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Pbx1 is a co-factor for Cdx-2 in regulating proglucagon gene expression in pancreatic A cells

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Abstract

A number of Hox and Hox-like homeodomain (HD) proteins have been previously shown to utilize members of the TALE HD protein family as co-factors in regulating gene expression. The caudal HD protein Cdx-2 is a transactivator for the proglucagon gene, expressed in pancreatic A cells and intestinal endocrine L cells. We demonstrate here that co-transfection of the TALE homeobox gene Pbx1 enhanced the activation of Cdx-2 on the proglucagon promoter in either the pancreatic A cell line InR1-G9 or BHK fibroblasts. The activation was observed for proglucagon promoter constructs with or without the binding motifs for Pbx1. Furthermore, mutating the penta-peptide motif (binding motif for TALE HD proteins) on Cdx-2 substantially attenuated its activation on proglucagon promoter, but not on the sucrase–isomaltase gene (SI) promoter, or its own (Cdx-2) promoter; suggesting that Cdx-2 utilizes Pbx1 as a co-factor for regulating the expression of selected target genes. Physical interaction between Cdx-2 and Pbx1 was demonstrated by co-immunoprecipitation as well as GST fusion protein pull-down. We suggest that this study reveals a novel function for Pbx1 in pancreatic islet physiology: regulating proglucagon expression by serving as a co-factor for Cdx-2.

Keywords: Proglucagon; Cdx-2; Pbx1; Homeobox gene; GST pull down

1. Introuction

The proglucagon gene is expressed in pancreatic islet A cells, intestinal endocrine L cells and selected endocrine neurons in the brain (Kieffer and Habener, 1999). It encodes three major peptide hormones: glucagon, glucagon like peptide 1 (GLP-1) and glucagon like peptide 2 (GLP-2). These hormones exert opposite or overlapping physiological functions (Drucker, 2003; Drucker et al., 1996; Meeran et al., 1999; Turton et al., 1996; Tang-Christensen et al., 2001).

It has been revealed that a number of transcription factors are able to interact with the first 300 bp proglucagon 5′ flanking (promoter) region (Knepel et al., 1990; Philippe et al., 1988). These interactions are considered to play sophisticated roles in regulating proglucagon expression in temporal and spatial manners. The transcription factors include the helix–loop–helix proteins E12/E47, Beta2/neuroD, members of the hepatocyte nuclear family (HNF3α, 3β and 3γ) and homeodomain (HD) proteins (Gauthier et al., 2002; Hussain et al., 1997; Jin and Drucker, 1996; Laser et al., 1996; Sander et al., 1997). We reported previously that the caudal HD protein Cdx-2 binds to two AT rich motifs within the G1 enhancer element of the proglucagon promoter and activates its transcription (Jin and Drucker, 1996). In addition, over-expressing Cdx-2 in the pancreatic InR1-G9 cell line was shown to stimulate endogenous proglucagon mRNA expression (Jin et al., 1997; Trinh et al., 1999).

Cdx-2 plays critical roles in embryo implantation and embryogenesis (Chawengsaksophak et al., 1997). In adult

Abbreviations: CRE, cAMP response element; EMSA, electrophoretic mobility shift assay; Exd, Extradenticle; GLP-1, glucagon like peptide 1; GLP-2, glucagon like peptide 2; glu, proglucagon; HD, homeodomain; LUC, luciferase; PDGP, proglucagon derived peptide; SI, sucrase–isomaltase; TALE, three amino acid loop extension

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animals, Cdx-2 is abundantly expressed in differentiated non-endocrine intestinal epithelia, and may have more than a dozen other potential target genes. Furthermore, Cdx-2 was shown to regulate the expression of its own promoter (da Costa et al., 1999; Xu et al., 1999). Cdx-2 was found to activate its own promoter only when the examination was conducted in Cdx-2 expressing cell lines, but not in Cdx-2 non-expressing fibroblasts (Xu et al., 1999). Taylor et al. also reported that Cdx-2 activates the sucrase–isomaltase (SI) gene promoter only in the Cdx-2 expressing intestinal Caco-2 cell line, but not in NIH-3T3 fibroblasts. In contrast, Cdx-2 was shown to activate the expression of proglucagon promoter in Cdx-2 expressing pancreatic and intestinal endocrine cell lines, as well as in BHK and NIH-3T3 cells (Jin and Drucker, 1996). These observations suggest that Cdx-2 may require cell type specific co-factors to regulate the expression of some of its downstream target genes, such as SI and Cdx-2 itself. For regulating the expression of proglucagon promoter, Cdx-2 may utilize a ubiquitously expressed co-factor. Because many Hox and Hox-like HD proteins, including another pancreatic cell Hox-like HD protein Pdx-1, were shown to utilize the three amino acid loop extension (TALE) HD protein Pbx1 as the co-factor, we propose that ubiquitously expressed Pbx1 also serves as a co-factor for Cdx-2 in regulating the expression of the proglucagon gene. We present here our experimental evidence that support this hypothesis.

2. Materials and methods

2.1. Materials

Cell culturing medium, fetal bovine serum (FBS), oligonucleotides and TRIzol reagent for RNA extraction, were purchased from Invitrogen Life Technology Inc. (Burlington, Ontario, Canada). Glutathione beads and reduced glutathione, as well as α-^{32}P labeled dCTP were obtained from Amersham Pharmacia Biotech (Baie d'Urfe, Quebec, Canada). Protein A sepharose was purchased from Upstate Biotechnology Inc. (UBI, Lake Placid, New York, USA). Restriction enzymes and DNA modification enzymes were molecular biology grade and were purchased from several sources. Other chemical reagents were purchased either from Bishop Canada Inc. (Burlington, Ontario, Canada) or Sigma–Aldrich Canada Ltd. (Oakville, Ontario, Canada).

2.2. Plasmids construction

The generation of Cdx-2/Luciferase (-374Cdx-LUC) and proglucagon-LUC (Glu-LUC) fusion gene constructs (−476 and −82) has been previously described (Jin and Drucker, 1995). The human sucrase–isomaltase gene promoter (−183–+54)/LUC fusion gene construct (SI-LUC) was kindly provided by Claude Asselin (Wu et al., 1992). Hamster Cdx-2 cDNA was originally provided by Michael German (German et al., 1992). For this study, the coding sequence of Cdx-2 was sub-cloned into the pcDNA3.1 vector (Invitrogene Life Technology Inc.) as the wild type Cdx-2 expression plasmid, and into the pGEX4T-2 vector (Amersham Pharmacia Biotech) for generating wild type Cdx-2-GST fusion protein (Xu et al., 1999). Human Pbx1, Pbx2 and Pbx3 cDNAs were gifts from Cornelis Murre (van Dijk et al., 1995). For this study, the cDNA fragments of these three genes were inserted into the pcDNA3.1 expression plasmid. Four additional Cdx-2 expression plasmids were generated. They carry site mutations on the Cdx-2 coding region, as illustrated in Fig. 3A. These constructs were generated by PCR using Vent polymerase, and sub-cloned into the pcDNA3.1 vector. Three additional Cdx-2-GST fusion protein constructs were generated. They carry site mutations or deletions on the Cdx-2 coding region, as illustrated in Fig. 7A. PCR products for sub-cloning were verified by DNA sequencing.

2.3. Cell culturing, plasmid transfection and LUC reporter gene analysis

The cultivation of pancreatic cell lines InR1-G9 and In111 has been described previously (Jin and Drucker, 1996). The human colon cancer cell line SW480 and two fibroblast cell lines, COS-7 and BHK, were obtained from American Type Culture Collection (ATCC). These cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (Chen et al., 2005; Jin and Drucker, 1996). For LUC reporter gene analysis, cell line transfection was conducted using the method of calcium phosphate precipitation. Sixteen to 18 h after the transfection, cells were harvested for LUC reporter gene analysis (Jin and Drucker, 1995). The same calcium phosphate precipitation method was used to generate Cdx-2 or Pbx1 protein expression or over-expression in BHK or COS-7 fibroblasts for verifying the expression level of the Cdx-2 expressing plasmids and for co-immunoprecipitation analysis, respectively.

2.4. Western blotting, co-immunoprecipitation and GST fusion protein pull-down assay

The methods for whole cell protein extraction, nuclear protein extraction, generation of Cdx-2 antibody and Western blotting have been previously described (Jin and Drucker, 1996; Trinh et al., 1999; Xu et al., 1999; Chen et al., 2005). The anti-β-actin monoclonal antibody was purchased from Sigma–Aldrich Canada Ltd. The anti-Pbx1 polyclonal antibody was purchased from UBI. The anti-GST polyclonal antibody was obtained from Amersham Pharmacia Biotech.

Whole cell lysates of the pancreatic InR1-G9 cells were prepared using radioimmunoprecipitation assay (RIPA) lysis buffer for co-immunoprecipitation (co-IP) (Branch and Mills, 1995). Following the determination of protein concentration, InR1-G9 whole cell lysates were diluted with the RIPA lysis buffer to a final concentration of 1 μg protein per μl. After a "pre-clear procedure" with protein A sepharose beads, the samples were incubated with the anti-Pbx1 antibody at 4 °C for 24 h with gentle rotation. The immune-complexes were then captured by protein A sepharose beads, followed by Western blotting.

GST fusion proteins were generated in the BL-21 strain of *Escherichia coli* with 0.2 μM IPTG as the inducer, purified with glutathione beads, and eluted using reduced glutathione, as previously described (Xu et al., 1999). To verify the generation of expected size GST and Cdx-2-GST fusion proteins for each plasmid construct, purified GST or Cdx-2-GST fusion proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie blue staining. The method for the GST fusion protein pull-down assay has been previously described (Ma et al., 2003).

2.5. Densitometry analysis and statistics

All LUC reporter gene analyses in this study were performed at least in triplicate. Relative LUC activities were calculated in each set of experiments, normalized against total protein utilized in the LUC assay and the results of β-gal assay with the same sample. The results are presented as mean ± standard error of the mean (S.E.M.) ($n \geq 3$). Variations between two groups were analyzed by paired Student's t-test.

3. Results

3.1. Pbx1 enhances the activation on proglucagon promoter by Cdx-2

Cdx-2 was shown to bind to the G1 enhancer element (Jin and Drucker, 1996; Laser et al., 1996), while Pbx1 binds to G3, G5 and possibly the cAMP response element (CRE), located within the first 300 bp 5′ flanking region of the proglucagon gene (Fig. 1A; Herzig et al., 2000). The BHK fibroblasts, which do not express Cdx-2 or proglucagon (Jin and Drucker, 1996; Xu et al., 1999). We first asked the following two questions. (1) In BHK cells, whether Pbx1 on its own activate −476 GLU-LUC, which