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The Minimal Promoter Region of the Dense Core Vesicle Protein IA-2: Transcriptional Regulation by CREB

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Abstract

IA-2 is a transmembrane protein found in the dense core vesicles (DCV) of neuroendocrine cells and one of the major autoantigens in type 1 diabetes. DCV are involved in the secretion of hormones (e.g., insulin) and neurotransmitters. Stimulation of pancreatic β cells with glucose upregulates the expression of IA-2 and an increase of IA-2 results in an increase of the number of DCV. Little is known, however, about the promoter region of IA-2 or the transcriptional factors that regulate the expression of this gene. In the present study, we constructed eight deletion fragments from the upstream region of the IA-2 transcription start site and linked them to a luciferase reporter. By this approach we have identified a short bp region (–216 to +115) that has strong promoter activity. We also identified a transcription factor, CREB (cAMP Responsive Element Binding Protein), which binds to two CREB-related binding sites located in this region. The binding of CREB to these sites enhanced IA-2 transcription by more than 5 fold. We confirmed these findings by site-directed mutagenesis, chromatin immunoprecipitation (ChIP) assays and RNAi inhibition. Based on these findings we conclude that the PKA pathway is a critical, but not the exclusive signaling pathway involved in IA-2 gene expression.

Keywords

Dense-core vesicle; Insulin; IA-2; Promoter; PKA pathway; CREB binding site

Introduction

IA-2 and IA-2 β (also known respectively as ICA512 and Phogrin) are dense-core vesicle (DCV) transmembrane proteins and major autoantigens in type 1 diabetes [1, 2]. Human IA-2 and IA-2 β are 979 and 1015 amino acids, respectively, in length (GenBank acc. no.,

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NM_002846.3 and NM_002847.3). Both proteins are encoded by 23 exons and consist of an intracellular, transmembrane and luminal domain [3]. The genomic DNA sequence of the human IA-2 gene is 20kb, whereas that of IA-2 β spans approximately 1,000kb. Both genes are widely distributed in neuroendocrine cells throughout the body, and are abundantly expressed in the pancreatic islets, pituitary and hippocampus.

Recently we showed that deletion of the IA-2 and/or IA-2 β genes in mice results in a decrease in the number of DCV, impairs insulin secretion [4], leads to female infertility, alters behavior and results circadian dysregulation [5]. In contrast to the deletion of these genes, overexpression of IA-2 in insulin-secreting MIN6 cells results in an increase in the number of DCV and the secretion of insulin [6]. These findings show that IA-2 and IA-2 β are important molecules involved in the secretion of insulin and other neurohormones and neuropeptides.

Despite the extensive studies on the properties and function of IA-2, little is known about the promoter region or transcriptional factors that regulate the expression of this gene. In the present experiments, using deletion fragments from the 5'-upstream region of the IA-2 transcription start site, we identified a short sequence with strong promoter activity and transcriptional factors that bind to this region.

Materials and Methods

IA-2 promoter-luciferase assay

Eight consecutive human IA-2 promoter fragments (-1300 to +115) were cloned in the pGL3 vector to drive the expression of the firefly luciferase reporter gene (Promega, Madison WI, USA) [3]. All constructs were confirmed by Sanger sequencing. A dual-luciferase reporter system was used for promoter activity measurement according to the manufacturer's instruction (Promega). Renilla luciferase was used as an internal control for transfection efficiency. Normalized luciferase data (firefly/renilla) was compared with the empty pGL3-basic vector. The construct pIA2-8 or pGL3 vector served as a control for basal levels. The cell line HEK293 also was included as a control because endogenous IA-2 expression was not detected. In a separate experiment, inserts of pIA2-1, pIA2-2, pIA2-6, pIA2-7 and pIA2-8 also were inserted into the pGL4 luciferase reporter vector (Promega) for comparison of luciferase activities measured from the corresponding pGL3-based constructs. Unless otherwise indicated the pGL3 vector was used in all experiments.

Cell culture and transfection

Four cell lines (HEK293, U87MG, AtT20, and MIN6) were cultured in RPMI 1640 or Dulbecco's modified Eagle medium supplemented with 5.6 mM glucose and 10% fetal bovine serum as described previously [6, 7]. AtT20 cells were commercially obtained (ATCC, Manassas, VA). MIN6 cells were obtained from Dr. Jun-ichi Miyazaki [8] and used between passages 37~42, which responded to high-glucose by a 5~6 fold increase of insulin secretion and also responded to forskolin and IBMX treatment [9]. Cells were transfected with 3 μ g of appropriate luciferase reporter plasmid by lipofectamin2000 (Invitrogen Corp., Carlsbad, CA, USA), which resulted in approximately 50% transfection efficiencies at 48 h.

Plasmid coding for Renilla luciferase (0.03 µg) was co-transfected with each of the reporter gene constructs. After transfection, cells were cultured for 24–48 hours and lysed in Passive Lysis Buffer (Promega) for measurement of luciferase activity using a Microplate Luminometer (Berthold Technologies, Germany).

Site-directed mutagenesis of the IA-2 promoter

The GeneEditor site-directed mutagenesis system (Promega) was used to generate mutations in the sequence of Cre-L and Cre.2 binding sites. The sequences of the primers used for deletion-mutation are shown in Supplementary Table 1. Constructs were confirmed by Sanger sequencing at the Sequencing Core Facility of NIDCR/NIH.

ChIP assay and Western blots

Approximately 2×10^6 MIN6 cells were used per immunoprecipitation assay [7] with minor modifications. Briefly, nuclei were collected from formaldehyde-fixed cells by homogenization. DNAs were sonicated into 200–1000 bp. Precleared chromatin was immunoprecipitated with 10 µg of anti-CREB monoclonal antibody LB9 (Thermo Fisher Scientific, Rockford, IL, USA) or anti-p-CREB-1 (Ser133)-R rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Western blot was performed using the ECL Western Blotting System (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Immunocomplexes were collected into 50 µl of protein A/G-Sepharose beads and purified with Qiaquick (Qiagen, Germantown, MD, USA) PCR purification columns. Immunoprecipitated DNAs were analyzed by PCR with primers encompassing the IA-2 CRE-L site or the IA-2 CRE.2 site (Supplementary Table 1). In addition, as a negative control of the ChIP assay, a flanking region of the two CREB binding sites was examined with primers (Supplementary Table 1) located in exon 2 of IA-2 that does not contain a CRE site. To measure IA-2 protein levels in MIN6 cells, rabbit polyclonal antibody to IA-2 N-terminal region (amino acid residues 65–80, GenBank acc. no., NP_001186692) was obtained from Abgent (San Diego, CA, USA). A dominant band (about 90 kDa) of IA-2 was detected by the immune serum at 1:200 in Western blots. A mouse alpha-tubulin monoclonal antibody (Sigma, St. Louis, MO, USA) at 1:2500 dilution was used in Western blots as a loading control.

RNAi and real time-PCR

MIN6 cells were cultured in 12-well plates and transfected with final concentration 50 nM of Creb RNAi (Mm_Creb1_1, SI00959035, Qiagen, Valencia, CA, USA) or with an unrelated RNAi (Gapdh, Mm_Gapd_3, SI01009393, Qiagen) using HiPerfect Transfection Reagent (Qiagen). Incubation time (for 1h, 12h, 24h, or 48h) of the RNAi reagents in MIN6 cell was compared and optimized for the best inhibitory effect on CREB expression. No cell toxicity was observed for CREB RNAi. Total RNA was extracted using Trizol (Invitrogen) and Creb and IA-2 mRNA expressions were quantified using RT-PCR Kit (Applied Biosystems, Foster City, CA, USA). Real time-PCR was conducted as previously described [7]. Primers and probes of mouse IA-2 (Assay ID Details, Mm01258989_m1), Creb (Assay ID, Mm00501607_m1), and Gapdh (Assay ID, Mm99999915_g1) were purchased from Applied Biosystems. Real-time amplification of cDNAs was performed using the TaqMan Universal Master Mix and the primers in 9700HT real-time PCR Cyclers (Applied Biosystems).

Statistical analysis

All experiments were performed independently at least three times, and each experiment consisted of triplicate samples. The significance of differences between groups was analyzed by either student's *t* tests or one-way ANOVA and Bonferroni test.

Results

Minimal IA-2 promoter sequence

We previously isolated and sequenced a 3.9-kb 5'-upstream region of the human IA-2 gene, and found that the 1.3-kb proximal region possessed promoter activity in U87MG cells [3]. In the present study, we attempted to determine the minimal promoter region responding to high glucose or forskolin (a direct cAMP activator for increasing PKA and in turn CREB) stimulation. To do this, we constructed eight different lengths of IA-2-luciferase reporters ranging from -1300 to +115 bp (Fig. 1). Each of the plasmid DNAs was used to transiently transfect HEK293, U87MG, AtT20, and MIN6 cells. After 48 h in culture the cells were stimulated with either 16.7 mM of glucose or 10 μ M of forskolin (Fig. 1), and IA-2 promoter-luciferase activity was determined using the Microplate Luminometer. With high glucose, the activity of pIA2-2 (-826 to +115) reporter was increased >8-fold in MIN6 and AtT20 cells and about 5-fold in U87MG cells as compared to the basal reporter pIA2-8 (+1 to +115) or the embryonic kidney HEK293 cells. The activity of pIA2-1 (-1300 to +115) was substantially less than that of pIA2-2, suggesting the existence of potential transcriptional repressor(s) in the region of -1300 to -826. All the constructs from pIA2-2 to pIA2-7 showed some degree of responsiveness to glucose. In contrast, pIA2-8 did not respond to glucose indicating that there were no glucose-associated regulatory elements in that region of the promoter.

Forskolin produced even greater changes in luciferase activity than glucose. As seen in Fig. 1 (lower panel), forskolin significantly increased luciferase activity (10–16 folds) in MIN6 and AtT20 cells that had been transfected with the reporter plasmids from pIA2-1 to pIA2-6. No luciferase activity was observed in the cells that had been transfected with the reporter plasmids pIA2-7 or pIA2-8 (Fig. 1), indicating that the region from -94 to +115 bp did not contain regulatory elements for forskolin stimulation.

Because of lower background activity in the pGL4 vector as compared to the pGL3 vector, in a separate experiment the pGL4 vector was used with inserts from pIA2-1, pIA2-2, pIA2-6, pIA2-7 and pIA2-8. The pGL4 constructs yielded results similar to what we obtained with the pGL3 construct although the fold changes were slightly lower (Supplementary Figure 1).

To see if forskolin, which our experiments showed activated the IA-2 promoter constructs, would actually increase endogenous IA-2 levels, MIN-6 cells were treated with forskolin and at different times thereafter IA-2 mRNA was measured by real-time PCR (Fig. 2). By 1 hour, IA-2 mRNA levels were increased 1.5- to 2-fold, and by 24 to 48 hours, they were increased 6-fold. Since forskolin is a known activator of cAMP, we tested the ability of IBMX (3-isobutyl-1-methylxanthine), another known activator of cAMP, to increase IA-2 mRNA. By 1 hour, IA-2 mRNA levels were increased about 2-fold, and by 24 to 48 hours

they increased 3.0- to 3.5-fold, consistent with previous studies [6, 10–12]. Glucose served as a positive control and PMA (Phorbol 12-myristate 13-acetate), which does not activate cAMP, served as a negative control.

CRE transcription binding sites and mutagenesis of these sites

Using the comparative promoter sequence analysis program (TRES)[13], we analyzed the IA-2 promoter sequences in several mammalian species to look for evolutionary conserved transcription binding sites. Multiple sequence alignment identified two potential CRE binding sites (e.g., CRE-L and CRE.2) in the –216 to –128 region of human (h), rat (r), and mouse (m) IA-2 (Fig. 3). In addition, two conserved SP1 binding sites were found in the –128 to –40 region of these three species.

To determine whether the two CRE binding sites were functional, we mutated CRE-L or CRE.2 or both, and transfected each of the plasmids into MIN6 cells. After 48 h treatment with or without forskolin and/or IBMX, IA-2 promoter luciferase activity was measured. The activity of IA-2 luciferase in wildtype promoter was increased about 16-fold by forskolin and about 8-fold by IBMX over basal activity (Fig. 4 upper panel). The IA-2 luciferase activity, however, was significantly decreased when the CRE-L or the CRE.2 binding sites were mutated. When MIN6 cells were transfected with both mutated CRE-L and mutated CRE.2, IA-2 luciferase activity was reduced to a level near that of the basal control, pIA-2-7. These observations indicate that the CRE-L and CRE.2 binding sites are required for forskolin- or IBMX- induced promoter activity.

Endogenous CREB protein binds to CRE binding sites

To see whether CREB protein actually binds to the CRE binding sites of the IA-2 promoter in MIN6 cells, we used chromatin immunoprecipitation (ChIP) assay. After immunoprecipitation with CREB antibody, precipitated DNAs were PCR-amplified by specific primers for the CRE-L- or CRE.2-containing regions of the IA-2 promoter. As seen in Fig. 4 (lower panel), both CRE-containing fragments, but not the flanking regions that do not contain the CRE binding site, were efficiently precipitated and amplified. These findings show that the CRE binding sites and the transcriptional factor CREB play an important role in regulating the expression of IA-2.

Knockdown of CREB affects IA-2 promoter activity

To confirm the effect of CREB on IA-2 promoter activity, we knocked down CREB expression in MIN6 cells by RNAi. After 48 h treatment with CREB-specific RNAi, endogenous CREB and IA-2 levels were evaluated by real-time PCR. Figures 5 (upper-left and lower-left panels) shows that both CREB mRNA and IA-2 mRNA were significantly reduced in MIN6 cells, thereby supporting the argument that the expression of CREB affects endogenous IA-2 expression at the level of transcription. Western blotting with anti-CREB antibody also showed that the CREB protein level was significantly decreased in MIN6 cells by CREB RNAi treatment, but not by Gapdh RNAi (Fig. 5 upper-right panel). Western blotting with anti-IA-2 antibody also showed reduced IA-2 protein level (Fig. 5 upper-right panel). As expected, the IA-2 luciferase activity in the RNAi treated MIN6 cells also was significantly decreased (Fig. 5 lower-right panel).

Discussion

As a unique transmembrane protein of DCV, IA-2 has been implicated in insulin secretion and DCV stability [4]. Overexpression of IA-2 increases DCV number and insulin secretion [6], whereas deletion of IA-2 decreases DCV number and insulin secretion [4]. In the present study, using deletion fragments of IA-2 from the upstream region of the IA-2 transcription start site, we identified a short fragment (–216 to +115) that has strong promoter activity when stimulated, in particular, with forskolin. The importance of CREB as a critical transcription factor [14] in the –216 to +115 fragment is strongly supported by the evolutionary conservation in humans, rats and mice of two CREB binding sites in this region (Fig. 3) and by the data described in this paper using site-directed mutagenesis, chromatin immunoprecipitation (ChIP) and RNAi inhibition.

During the course of this work, however, we observed that the knockdown of CREB by RNAi did not completely abolish endogenous IA-2 mRNA expression (Fig. 5 middle panel) or IA-2-promoter-luciferase activity (Fig. 5 lower panel). This suggested that additional transcriptional factor(s) might be involved. One such transcriptional factor could be ATF (Fig. 3), which is known to bind to the CRE-L binding site, at least, in the insulin promoter [15, 16]. Whether ATF actually is induced by forskolin and binds to the CRE binding site of the IA-2 promoter, however, requires further investigation.

Of particular interest, an even shorter upstream region (–94 to +115), which does not have CREB binding sites and does not respond to forskolin, still responds to glucose (Fig. 1). This –94 to +115 region, however, has one or more glucose-sensitive sites. For example, an SP1 binding site is located in PIA-2-7 (–84 to –40 region) and the binding of the SP1 transcription factor is known to make it sensitive to high glucose particularly in TATA-less genes such as IA-2 [3, 17]. Other SP1 binding sites, such as a short fragment containing glucose-elements (GRE) in the promoter of pyruvate carboxylase (from nt –546 to –399), were recently identified to regulate high glucose-induced gene expression [18]. This argues that glucose and forskolin regulate the transcriptional activity of the IA-2 through different signaling pathways. Alternatively, cross-talk between glucose and cAMP pathway may synergistically regulate the expression of IA-2. For instance, glucose has been found to promote CREB activity by inducing TORC2 nuclear relocalization, which is required for the cAMP-mediated activation of CREB in beta cells and islets [19].

In conclusion, both forskolin and IBMX are known to increase cAMP and that cAMP can stimulate PKA-mediated phosphorylation of the transcription factor CREB [20]. The binding of CREB to the CRE-L and CRE.2 binding sites results in the activation of the IA-2 promoter and an increase in IA-2 mRNA and IA-2 protein. The widespread distribution of CREB and CREB-binding sites throughout the genome [21] may explain the high levels of IA-2 seen in some tissues [22, 23] and tumors [24, 25] where DCV have not been detected. Viewing the IA-2 promoter region from an even broader perspective, when the 5′-upstream region from –1300 to +115 was examined by the TRES program, a number of other potential transcription binding sites and CREB activators were identified [26–30]. Thus, other transcription factors, depending on their concentration and the tissue type, also may

play a role in the regulation and expression of the 5'-upstream region (−1300 to +115) of the IA-2 gene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Transcriptional activity of the IA-2 promoter. Various lengths of the 5'-upstream IA-2 promoter region (from -1300 to +115 bp) were constructed and cloned into the luciferase reporter pGL3 vector, and transfected into HEK293, U87MG, AtT20, and MIN6 cells. The pRL-TK vector, coding for Renilla luciferase, was co-transfected as an internal reference, and luciferase values were normalized to Rluc activity. Transfection was carried out in triplicate and each experiment was repeated three times. Cells were treated with either (upper panel) high glucose (16.7 mM) or (lower panel) forskolin (10 μ M). Data (mean $\pm$ SE) are presented as luciferase activity relative to that of the promoter-less pGL3 basic vector

(i.e., pIA2-8) in each cell line. Three independent experiments were performed. The X-axis of the graph indicates fold-activation over normalized luciferase activities.

Fig. 2.

Effects of different secretagogues on IA-2 mRNA levels in MIN6 Cells. MIN6 cells were treated with forskolin (10 μ M), IBMX (100 μ M), high glucose (16.7 mM), or PMA (1 μ M), and samples were collected at 1 h, 12 h, 24 h, and 48 h. Endogenous IA-2 mRNA levels were determined by Real-Time PCR. IA-2 mRNA level at basal condition was estimated to be about 10-fold greater than Gapdh (control). Data (mean $\pm$ SEM) from three independent experiments after being normalized to Gapdh mRNA levels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. basal level (i.e., the IA-2 expression level before the stimulators were added), calculated by the student's t test.

hIA-2	-216	CTCTTACGCCATATG	TGACGCAA	ATTAGCTTACGAGCCGGGGGT
rIA-2	-214	CTCTTACGCCA.GTAT	TGACGCAA	ATAGGCCTAAGAGCCGGGGGT
mIA-2	-212	CTCTTACGCCA.GTAT	TGACGCAA	ATAGGCATAAGAGCCGGGGGT
CRE-L/ATF				
hIA-2	-172	GGAGGTG	TCGTCA	GCACCCGCCTGCGGTTGGCCGCGTTGCAGCG
rIA-2	-170	GGAGATG	TCGTCA	TTGCACGCCTGCAGTTGGCCGGGTTCGCCTCA
mIA-2	-168	GGAGATG	TCGTCA	TTGCACGCCTGCAGTTGGCCGGGTTCGCCTCA
CRE.2				
hIA-2	-128	AGAGGGGCATGTAGAC	CCCGCC	TCGTAGGGAGGTGGGGAGCGGC
rIA-2	-126	AGAGGGGCAAGTAGAC	CCCGCC	TTGGAGGTGGGTGGGGAGTGGC
mIA-2	-124	AGAAGGGCAACTAGAC	CCCGCC	TTGGAGGTGGGTGGGGAGTGGC
SP-1				
hIA-2	-84	AAGCC	CCGCCT	CAGCCCCTCTGGCAGGCTCCCGCCAGCGTCGCT
mIA-2	-82	AGACT	CCGCCT	AGGCTCCT.GGGCTGACTCCCACCAACGTCGCC
rIA-2	-80	AGACT	CCGCCT	AGGCTCCT.GGGTTGACTCCCACCAACGTCGCT
SP1-TPI				
hIA-2	-40	GCGGCTCCGGCCCCGGGAGCGAGCGCCCGGAGCTCGGAAAG	ATG	
rIA-2	-38	GAGACTCTGGCCGGAGAGCGAGAGCTCAGAGCCGGGAAGG	ATG	
mIA-2	-36	GAGACTCTGGCCGGAGA..GAGAGCTCAGACCCGGGAAGG	ATG	
Start site				

Fig. 3.

Analysis of IA-2 promoter sequence. Multiple sequence alignment (human, rat, and mouse) revealed two evolutionarily conserved CRE binding sites in the proximal 5'-terminal region of IA-2. The CRE-L/ATF binding site (TGACGCAA) is located at -194 to -201 and the CRE.2 (TCGTCA) binding site is located at -160 to -165. These two motifs are spaced 28 base pairs apart. In addition, SP1 and SP1-TPI binding sites were found between -107 to -112 and -74 to -79, respectively.

Luciferase activities

ChIP analysis

Fig. 4.

CRE binding sites and IA-2-luciferase expression. Upper panel: Effect of mutated CRE binding sites on IA-2-luciferase expression in MIN6 cells treated for 48 h with forskolin or IBMX. Data shown as fold change of luciferase activity relative to that of the pIA2-7 pGL3 construct. Renilla luciferase vector was co-transfected as an internal control. Three independent experiments were performed. Luciferase activity of single and double CRE mutated IBMX-treated cells (grey) and single and double CRE mutated forskolin-treated cells (black) as compared to unmated cells. The P-values (**P < 0.01, ***P < 0.001) were

obtained by one-way ANOVA and Bonferroni test. Lower panel: CREB protein binds to the IA-2 promoter. By ChIP analysis, chromatin isolated from MIN6 cells was treated with CREB antibodies (CREB Ab) or control IgG. Input DNA was serially diluted and served as a positive control for PCR analysis. Both CRE regions of the IA-2 promoter were co-immunoprecipitated by CREB antibody. The flanking region of both CRE regions that do not contain a CRE site, served as the negative control.

Fig. 5.

Effect of RNAi-mediated suppression of Creb on IA-2 mRNA levels. MIN6 cells were transfected with 100 ng of Creb siRNA or Gapdh siRNA and incubated with forskolin (10 μ M) for 48 h. Total mRNA was extracted for measuring Creb (upper-left panel) and IA-2 expression (lower left panel) by real-time PCR. The Western blots showed that CREB and IA-2 proteins (upper-right panel) were reduced by about 40–50% as determined by NIH ImageJ analysis. Effect of knockdown of Creb in MIN6 cells (lower-right panel) on the luciferase activity of the IA-2 construct pIA-2-6 (–216 to +115). The pIA-2-7 construct, which does not contain CREB binding sites, was used as the control. Values represent the mean $\pm$ SEM. Three independent experiments were performed. The P-values (**P < 0.01; *** P < 0.001) were calculated by the student's *t* test.