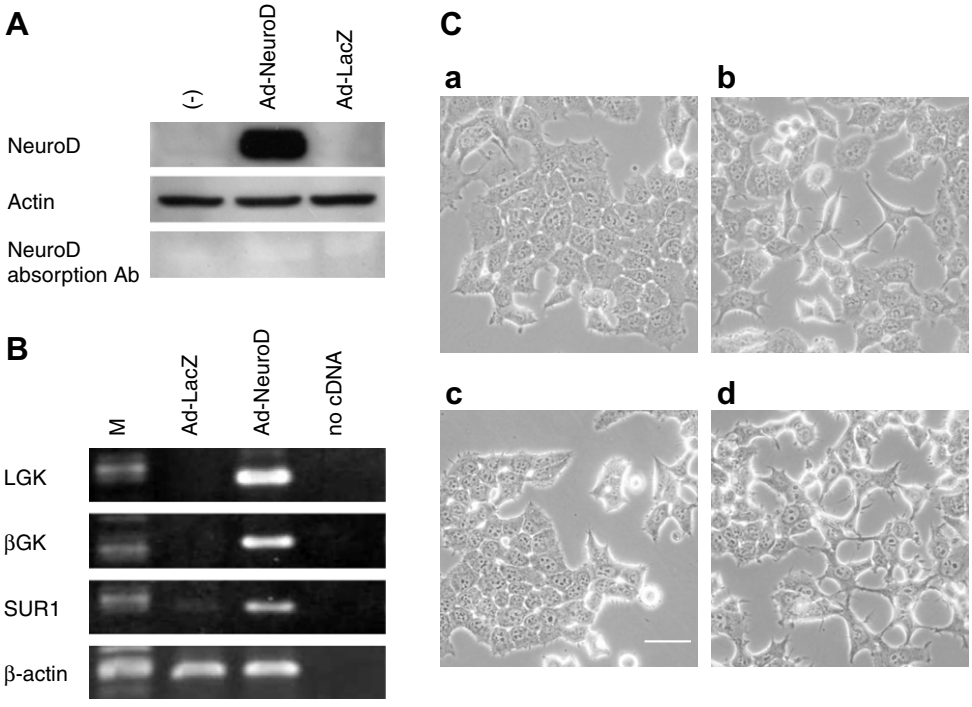

Fig. 1. Validation of adenovirus and morphological changes of the cells. (A) NeuroD expression by Ad-NeuroD infection. Intestine 407 cells were infected with Ad-NeuroD or Ad-LacZ (25 moi). NeuroD protein was detected by SDS–PAGE and immunoblotting. (−), without infection. (B) Induction of glucokinase and SUR1 by NeuroD overexpression. Total RNAs were isolated 48 h after infection. Gene expressions of glucokinase and SUR1 were assessed by RT-PCR. Lane M, molecular weight marker (Hinc II-digested ϕX174 DNA fragments); LGK, liver-type glucokinase; βGK, β-cell-type glucokinase. (C) Phase-contrast photomicrographs of NeuroD-infected cells 48 h after infection. Cells grown on a tissue culture dish were infected with Ad-LacZ (25 moi (a) and 100 moi (c)) or Ad-NeuroD (25 moi (b) and 100 moi (d)). Cells were photographed through a microscope. Bar, 50 μm.