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Insulinoma Cells Contain an Isoform of Ca^{2+}/Calmodulin-Dependent Protein Kinase II δ Associated with Insulin Secretion Vesicles*

M. MÖHLIG, S. WOLTER†, P. MAYER, J. LANG, M. OSTERHOFF, P. A. HORN†‡, H. SCHATZ, AND A. PFEIFFER

Department of Internal Medicine (M.M., S.W., P.M., M.O., P.A.H., H.S., A.P.), BG Kliniken Bergmannsheil, University of Bochum, Medical School, D-44789 Bochum, Germany; Division of Clinical Biochemistry (J.L.), University of Geneva, Medical School, CH-1211 Geneva, Switzerland

ABSTRACT

The Ca^{2+}/calmodulin dependent protein kinase II (CaM kinase II) is thought to play an important part in glucose-stimulated insulin secretion. To determine which of the known subtypes (α, β, γ, δ) occur in insulin-secreting cells, we amplified all types of CaM kinase II by RT-PCR and found the β_3-, γ-, δ_2- and δ_6-subtypes in RINm5F insulinoma cells. None of the other 8 δ-subtypes was present. Antibodies generated against the bacterially expressed association domain of the δ_2-subtype recognized the recombinant γ and δ-subtypes. In INS-1 and RINm5F cells, as well as freshly isolated rat islets, only a 55-kDa protein corresponding in size to the δ_2-subtype expressed in NIH3T3 fibroblasts was detected. The δ_2-subtype therefore appears to represent the predominant subtype of CaM kinase II present in insulin secreting cells. The enzyme was primarily associated with cytoskeletal structures, and very little was present in the soluble compartment or detergent soluble fraction in INS-1- or RINm5F-cells. An analysis of its subcellular distribution was performed by sucrose and Nycodenz density gradient fractionation of INS-1 cells and detection of CaM kinase II δ by immune blots. The enzyme codistributed with insulin used as a marker for secretory granules but not with the lighter synaptic-like microvesicles detected with an antibody against synaptophysin, plasma membranes (syntaxin 1), lysosomes (arylsulfatase), or mitochondria (cytochrome *c* oxidase).

CaM kinase II δ_2 thus is identified as the subtype associated with insulin secretory granules and is likely to be involved in insulin secretion. (*Endocrinology* **138:** 2577–2584, 1997)

THE EXOCYTOSIS of insulin in β-cells is closely related to intracellular elevations of Ca^{2+} (1, 2). Ca^{2+} dependent events are thought to be involved in the final steps of insulin granule maturation that involves movement towards and fusion with the plasma membrane. One component of these events appears to be Ca^{2+}/calmodulin dependent protein kinase II (CaM kinase II) as suggested by a strong inhibition of insulin exocytosis by a peptide inhibitor of CaM kinase II (3) and by specific CaM kinase II antagonists (4). Moreover, glucose stimulation of islets caused an autophosphorylation of CaM kinase II, suggesting that an activation of this enzyme is involved in insulin secretion (5). In the toadfish endocrine pancreas, a CaM kinase II was demonstrated on insulin secretory granules (6). CaM kinase II was shown to phosphorylate proteins present in insulin secretory vesicles (7–9). Because on one hand CaM kinase II appears to be involved in insulin secretion and on the other hand different subtypes have been described recently, it should be of interest to determine the subtypes present in insulin secreting cells. The subtypes might differ in their subcellular localization and as a consequence their function may be altered particularly with regard to insulin secretion.

The multifunctional CaM kinase II is present in most cell types and is an important mediator of Ca^{2+}-dependent signal transduction (10–12). Four isoenzymes have been cloned and characterized named α, β, γ and δ (10, 13–16). These isoforms are highly conserved and have over 90% amino acid sequence homology in the N-terminal kinase domain and in the regulatory domain comprising the Ca^{2+}/calmodulin binding sequence that is located in the mid of the molecule (12).

The association domain comprises the C-terminal 2/5 of CaM kinase II and is less conserved among the different isoenzymes. In fact, for each isoform 2–10 splice variants have been described that appear to be important for the subcellular localization of the enzyme as indicated by the work of Srinivasan and colleagues (17–24).

In the present report, we investigated which of the isoenzymes of CaM kinase II occur in insulin secreting cells and define the splice variants of CaM kinase II δ present. We moreover developed an antibody against the bacterially expressed association domain of CaM kinase II δ and characterized protein expression of the δ isoforms as well as its subcellular distribution.

Materials and Methods

Male Wistar rats weighing 200–350 g were obtained from a local breeding company. Chemicals were from Merck (Darmstadt, Germany) or Boehringer (Ingelheim, Germany) and were of analytical or higher grade except when noted otherwise. Calmodulin was from Pharmacia/LKB (Bromma, Sweden). [α^{35}S]dATP and [γ^{32}P]ATP were from Du Pont

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Address all correspondence and requests for reprints to: Prof. Dr. Andreas Pfeiffer, Medizinische Klinik und Poliklinik, Berufsgenossenschafliche Kliniken Bergmannsheil, Universitätsklinik, Bürkle de la Camp-Platz 1, D-44789 Bochum, Germany. E-mail: Andreas.Pfeiffer@rz.ruhr-uni-bochum.de

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TABLE 1. PCR protocols

	Primer pair 1	Primer pair 2	Primer pair 3
Left primer	5′-CAGCAGGCGTGGTTTGGTTT-3′	5′-CAGCAGGCGTGGTTTGGTTT-3′	5′-GGAGGAGGAGGAAGAAGGT-3′
bp-position	502	502	−200
Right primer	5′-TGTGTCCCATTCTGGTGATG-3′	5′-GAGGCCGCGGTGGACATTGT-3′	5′-CACCAGCAAGATGTAGAGCA-3′
bp-position	720	992	627
$MgCl_2$-concentration	3.8 mM	3.8 M	1.8 M
Annealing temperature	60 C	60 C	57 C
Length of amplificate	219 bp subtypes α, γ, δ	491 bp subtypes β_1, β_2	827 bp subtype δ
5′-elongation of left primer			
right primer			

de Nemours (Dreieich, Germany). INS-1 cells (25) were kindly provided by C. Wollheim (Geneva, Switzerland). Human CaM kinase γ_B complementary DNA (cDNA) was a gift from H. Schulman (Stanford, CA).

Enzyme preparation

Rats were anesthetized with diethyl ether and killed by cervical dislocation. RINm5F and INS-1 cells where cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD) containing additions as described (25, 26). Tissues or cells were homogenized in ice-cold homogenization buffer using a Polytron. The buffer consisted of 50 mM Tris/HCl pH 7.4, 2 mM EDTA, 10 mM EGTA, 1 mM phenyl methyl sulfonyl fluoride (PMSF) added freshly, 1 mM dithiothreitol, 100 μM leupeptin, 0.3 μM aprotinin.

Assay of kinase activity

CaM kinase II assays were performed using autocamtide II (KKALRRQETVDAL) as substrate (27) in 50 μl volume containing 10 μl enzyme preparation (1–5 μg protein), 50 mM Tris, pH 7.5, 20 μM ATP, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$ in excess of EGTA, 1 μM calmodulin, 1 mg/ml BSA, 0.2–2 μCi [γ^{32}P]ATP/assay and 1–40 μM autocamtide II. Controls contained calcium but no calmodulin. By determining background in the presence of Ca^{2+}, only calmodulin-dependent activity was assayed and interference by Ca^{2+}-activated calmodulin-independent kinases was eliminated. The assay was started by addition of either enzyme preparation or [γ^{32}P]ATP. The reaction was linear over 5 min at 21 C. After 3 min, the reaction was stopped with 25 μl 20% TCA, and an aliquot was applied to Whatman P81 phosphocellulose paper that was washed according to Roskowski (28) and dried before determination of radioactivity by Cerenkov-counting in a Beckmann LS 6000 counter.

RT-PCR

Total RNA was purified from RINm5F-cells by the method described by Chomczynski and Sacchi (29), and about 1 μg was used for reverse transcription using M-MLV reverse transcriptase (200 U, Life Technologies) and random hexamer primers (Boehringer).

PCR was performed with *Taq*-DNA-polymerase (Cetus or Boehringer) over 30 cycles either with two primer pairs specific for the β-subtypes (pair 2 and 6) or with a primer pair allowing amplification of the α-, γ- and δ-subtype (pair 1).

For cloning of the complete cDNA sequence of the subtype δ, three overlapping PCR-amplificates (pairs 3–5) were restricted in the overlapping region by Acy I or *Sst*I and religated with T4-DNA-ligase. The result was controlled by sequencing. To facilitate cloning and to obtain more positive clones, the CloneAmp-Kit (Life Technologies) was used for primer-pair 5. After PCR, the samples were separated on a 1.5% agarose gel.

Primer sequences, annealing temperatures, and final Mg^{2+}-concentrations are shown in Table 1.

PCR products of primer pairs 3 and 4 were subcloned into pBluescript 2 KS (Stratagene, Heidelberg, Germany), and the amplificate of primer pair 5 was subcloned into pAmp 1 (Life Technologies). Transformation followed the method of Chung (30).

Sequencing

Sequencing was performed after subcloning by the didesoxynucleotide-chain-termination-method described by Sanger (31) using Sequenase 2.0 (USB) and [α^{35}S]dATP. Both strands were sequenced.

Restriction analysis of the PCR products

To determine the subtypes present, restriction analysis of amplificate 1 (Table 1) was performed using different endonucleases (Bst XI, *Eco*RV, *Dra*II, *Hae*III, *Mae*II, and *Xho*II). This approach is based on the differences of the sequences of the subtypes α, γ and δ. The expected restriction fragments are shown in Table 2. Before restriction analysis, PCR product 1 was purified by agarose gel-electrophoresis using a glass-matrix (Gene-Clean, Bio 101). Restriction products were separated on polyacrylamide gels. Silver staining was used for detection (see Fig. 2).

A further primer pair 6 (Table 1) was designed for subtype β_3 (32) that amplified a 215-bp sequence as predicted and yielded the correct fragments after restriction with *Alu*I (116/84/15), *Fok*I (120/95) and *Mbo*II (108/107).

Bacterial expression and generation of polyclonal antibodies

To obtain an antigen for antibody production the C-terminal 194 amino acids of CaM kinase II subtype δ_2 [longer amplificate of primer pair 5, nomenclature according to (19)] were cloned into a bacterial expression vector pQE 31 (Quiagen, Hilden, Germany) by restriction with *Bam*HI and *Psp*AI. Using the N-terminally fused hexahistidine tag, the bacterially expressed protein was purified by denaturing chromatography on a metal chelate-affinity column loaded with Ni^+ according to the instructions of the producer. The purified protein was used as immunogen for sc immunization of three rabbits in the presence of complete Freund's adjuvans. Four booster immunizations with incomplete Freund's adjuvans followed. The resulting antiserum recognized 500 fg/lane of the protein used for immunization on Western blots.

To assess the cross-reactivity of the antibodies to the γ-subtype, the cDNA of human CaM kinase II γ_B (20) that is 100% homologous to the rat protein sequence was cloned into the *Bam*HI and *Sac*I sites of pQE 30 (Quiagen) and expressed in bacteria. The antibody recognized the γ_B-isoform in immune blots of bacterial lysates.

Immune blot

Cells or tissues were homogenized in homogenization buffer as described above. Quantification of protein content was performed according to Bradford using the modification of Stoscheck (33). The homogenized protein was denatured for 5 min at 94 C in SDS-sample buffer (0.0625 M Tris-HCL pH 6, 8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0,002% bromphenolblue, 5 M urea) just before SDS-gel-electrophoresis according to the method of Laemmli (34). Molecular weight markers were from Sigma or from biotinylated ECL-mol wt markers from Amersham. Semidry protein transfers onto PVDF-membranes (Immobilon P, Millipore) were done as described by Burnette (35). Transfer efficiency was checked by Ponceau-staining of the membrane. After blocking in standard PBS containing 5% nonfat dry milk (Glueckskiee GmbH, Munich, Germany) and 0,05% Tween-20 the membrane was incubated in a

TABLE 1. Continued

Primer pair 4	Primer pair 5	Primer pair 6
5′-CAGCAGGCGTGGTTTGGTTT-3′	5′-CAACTATGCTGGCTACGAGA-3′	5′-GTCTGCCAACGATCCACGGT-3′
502	917	814
5′-CCATGTACTGTGTGAGCCGA-3′	5′-GACGTGGCACTGTTGACAAT-3′	5′-CTGGTGATGGCCGAGCTGTT-3′
1417	1647	1028
1.8 M	3.8 M	1.8 M
55 C	59 C	61 C
916 bp subtype δ	731 bp subtype δ	215 bp subtype β_3 287 bp subtypes β_1, β_2
	CUACUACUACUA- CAUCAUCAUCAU-	

bp-1 is start of translation.

1:500 dilution of the immune serum in PBS containing 0.5% nonfat dry milk. After two wash steps with standard TBS protein A conjugated to alkaline-phosphatase (Calbiochem) was used for detection. Visualization was done by BCIP/NBT staining (bromchlorindoylphosphate/nitroblue-tetrazolium) according to a standard protocol (36). Specificity of detection was demonstrated by preadsorption of the antibody with the immunogen over 1 h at 4 C.

The detection of cellular marker enzymes by immune blots and the detection CaM kinase II (see Figs. 3b and 5a) was performed with the ECL system from Amersham using streptavidin-HRP for detection as described (37).

Subcellular distribution

RINm5F- or INS-1 cells were homogenized in the buffer described above by ultrasonic waves. One hundred micrograms of the homogenized protein were ultracentrifuged at 100,000 × *g* for 1 h. The cytosolic fraction was the supernatant. The pellet was extracted with 0.5% NP-40 that brings the membrane fraction into the soluble supernatant phase after 100,000 *g* for 1 h centrifugation. The detergent insoluble fraction was pelleted. This fraction contains mostly components of the cytoskeleton. All fractions were chloroform/methanol precipitated and afterwards completely dissolved and heat denatured in SDS-buffer. By this approach the complete fractions were applied onto a SDS-gel, leading to a relative distribution (see Fig. 4).

Sucrose gradients and Nycodenz step gradients

10^8 INS-1 cells were disrupted by nitrogen cavitation and separated on a 0.25–2.0 M sucrose gradient as described recently (37, 38). Synaptophysin was determined by immune blot using the monoclonal SVP 38 antibody (Sigma) and syntaxin 1 using a monoclonal antibody from Sigma (37). The blots were quantified by densitometry with a laser densitometer (Molecular Dynamics, Sunnyvale, CA). The linearity of the signal was as detailed previously (37). Arylsulfatase and cytochrome *c* oxidase were determined as described by Storrie and Madden (39). Four sucrose gradients were analyzed with similar results. In two experiments, Na^+/K^+-ATPase was also determined by immune blots as described (37) and used as a plasma membrane marker with similar results.

Nycodenz (Nyegaard Diagnostica, Norway) step gradients (40) were performed using a detailed protocol kindly provided by B. A. Wolf as described (41). Briefly, 27.6% (wt/vol) Nycodenz stock solution was mixed with homogenization medium (0.3 M sucrose, 10 mM *N*-morpholino-ethanesulfonic acid, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.5) in the ratio 64/36 (A), 32/68 (B), and 16/84 (C). INS-1-cells were homogenized with a tight fitting glass Teflon potter in homogenization medium and layered over the Nycodenz media in a centrifugation tube. After spinning 100,000 × *g* for 60 min in a swing out rotor the interphase between A/B containing the enriched insulin granules and the interphases B/C and C/homogenate (containing enriched plasma membranes) were collected by centrifugation washed by resuspension and centrifugation in 0.25 M sucrose, 10 mM *N*-morpholino-ethanesulfonic acid, pH 6.5, and analyzed by Western blot as described above. Determination of arylsulfatase and cytochrome *c* oxidase (39) as lysosomal and mitochondrial markers showed a 2- to 4-fold reduction in the granule fraction relative to the initial homogenate. Insulin was enriched 8-fold in the granule relative to the homogenate fraction. Insulin was determined by RIA using antibodies against human insulin (Sigma) and [^{125}I]human insulin with rat insulin as standard (Novo, Copenhagen, Denmark) as described (42).

TABLE 2. Restriction pattern of the 219 bp amplification product of primer pair 1 according to the subtypes of CaM kinase II present

Endonuclease	Length of restriction products, bp	Restrictable subtype
*Bst*XI	140/79	β
	140/79	δ
*Eco*RV	132/87	β
	132/87	γ
*Dra*II	156/63	α
	185/34	β
*Hae*III	125/94	α
	120/67/32	β
	125/94	γ
*Mae*II	162/57	δ
*Xho*II	156/63	γ
	156/63	δ

Islet preparation

Islets were prepared by the collagenase method as described (43). Collagenase (0.3 mg/ml, Serva, Heidelberg, Germany) in RPMI 1640 was injected into the ductus pancreaticus of 200 g anesthetized Wistar rats. After digestion and repeated centrifugation islets were hand picked under a stereo microscope. For each experiment 500–800 islets were homogenized and analyzed by immune blot as described above.

Results

To identify the subtypes of CaM kinase II present in RINm5F cells, we first searched for expression of messenger RNAs (mRNAs) of the four isoforms of CaM kinase II α through δ. Three pairs of PCR primers were designed that were expected to amplify all CaM kinase II subtypes. The primer pairs yielded amplificates of the expected length of 491 bp for the β_1- and β_2-specific primer pair 2, 215 bp for the β_3-primer pair 6 and 219 bp for primer pair 1 (Table 1). The β_1/β_2-specific primer pair amplified the expected 491 bp fragment in rat brain mRNA but not in insulinoma cells suggesting the absence of CaM kinase β_1/β_2 (Fig. 1, lanes 1–4). A primer pair designed to detect expression of the β_3-subtype amplified the expected 215 bp sequence in cDNA from RINm5F cells (Fig. 1, lanes 5–7). Digestion with *Alu*I, *Fok*I, and *Mbo*II yielded the expected fragments, confirming that the correct sequence was amplified (data not shown). Primer pair 1 was expected to amplify CaM kinase II subtypes α, γ and δ. Rat brain was used to demonstrate that these CaM kinase II subtypes can be shown by this approach

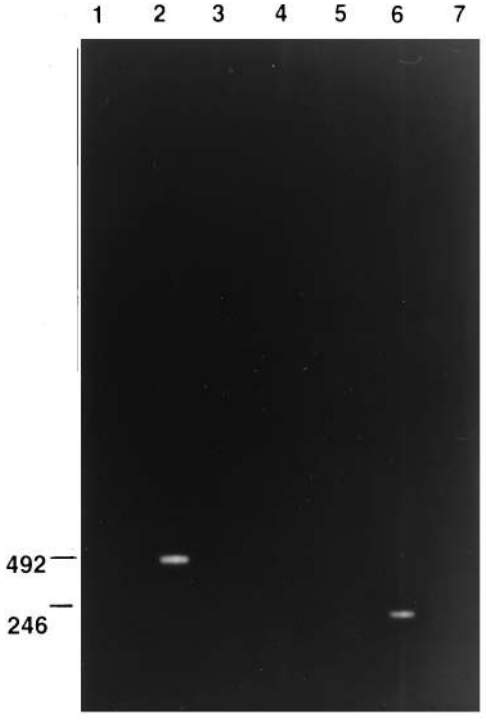

FIG. 1. Detection of CaM kinase β mRNA by RFPCR. The figure shows an ethidium bromide stained agarose gel of a RT-PCR using the CaM kinase II β1/2 -specific primer pair 2 (Table 1) that yielded an amplification product of 491 bp from rat brain cDNA (lane 2). Lane 1 shows a control without RT and lane 3 is a blanc without RNA or DNA. No amplificate was obtained from RINm5F-cell cDNA (lane 4). Lanes 5–7 show a RT-PCR of RINm5F-cell cDNA using the primer pair for β_3 (pair 6). An amplificate of the expected length of 215 bp was obtained (lane 6). Lane 5, Without reverse transcriptase. Lane 7, RT-PCR but no DNA or RNA added.

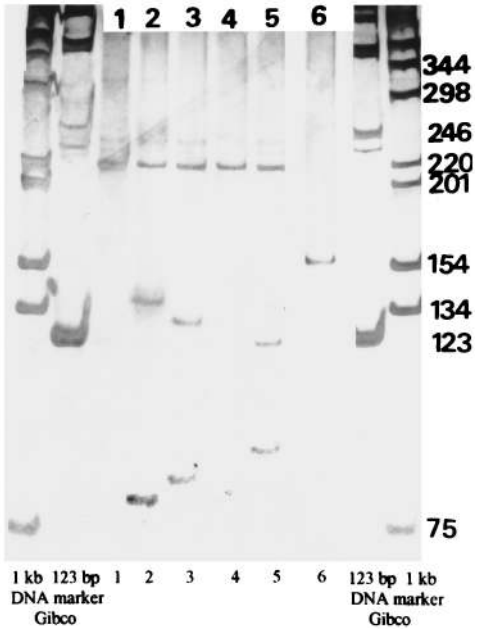

FIG. 2. Restriction analysis of primer pair 1 by silver stained PAGE. Lane 1 shows the unrestricted product followed by digestion with *Bst*XI, *Eco*RV, *Dra*II, *Hae*III, and *Xho*II (lanes 2–6). The length of Life Technologies 1 kb and 123 bp markers are shown to the right. For explanation see *Results* section.

although there were mismatches with the α- and δ-subtypes (44). The subtypes expressed were determined by analyzing the restriction pattern that differs between the subtypes due to minor sequence variations (Table 2). The absence of restriction of the amplification product of primer pair 1 by *Dra*II in insulinoma cells suggested the absence of the CaM kinase II subtype α. The fragment pattern of the amplificate of primer pair 1 obtained with the restriction enzymes *Bst*XI and *Eco*RV and the complete cut of *Xho*II suggested the presence of γ and δ subtypes in insulinoma cells (Fig. 2 and Table 2). The restriction patterns obtained with the remaining enzymes confirmed this result.

A total of 10 splice variants of CaM kinase II δ has been described (18, 19, 23, 45). We now examined which of these splice variants occur in RINm5F-insulinoma cells and which are absent. To this end, the C-terminal region containing both variable domains was amplified by PCR (primer pair 5) and subcloned for detailed analysis by sequencing.

Of the known subtypes of CaM kinase II δ, the mRNAs of only two subtypes were detectable in RINm5F cells: both completely lacked the upper variable domain (bp 985-1086). The C-terminal variable domain (bp 1536–1624) was either present or deleted. Taken together, these results demonstrate the presence of the subtypes δ_2 and δ_6 according to the previously published nomenclature (19).

The demonstration of CaM kinase II δ-mRNA does not necessarily prove the expression of the protein. We therefore raised polyclonal antibodies against the association domain of CaM kinase II δ_2 that was generated by expression of the C-terminal 194 amino acids in bacteria. The resulting antibody recognized a 55-kDa protein in Western blots of RINm5F cell homogenate. The 55-kDa band was completely eliminated by pretreatment of the antiserum with the immunizing protein (Fig. 3a). The protein corresponds in size exactly to CaM kinase II δ_2 expressed in NIH3T3 cells using an ecotropic retroviral expression system (detailed report in preparation). Subtypes δ_6 and γ (21) expected to migrate at 53 kDa or at approximately 59 kDa, respectively, were not detected at the protein level. The antibody detected bacterially expressed CaM kinase II γ_B (see *Materials and Methods*).

In conclusion, the major subtype of CaM kinase II present in RINm5F cells appeared to be the subtype δ_2. It was therefore of interest to determine whether the same isoform was present in the more differentiated cell line INS-1 and in normal rat islets. Immune blots comparing RINm5F with INS-1 cells and with normal freshly prepared islets showed that an immunoreactive band of the same size was present in all three cell types. Preadsorption of the antibody with the immunizing protein strongly reduced staining of the 55-kDa band, suggesting that CaM kinase II δ_2 was detected (Fig. 3b). Remarkably, the expression level of CaM kinase II δ was markedly higher in Islets and in INS-1 cells as compared with RINm5F-cells.

To characterize the subcellular distribution of CaM kinase II δ_2, INS-I cells were fractionated into cytosol and a membrane-cytoskeletal fraction. The pellet was extracted with 0.5% NP-40 detergent leaving a cytoskeletal fraction. The entire protein of each fraction, obtained from a total of 100 μg of cell-protein, was analyzed by immune blot to enable a

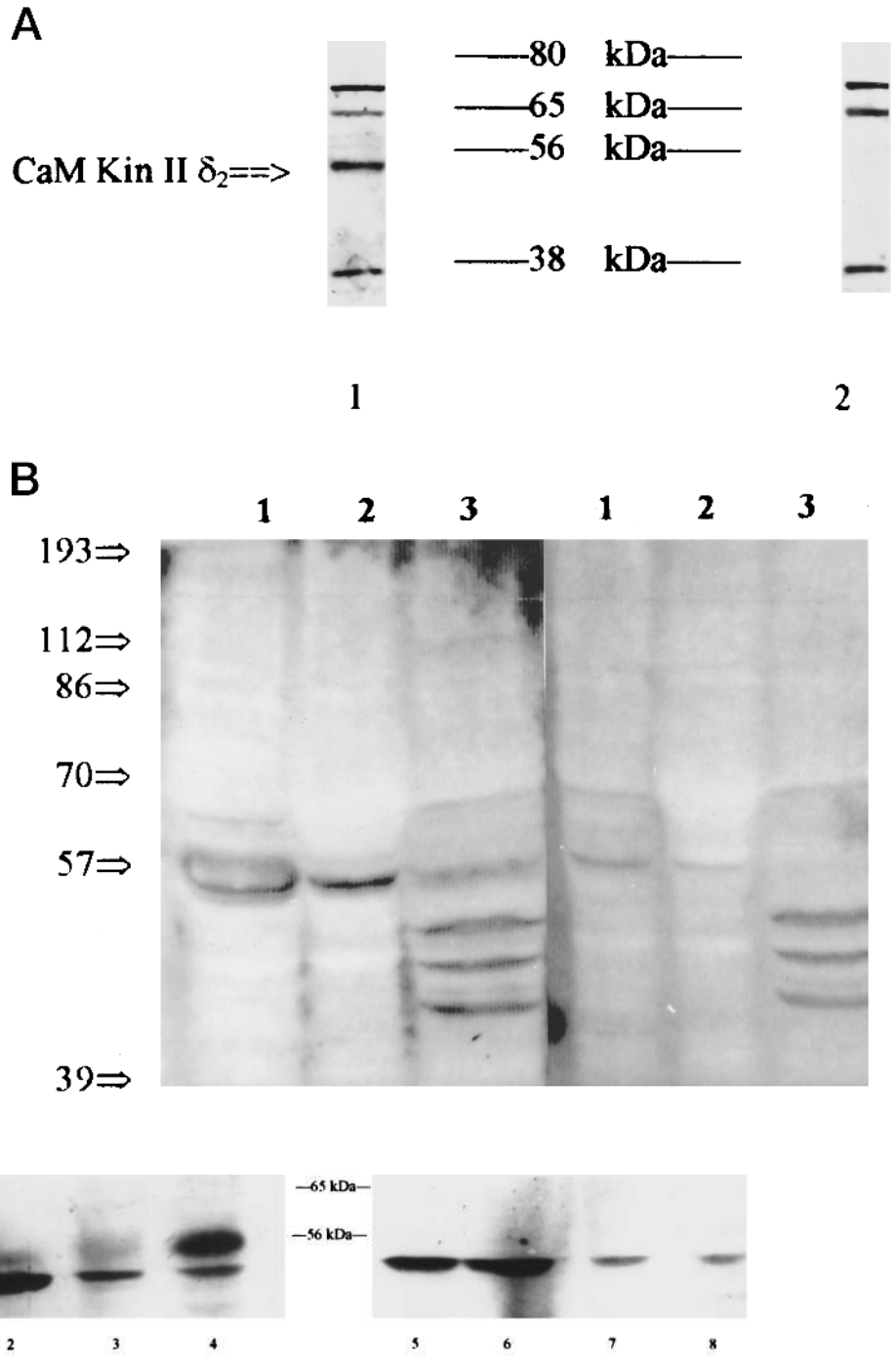

FIG. 3. a, Immune blot with the antibody against CaM kinase II δ (BCIP/NBT stained). Twenty micrograms of RINm5F-cell homogenate were separated by SDS-PAGE. The right lane 2 shows a control after preadsorption of the immune serum with the immunogen. A 55-kDa protein was apparent corresponding in size to the subtype δ_2 (lane 1). b, Immune blot with the antiserum against CaM kinase II δ_2 of INS-1 cells (lane 1), rat pancreatic islets (lane 2), and RINm5F cells (lane 3). Twenty micrograms of protein were analyzed per lane. The Amersham ECL system was used for detection. The *right panel* shows the corresponding assay after preadsorption of the immune serum with the immunogen. The specific band migrating at 55 kDa represents CaM kinase II δ_2.

FIG. 4. Subcellular distribution of CaM kinase II δ_2 in INS-1 cells. One hundred micrograms of INS-1 cell homogenate (lane 1) were fractionated into cytosolic- (lane 2), membrane- (lane 3), and cytoskeletal fraction (lane 4). The complete fractions were separated by SDS page. BCIP/NBT was used for staining. Lane 1 was loaded with 20 μg of complete cell homogenate. Lanes 5–8 correspond to lanes 1–4 except for preadsorption of the immune serum with the immunogen.

quantitative comparison of the amount of CaM kinase II in each subcellular compartment (Fig. 4). The majority of CaM kinase II δ_2 was located in the cytoskeletal fraction, and only very little of the protein was cytosolic or in the detergent soluble membrane compartment. Similar results were also obtained by analyzing 20 μg of protein/fraction.

The amount of CaM kinase II enzyme activity was measured in these fractions using the highly specific substrate autocamtide II (27, 46). The activity of CaM kinase II in the crude cytosolic fraction of RINm5F cells at saturating concentrations of autocamtide II was slightly below 10 pmol/μg·min and 27 pmol/μg·min in the cytoskeletal fraction. The solubilized membrane fraction did not contain reproducibly measurable CaM kinase II activity .

To investigate the subcellular distribution of CaM kinase II δ in INS-1 cells, we employed linear 0.25–2.0 M sucrose

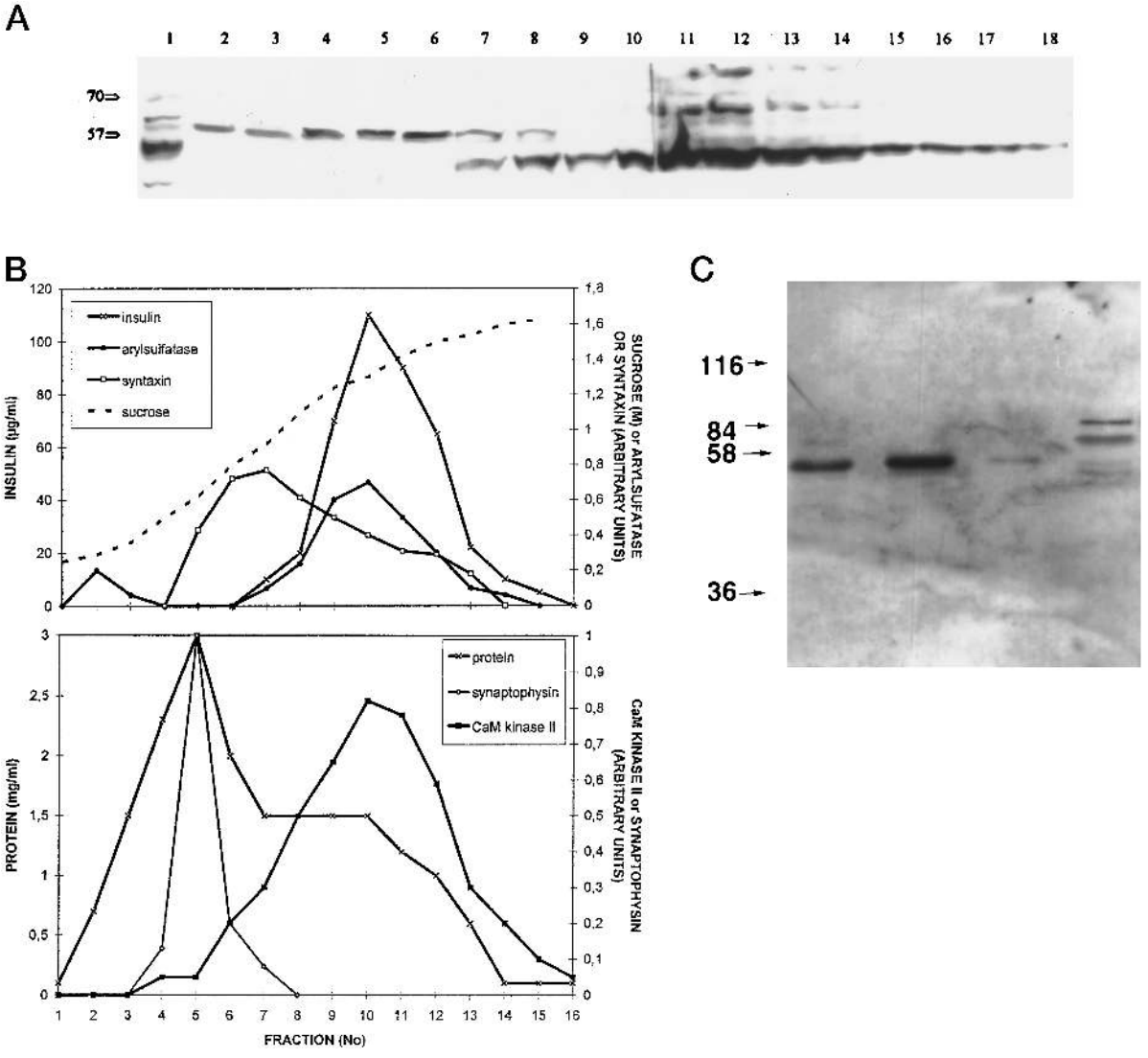

FIG. 5. a, Immune blot of INS-1 cell homogenate fractionated by 0.25–0.4 M sucrose density gradient centrifugation using the anti-CaM kinase II δ immune serum and the Amersham ECL system for detection. Twenty microliters of each gradient fraction were separated on SDS page. Lane 1 contains the unfractionated homogenate. Lane 2 corresponds to the first fraction of the gradient. The band migrating at 55 kDa was eliminated by preadsorption of the antibody with the immunogen and corresponds to CaM kinase II. b, Diagram of marker proteins of fractions of the sucrose gradient. The *upper panel* shows insulin content, arylsulfatase, syntaxin 1, and sucrose. The *lower panel* synaptophysin, protein concentration, and CaM kinase II δ immunoreactivity. Sucrose was determined by diffraction. For further details see *Materials and Methods*. c, Immune blot of CaM kinase II in INS-1 cells fractionated by Nycodenz gradient centrifugation. The Amersham ECL system was used for visualization. Lane 1 shows 36 µg of complete cell homogenate; lane 2, 12 µg of interphase A/B containing insulin granules; lane 3, 12 µg of interphase B/C; and lane 4, 24 µg of the interphase C/homogenate.

gradients (n = 4). This method is well established for INS-1 cells and allows a good separation of insulin secretory granules from synaptic-like microvesicles and other membranes (37). Synaptotagmin was used as a marker for plasma membranes, synaptophysin as a marker for synaptic-like microvesicles, and arylsulfatase was used as a lysosomal marker (Fig. 5, a and b). The insulin content served as a marker for dense-core granules. CaM kinase II δ comigrated closely with the fractions highly enriched in insulin secretory granules (Fig. 5, a and b). The other fractions, in particular synaptic-like microvesicles and plasma membranes, had a low content of CaM kinase II. Unfortunately, the lysosomal marker arylsulfatase comigrated with insulin granules (Fig. 5b).

To achieve a clear separation of lysosomes and mitochondria from insulin granules, the Nycodenz step gradient technique was employed (40, 41). This allowed an 8-fold enrichment of insulin relative to the initial homogenate while arylsulfatase and cytochrome *c* oxidase were reduced to less than half the content of the original homogenate. CaM kinase II δ_2 was 6- to 8-fold enriched in the insulin granule fraction and almost undetectable in the plasma membrane fraction (Fig. 5c).

Discussion

Our study demonstrates the expression of CaM kinase II β_3-, γ- and δ_2 and δ_6-mRNA in RINm5F insulinoma cells and

largely excludes the presence of α and $\beta_{1/2}$-subtypes. The occurrence of these subtypes might have been expected because a previous study indicated expression of γ and δ types of CaM kinase II in peripheral tissues, whereas the α- and $\beta_{1/2}$-subtypes were found in neuronal structures (10). The β_3-subtype was described in HIT T15 and MIN6-insulinoma cells (32). We did not employ isolated rat islets for these experiments because islets contain non-β-cells, the mRNA of which will easily be amplified by RT-PCR.

We have further characterized variants of CaM kinase II δ at the RNA level in insulinoma cells. One variant type expressed, CaM kinase II δ_2, lacked 34 amino acids immediately C-terminal to the calmodulin binding domain, whereas the other, CaM kinase II δ_6, lacked this upper variable domain and additionally the C-terminal 21 amino acids that distinguishes the originally described δ-type from all other isoforms of CaM kinase II α to γ (10, 18, 19).

The presence of particular splice variants may be of relevance regarding the role of CaM kinase II in insulin secretion. In brain synaptosomes for example, CaM kinase II α and β are present and CaM kinase II α regulates the phosphorylation of synapsin I (47). Synapsin appears to anchor vesicles to cytoskeletal components, particularly to actin, and probably prevents the final steps of vesicle transfer from this pool to the pool immediately available for release (48). CaM kinase II α binds to synapsin I and releases vesicles from the cytoskeletal pool by phosphorylation of synapsin I. Although CaM kinase II β is largely homologous to CaM kinase II α outside the variable domains, it neither binds to nor phosporylates synapsin I (47). The presence of the CaM kinase II β-specific variable domains, therefore, may prevent association to structures of the cytoskeleton. The work of Srinivasan and co-workers (17) underlines the importance of the association domain for intracellular targeting. Moreover, CaM kinase II δ_2 might function similiarly to brain CaM kinase II α in insulin secretory cells. In fact, Matsumoto and co-workers (9) recently characterized a synapsin-1-like protein in MIN-6 insulinoma cells that was a substrate of CaM kinase II and became rapidly phosphorylated upon stimulation of the cells with glucose, KCl or tolbutamide.

To investigate the expression and subcellular localization of CaM kinase II δ in more detail, we raised antibodies against the C-terminal association domain of CaM kinase II δ_2 comprising 196 amino acids. We used this approach upon failure to obtain high affinity antibodies against the δ-specific C-terminal 15 amino acids after immunization of four rabbits. The antibodies against the rat CaM kinase II δ-association domain were of high affinity and recognized the eukaryotic expressed subtype δ_2, several splice variants of the δ-isoform extracted from different tissues in Western blots (Möhlig, M., P. A. Horn, and A. Pfeiffer, manuscript in preparation) and the bacterially expressed γ_B subtype. RINm5F- and INS-1-insulinoma cells contained a single compatible band at 55 kDa that corresponds to the predicted size of the δ_2-subtype and exactly comigrates with CaM kinase II δ_2 expressed in NIH 3T3 cells (data in preparation). The smaller δ_6-subtype and subtype γ were not detected, suggesting predominant expression of the δ_2-subtype. At present, it is not clear whether the β_3-isoform (32) is expressed at the protein level, as we do not know the cross-reactivity of our antibody with this isoform. The β_3-isoform would be expected to migrate at approximately 60 kDa (32).

Our data agree excellently with those of Matsumoto and co-workers (9), who observed a 55-kDa isoform of CaM kinase II in MIN-6 cells using a nonspecific antibody generated against rat brain CaM kinase II. Quite remarkably, these authors did not observe other isoforms of CaM kinase II with this nonspecific antibody supporting the notion that the δ_2-isoform appears to be the predominant subtype expressed in MIN-6 cells. Norling and co-workers (49) purified a 54 kDa and 57 kDa CaM kinase II activity from RINm5F-cells. The 54-kDa CaM kinase II activity agrees excellently with δ_2-subtype, whereas the nature of the 57 kDa activity remains to be identified.

Insulinoma cell lines may show altered expression of proteins characteristically expressed in islets. The observation that CaM kinase II δ_2-like immunoreactivity identical to that in INS-1 and RINm5F-cells is present in islets suggests that this isoform is strongly expressed in islets. Remarkably, the expression level of CaM kinase II δ_2 was markedly higher in islets and INS-1-cells compared with RINm5F-cells. RINm5F-cells have a greatly reduced insulin content compared with INS-1 cells and islet β-cells. Therefore, the insulin content appears to parallel the CaM kinase II δ_2 content that might be expected in view of the association of the enzyme with insulin granules.

Intracellular targeting may enable protein kinases with broad substrate specificities such as CaM kinase II to achieve selectivity of action *in vivo*. A study of the subcellular localization of the enzyme showed that most of the immunoreactivity was present in the detergent insoluble cytoskeletal compartment, whereas little immunoreactivity was present in the membrane and cytosolic fraction. This was observed in RINm5F and INS-1 cells and agrees with data from Norling and co-workers (49) on the distribution of the CaM kinase II activity. The cytoskeletal association differs from the distribution of CaM kinase II in other cell types where the majority of the enzyme is present in the cytosolic compartment (12). However, the distribution in INS-1 cells is comparable with that in brain synaptosomes that contain CaM kinase II predominantly associated with the cytoskeleton (50), where it is thought to direct synaptic vesicle traffic and function (48) as discussed above.

A more detailed analysis of the subcellular localization showed comigration of CaM kinase II δ with insulin secretion granules in INS-1 cells. This suggests that the enzyme may be either a component of insulin containing dense core granules or of associated proteins. In fact, this localization fits very well with the demonstration of a synapsin-like protein in β-cells that may be expected to be associated with secretory granules similar to its function in synapses. Moreover, the association with cytoskeletal elements may point to a role of CaM kinase II in the transport or release of vesicles from such structures. For example, actin filaments are involved in exocytosis in endocrine cells as shown by a stimulation of this process by low concentrations of reagents that depolymerize actin and by inhibition of exocytosis by antibodies against the actin-organizing protein fodrin (51) or an actin disrupting botulinum toxin (52).

The identification of the CaM kinase II δ_2 as the major

isoform in insulinoma cells and of its association with insulin secretory vesicles will allow both: overexpression and suppression studies that should allow a more precise investigation of the role of CaM kinase II in insulin secretion.

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