Regulation of the Diabetes-Associated Autoantigen IA-2 in INS-1 Pancreatic β-Cells

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IA-2, a member of the protein tyrosine phosphatase family, represents a major target autoantigen in type 1 diabetes. To study the regulation of IA-2 gene expression, we used INS-1 insulinoma cells to analyze β-cell signal transduction pathways as well as the effect of metabolic and hormonal factors involved in the regulation of the insulin secretory pathway. Quantitative competitive reverse transcriptase–polymerase chain reaction revealed that an increase of cellular cAMP mediated by forskolin (10 µmol/l, 24 h) or 3-isobutyl-1-methylxanthine (100 µmol/l, 24 h) induced maximal stimulation of IA-2 mRNA levels (451 ± 85 and 338 ± 86% compared with basal conditions; P < 0.001). In contrast, activation of protein kinase C (PKC) by short-term treatment with phorbol 12-myristate 13-acetate (100 µmol/l, 24 h) did not alter IA-2 expression, whereas depletion of PKC by prolonged culturing (24 h) exerted a significant inhibition (57 ± 24%; P < 0.05). cAMP-dependent upregulation was confirmed by the findings that glucagon (10 µmol/l, 24–48 h) increased levels of IA-2 mRNA (190 ± 35%; P < 0.05), whereas short-term incubation with high glucose concentration showed no effect. However, prolonged incubation in high glucose (21 mmol/l) induced a time- and dose-dependent increase of IA-2 mRNA expression, reaching maximal values after 144 h (285 ± 68%; P < 0.05). These studies demonstrate that stimuli of insulin secretion that operate by activation of adenylate cyclase generating cAMP significantly increase IA-2 gene expression. In contrast, activation of PKC by high glucose concentration or PMA exerted no effect, suggesting that IA-2 gene expression is not simply coupled to insulin secretion, but may be involved in the fine regulation of β-cell function. These findings may important to clarify the function of IA-2 in β-cells and elucidate mechanisms involved in the induction of autoimmunity to IA-2. Diabetes 49:1137–1141, 2000

Type 1 diabetes results from the destruction of pancreatic β-cells mediated by a chronic autoimmune process. Recently, one member of the receptor-type protein tyrosine phosphatase (PTP) family, designated as IA-2 or ICA512, has been identified as a major diabetes-specific autoantigen (1–3). Autoantibodies to IA-2 were observed in 50–70% of newly diagnosed patients with type 1 diabetes (4,5) and in 49–64% of prediabetic subjects at high risk for rapid development of the disease (6–8). In addition, a specific T-cell response has been described in 42–60% of diabetic patients, suggesting that IA-2 is a dominant target of humoral and cellular autoimmunity (8–11).

IA-2 is predominately expressed in neuroendocrine cells, including the brain, pituitary, and pancreatic β-cells, and is highly conserved in evolution. The human IA-2 gene encodes a transmembrane protein of predicted molecular weight (Mr) 105 kDa, which is processed in vivo to a glycosylated mature protein of Mr, 65–70 kDa. In the pancreatic β-cells, IA-2 is localized in the membrane of insulin secretory vesicles, suggesting that it may be important for maturation or trafficking of insulin granules (12). IA-2 is characterized by an intracytoplasmic domain containing 1 PTP motif, 1 transmembrane region, and an extracytoplasmic part directed to the luminal site of the insulin granula that harbor the receptor-like domain (1,12). Thus far, no enzymatic activity has been detected using common PTP substrates, and no ligand has been identified interacting with the receptor-like domain of IA-2 (13). Therefore, the biological function of IA-2 is still unknown.

In the present study, we investigated the regulation of IA-2 gene expression in the glucose-sensitive insulinoma cell line INS-1. By means of quantitative competitive reverse transcriptase–polymerase chain reaction (RT-PCR), we demonstrate that IA-2 gene expression is regulated by the 2 main transduction systems, an increase in cAMP levels, and the activation state of the protein kinase C (PKC), which are both involved in the coordinated β-cell function.

RESEARCH DESIGN AND METHODS

Cell culture. INS-1 cells (a clonal glucose-sensitive rat insulinoma cell line) were a gift from Prof. C.B. Wolfsheim (University of Geneva, Switzerland) (14). Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mmol/l glutamine, and 50 µmol/l 2-mercaptoethanol (complete medium) and propagated by weekly subculture. Their ability to respond to glucose was constant (1.9-fold stimulation of the insulin release) between passages 90 and 98 used in the present experiments. INS-1 cells were plated at 1 × 10^6 in 5-ml tissue culture flasks in complete medium. After 3 days of culture, cells were fed with low glucose (7 mmol/l) complete medium (basal medium) and cultured for an additional 3 days before addition of test agents. The first set of experiments was performed to study the effects of second messenger systems involved in insulin secretion. Cells were incubated in basal medium.
supplemented with phorbol 12-myristate 13-acetate (PMA) (1 µmol/l, 6-48 h), forskolin (10 µmol/l, 6-48 h), and 3-isobutyl-1-methylxanthine (IBMX) (100 µmol/l, 24 h) (Sigma, Deisenhofen, Germany). To study the influence of metabolic and endocrine factors on the regulation of IA-2 gene expression, cells were stimulated with glucose (7, 10, 15, or 21 mmol/l), glucagon (1 or 10 µmol/l) (Bachem, Heidelberg, Germany), or somatostatin (1 or 10 µmol/l) (Sigma) for 24-48 h. In some experiments, stimulation with glucose was extended to 72 and 144 h. Glucose, forskolin, IBMX, PMA, and glucagon promoted insulin release under our experimental conditions (data not shown). Viability of INS-1 cells was found to be >95% as assessed by trypan blue staining at the end of the stimulation period.

The agent with the strongest stimulatory effect (forskolin) was also tested in experiments using freshly isolated islets of Langerhans. Islets were isolated from Wistar rats (150 g) by collagenase digestion followed by separation on a discontinuous Ficoll density gradient (Pharmacia, Freiburg, Germany), with relative densities of 1.088, 1.069, 1.056, and 1.000. Purified islets were collected at the 1.069–1.056 interface, washed 3 times in RPMI 1640, and cultured for 24 h in basal medium (7 mmol/l glucose). Then islets (n = 200) were stimulated for 24 h with 10 µmol/l forskolin.

RNA extraction and cDNA synthesis. Total RNA was extracted from INS-1 cells or isolated islet cells by High Pure RNA Isolation Kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s instructions. After spectrophotometric measurement of RNA levels, 4 µg total RNA was used to synthesize first-strand cDNA (Superscript RT; Gibco, Karlsruhe, Germany) in a reaction of 40 µl.

Quantitative competitive RT-PCR. mRNA expression was analyzed by quantitative competitive RT-PCR technique using competitor fragments (CFs) for β-actin and IA-2. To generate specific CFs, we first cloned IA-2 cDNA fragments from INS-1 cells by RT-PCR. PCR products were cloned into the pGEM T vector (Promega, Mannheim, Germany) and sequenced using an automated sequencing apparatus (ABI; Applied Biosystems, Foster City, CA). Sequencing revealed 100% identity to the published sequence of rat IA-2 (bp 1642–2463; GenBank accession number D38222) and rat β-actin (bp 827–1583; accession number V01217), respectively. IA-2 CF was constructed by digestion of the IA-2 cDNA clone with restriction endonuclease Ava II followed by limited digestion with Exonuclease III (50 s) and S1 nuclease. After religation with T4-DNA ligase, the construct was sequenced to confirm appropriate internal deletion (100 bp for IA-2). The same procedure was performed with the β-actin clone using HindIII restriction enzyme (internal deletion 144 bp). The resulting CFs differ only in size from the cDNA clones and possess the same 5‘ and 3‘ sequences.

Competitive PCR was performed by the coamplification of known amounts of CF (0.003 fmol of β-actin CF and 0.05 amol of IA-2 CF) with unknown amounts of cDNA using specific primer pairs that span 1 or more introns to exclude amplification of genomic DNA (β-actin, 5’-CTATCGGCAATGAGCCGG TCTC-3‘ and 5’-GTGTAAGCTGCACTGGCAAG-3‘; IA-2, 5’-GAGTTGGGG GGTGCGCTTG-3’ and 5’-CAGTAAACAGTACAGCTGCAAG-3‘). Each PCR reaction contained 1.5 mmol/l MgCl2, 200 µmol/l dNTP, 1 µmol/l of each primer, 0.86 U High Fidelity DNA polymerase (Boehringer Mannheim), 1 µl CF, and cDNA in adjusted dilutions. Direct sequencing of the IA-2 CF and β-actin CF was performed with the ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA).

Northern blotting. For Northern blot analysis, 4 µg total RNA of each sample was heated to 65°C for 10 min and then applied to a 1% agarose gel containing 5% formaldehyde. The integrity of the RNA was assessed by ethidium bromide staining. RNA was blotted onto a nylon membrane (Hybond-N+; Amersham, Braunschweig, Germany), crosslinked to the membrane, and prehybridized with DIG Easy Hybridization Kit (Boehringer Mannheim) for 2 h at 68°C. Then, the probes were hybridized simultaneously with a digoxigenin (DIG)-labeled 822-bp fragment of rat IA-2 cDNA and a DIG-labeled 757-bp fragment of rat β-actin cDNA on the same blot for 18 h at 68°C in DIG Easy Hybridization Kit. The membranes were washed twice with 2x sodium chloride–sodium citrate (SSC) (300 mmol/l NaCl, 30 mmol/l C6H5O7Na3, pH 7.0), 0.1% SDS at room temperature and twice with 0.1 x SSC, 0.1% SDS at 68°C, followed by staining with an anti-DIG alkaline phosphatase labeled antibody and disodium 3-(4-methoxyxypropyl)2-dioxetane-3,2-(5-chloro)tricyclo[3,3.1.17]decane-14-y)phenyl phosphate. The relative densities of 1.088, 1.069, 1.056, and 1.000 were set equal to 100%.
phosphate solution (both purchased from Boehringer Mannheim, Mannheim, Germany). Chemiluminescent signals were detected and quantified by the Lum-I-mager system. Calibration of the RNA content was performed by normalization of the signals to the strength of the β-actin signal. 

**Statistical analysis.** mRNA levels are given as means ± SD of at least 3 independent experiments. Differences between mRNA levels and insulin secretion were analyzed by unpaired Student’s t test (SPSS software, Chicago, IL).

**RESULTS**

**Effects of cAMP and PKC on IA-2 gene expression.** To determine the potential intracellular mechanisms involved in the regulation of IA-2 mRNA levels, we studied the effects of forskolin and IBMX, which raises the cellular cAMP levels, and PMA, which activate PKC. First, we determined the changes of the relative IA-2 mRNA amounts in INS-1 cells after stimulation with 10 μmol/l forskolin or 1 μmol/l PMA for 24 h using Northern blot analyses. As shown in Fig. 2, INS-1 cells express an IA-2–specific mRNA species, which was significantly upregulated after treatment with forskolin (5.5-fold stimulation).

After incubation with PMA, a slight decrease of IA-2 mRNA levels (1.3-fold inhibition) was detected. These data were confirmed using the competitive RT-PCR technique. There was a slight increase of IA-2 mRNA in INS-1 cells that were cultured for 6 h with 10 μmol/l forskolin (118 ± 23%), which reached significant levels after 24 h (451 ± 85%) and gradually further increased at 48 h (P < 0.001) (Fig. 1B and Fig. 3). A strong stimulatory effect (338 ± 86%) was also observed in INS-1 cells that were treated with 100 μmol/l IBMX, suggesting a cAMP-mediated stimulation of IA-2 gene expression. To assess whether this effect was limited to INS-1 cells, we cultured freshly isolated rat islet cells with 10 μmol/l forskolin for 24 h. As illustrated in Fig. 4, forskolin significantly stimulated IA-2 gene expression in rat islet cells to 268 ± 46% compared with basal conditions (P < 0.01).

In line with the results obtained by Northern blotting, PMA exhibits an inhibitory effect on IA-2 gene expression. Treatment with 1 μmol/l PMA for different periods of time revealed that mRNA levels were not altered after 6 h, but they were significantly decreased after exposure for 24 and 48 h (57 ± 24 and 52 ± 19% respectively; P < 0.05) (Fig. 1C).

Because Northern blotting has a limited sensitivity and does not allow for a precise quantification of mRNA levels, further experiments were performed using the competitive RT-PCR technique.

**Regulation of IA-2 mRNA levels by glucose.** Exposure of INS-1 cells to various glucose concentrations showed a dose- and time-dependent increase of IA-2 mRNA levels. The study of the kinetics of glucose stimulation, performed at 21 mmol/l glucose, revealed a progressive increase in IA-2 expression over a time period of up to 6 days. A slight increase in IA-2 expression could be detected after 72 h (145 ± 47%), reaching maximal levels (285 ± 68%) after incubation of INS-1 cells for 6 days at high glucose concentration (21 mmol/l). When the glucose stimulus was discontinued for 48 h, mRNA levels of IA-2 returned to prestimulatory values (Fig. 1D). Long-term exposure to 10 or 15 mmol/l glucose resulted in a nonsignificant increase of IA-2 mRNA (114 ± 21 and 172 ± 26%) compared with the rate found under basal conditions (Fig. 1E).

**Influence of endocrine factors on IA-2 mRNA levels.** To analyze whether hormonal factors regulate IA-2 gene expression, we stimulated INS-1 cells for 24–48 h with glucagon and somatostatin, respectively. As illustrated in Fig. 1F, addition of 1 μmol/l glucagon caused a 190 ± 35% increase in IA-2 expression (P < 0.05). Higher glucagon concentrations (10 μmol/l) did not lead to further upregulation of IA-2 mRNA levels. In contrast, somatostatin treatment at concentrations of 1 or 10 μmol/l did not alter IA-2 expression (Fig. 3).
IA-2 represents a major target of the humoral and cellular autoimmune response in type 1 diabetes. With respect to the localization of IA-2 in insulin granula, it has been suggested that IA-2 is involved in the insulin secretory pathway. To test this hypothesis, we here demonstrate for the first time data on the regulation of IA-2 gene expression. We studied the classical signal transduction pathways of insulin secretion, including stimulation with glucose, an increase in cAMP levels, and the modulation of PKC activity. cAMP was found to strongly activate transcription of the IA-2 gene in INS-1 cells, whereas short-term activation of PKC exerted no significant effect and depletion of PKC was associated with significant inhibition of IA-2 expression. In addition, acute exposure to high glucose did not alter IA-2 expression. Interestingly, in INS-1 cells IA-2 may be not regulated by signals of insulin exocytosis mediated by a rise in cytoplasmic Ca\(^{2+}\) concentration.

In the present study, we examined the action of different secretagogues and inhibitory agents that contribute to the control of the fine-tuning of pancreatic β-cell function. Although IA-2 is expressed in only low amounts in islets of Langerhans and insulinoma cells (17), the development of a competitive RT-PCR technique allowed us to quantify IA-2 mRNA levels with high sensitivity. In the first set of experiments, we analyzed different transduction systems, the rise in cAMP and the PKC signaling system, which both have been shown to be involved in the modulation of insulin secretion in normal islets and INS-1 cells (18–21). We here demonstrate that an increase of intracellular cAMP levels, induced either by the activation of adenylate cyclase (forskolin) or by the inhibition of phosphodiesterase (IBMX), results in a strong upregulation of IA-2 gene expression in INS-1 cells. These data were supported by the fact that glucagon, which acts through the cAMP-mediated pathway, also induced a significant upregulation of IA-2 mRNA. Because forskolin exhibits similar effects in isolated rat islet cells, our data suggest that cAMP is the major player of signal transduction to stimulate IA-2 gene expression. The lower amount of stimulation observed in islets compared with INS-1 cells may be explained by an increased basal stimulation mediated by glucagon, secreted from α-cells in the rat islets. cAMP exerts its effect as a potent inhibitor of nutrient-induced insulin release by the activation of protein kinase A, resulting in phosphorylation of several cellular proteins (19,22). Recently, Xie et al. (23) described several binding sites for transcriptional factors in the 5′-flanking region of the IA-2 gene, including cAMP response element (CRE), E2F, HSF, YY1, and Sp1. Although we have not directly analyzed the specific mechanisms by which cAMP stimulates IA-2 gene expression, changes in cAMP levels may regulate directly IA-2 mRNA expression on the transcriptional level through the phosphorylation of the CRE-binding proteins or activate cAMP-dependent secondary transduction signals (24–26).

To investigate the effect of the PKC signaling pathway, we studied IA-2 expression under conditions thought to activate (short-term treatment with phorbol ester) or deplete/inactivate PKC (phorbol ester treatment for >18 h) (20,27,28). In pancreatic β-cells, PKC is activated by glucose and Ca\(^{2+}\)-mobilizing agents, resulting in a series of cellular events involved in the modulation of the insulin response (29). We here observed that activation of PKC for 6 h did not influence IA-2 mRNA expression. It is important to note that our experiments did not exclude the possibility that long-term activation of PKC can stimulate IA-2 expression. However, we clearly demonstrated that conditions accompanied by PKC depletion or inactivation resulted in a significant downregulation of IA-2 expression. With respect to the complex interaction of PKC with multiple substrates (30), the detailed molecular mechanisms involved in the inhibition of IA-2 expression remained to be determined in further studies.

Because PKC is involved in insulin secretion stimulated by glucose, we next investigated the effect of glucose as the principal β-cell secretagogue. The results of this study indicate that glucose—in a dose- and time-dependent manner—enhanced IA-2 mRNA levels in INS-1 cells. This effect was, in contrast to the immediate stimulation of insulin secretion (data not shown), only observed after prolonged exposure to high glucose. Thus, our findings suggest that the upregulation of IA-2 expression is not simply coupled to increased insulin secretion. The long lag period of the glucose-induced upregulation of IA-2 gene expression is in contrast to the regulation of the insulin gene and enzymes involved in the glucose metabolism, which are upregulated within a few hours after exposure to supraphysiological glucose concentrations (31,32). The question arises as to how the enhanced expression of IA-2 relates to the long-term exposure of INS-1 cells to elevated glucose. Prolonged exposure to high glucose has been reported to induce a marked increase in metabolic activity and promote several signals involved in trophic effects in islet cells and INS-1 cells, including an increase in glucokinase activity, glycogen deposition, lipogenesis, the induction of glycolytic genes, increased cell proliferation, and a decrease of glucose-induced insulin release (33–35). Because we normalized our probes for β-actin mRNA levels by competitive RT-PCR, differences between 24-h and 6-day cultures resulting from increased cell numbers at high glucose levels can be excluded. We speculate that high glucose concentrations may induce as-yet-unknown glucose-responsive factors involved in the adaptation of islet cells to chronic hyperglycemia. In neurons, several members of the receptor-type PTP family were shown to be crucially involved in cell growth and differentiation (36). Whether IA-2 has similar functions in β-cells has to be answered in further studies.

In conclusion, we have demonstrated for the first time that IA-2 gene expression is regulated through the cAMP and PKC transduction systems in INS-1 cells. The coordinated regulation of IA-2 expression supports the suggestion that IA-2 may be important for β-cell function. Our findings may encourage studies to identify the as-yet-unknown potential IA-2 substrates and receptor ligands. In addition, the present data may provide the basis to elucidate the physiological function of IA-2 and mechanisms involved in the induction of autoimmunity to IA-2.

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