Isolation and characterization of an alternatively spliced variant of transcription factor Islet-1

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Abstract

The LIM homeodomain protein Islet-1 (Isl1), one of the earliest markers for motor neuron differentiation, is also expressed in all classes of islet cells in the pancreas. Isl1 is known to bind and regulate the promoters of the insulin, glucagon and somatostatin genes. In this study, we describe isolation of a novel isl1 cDNA species from the mouse islet β cell line βTC6, which arose from the utilization of an alternative splicing acceptor site in the fifth exon. This shorter cDNA encodes an Isl1 isoform (Isl1-β) lacking the carboxy-terminal 23 amino acids of the previously reported product Isl1-α. Although the level of isl1-β mRNA is much lower than that of isl1-α, isl1-β is preferentially expressed in murine insulinoma cell lines but not in glucagonoma cell line. Upon transient transfection, both Isl1-α and Isl1-β accumulate in the nuclei of murine insulinoma cells. We found that Isl1-β is a relatively more potent transcriptional activator of the insulin promoter than Isl1-α and that the Isl1-α isoform undergoes phosphorylation. Therefore, the transcriptional activity of Isl1 is potentially regulated by the alternative splicing of its mRNA and by phosphorylation.

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Introduction

Islet-1 (Isl1) was originally isolated as a transcription factor that binds to an islet β cell-specific enhancer element in the insulin gene (Karlsson et al. 1990). Isl1 is expressed in β cells as well as in the other classes of pancreatic endocrine cells (α, δ and γ cells) (Dong et al. 1991, Thor et al. 1991), and it was also shown to bind and regulate the α and δ cell-specific hormone genes glucagon (Wang & Drucker 1995) and somatostatin (Leonard et al. 1992) respectively. Isl1 is also expressed in developing neurons in the spinal cord, where it serves as an early marker of motor neuron differentiation (Ericson et al. 1992, Tsuchida et al. 1994). In accord with its expression profile, homozygous deletion of the isl1 gene in mice results in the ablation of islets and motor neurons (Pfaff et al. 1996, Ahlgren et al. 1997). In addition, heterozygous nonsense mutations have been found in the isl1 gene in patients with type 2 diabetes (Shimomura et al. 2000).

The Isl1 protein contains two LIM domains (LIM1 and LIM2) at the amino terminus and a homeodomain (HD) in the central region (see Fig. 1). The HD is responsible for the binding of Isl1 to target AT-rich DNA sequences, and the LIM domains are zinc-binding motifs that mediate interactions with other proteins such as NLI/Ldb1 (Sanchez-Garcia et al. 1993, Jurata et al. 1996). In contrast, the function of the carboxy-terminal region is not clearly defined. It is thought to serve as a transactivator domain, because a truncated Isl1 product (Isl1–310X) that has been found in type 2 diabetes patients and which lacks the carboxy-terminal 40 amino acids has been shown to be a weaker transactivator than wild type Isl1 (Shimomura et al. 2000).

In this manuscript, we describe the isolation of an alternatively spliced isl1 cDNA variant from the mouse pancreatic β cell line βTC6. Its product, Isl1-β, lacks 23 amino acids that are present in the carboxy-terminal region (amino acids 256–278) of the previously reported Isl1 (Isl1-α). Unexpectedly,
the novel Isl1-β isoform is a relatively more potent transactivator of the insulin promoter than Isl1-α, suggesting a new regulatory role for the carboxy-terminal region of Isl1.

Materials and methods

Cells

MIN6 cells (Miyazaki et al. 1990) were a generous gift from Dr Jun-ichi Miyazaki (Osaka University). βTC6 and αTC1 clone 9 (hereafter αTC1) cells were purchased from the American Tissue Culture Collection. MIN6 and βTC6 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal calf serum, and αTC1 cells were grown in F12K medium supplemented with 10% fetal calf serum.

isl1 cDNA isolation and RT-PCR analysis

One microgram of total RNA isolated from βTC6 cells using TRIZOL Reagent (Invitrogen) was subjected to a reverse transcription reaction (50 °C for 1 h) using SuperScript II reverse transcriptase (Invitrogen) and a specific anti-sense primer (isl1-R; 5’-agaccgcgtgTTCCCTCATGCTCAAT AGGAC-3’), which is complementary to the 3’ end of isl1 open reading frame. isl1 cDNAs containing the entire open reading frame were then amplified by PCR using LA-Taq DNA polymerase and 1× GC buffer (Takara, Kyoto, Japan). The primers used were isl1-F1 (sense strand; 5’-gggaggccCTT ACAGATATGGGGAGACATGGG-3’), which corresponds to the region around the first ATG of isl1, and anti-sense isl1-R primer described above. The PCR cycles were 40 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 2 min.

The PCR products were digested by StuI and SacII, linked to a double-stranded oligonucleotide encoding the FLAG-tag sequence (5’-ctagattcatTAATGATGACAGCATAAGGG AGG-3’ and 5’-CCTCGCTTTAGTTCGTCGTCG CAT CCTGTAATCCCATTgta-3’), and cloned into the SpeI-SacII-digested vector pGEM-T-easy (Promega) to generate pGEM-T-easy/FLAG-isl1-α and pGEM-T-easy/FLAG-isl1-β. The nucleotide sequence of isl1-β is deposited in GenBank (accession number AB104633).

For isl1 mRNA expression analysis, total RNA (1 µg) was subjected to RT-PCR using the Titan One-Tube RT-PCR System (Roche). The primers
were isl1-F2 (sense strand, 5′-AAGGAGCAACTAGTGAGATGACGGG-3′), which corresponds to the exon 4 encoding the HD of isl1, and the isl1-R primer described above. The PCR cycles were 40 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 60 s.

**Immunofluorescence staining**

Isl1-α and Isl1-β expression plasmids were constructed by inserting partially digested NotI fragments excised from pGEM-T-easy/FLAG-isl1-α and pGEM-T-easy/FLAG-isl1-β into the mammalian expression vector pHygEF2. MIN6 cells grown on collagen-coated cover slides (Iwaki, Tokyo) were transfected with 3·2 µg of plasmids (pHygEF2/FLAG-isl1-α or pHygEF2/FLAG-isl1-β) using 8 µl Lipofectamine 2000 reagent (Invitrogen). Twenty-four hours after transfection, the cells were fixed with PBS containing 3% formaldehyde at room temperature for 15 min and treated with 0·25% Triton X-100 in PBS for an additional 15 min. The cells were then stained with anti-FLAG (M2) monoclonal antibody and Alexa Fluor 488-labeled anti-mouse IgG serum (Molecular Probes) and 4,6-diamidino-2-phenylindole.

**Luciferase assay**

The reporter plasmid h-ins-p-luc, containing the human insulin promoter region, was described previously (Kataoka et al. 2002). NIH3T3 cells grown in a 35 mm dish were washed with PBS, and were added with 200 µl ice-cold buffer A (10 mM Hepes pH 7·9, 10 mM KCl, 1·5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 0·1% Nonidet P40 and protease inhibitors). The cells were scraped, transferred into a microtube, and centrifuged at 2500 g for 1 min at 4 °C. The pellet was re-suspended in 20 µl buffer B (20 mM Hepes, pH 7·9, 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol and protease inhibitors) and incubated on ice for 10 min. After centrifugation at 15 000 g for 10 min, the supernatant (nuclear extract) was collected. Four microliters of the extract were diluted to final concentration of 2500 g for 1 min at 4 °C. The pellet was re-suspended in 20 µl buffer B (20 mM Hepes, pH 7·9, 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol and protease inhibitors) and incubated on ice for 10 min. After centrifugation at 15 000 g for 10 min, the supernatant (nuclear extract) was collected. Four microliters of the extract were diluted to final concentration of 25 mM Tris (pH 7·9), 100 mM NaCl, 5 mM MgCl2 and 8 mM dithiothreitol, added with 20 units CIAP (Takara), incubated at 30 °C for 1 h, and then subjected to Western analysis.

**Results**

**Isolation of an alternatively spliced variant of isl1 cDNA**

We molecularly cloned isl1 cDNAs from total RNA prepared from the murine insulinoma cell line.
βTC6 by using an RT-PCR strategy. Among four clones analyzed by nucleotide sequencing, one clone was found to contain an internal in-frame deletion of 69 nucleotides compared with the previously reported isl1 cDNA. As schematically shown in Fig. 1 (upper panel), this cDNA encodes a protein that lacks 23 amino acids that correspond to amino acids 256–278 of the previously described Isl1. Hereafter, we call this shorter Isl1 isoform Isl1-β (GenBank accession number AB104633), and refer to the previously described isoform as Isl1-α. A comparison of the nucleotide sequences of the isl1-α and isl1-β cDNAs with the corresponding mouse genomic sequence revealed that these two mRNA species were generated by the usage of an alternative splicing acceptor site in the fifth exon (Fig. 1, lower panel).

The isl1-β isoform is preferentially expressed in murine insulinoma cell lines

We examined the mRNA expression profile of the two isl1 isoforms. Total RNAs were isolated from mouse glucagonoma (αTC1 clone 6) and insulinoma (βTC6 and MIN6) cell lines grown in the presence of low (1 mM) or high (20 mM) concentrations of glucose and were analyzed by RT-PCR using specific primer pairs. As shown in Fig. 2, both the isl1-α and isl1-β isoforms are expressed in βTC6 and MIN6 cells, and isl1-α is abundant. In contrast, the isl1-β isoform is barely detectable in αTC1 cells, suggesting that it is preferentially expressed in insulinoma cells. Although a high concentration of glucose activates transcription of the insulin gene in these insulinoma cell

Figure 3 Nuclear localization of Isl1 isoforms in MIN6 cells. Expression vectors encoding FLAG-tagged Isl1-α and Isl1-β were transfected into MIN6 cells. The cells were fixed and stained with anti-FLAG antibody and Alexa Fluor 488-labeled anti-mouse IgG antiserum (upper panels) and with 4,6-diamidino-2-phenylindole (DAPI) (lower panels).
lines, the level of glucose in the culture medium had no effect on the levels of expression and on the relative amounts of the two isl1 isoforms.

**Isl1-β is a more potent transactivator than Isl1-α**

We next tested whether the two Isl1 isoforms are functionally different. For this purpose, we constructed mammalian expression vectors encoding FLAG-tagged Isl1-α and Isl1-β. MIN6 cells transfected with these constructs were stained with anti-FLAG antibody. Both Isl1-α and Isl1-β were observed to predominantly localize in the nucleus (Fig. 3).

We then transfected both expression vectors into NIH3T3 fibroblast cells together with an insulin promoter-luciferase reporter plasmid. In accord with previous data demonstrating that the transactivation potential of Isl1-α is modest (German et al. 1992, Shimomura et al. 2000), we observed a marginal but significant activation of the insulin promoter by Isl1-α in a dose-dependent manner (Fig. 4A). We also observed dose-dependent transactivation by Isl1-β to a reproducibly greater extent than by Isl1-α. We confirmed the levels of expression of Isl1-α and Isl1-β by Western blot analysis (Fig. 4B). Although Isl1-β accumulated at slightly higher levels than Isl1-α, Isl1-β appeared to be a relatively stronger transactivator of the insulin gene promoter than Isl1-α (for example, compare Isl1-α 1·0 µg and Isl1-β 0·5 µg, Fig. 4A and B, lanes 4 and 6). Thus, the transactivation activity of Isl1-β is relatively higher than that of Isl1-α.

**The Isl1-α isoform is phosphorylated**

We noticed that FLAG-tagged Isl1-α but not Isl1-β migrated as a doublet, as shown by Western blot analysis (see Fig. 4B, lane 4). One plausible explanation for this observation is that Isl1-α is modified, most likely by phosphorylation. To test this hypothesis, we prepared nuclear extracts from NIH3T3 cells transfected with Isl1-α and Isl1-β. The extracts were treated with CIAP and subjected to SDS-PAGE/Western blot analysis. As shown in Fig. 5, treatment with CIAP caused the more slowly migrating form of Isl1-α to disappear and...
the more rapidly migrating form to accumulate, indicating that Isl1-α is phosphorylated. In contrast, the mobility of Isl1-β was not affected by CIAP treatment. These results indicate that Isl1-α undergoes phosphorylation that can be distinguished by mobility on SDS-PAGE.

Discussion

We isolated a novel splicing variant of isl1 cDNA and analyzed the function of its product, Isl1-β, in comparison with the previously reported Isl1-α. Although the isl1-β isoform represents only a small portion of total isl1 mRNA, it was specifically detected in murine insulinoma cell lines but not glucagonoma cell line, suggestive of a cell lineage-specific regulation of Isl1 splicing.

We demonstrated here that Isl1-α is phosphorylated. The 23 amino acid region contains two threonine and one serine residues, two of which (Thr263 and Ser269) are each followed by a proline residue and may be targets for MAP kinase family members (Davis 1993). However, from our analysis using CIAP, we cannot conclude that these amino acid residues in the 23 amino acid region are phosphorylated, because there remains a possibility that this region is just required to regulate the phosphorylation of other sites of the protein. Furthermore, we cannot exclude the possibility that Isl1-β is phosphorylated in a manner that does not affect protein mobility with respect to SDS-PAGE.

Although the role of phosphorylation in Isl1 activity is not clear at present, this modification possibly affects the transactivation properties of Isl1, because Isl1-β is a relatively more potent transactivator than Isl1-α. One possible explanation for this is that the transactivation function of Isl1-α is negatively regulated by phosphorylation and that Isl1-β escapes this regulation. Previous work has demonstrated that the carboxy terminus (amino acids 310–349) serves as a transactivator domain (Shimomura et al. 2000). The 23 amino acid region present in Isl1-α but not Isl1-β (amino acids 256–278) is adjacent to this transactivator domain and may thus exert a negative regulatory effect on it. Therefore, Isl1 transactivation activity may be regulated in two ways, by alternative splicing and by phosphorylation. However, as far as we have tested, the isl1-β isoform represents only a small portion of total isl1 mRNA in murine insulinoma cell lines. A more detailed expression profile of the two isoforms in pancreatic endocrine cell types, as well as in neuronal tissues, awaits the availability of isoform-specific antibodies, which in turn will clarify the physiological relevance of the isl1-β isoform.

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