Expression of Neuronal Traits in Pancreatic Beta Cells

IMPLICATION OF NEURON-RESTRICTIVE SILENCING FACTOR/REPRESSOR ELEMENT SILENCING TRANSCRIPTION FACTOR, A NEURON-RESTRICTIVE SILENCER*

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Pancreatic beta cells (insulin-producing cells) and neuronal cells share a large number of similarities. Here, we investigate whether the same mechanisms could control the expression of neuronal genes in both neurons and insulin-producing cells. For that purpose, we tested the role of the transcriptional repressor neuron-restrictive silencing factor/repressor element silencing transcription factor (NRSF/REST) in the expression of a battery of neuronal genes in insulin-producing cells. NRSF/REST is a negative regulator of the neuronal fate. It is known to silence neuronal-specific genes in non-neuronal cells. We demonstrate that, as in the case of the neuronal pheochromocytoma cell line PC12, mRNA coding for NRSF/REST is absent from the insulinoma cell line INS-1 and from three other insulin- and glucagon-producing cell lines. NRSF/REST activity is also absent from insulin-producing cell lines. Transient expression of REST in insulin-producing cell lines is sufficient to silence a reporter gene containing a NRSF/REST binding site, demonstrating the role of NRSF/REST in the expression of neuronal markers in insulin-producing cells. Finally, by searching for the expression of NRSF/REST-regulated genes in insulin-producing cells, we increased the list of the genes expressed in both beta and neuronal cells.

It is now well established that, in spite of different embryological origins, beta and neuronal cells share a large number of similarities. Indeed, molecules such as glutamic acid decarboxylase (1), tyrosine hydroxylase (2), dopamine β-hydroxylase (3), type II voltage-dependent sodium channel (4), glutamate receptor (5), neurofilament proteins (6, 7), receptors for neuropeptides (8, 9), and thyrotrpin-releasing hormone (10, 11) have been shown to be expressed in both beta and neuronal cells. The regulation of the expression of neuronal markers in beta cells at the molecular level is not understood. Theoretically, the expression of the same gene in two different cell types can be explained by either the presence in both cell types of specific transcriptional activators or the absence of specific transcriptional repressors. At least three specific transcriptional activators, Islet-1 (12), Pax-6 (13), and Beta2 (14), are expressed in both beta and neuronal cells. To our knowledge, there are no data demonstrating the role of transcriptional repressors in the expression of neuronal molecules by beta cells.

Recently, progress has been made on the mechanisms underlying the specific expression of genes in neurons. It has indeed been demonstrated that a large battery of neuron-specific genes was regulated by one silencer protein. This repressor, named NRSF/REST, 1 is present in non-neuronal cells and absent from neuronal cells (15, 16). It was cloned according to its ability to bind a 24-bp cis-element necessary and sufficient for silencing neuron-specific genes such as the type II voltage-dependent sodium channel gene and the SCG10 gene, another neuron-specific gene. This 24-bp cis-element, which represents a binding site for NRSF/REST, has been found in 14 additional neuron-specific genes (16). While looking at the group of neuron-specific genes under the control of NRSF/REST, it was interesting to note that a large subgroup, including the NMDA receptor, dopamine β-hydroxylase, neurofilaments, and type II voltage-dependent sodium channel had previously been detected in beta cells in different species.

The aim of the present work was thus to define whether NRSF/REST could be implicated in the expression of neuron-specific genes by beta cells. We first demonstrated that INS-1 cells, deriving from a rat insulinoma, express type II voltage-dependent sodium channel and glutamate receptor mRNAs previously detected in other murine beta cell lines (4, 5) and known to be under the control of NRSF/REST in neuronal cells. Thus, INS-1 cells represent an interesting experimental system to define the role of NRSF/REST in the expression of neuron-specific genes by beta cells. We next studied NRSF/REST mRNA and activity in beta cell lines. Our results demonstrate that NRSF/REST is absent from INS-1 cells and from three other islet cell lines, suggesting that some identical mechanisms control the expression of neuronal traits in beta and neuronal cells. Finally, we searched for the expression in beta cells of additional genes known to be silenced by NRSF/REST in non-neuronal cells. We demonstrated the expression in INS-1 cells of three such genes previously not known to be transcribed in insulin-producing cells. NRSF/REST is thus a key factor in the control of neuronal markers in beta cells.

EXPERIMENTAL PROCEDURES

Cell Culture—The following cell lines were used in this study: RINm5F and INS-1 (rat beta cell line) (17, 18), beta TC3 (mouse beta cell line) (19), alpha TC (mouse alpha cell line) (20), SOX-6 and BxPC-3 (human pancreatic adenocarcinoma), PC12 (rat pheochromocytoma cell line) (21), C6 (rat glioma cell line) (22), NIH3T3 (mouse fibroblastic cell line, ATCC CRL1658), and A875 (human melanoma cell line) (23). They were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. For INS-1 cells, the medium was supplemented with 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol (24). For PC12 cells, the medium was supplemented with 5% horse serum. Cultures were incubated at 37 °C in a humidified atmosphere of 95% air, 5% CO2.

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1 The abbreviations used are: NRSF, neuron-restrictive silencing factor; REST, repressor element silencing transcription factor; bp, base pair(s); RT, reverse transcriptase; PCR, polymerase chain reaction; NDMA, N-methyl-o-aspartate.
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RNA Isolation and Amplification by RT-PCR—Cytoplasmic RNA was prepared as described previously (8). For the preparation of mRNA, RNA was treated with DNase I. First strand cDNA was prepared from 5 μg of DNase-treated RNA using random hexamer primers, 200 μM of each dNTP, and 100 units of reverse transcriptase (SuperScript, Life Technologies, Inc.). The reaction was also carried out in the absence of reverse transcriptase to ensure that the amplified material derived from RNA. The oligonucleotides used for amplification and the PCR conditions are described in Table I. Although the sequence of human and mouse NRSF/REST was available, the sequence of rat NRSF/REST was not published. To define the expression of NRSF/REST in different rat cell lines, we used primers corresponding to two sequences conserved between human and mouse NRSF/REST. The PCR products were analyzed by electrophoresis on a 2% agarose gel, subcloned using the TA cloning system in pCR™ (Invitrogen) vectors, and sequenced using the fmol™ DNA sequencing system (Promega) according to the manufacturer’s protocol.

Northern Blot Analysis—10 μg of RNA were analyzed by electrophoresis in a 1% agarose-formaldehyde gel and transferred to Hybond-N nylon membranes. Membranes were cross-linked by exposure to UV light. Hybridization was carried out according to the method of Church and Gilbert (25). The inserts used as probes to study the expression of NRSF/REST and SCG10 mRNAs were prepared by RT-PCR from cDNA derived from C6 and PC12 cells, respectively, as described above and were labeled by random priming. Membranes were prehybridized at 65 °C in 7% lauryl sulfate, 200 mM phosphate buffer, pH 7.2, 1 mM EDTA, 1% bovine serum albumin and then hybridized with the labeled probe for 16 h at 65 °C. Filters were then washed three times for 15 min at 65 °C in 0.5 × SSC containing 0.1% SDS. Autoradiography was performed at −70 °C with Fuji RX film.

Gel Shift Assay—Nuclear protein extracts were prepared from 4 × 10⁶ cells according to Andrews and Faller (26). Two different probes were used: a 72-bp fragment containing two copies of the silencing element present in the SCG10 gene (16) or a 34-bp fragment representing a binding site for the ubiquitously expressed transcriptional factor nuclear factor 1. The probes were labeled with [γ-32P]dCTP using the Klenow fragment of Escherichia coli DNA polymerase I and purified by electrophoresis on an 8% polyacrylamide gel. Gel shift assays were performed in a 20-μl final volume of binding buffer containing 20 μM Hepes (pH 7.6), 10 mM glycerol, 1 mM dithiothreitol, 2.5 mM MgCl₂, 250 mM KCl, and 150 μg/ml poly(dI-dC). The extracts (5 μg of nuclear protein) were preincubated in the binding buffer for 10 min at 4 °C and then incubated for 10 min at room temperature with 2 × 10⁶ cpm of labeled DNA probe in the presence or absence of competitor. Electrophoresis was performed on a 4% polyacrylamide gel in 0.25 × Tris borate/EDTA for 2 h at 150 V. The gels were dried and subjected to autoradiography.

Cell Transfections—INS-1 cells were cotransfected with a type II promoter reporter plasmid containing a NRSF/REST-binding site (27) and either a REST expression plasmid (REST-Express) (15) or carrier DNA. Transfections were performed with Lipofectamine (Life Technologies, Inc.) according to the manufacturer’s protocol. Briefly, cells were grown in 60-mm dishes to 50% confluence, washed twice with RPMI 1640 medium without serum and antibiotics, and the medium was replaced with 3 ml of serum-free medium without antibiotics containing 6 μg of Quagen column-purified DNA and 12 μl of Lipofectamine. 6 h later, the transfection medium was removed and replaced with RPMI 1640 medium supplemented with serum, and the cells were harvested 48 h later. Cell extracts were assayed for chloromphenicol acetyltransferase activity as described previously (7).

RESULTS

Expression of N-Methyl-D-aspartate Receptor mRNA, Type II Voltage-dependent Sodium Channel mRNA, and Dopamine β-hydroxylase mRNA in INS-1 Cells—We first defined whether genes that contain in their promoter region a cis-element that represents a binding site for NRSF/REST were expressed in the beta cell line INS-1. The expression of three such genes, N-methyl-D-aspartate receptor, type II voltage-dependent sodium channel, and dopamine β-hydroxylase, was studied by RT-PCR. For that purpose, cDNAs prepared from INS-1 cells were amplified by PCR using specific oligonucleotides (Table I). cDNAs prepared from C6 and NIH3T3 were used as negative controls, while cDNA from PC12 cells were used as positive controls. The PCR was also performed on samples prepared from INS-1, C6, NIH3T3, and PC12 RNAs from which reverse transcriptase was omitted during the reverse transcription reaction but which were otherwise processed identically to the first samples. The products of amplification were analyzed by 2% agarose gel electrophoresis. As shown in Fig. 1, predicted size DNA fragments corresponding to N-methyl-D-aspartate receptor, type II voltage-dependent sodium channel, and dopamine β-hydroxylase were amplified from INS-1 and PC12 cells. No amplification was obtained when cDNAs derived from C6 or NIH3T3 cells were used. PCR was performed in parallel using cyclophilin primers as a control. Thus, INS-1 cells express a panel of genes whose expression is REST-dependent in neuronal cells.

NRSF/REST mRNA Is Absent from Beta Cell Lines—We next studied by RT-PCR whether NRSF/REST mRNA was present in INS-1 cells (Fig. 2). We used a set of primers able to amplify NRSF/REST from rat, mouse, and human. As expected, a single band of the predicted size was amplified from both C6 and NIH3T3 cDNAs. This band was absent when INS-1 and PC12 cDNA were used (Fig. 2). NRSF/REST expression was then examined by Northern blot analysis in a panel of pancreatic cell lines to confirm the data obtained with INS-1 cells. As expected, a positive signal of the predicted size (7.6 kilobase pairs) was observed in the non-neuronal cell lines C6 and NIH3T3, whereas no signal was detected in the pheochromocytoma cell line PC12 (Fig. 3). Although a signal identical to the one detected in C6 and NIH3T3 cells was found in the two exocrine cell lines SOX-6 and BxPC-3, no signal was detected in the glucagon-producing cell line alpha TC in the insulin-producing cell lines RINm5F, INS-1, and beta TC (Fig. 3).
The quality of the cDNAs.

Reversetranscriptase.Cyclophilin-specificprimerswereusedtocontrol
described total RNA in the presence (–RT) or in the absence (+RT) of reverse transcriptase. Cyclophilin-specific primers were used to control the quality of the cDNAs.

Taken together, these data demonstrate the absence of NRSF/REST mRNA expression in a panel of endocrine pancreatic cell lines.

**NRSF/REST Activity Is Absent from INS-1 and RIN Cells**—To demonstrate the absence of NRSF/REST activity in nuclear extracts prepared from beta cell lines, gel shift analysis was performed using a NRSF/REST binding site as a probe. Results were compared with those obtained with NIH3T3 nuclear extracts, which are known to contain NRSF/REST activity, and with PC12 nuclear extracts, which do not contain NRSF/REST activity. As expected, a DNA-protein complex was detected using nuclear extracts prepared from NIH3T3 cells (Fig. 4A, lane 11). Formation of this complex was inhibited with an excess of unlabeled competitor (Fig. 4A, lane 12) but not when a similar excess of irrelevant competitor was added (Fig. 4A, lane 13). No specific complex was detected when nuclear proteins from RINm5F or INS-1 cells were used (Fig. 4A). The faint shifted band obtained with RINm5F and INS-1 nuclear extracts was not specific. Indeed, it had a different migration profile than the specific complex found with NIH3T3 nuclear extracts, and it could not be inhibited with an excess of unlabeled competitor. Moreover, this nonspecific complex detected in RINm5F and INS-1 cells was also found in NIH3T3 cells upon longer exposure (data not shown). Control experiments using a probe representing a binding site for the ubiquitously expressed transcriptional factor nuclear factor 1 indicate the quality of the nuclear extracts prepared from the different cell types (Fig. 4B).

**Expression of REST in INS-1 Cells Is Sufficient to Silence Reporter Genes Containing a Binding Site for NRSF/REST**—To determine whether NRSF/REST was necessary to silence neuronal genes in insulin-producing cells, INS-1 cells were cotransfected with a type II promoter reporter plasmid containing a NRSF/REST binding site (repressor element 1-chloramphenicol acetyltransferase) (27) and with either a REST expression plasmid (REST-Express) (15) or carrier DNA. As shown in Fig. 5, in INS-1 cells chloramphenicol acetyltransferase activity from the reporter gene repressor element 1-chloramphenicol acetyltransferase was as high as that found when a Rous sarcoma virus-chloramphenicol acetyltransferase plasmid was used (Fig. 5, compare lanes 1 and 2 with lanes 6–8). This activity was strongly decreased when the REST expression plasmid (REST-Express) was cotransfected (Fig. 5, compare lanes 3–5 with lanes 6–8). Identical results were ob-

**Identification in Insulin-producing Cells of Additional “NRSF/REST-dependent Genes”**—We next tested the expression in INS-1 cells of other NRSF/REST-dependent genes whose expression had not been demonstrated previously in pancreatic endocrine cells. The expression of three such genes was tested: SCG10 (28), synapsin I (29), and the β2 subunit of neuronal nicotinic acetylcholine receptor (30). As shown in Fig. 6A, predicted size DNA fragments corresponding to all three genes were amplified from INS-1 and PC12 cells. The fragments amplified from INS-1 were cloned, and their sequences were shown to be identical to the expected sequences. No amplification was obtained when cDNAs derived from C6 or NIH3T3 cells were used. To determine in a more quantitative fashion the expression of these NRSF/REST-dependent genes in INS-1 cells, the level of expression of SCG10 mRNA INS-1 cells was studied by Northern blot analysis and compared to the level found in PC12 cells, a cell line previously used as a model system to study SCG10 expression (31), and to the level found in C6 cells, which was used as a negative control. As shown in Fig. 6B, although no signal was detected in C6 cells (as expected), nearly identical signals were detected in RNA derived from INS-1 and PC12 cells, demonstrating the high level of SCG10 mRNA in INS-1 cells.

**DISCUSSION**

Here, we report that the same silencing mechanism controls the expression of a panel of neuron-specific genes in neuronal and insulin-producing cells. Indeed, as in the case of neuronal cells, the repressor NRSF/REST is absent from beta cell lines as demonstrated by RT-PCR, Northern blot analysis, and gel shift assay. Moreover, the expression of a NRSF/REST cDNA in beta cell lines is sufficient to silence a reporter gene containing a binding site for this repressor. Finally, based on the hypothesis that the absence of NRSF/REST expression would be sufficient to allow the expression of NRSF/REST target genes in insulin-producing cells, we demonstrate the expression in insulin-producing cells of three genes whose expression had not been demonstrated previously in this cell type.

It is now well known that beta and neuronal cells share a large number of similarities. The expression of different enzymes implicated in the synthesis of γ-aminobutyric acid or catecholamines, of specific cell-surface receptors for growth factors and amino acids, of specific intermediate filamentous such as neurofilament, and of hormones such as thyrotropin-releasing hormone is shared by neuronal and beta cells at different stages of their development (1, 6, 8, 10, 32). Moreover, beta cells also resemble neurons by being electrically excitable and by responding to hormonal stimuli and glucose by depolarization and exocytosis in a process similar to neurotransmitter release from synaptic vesicles (33). These phenotypical similarities between these two cell types and the fact that transgenic mice

**FIG. 1. Expression of NMDA receptor 1 (NMDAR1), brain type II sodium channel (Na Ch, Type II), and dopamine β-hydroxylase (DBH) mRNAs in INS-1 cells.** Shown are ethidium bromide stains of 1% agarose gels containing PCR products of reverse-transcribed total RNA in the presence (+RT) or in the absence (–RT) of reverse transcriptase. Cyclophilin-specific primers were used as controls.

**FIG. 2. PCR analysis of NRSF/REST mRNA expression in INS-1 cells.** Shown are ethidium bromide stains of 1% agarose gels containing PCR products of reverse-transcribed total RNA in the presence (+RT) or in the absence (–RT) of reverse transcriptase using NRSF/REST primers. Cyclophilin-specific primers were used as controls.
harboring a hybrid gene utilizing insulin gene regulatory information transiently express the transgene in cells of the neural tube and in the neural crest suggest that these two cell types have identical embryological origins (34). However, experiments based mainly on chimeric embryo constructs argue against this hypothesis (35). Another explanation for the large number of similarities shared by beta and neuronal cells could be the fact that these two cell types express identical transcriptional factors, which seems to be the case. NeuroD/BETA2 is an example of a transcriptional activator expressed in beta and neuronal cells (14, 36). NeuroD/BETA2 is a basic helix-loop-helix protein transiently expressed in a subset of neurons and able to convert presumptive epidermal cells into neurons (36). It is also expressed in pancreatic beta cells, where it acts as a transactivator of the insulin gene (14). Thus, similarities between beta and neuronal cells could be explained by the expression in both cell types of identical transactivators. We demonstrate in the present work that these similarities can also be due to the lack of expression in these two cell types of a specific transcriptional repressor such as NRSF/REST.

We also demonstrate that NRSF/REST is absent from a glucagon-producing cell line, this common lack of expression of NRSF/REST in beta and alpha cells is not surprising. Indeed, it is well established that beta and alpha cells have a common origin. For example, during development bipotential cells containing both insulin and glucagon are present in the pancreatic bud (34). Moreover, it is known that beta and alpha cells express identical transcriptional activators such as NeuroD/BETA2 (14), the insulin E-box binding activity 3a1 (37), and the homeobox gene islet-1 (12). Although the role of these transactivators in beta cells is now well described, their function in alpha cells is not known. Finally, it has recently been reported that alpha cells also express neuronal markers such as neurofilaments (38). Thus, identical transcriptional silencing mechanisms regulate the expression of specific genes in both insulin-producing and glucagon-producing cells.

In the case of NRSF/REST, one transcriptional factor controls the tissue-specific expression of a battery of neuron-specific genes in insulin-producing cells. Insulin-producing cells are also known to express other neuron-specific genes. For
example, trkA and trkB, two members of the family of the receptors for neurotrophins, are expressed in INS-1 cells (8, 32). Moreover, mRNA coding for trkB, the receptor for brain-derived neurotrophic factor, is also present in INS-1 cells. Thus, it could be possible that the expression of these different trk members is under the control of a unique regulator. Based on the fact that REST is highly expressed in dorsal root ganglia (15), a tissue in which trkA receptor is also present, it does not seem plausible that NRSF/REST is implicated in the control of trk members in beta cells. It would thus be interesting to define common boxes in the promoter/enhancer regions of the different trk members and to characterize tissue-specific activators or repressors able to bind these conserved elements.

Recently, different studies have been performed to search for beta cell-specific genes. Indeed, because type I diabetes results from an autoimmune attack that may be directed at molecular targets specifically expressed by beta cells, it is now important to find putative targets (39). Different strategies have been used to compile a molecular inventory of beta cells. Takeda et al. (40) have constructed a cDNA library from human pancreatic islets and randomly sequenced 1000 clones. Tanaka et al. (41) have sequenced nearly 2000 clones derived from cDNA libraries prepared from a mouse beta cell line. Finally, Neophitou et al. (42) have used a subtractive cloning approach between insulin- and glucagon-producing cell lines to identify mRNAs specifically expressed in pancreatic beta cells. Although these studies gave interesting results, they are time consuming and require a large number of clones to be sequenced before it is possible to find one with some specific expression in beta cells. Here, by searching for the expression in insulin-producing cells of NRSF/REST targets characterized previously in neuronal cells, we were able to demonstrate the expression in INS-1 cells of three genes that, to our knowledge, had not previously been defined in this cell type. Moreover, these three genes have a high degree of cell type specificity. This strategy of searching for targets of transcriptional factors represents an alternative way to define genes specifically expressed in insulin-producing cells.

There are other transcriptional factors that are specifically expressed in beta and neuronal cells. In addition to NeuroD/BETA2, islet-1 is expressed in a subset of neurons, including motor neurons (43). Moreover, the homeobox-containing gene Pax-6 has been shown to be expressed in neurons and pancreatic endocrine cells (13). Targets for these transcriptional factors are not yet known. It will be interesting to determine whether these targets, once they are defined, are expressed in beta cells. This will increase the number of markers specifically shared by beta and neuronal cells and putatively implicated in type I diabetes.

In conclusion, we demonstrate that identical molecular mechanisms control the expression of neuronal markers in beta and neuronal cells, leading to the expression in beta cells of neuronal markers. The function of these neuronal markers in beta cells is not yet clear. They could be implicated in beta cell development (6). By generating transgenic mice expressing NRSF/REST in beta cells, we should be able to further the understanding of the role of these neuronal markers in beta cells.

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