Cloning from Insulinoma Cells of Synapsin I Associated with Insulin Secretory Granules*

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Synapsin I is a synaptic vesicle-associated protein involved in neurotransmitter release. The functions of this protein are apparently regulated by Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaM kinase II). We reported evidence for CaM kinase II and a synapsin I-like protein present in mouse insulinoma MIN6 cells (Matsumoto, K., Fukunaga, K., Miyazaki, J., Shichiri, M., and Miyamoto, E. (1995) Endocrinology 136, 3784–3793). Phosphorylation of the synapsin I-like protein in these cells correlated with the activation of CaM kinase II and insulin secretion. In the present study, we screened the MIN6 cDNA library with the full-length cDNA probe of rat brain synapsin Ia and obtained seven positive clones; the largest one was then sequenced. The largest open reading frame deduced from the cDNA sequence of 3695 base pairs encoded a polypeptide of 670 amino acids, which exhibited significant sequence similarity to rat synapsin Ib. The cDNA contained the same sequence as the first exon of the mouse synapsin I gene. These results indicate that synapsin Ib is present in MIN6 cells. Synapsin I was expressed in normal rat islets, as determined by reverse transcriptase-polymerase chain reaction analysis. Immunoblot analysis after subcellular fractionation of MIN6 cells demonstrated that synapsin Ib and \(\delta\) subunit of CaM kinase II co-localized with insulin secretory granules. By analogy concerning regulation of neurotransmitter release, our results suggest that phosphorylation of synapsin I by CaM kinase II may induce the release of insulin from islet cells.

Intracellular Ca\(^{2+}\) has been reported to play a critical role in insulin secretion. Glucose seems to elevate intracellular Ca\(^{2+}\) concentrations via influx through voltage-dependent Ca\(^{2+}\) channels. The Ca\(^{2+}\) influx primarily occurs as a result of the membrane depolarization secondary to closure of ATP-sensitive K\(^+\) channels (1–3). After elevation of intracellular Ca\(^{2+}\) levels, effector molecules are activated; these include calmodulin (CaM)\(^3\) and protein kinase C. CaM is a ubiquitous Ca\(^{2+}\)/calmodulin-dependent protein kinase; RT, reverse transcriptase; PCR, polymerase chain reaction; NRSF, neuron restrictive silencer factor; REST, RE-1-silencing transcription factor; MES, 2-(N-morpholino)ethanesulfonic acid.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\(^{TM}\) / EBI Data Bank with accession number(s) AF085809.

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\(^*\) The abbreviations used are: CaM, calmodulin; CaM kinase, Ca\(^{2+}\)/calmodulin-dependent protein kinase; RT, reverse transcriptase; PCR, polymerase chain reaction; NRSF, neuron restrictive silencer factor; REST, RE-1-silencing transcription factor; MES, 2-(N-morpholino)ethanesulfonic acid.
the inhibition of the interaction between synaptic vesicles and plasma membranes and thereby induces concomitant changes in Ca^{2+}-dependent neurotransmitter release.

We reported elsewhere the existence of CaM kinase II and its endogenous substrate, an 84-kDa protein in mouse insulinoma MIN6 cells (13). As the 84-kDa protein had several biochemical and immunochemical properties in common with synapsin I, we tentatively termed it a "synapsin I-like protein." To clarify whether or not the synapsin I-like protein is a new isoform of synapsin I expressed in endocrine cells, we isolated and sequenced the cDNA clones that hybridized to rat brain synapsin I cDNAs from a cDNA library of MIN6 cells. Sucrose density gradient fractionation of MIN6 cells revealed that the protein and δ subunit of CaM kinase II (CaM kinase II δ) co-localizes with insulin secretory granules. We also found that the protein is present in other endocrine cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following chemicals and reagents were obtained from the indicated sources: Dulbecco's modified Eagle's medium, Nissui Pharmaceutical Co. (Tokyo, Japan); RPMI 1640 medium, Life Technologies, (Gaithersburg, MD); fetal bovine serum, JRH Biosciences (Lenexa, KS); [α-32P]dCTP, Amersham Pharmacia Biotech (Tokyo, Japan); [γ-32P]ATP and [32P]i-protein A, NEN Life Science Products; bovine serum albumin, the antibody against synaptophysin (monoclonal), and mouse IgG (polyclonal), Sigma; (γ-amidinophenyl)methanesulfonyl fluoride hydrochloride, Wako Pure Chemical Industries (Osaka, Japan); MES, Dejindo Laboratories (Kumamoto, Japan); Phadeseph Insulin, Kabi Pharmacia Diagnostics (Uppsala, Sweden). Polyclonal antibody against carboxyl-terminal peptide derived from the subunit of CaM kinase II (CaM kinase II δ) co-localizes with insulin secretory granules. We also found that the protein is present in other endocrine cells.

**Cell Culture**—MIN6 cells, αTC-6 cells, and AtT-20 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 25 mM glucose, 50 IU of penicillin/ml, 50 μg of streptomycin/ml, 5 μM 2-mercaptoethanol, and 15% heat-inactivated fetal bovine serum, as described (20–22). Hamster insulinoma In-R1-G9 cells and HIT-T15 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU of penicillin/ml, and 100 μg of streptomycin/ml (23). Bovine adrenal chromaffin cells were kindly provided by Dr. N. Yanagihara (19).

**Northern blot analysis**—Total RNA was prepared from rat brain, mouse brain (C57BL/6), and MIN6 cells as described (24). Rat synapsin I and II cDNAs were kindly provided by Dr. K. Araki (Purdue University). For RNA transfer blot, 5, 5, and 20 μg of total RNAs from rat and mouse brains and MIN6 cells, respectively, were denatured with formaldehyde, electrophoresed on a 1% agarose gel, and transferred to a nylon membrane. Following overnight hybridization, the membranes were washed once in each of the following solutions prior to x-ray film.

**Isolation of cDNA Clones**—About 1.1 × 10^6 independent XZip phage clones of the mouse insulinoma MIN6 cDNA library, kindly provided by Dr. H. Ishihara (University of Tokyo) (25), were screened with the full-length fragment of the cDNA-encoding rat brain synapsin I as a probe. The probe was labeled with [α-32P]dCTP to a specific activity of 2.9 × 10^9 cpm/μg with the Random Primer Kit (Takara Biomedicals), as described by the manufacturer. The plaque hybridization assay was performed in 0.9 M NaCl, 50 mM NaH2PO4, 5 mM EDTA (pH 7.7), 0.1% bovine serum albumin, 0.1% Ficol 400, 0.1% polyvinylpyrrolidone, and 0.1% SDS in the presence of 100 μl of denatured herring DNA at 65 °C. The final wash of membranes was carried out in 0.15 M NaCl, 15 mM sodium citrate, and 0.1% SDS at 60 °C for 60 min. The plaque of the phagemid containing cDNA inserts were recovered from purified phage clones following the procedures outlined in the Rapid Excision Kit (Stratagene, La Jolla, CA).

**DNA analyses**—The nucleotide sequence was determined using the DyeDeoxy Terminator Cycle Sequencing Kit or PHRIS Sequenase® Terminator Double-Stranded DNA Sequencing Kit and model 373S DNA sequencer (Applied Biosystems Japan, Chiba, Japan).

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analyses**—Islets were isolated by collagenase digestion from the pancreas of Wistar rats, as described (26). Although the preparation of islets would contain non-beta cells, they would be less than 10–20% of all the islet cell population, as described (27, 28). Therefore, more than 80% would be beta cells. Total cellular RNA was extracted from isolated islets using a TriZol Total RNA Isolation Kit (Life Technologies, Inc.), according to the manufacturer's protocol. The total RNA was reverse-transcribed using an oligo(dT) primer (Promega) and Moloney murine leukemia virus transcriptase (Life Technologies, Inc.). Primers used for the cDNA synthesis were designed to span alternatively spliced fragments thereby allowing size discrimination between synapsin Ia and Ib. Primers used to amplify synapsin I cDNA were 5′-AGGCTACCGT-CAGGCATCTACTC-3′ and 5′-TCACCTCATTCTCGTAAAGG-3′ (nucleotides 1765–1783 and 2192–2199, a 350-base pair fragment of synapsin Ia or nucleotides 1759–1783 and 2082–2101, a 342-base pair fragment of synapsin Ia (29). PCR amplification consisted of a 5-min start at 95 °C, followed by 35 cycles at 95 °C for 45 s, 65 °C for 45 s, 72 °C for 2 min, and a final cycle at 72 °C for 5 min. For sequencing of PCR products, each PCR fragment was purified by agarose gel electrophoresis and ligated into the pCR® II cloning vector following procedures outlined in the original TA Cloning Kit (Invitrogen, Carlsbad, CA).

**Equilibrium Sucrose Density Gradients**—MIN6 cells grown in ten 100-mm dishes to 90% confluency were harvested by trypsinization, pelleted by centrifugation, resuspended in 5 volumes of the homogenizing buffer (0.25 M sucrose, 10 mM EDTA, 10 mM MES (pH 6.5), 1 mM (p-amidinophenyl)methanesulfonyl fluoride, 0.1 mM leupeptin, 75 μM pepstatin A, and 0.1 mg of aprotinin/ml), and homogenized in a Teflon glass homogenizer. The homogenate was centrifuged at 60,000 × g at 4 °C for 10 min, and the resulting supernatant was loaded onto the top of a continuous sucrose density gradient (0.5–2 M sucrose, 10 mM EDTA, and 10 mM MES, pH 6.5) and then centrifuged at 100,000 × g at 4 °C for 6 h. The resulting pellets were isolated using the insulin assay kit (Phadeseph Insulin).

**Preparation of the Cytosol and Particulate Fractions from Various Cultured Cells and Freshly Obtained Bovine Adrenal Medulla**—The cells and tissue of bovine adrenal medulla were homogenized with 5 volumes of 20 mM Tris-HCl (pH 7.5), 4 mM EGTA, 1 mM dithiothreitol, 0.1 mM leupeptin, 75 μM pepstatin A, and 0.1 μg of aprotinin/ml. The homogenate was centrifuged at 16,350 × g for 20 min at 4 °C using a desk top centrifuge. The resulting supernatant was used as the crude cytosol fraction. The pellet was homogenized with 5 volumes of 100 mM HEPES (pH 7.5), 0.1% Triton X-100 (vv), 4 mM EGTA, 1 mM dithiothreitol, 0.1 mM leupeptin, 75 μM pepstatin A, and 0.1 mg of aprotinin/ml. The homogenate was centrifuged at 16,350 × g for 20 min at 4 °C using a desk top centrifuge. The resulting supernatant served as the crude particulate fraction.

**RESULTS**

**Isolation and Characterization of Synapsin I cDNA from MIN6 Cells**—In foregoing work, we identified the synapsin I-like protein in MIN6 cells (13). If the protein is an isoform of synapsin I expressed in insulinoma cells, cDNA clones corresponding to full-length nucleotides of synapsin Ia or Ib could hybridize to the mRNA of the protein. In fact, we noted the expression of 3.7-kilobase mRNA in MIN6 cells by Northern blot analysis with either cDNA probe (Fig. 1). No other mRNA bands were detected, even under conditions of low stringency (data not shown). Because the synapsin I-like protein is the major protein that immunoreacted with the anti-synapsin I antibody (13), it seems possible that the synapsin I-like protein originated from the 3.7-kilobase mRNA. To clarify this point, we cloned the cDNA that hybridized with rat synapsin I cDNA.

Approximately 1.1 × 10^5 independent XZip phage clones from a cDNA library of MIN6 cells were screened with the 32P-labeled synapsin Ia cDNA probe, and seven positive clones...
were obtained. The cDNA inserts from these clones were further characterized by restriction mapping analysis and partial sequence determination of their 5′-ends. One of these clones, designated sim5, contained a full-length open reading frame and was further analyzed (Figs. 2 and 3). The largest open reading frame deduced from the 3695-base pair sequence of the sim5 cDNA encoded a polypeptide of 670 amino acids, which exhibited a significant sequence similarity to rat synapsin Ib (97.8% identity). All seven serine residues phosphorylated in sim2 mouse sequence (Fig. 2). Furthermore, sim5 contained a nucleotide sequence identical to the first exon of mouse brain synapsin I gene (33) (Fig. 3A). Thus, we concluded that sim5 originated from the gene that encodes mouse brain synapsin I. Eleven amino acids were different from rat brain synapsin Ib, and three additional amino acids (Gln421, Pro422, and Thr 601) were generated (29). These two isoforms differed in their carboxyl termini, and this alternative splicing mechanism was reported to be conserved well among human, rat, and bovine cells (17). In addition to the synapsin I-like protein, a autoradiograph of the immunoblot analysis showed another faint band at position ~88 kDa on SDS-polyacrylamide gel electrophoresis after long exposure to the x-ray film (data not shown). Therefore, we investigated the possibility that synapsin Ia might be present in MIN6 cells. Using primers spanning the splice acceptor site on the primary RNA transcript, we found that one clone designated sim2 had 38 nucleotides that were not present in rat brain synapsin Ib mRNA (Fig. 3B). The 38 nucleotides were the same as those expressed in rat brain synapsin Ia. sim2 did not contain the complete open reading frame of synapsin Ia, yet when the 38 nucleotides were inserted into the sim5 sequence and transferred to amino acids, the sequence homology between rat brain synapsin Ia and the putative full-length sim2 transcript was 94.2% (data not shown). These results strongly suggest that the mRNA of mouse synapsin Ia is also expressed in MIN6 cells.

Expression of Synapsin I mRNA in Rat Isolated Islets—To determine whether rat pancreatic islets express synapsin Ia and/or Ib, we prepared total RNA from rat islets. When the total RNA from MIN6 cells and rat islets was subjected to RT-PCR, two amplified products were obtained (Fig. 4). When PCR amplification was performed without reverse transcriptase reaction, these two bands were not detected. When genomic DNA from rat liver was used as a template, an approximately 1330-base pair band was amplified. All three products hybridized with the synapsin Ib cDNA probe (data not shown). These observations mean that the former two products were not amplified from genomic DNA. When we subcloned the two products for sequencing, we confirmed that they were the predicted fragments of synapsin Ia and Ib (data not shown).

Co-localization of Synapsin I, CaM Kinase II δ, and Secretory Granules in MIN6 Cells—In neuronal cells, peptide and nonpeptide neurotransmitters are packed in large density core vesicles and synaptic vesicles, respectively, and synapsin I was reported to be associated with synaptic vesicles rather than large density core vesicles (34). Large density core vesicles were considered to be counterparts of the secretory granules in endocrine cells. In addition to insulin secretory granules, islet beta cells were seen to have synapsin vesicle-like microvesicles, which contain γ-aminobutyric acid and glutamic acid decarboxylase (35). From these data, we considered the possibility that synapsin I in MIN6 cells is associated with microvesicles but not with insulin secretory granules. We then carried out equilibrium sedimentation through a linear 0.5–2.0 m sucrose gradient using the MIN6 cell extracts (Fig. 5). Individual fractions were collected, and immunoblot analysis was done with antibodies against synapsin I, synaptophysin, chromogranin A, and CaM kinase II δ. The insulin secretory granule fraction was identified based on measurements of immunoreactive insulin. Fig. 5 summarizes the results of one of four independent experiments. Synapsin I was present in relatively dense fractions, and synaptophysin, a marker for endosomes and small density vesicles, was mainly observed in distinct membrane fractions from synapsin I. A minor peak of synaptophysin was also observed in the insulin secretory granule fraction. Moreover, most of the immunoreactive insulin (Fig. 5B) and the immunoreactivity of chromogranin A, a marker for large secretory granules (36) (data not shown) and CaM kinase II δ, were detected in the same fractions as synapsin I. Thus, synapsin I was co-fractionated with insulin secretory granules in MIN6 cells. Co-localization of synapsin I with insulin secretory granules was observed in an independent beta cell line, HIT-T15 cells (data not shown).

Expression of Synapsin I in Endocrine Cells—We then asked if synapsin I occurs in other endocrine cells. Fig. 6 shows immunoblot analysis of synapsin I in various rodent endocrine cell lines, bovine adrenal chromaffin cells, and adrenal medulla. One immunoreactive protein band migrating at the same position as rat brain synapsin Ib was observed mainly in the particulate fractions from αTC cells and AtT-20 cells. More than four proteins were detected in both cytosol and particulate fractions from In-R1-G9 cells; one migrated at the same position as synapsin Ib, and properties of other immunoreactive proteins were not clear. Protein bands of lower molecular masses were considered to be degraded products. In bovine adrenal chromaffin cells, the two immunoreactive bands observed around the position of synapsin Ib were exclusively present in the particulate fraction. Two immunoreactive bands were also observed in the particulate fraction when freshly isolated bovine adrenal medulla was examined by immunoblot analysis (Fig. 6B). Synapsin I was observed in the cytosol fractions of αTC cells, In-R1-G9 cells, AtT-20 cells, and bovine adrenal medulla. Although small particles are not included in the crude cytosol fraction by the preparation method, the hypertonic conditions would result in the inclusion of the loosely
bound proteins of the particulate fraction in the cytosol fraction.

DISCUSSION

When we reported evidence for the existence of synapsin I-like protein in MIN6 cells, the unsolved question was whether or not the protein was a new isoform of synapsin I (13). Although synapsin I was not detected in any significant amount in non-neuronal cells by immunoblots, immunohistochemical, and Northern blot analyses and was considered to be neuron-specific (37, 38), the antibody we used revealed a significant amount of the protein in MIN6 cells. In previous work, the electrophoretic mobility of the protein by SDS-polyacrylamide gel electrophoresis differed from either synapsin Ia or Ib purified from rat brain. These findings raised the possibility that a new isoform of synapsin I was present in MIN6 cells. In attempts at clarification we isolated cDNA clones of the protein from a cDNA library of MIN6 cells. Sequence analyses of the cDNA clones revealed the expression of synapsin I. Compared with rat brain synapsin Ib, 3 additional amino acids (Gln421, Ser422, Gln423) were found in the cDNA clones. These findings suggest that a new isoform of synapsin I is present in MIN6 cells.

Fig. 3. Analyses of clones of synapsin I obtained from MIN6 cDNA library. A, comparison of the nucleotide sequences of sim5 with those of the first exon from mouse synapsin I (m.synI) (31). B, comparison of the nucleotide sequence of sim2 with those of sim5, rat synapsin Ia (Syn Ia), and rat synapsin Ib (Syn Ib) (29). The sim2 clone contains 38-base nucleotides deleted by alternative splicing in mRNA of rat synapsin Ib.
carried out partial sequencing of other clones around the position indicated that synapsin Ia may be expressed in MIN6 cells. We diograph was subjected to long exposure to x-ray film. This slightly higher position than the major band when the autoradiograph was subjected to long exposure to x-ray film. This indicated that synapsin Ia may be expressed in MIN6 cells. Another faint band was detected at a higher position than did rat synapsin Ib. Immunoblot analysis of MIN6 cells showed one major band of synapsin I, which was considered to be synapsin Ib, based on the sequence analysis. Another faint band was detected at a slightly higher position than the major band when the autoradiograph was subjected to long exposure to x-ray film. This indicated that synapsin Ia may be expressed in MIN6 cells. We carried out partial sequencing of other clones around the possible splicing site and found that one clone designated as sim2 had the sequence of the synapsin Ia cDNA. These findings suggested that synapsin Ia is a minor isoform in MIN6 cells. This preferential expression of two isoforms was in accord with findings that they are differentially expressed among synapses (29, 39). Functional differences in the synapsin isoforms remain to be elucidated.

Synapsin I has been considered a neuron-specific phosphoprotein, although trace positive immunoreactivity of synapsin I was noted in some endocrine cells by immunoblot, immunohistochemical, and Northern blot analyses (29, 33). The synapsin I gene promoter contained a sequence motif similar to the neuron restrictive silencer element/repressor element-1 (40, 41), and the neuron-specific expression of synapsin I was reported to be under the control of a neuron-specific silencer element and trans-activating factor (42). A zinc finger protein termed neuron restrictive silencer factor (NRSF)/RE-1-silencing transcription factor (REST) was expressed only in non-neuronal cells that bind to this motif and function as a trans-criptional repressor (43, 44). It was demonstrated that mRNA coding for NRSF/REST is absent in the insulinoma cell line INS-1 and in three other insulin- and glucagon-producing cell lines (45). In these cells, NRSF/REST activity was absent, and transient expression of NRSF/REST was sufficient to silence a reporter gene containing a NRSF/REST binding site. Our data demonstrated the expression of synapsin I in normal rat islets, endocrine cell lines, and bovine adrenal chromaffin cells and thereby provided further support for the NRSF/REST hypothesis. It would be important to show that REST is absent in MIN6 cells.

We demonstrated the expression of synapsin I in isolated islets from rat pancreas by RT-PCR with RNA from islets (Fig. 4). Because sympathetic and parasympathetic neurons innervate pancreatic islets, there may be contamination of nerve endings in the fraction of isolated islets. However, this is not likely because synapsin I is translated from its mRNA in the cell body and is transported to nerve endings.

In neuronal cells, two types of vesicles have been described; one is synaptic vesicles that contain non-peptide neurotransmitters, and the other is large dense core vesicles that contain peptide neurotransmitters. In neuroendocrine and endocrine cells, secretory granules and small synaptic-like vesicles are thought to be counterparts of large dense core vesicles and synaptic vesicles, respectively. In addition to secretory granules, pancreatic beta cells have small synaptic-like vesicles that contain y-aminobutyric acid (35). Although synapsin I has been reported to be associated only with synaptic vesicles or synaptic-like microvesicles in rat and bovine neurohypophysis (46), we found that synapsin I was not present in fractions that contained synaptic-like microvesicles, as determined by immunoblot analysis using the anti-synaptophysin antibody but was

![Image](https://via.placeholder.com/150)
associated with secretory granules. In agreement with our observation, synaptic-like microvesicles in pinealocytes were not found to interact with synapsin I, as demonstrated immunohistochemically (47). In endocrine and neuroendocrine cells, synapsin I can be absent on synaptic-like microvesicles. In this context, it is important that ribbon synapses in cone, rod, and bipolar cells of the retina with synaptic-like microvesicles do not contain synapsin I (48).

One of the functional roles of synapsin I in neuronal cells was reported to be the regulation of neurotransmitter release (17). Greengard et al. (16) hypothesized that synapsin I forms the link between the vesicle membrane and a primarily actin-based cytoskeleton, thus preventing the vesicles from moving to the presynaptic plasma membrane. The increase in cytosolic Ca$^{2+}$ levels causes phosphorylation of synapsin I by CaM kinase II, which results in the release of vesicles from the cytoskeletal network and transfer of the vesicles from a resting to an active pool. In pancreatic beta cells, the actin network beneath the plasma membrane may prevent the interaction between secretory granules and plasma membranes (49). By analogy with neuronal granules, synapsin I in beta cells may possibly function as a linker of insulin granules and the cytoskeletal network.

CaM kinases were reported to be present in pancreatic beta cells, including CaM kinase II (8), CaM kinase III (7), and myosin light chain kinase (5). Among these, only CaM kinase II was reported to be present in particulate fractions, which contained insulin secretory granules (7). CaM kinase activity and endogenous substrates have been shown to be present in insulin secretory granules from toadfish (50). Furthermore, in other insulinoma INS-1 cells, the CaM kinase II $\delta$ was seen to be associated with insulin secretory granules (51). We also confirmed the association of CaM kinase II $\delta$ with insulin secretory granules in MIN6 cells by sucrose gradient subcellular fractionation and immunoblot analysis with an antibody specific to CaM kinase II $\delta$ (Fig. 5). Taken together, it is plausible that CaM kinase II is present on the surface of insulin secretory granules. In the present work, we observed that synapsin I co-localizes with secretory granules. In the brain, synapsin I has been reported to be complexed with CaM kinase II $\alpha$ on the synaptic vesicle surface (52). We suggest that synapsin I may be associated with CaM kinase II $\delta$ on insulin secretory granules.

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