IB1, a JIP-1-related Nuclear Protein Present in Insulin-secreting Cells*

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JIP-1 is a cytoplasmic inhibitor of the c-Jun amino-terminal kinase activated pathway recently cloned from a mouse brain cDNA library. We report herein the expression cloning of a rat cDNA encoding a JIP-1-related nuclear protein from a pancreatic β-cell cDNA library that we named IB1 for Islet-Brain 1. IB1 was isolated by its ability to bind to GTII, a cis-regulatory element of the GLUT2 promoter. The IB1 cDNA encodes a 714-amino acid protein, which differs from JIP-1 by the insertion of 47 amino acids in the carboxy-terminal part of the protein. The remaining 667 amino acids are 97% identical to JIP-1. The 47-amino acid insertion contains a truncated phosphotyrosine interaction domain and a putative helix-loop-helix motif. Recombinant IB1 (amino acids 1–714 and 280–714) was shown to bind in vitro to GTII. Functionally IB1 transactivated the GLUT2 gene. IB1 was localized within the cytoplasm and the nucleus of insulin-secreting cells or COS-7 cells transfected with an expression vector encoding IB1. Using a heterologous GAL4 system, we localized an activation domain of IB1 within the first 280 amino acids of the protein. These data demonstrate that IB1 is a DNA-binding protein related to JIP-1, which is highly expressed in pancreatic β-cells where it functions as a transactivator of the GLUT2 gene.

In an attempt to identify DNA-binding proteins necessary for proper β-cell-specific expression of genes in the endocrine pancreas, we initiated the characterization of the promoter of the GLUT2 gene. GLUT2, a facilitated glucose transporter isofrom, is a membrane protein present in pancreatic β-insulin-secreting cells, the basolateral membrane of intestinal and kidney absorptive cells, in hepatocytes, and in a subset of neurons (1–3). In several experimental models of diabetes, GLUT2 expression is dramatically reduced in pancreatic β-cells, and it has been suggested a role for GLUT2 in the pathogenesis of the disease (4–10). We and others have shown that a fragment of the GLUT2 promoter displayed glucose responsiveness when transfected into differentiated insulin-producing cells or into hepatocytes (11–13). Important cis-regulatory sequences were identified within this promoter region, including functionally responsive PDX-1 and cyclic AMP-responsive elements and three cis sequences named GTI, GTII, and GTIII (13–15). The minimal promoter region containing GTI, GTII, and GTIII is both sufficient and necessary to confer pancreatic expression to a reporter gene in vitro or in vivo in transgenic mice (14, 16). Nuclear proteins specifically expressed in pancreatic β-cells interact with the GTII sequence (14).

In this report, we describe the expression cloning of a GTII-binding protein from a pancreatic β-cell cDNA library. The gene encodes a cDNA abundantly expressed in the pancreatic islets and in the brain, which was named IB-1 for Islet-Brain 1 (17). A GeneBank™ data base search with the IB1 cDNA revealed that IB1 is a rat homologue of the murine cytoplasmic inhibitor of the c-Jun amino-terminal kinase (JNK)3-activated pathway termed JIP-1 (18). IB1 differs, however, from JIP-1 by the insertion of a 47-amino acid region in its carboxy-terminal part. This insertion encodes a phosphotyrosine interaction domain (PID) and a helix-loop-helix motif (HLH). Furthermore, IB1 is a cytoplasmic and nuclear DNA-binding protein, which functions as transactivator of the GLUT2 gene.

MATERIALS AND METHODS

Construction of an INS-1 cDNA Expression Library and Cloning of the IB1 cDNA—An oligo(dT)-primed cDNA was generated from 10 μg of poly(A)+ RNA obtained from the differentiated INS-1 insulin-secreting cell line using a cDNA synthesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The cDNAs were cloned into the EcoRI and Xhol sites of the XZap Express expression vector (Stratagene). A total of 2 × 10^6 colonies were screened by the procedure described by Singh et al. (19) using as probe concatenated GTII oligonucleotides (14). One GTII-interacting positive clone was obtained from the screening and excised. The resulting cDNA in the pBKS plasmid (pBKS-IB1) was sequenced in both 5’ and 3’ orientations.

Cell Lines, Plasmid Constructions, Transient Transfections, and Luciferase Assays—The transplantable x-ray induced rat insulinoma INS-1 cell line was kindly provided by Asfari et al. (20) and grown as described. The mouse insulin-producing βTC3 cell line and the kidney-derived COS-7 cell line were cultured as described previously (21, 22).

The eukaryotic expression vector encoding IB1 was constructed by inserting the IB1 cDNA into the NheI/Xhol sites of the CMV-driven plasmid PBKS (Stratagene) to generate the pCMV-IB1 vector. Polymerase chain reaction mutagenesis was used to add a Flag epitope (Eastman Kodak Co.) in the pCMV-IB1 construct 3’ of the initiating methionine. The ~338 bp of the murine GLUT2 promoter (14) were cloned 5’ of a luciferase gene (pGL3Basic vector, Promega, Madison, WI). For the GAL4 constructs, the IB1 cDNA was introduced into the pSG147 vector, in frame with the GAL4 DNA-binding domain (aa 1–147) of this vector (23). The luciferase reporter construct used in the GAL4 system was obtained by linking five copies of the GAL4 DNA-binding sites (5 × GAL4 DNA-binding sites) 5’ to the minimal herpes simplex virus thymidine kinase promoter in the pGL3-TK plasmid (Promega). All constructs were transiently transfected using the cationic reagent DOTAP (Boehringer Mannheim, Mannheim, Germany) as described previously (14). Luciferase activities were measured according to the protocol of Brasier et al. (24).

SouthWestern Experiments, In Vitro Transcription and Translation, RNA, and Northern Blot Analysis—Nuclear and cytoplasmic extracts were prepared according to the method of Dent and Latchmann (25). The SouthWestern experiments were conducted as described (14). The in vitro translation experiments were performed with the pBKS-IB1 plasmid as template using the coupled transcription-translation kit (TNT) from Promega and according to the manufacturer’s instructions.
in the presence of [35S]methionine. The RNA isolation and Northern blot analysis from rat tissues or cell lines were conducted exactly as described previously (14). The rat pancreatic islets were isolated by the method of Gotto et al. (26).

Identification of Anti-IB1 Antiserum—Anti-IB1 antiserum was prepared using a cDNA fragment encoding the first 280 amino acids of the protein. This fragment was inserted into the His-tagged pQE-9 expression vector (Qiagen, Basel, Switzerland), expressed, and purified through a Ni²⁺-containing column following instructions from the manufacturer. Purified material was used to elicit polyclonal antibodies in rabbits. To affinity-purify the antibodies, the Ni²⁺-column-purified 1–280 aa of the recombinant protein were immobilized onto a nitrocellulose membrane, and the rabbit antiserum (diluted 1:50 in phosphate-buffered saline) was incubated with this membrane. The membrane was then washed in phosphate-buffered saline buffer, and the anti-IB1 antibody was eluted by 0.2 M Tris-glycine, pH 2.8, followed by neutralization at pH 7.5. The preimmune serum was treated in a similar fashion to provide an immune control.

RESULTS AND DISCUSSION

Identification of a JIP-1-related Protein in Pancreatic β-Cells

A DNA binding activity to GTII was shown to be restricted to insulin-secreting cells (INS-1 and βTC3) (14). A poly(dT)-primed INS-1 cDNA expression library was constructed and screened by the procedure described by Singh et al. (19) using a concatenated GTII oligonucleotide probe. One positive clone was isolated from a primary screen of approximately 2 × 10⁹ plaques. The 2,953-bp-long insert encoded a large open reading frame of 714 amino acids and was termed IB1, for Islet-Brain 1, as its expression was primarily restricted to these two tissues, as discussed below (17). A GeneBank™ data base search revealed that IB1 is a rat homologue of the recently identified JIP-1 protein (18). JIP-1 is a cytoplasmic inhibitor of the JNK-activated pathway, which was cloned from a mouse brain cDNA library using a two-hybrid system (18). Amino acid and nucleic acid comparison of mouse JIP-1 and rat IB1 showed that the proteins are almost identical (97% identity) with the exception of a 47-amino acid addition in the carboxy-terminal part of IB1. As depicted in Fig. 1, this 47-amino acid insertion contains a putative helix-loop-helix domain as well as a PID. PID domains are an average length of 100–160 amino acids and consist of four conserved blocks (27, 28). The first block of the putative PID domain of IB1 is contained in the 47-amino acid insertion and therefore is absent from the JIP-1 protein. JIP-1 was shown to be a cytoplasmic protein that caused cytoplasmic retention of JNK and that inhibited the JNK-regulated gene expression (18). As JNK binds in the nuclei to the transcription factors c-Jun and ATF2, the sequestration of JNK by JIP-1 in the cytoplasm inhibits the JNK signaling pathway. One may speculate that the insertion of the 47 amino acids in the JIP-1 protein, which creates a HLH and a PID domain, will allow protein-protein interactions, possibly with other members of the tyrosine kinase signaling pathway or with transcription factors.

By computer analysis using the SOPMA algorithm (Self Optimized Prediction Method of Alignments, CNRS, Lyon, France), two acidic helicoidal structures (aa 31–61 and 114–125) and a proline-rich region (aa 292–366) in the amino-terminal part of IB1 were also predicted, which could act as transactivation domains (29). Putative nuclear localization signals were also localized at aa 163–190 and 242–270 (30).

IB1 Is Expressed in Pancreatic Insulin-secreting Cells—IB1, as JIP-1, was highly expressed in the brain and, to a lower extent, in the kidney (17, 18). In addition, IB1 was also abundantly expressed in several insulin-secreting cell lines (INS-1, RIN5F) as well as in freshly isolated rat pancreatic islets, but not in the liver or in RNA prepared from whole pancreas, since the pancreatic islets represent a small proportion of the organ (Fig. 2, A and B). In pancreatic islets, IB1 expression was not regulated by increasing the glucose concentration in the incubation medium from 2.8 to 30 mM (Fig. 2B). Affinity-purified antibodies detected a 120-kDa protein in nuclear extracts prepared from βTC3 cells and in crude cellular extracts prepared from freshly isolated pancreatic islets (Fig. 2, C and D). This 120-kDa protein comigrated with the product obtained by in vitro transcription-translation of the IB1 cDNA in the presence of [35S]methionine (data not shown). We could also detect the IB1 protein in both nuclear and cytoplasmic extracts obtained from COS-7 cells transiently transfected with the CMV-driven IB1 cDNA (Fig. 2E).

To gain further insight into the tissue and cellular localization of IB1 within the pancreas, immunohistochemistry studies were performed on mouse islets and βTC3 cells. Affinity-purified antibodies raised against IB1 detected this factor in the pancreas islet as well as in the nuclei and the cytoplasm of βTC3 cells (Fig. 3, A and C). To confirm the specificity of the anti-IB1 antibodies in immunocytochemistry, a construct was generated that includes a Flag epitope located NH₂-terminal to the IB1 protein expressed under the control of a CMV promoter. This construct was transiently transfected into COS-7 cells, and the translated product was immunodetected with an
anti-Flag antibody subsequently visualized by fluorescein isothiocyanate staining (Fig. 3E) or with the anti-IB1 antibody detected using an anti-rabbit Texas Red-labeled antibody (Fig. 3F). The IB1 protein, in transfected COS-7 cells, was detected with both the anti-Flag and the anti-IB1 antibodies in the cytoplasm and the nuclei of COS-7 cells.

The IB1 Protein Binds through Its Carboxyl-terminal Domain to GTII—The IB1 cDNA was cloned 3' to a CMV promoter and transiently transfected into COS-7, a cell line that does not express endogenous IB1. Crude cellular extracts prepared from these transfected cells were then analyzed by the SouthWestern technique using the GTII probe. A 120-kDa GTII-binding protein was detected by SouthWestern (Fig. 4), and this size product was similar to the one obtained by Western blotting with the α-IB1 antibody. E, a plasmid containing the IB1 cDNA driven by a CMV promoter or its parent vector was transiently transfected into COS-7 cells and cytoplasmic (CE) or nuclear (NE) extracts prepared 48 h after transfection. By Western blot analysis, IB1 is detected in the cytoplasm and the nucleus of the transfected cells. 

Interference with endogenous IB1 protein interacting with GTII and/or heterodimerization of IB1 with related factors, aa 1–268 and 1–714 of IB1 were fused with the GAL4 DNA-binding domain. The GTII-binding sites of the reporter gene include only the amino-terminal part (aa 1–280) or the COOH-terminal part (280–714) of the protein, and bacterially produced recombinant IB1 proteins were obtained from these plasmids. The 280–714-aa protein, but not the 1–280-aa protein, was able to bind to the GTII cis sequence when tested by SouthWestern analysis implying that the carboxyl end of the protein contains the DNA-binding domain of IB1 (data not shown).

Transcriptional Activation by IB1—Transcriptional activation by IB1 was assessed by cotransfection experiments in the insulin-secreting cell line βTC3, an IB1 expression vector (pCMV-IB1), and the proximal region of the GLUT2 promoter (−338 bp) linked to a luciferase reporter gene. Overexpression of IB1 transactivated the GLUT2 promoter 1.6 (±0.1)-fold when compared with a cotransfection with the expression vector lacking the IB1 cDNA (PBKS). This effect was absent with the promoterless reporter construct (pGL3). To avoid possible interference with endogenous IB1 protein interacting with GTII and/or heterodimerization of IB1 with related factors, aa 1–268 and 1–714 of IB1 were fused with the GAL4 DNA-binding domain. The GTII-binding sites of the reporter gene were replaced with GAL4-binding sites linked to a minimal thymidine kinase-luciferase gene. As shown in Fig. 5, the amino-terminal part of IB1 was sufficient to confer transactivating
functions to this heterologous GAL4 system (9.0 ± 0.4 and 7.5 ± 1.2 versus control with the 1–268 and 1–714 GAL4 constructs, respectively.

In summary, we describe herein the expression cloning, using a cis-regulatory element of the GLUT2 promoter, of a protein preferentially expressed in the brain and the insulin-secreting cells named IB1 (17). The rat IB1 amino acid sequence is 97% identical to the recently described murine JIP-1 cytoplasmic protein (18). JIP-1 and IB1 differ mainly by sequence is 97% identical to the recently described murine JIP-1 cytoplasmic protein (18). JIP-1 and IB1 differ mainly by

Further work will be needed to identify other potential partners of the IB1/JIP-1 proteins, either other transcriptional factors or other members of the JNK signaling pathway. It remains to be elucidated whether IB1/JIP-1 plays a differential functional role in the insulin-secreting cells, in particular in the control of glucose-induced insulin secretion.

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