Evidence That Casein Kinase 2 Phosphorylates Hepatic Microsomal Calcium-Binding Proteins 1 and 2 but Not 3

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ABSTRACT: We have extensively purified three of the hepatic microsomal intralumenal Ca^{2+}-binding proteins, CBP1, CBP2, and CBP3, which were originally described by Van *et al*. [(1989) *J. Biol. Chem. 264,* 17494−17501]. These apparently homogeneous preparations showed only single $^{45}Ca^{2+}$ binding bands. On the basis of the peptide sequence, CBP2 was found to be highly homologous with the previously described protein ERp72. Similarly, CBP3 was identical to calreticulin and CBP1 had some homology to calmodulin. Contrary to the report of Van *et al*. (1989), we found that CBP2 had little thiol:protein disulfide oxidoreductase activity. Of the three purified preparations, only CBP2 exhibited apparent intrinsic protein kinase activity. This activity was found to be due to contamination of the CBP2 preparation by an extremely low concentration of tightly bound casein kinase 2 (CK2). In line with this observation, the phosphorylation was inhibited by heparin, removed by antibody to CK2, and stimulated by spermine. Furthermore, CBP2 was readily phosphorylated *in vitro* by added CK2 but only slowly phosphorylated by several other protein kinases. Thus, the persistence of CK2 in a highly purified preparation of CBP2 along with several other lines of evidence presented in this study might suggest that the protein CBP2 is a physiologically relevant substrate for CK2. Furthermore, these data suggest that CK2 might be localized in the lumen of the endoplasmic reticulum and that the phosphorylation of CBP2 in the lumen may play a role in the chaperone activity attributed to this protein.

Van *et al*. (1989) have reported the purification of four proteins from the hepatic endoplasmic reticulum which are involved in the intralumenal binding of Ca^{2+}. They designated them as CBP1−4.[1] These proteins are thought to serve to reduce the intralumenal concentration of Ca^{2+} and thereby reduce the energy required for Ca^{2+} sequestration by the endoplasmic reticulum. On the basis of the N-terminal amino acid sequence, both we and Perrin *et al*. (1991) have found that CBP3 is calreticulin, the major intralumenal Ca^{2+}-binding protein in the endoplasmic reticulum of the liver.

In their original study, Van *et al*. (1989) did not report sequences for CBP1 and CBP2, but recently they have reported that CBP2 was identical to ERp72, a protein of unknown function from the endoplasmic reticulum of mouse liver (Van *et al*., 1993; Rupp *et al*., 1994; Mazzarella *et al*., 1990). We have independently observed this same homology (Chen *et al*., 1993). ERp72 has three sets of the peptide motif -EFYAPNCGHCK-. This motif is thought to be characteristic of thioredoxin and the thiol:protein disulfide oxidoreductases. The latter are also designated as protein disulfide isomerases (Goldberger *et al*., 1963; Van *et al*., 1993; Mazzarella *et al*., 1990; Srivastava *et al*., 1991). In line with this homology, Soling's group has reported that CBP2 had thiol:protein disulfide oxidoreductase activity (Van *et al*., 1993; Rupp *et al*., 1994). On the other hand, in our previous studies we identified only two isoforms of thiol: protein disulfide oxidoreductases in microsomal preparations, suggesting that ERp72 is not primarily an oxidoreductase (Srivastava *et al*., 1991).

More recently, Nigam and co-workers (Nigam *et al*., 1994; Kuznetsov *et al*., 1994) have suggested that a number of these intralumenal proteins, including ERp72 and calreticulin, are molecular chaperones. In their studies they reported that ERp72 and calreticulin bound to a variety of denatured proteins which had been linked to Sepharose. Furthermore, they found that the release of these microsomal proteins from the chromatography column required the hydrolysis of ATP. This would suggest that the release of the CBPs only occurs

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[1] Abbreviations: ATP, adenosine triphosphate; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CBP1 − 4, microsomal Ca^{2+}-binding proteins 1−4; CK1, casein kinase 1; CK2, casein kinase 2; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid, trisodium salt; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FPLC, fast protein liquid chromatography; GSH, reduced glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, *N*-(3-sulfopropyl)morpholine; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol) 6000; PVDF, Poly(vinylidene difluoride) membranes; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

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after their phosphorylation. For many of these microsomal proteins, Ca^{2+} was found to stimulate this ATP-dependent release (Nigam *et al*., 1994). Recently, Nauseef *et al*. (1995) have also presented evidence that calreticulin functions as a molecular chaperone.

In the current study we have modified the procedure of Van *et al*. (1989) for the purification of CBP1−3 by the addition of more chromatography steps. With these added steps we have purified both CBP1 and CBP2 to apparent homogeneity. In agreement with their recent reports, we have found that CBP2 is identical to ERp72 (Van *et al*., 1993; Chen *et al*., 1993; Mazzarella *et al*., 1990), but we have found that it has little thiol:protein disulfide oxidoreductase activity when compared to the two isoforms which we have previously purified and characterized (Srivastava *et al*., 1991). Furthermore, we have found that, contrary to the report of Van *et al*. (1989), the purified preparation of CBP2 is readily phosphorylated by the addition of ATP. On the other hand, the purified preparation of CBP1 was not autophosphorylated but was phosphorylated by the purified CBP2 preparation. On the basis of inhibitor, stimulator, and antibody studies we believe that the phosphorylation of these two proteins was due to the presence of a minimal amount of tightly bound casein kinase 2 (CK2) in the CBP2 preparation. Furthermore, both CBP1 and CBP2 were readily phosphorylated by the addition of purified CK2. These data would suggest that CBP1 and CBP2 may be physiologically relevant substrates for CK2. Calreticulin was not phosphorylated by either the CBP2 preparation or CK2.

MATERIAL AND METHODS

Materials. The animals used in these studies were 250-g Sprague-Dawley male rats purchased from Harlan Laboratories. $^{45}Ca^{2+}$ was purchased from DuPont/NEN (Cambridge, MA) and [γ-^{32}P]ATP was from Amersham (Des Plaines, IL). Sodium dodecyl sulfate (SDS), acrylamide, *N*-ethylmaleimide (NEM), alkaline phosphatase, casein, calmodulin, reduced glutathione (GSH), heparin, spermine, protein kinases A and C, and a series of peptide molecular mass standards, including Try-Gly-Gly (T-9005) (295.3 Da), leucine enkephalin (L-9133) (555.6 Da), pepstatin A (P-4265) (685.9 Da), adrenocorticotropic hormone (fragment 11−24) (A-2532) (1652.1 Da), bradykinin (B-3259) (1660.2 Da), and [Ala-OH^{21}]-conantokin-T (C-2176) (2683.8 Da), were purchased from Sigma Chemical Co. (St. Louis, MO). Similarly, a random copolymer of Glu:Tyr (4:1), which is a specific substrate for tyrosine kinases, and rabbit muscle protein kinase A inhibitor were also obtained from Sigma Chemical Co. (St. Louis, MO). A specific peptide substrate for casein kinase 1 (CK1) (RRDDLHDDEEDEAMSITA) was a kind gift from L. A. Pinna.[2] A specific peptide substrate for CK2 (RRREEETEEE) was obtained from Peninsula Laboratories (Belmont, CA). The H7 inhibitor of protein kinases A and C was purchased from Calbiochem (San Diego, CA). Specific substrates for cdc2 were provided by Promega (Madison, WI). Monoclonal antibodies to phosphotyrosine (PY 20) were obtained from Zymed (South San Francisco, CA). All other chemicals were ACS reagent grade.

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Microsomal Preparation. The rats were killed by guillotine. Their livers were perfused with KCl−HEPES (150 mM/20 mM, pH 7.4), quickly removed, cooled on ice, and homogenized in the KCl−HEPES buffer (3 mL/g of liver). The homogenates were centrifuged at 9000*g* for 15 min and the supernatants were centrifuged at 140000*g* for 45 min. The pellets were resuspended in KCl−HEPES and recentrifuged at least twice and in some studies up to nine times. The final, washed pellets were kept frozen at −70 °C until use.

Protein Purification. The intralumenal microsomal proteins were solubilized by incubation for 30 min in Na_2CO_3−EDTA (100 mM 1 mM) at 4 °C, as were all subsequent steps (Van *et al*. 1989). The extract was centrifuged at 105000*g* for 60 min and the membrane pellet was discarded. The solubilized proteins were purified by a modification of the procedure of Van *et al*. (1989) in which additional chromatographic steps were included as described below (Chart 1).

After centrifugation of the extract, the supernatant was brought to pH 7.4 with HCl (1 M) and chromatographed on a DEAE-Sepharose CL-6B column (1 cm × 10 cm) in Tris−EDTA (10 mM 0.1 mM, pH 7.4). The CBP1−4 fractions were eluted with a NaCl step gradient (0.15 and 0.27 M). The protein peaks were rechromatographed on an FPLC MonoQ column (0.5 cm × 5 cm) (Pharmacia, Piscataway, NJ) in Tris-HCl−EDTA (10 mM 0.1 mM, pH 7.4) with a NaCl gradient (0−1 M).

Homogeneous preparations of CBP1 were obtained after chromatography of the appropriate MonoQ fraction on a Nucleopac column (Dionex, Sunnyvale, CA) in Tris−DTT (10 mM 1 mM, pH 7.3) and elution with a NaCl gradient (0−1 M).

The Ca^{2+}-binding peak containing CBP2 was suspended in Tris-HCl buffer (20 mM, pH 7.4) containing EDTA (0.1 mM) and DTT (1 mM) and chromatographed on an octyl-Sepharose column (1 cm × 10 cm) in Tris-HCl (10 mM, pH 7.4). Finally, in some studies, the fraction containing CBP2 was applied to a hydroxyapatite column (5 mm × 25 mm) (Bio-Rad, Richmond, CA) in Tris-HCl−DTT (20 mM 1 mM, pH 7.5) and eluted with a continuous gradient of K_2HPO_4 (0−0.5 M, pH 7.5).

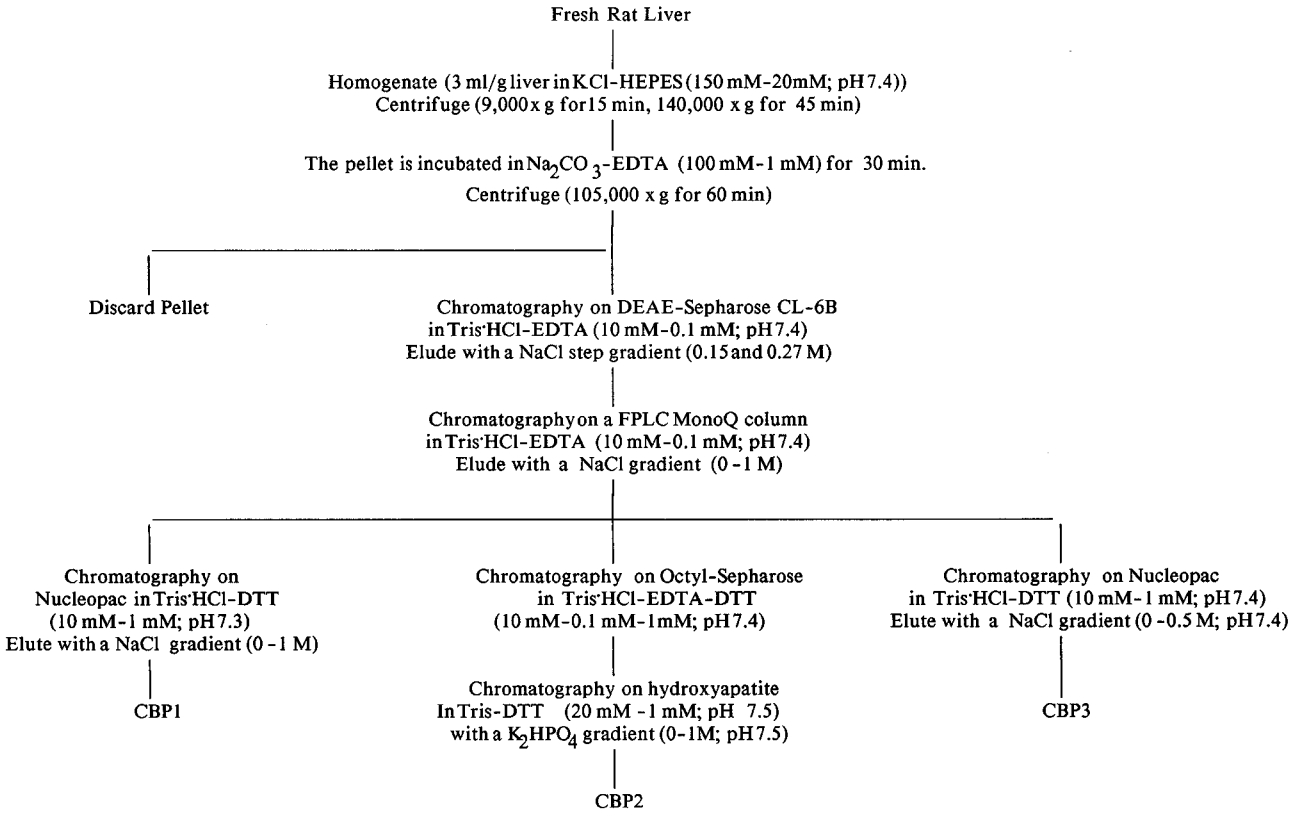
The CBP3 fraction obtained from the initial MonoQ step was purified to homogeneity by chromatography on a Nucleopac column (Dionex, Sunnyvale, CA) in Tris−EDTA (10 mM/1 mM, pH 7.4) and eluted with a NaCl gradient (0−0.5 M, pH 7.4).

CK2 was purified from rat liver as previously described (Goueli *et al*., 1986). The cdc2 was obtained from Promega (Madison, WI).

Antibody Studies. Polyclonal antibodies to the purified protein fractions were developed in laying hens as described by Damiani *et al*. (1988). The protein (2 μg) was suspended in a mixture of NaCl−Tris (0.1 M 20 mM, pH 7.4) (0.1 mL) and complete Freund's adjuvant (0.1 mL). This mixture was injected into the hens at several subcutaneous and intramuscular sites. At 2 and 3 weeks the immunizations were repeated except that incomplete Freund's adjuvant was substituted for the complete Freund's adjuvant.

Beginning 1 month after the first injection, eggs were collected daily. The yolks were separated from the whites and diluted in NaCl−Na_2HPO_4 (0.1 M 10 mM, pH 7.5) (PBS). Most egg yolk proteins were precipitated with poly-

Chart 1: Preparation of Homogeneous CBP1−3



(ethylene glycol) 6000 (PEG) (final concentration of 3.5%) in PBS (Damiani *et al*., 1988). The suspension was centrifuged for 20 min at 12000*g* and the antibodies were precipitated with PEG (final concentration of 11%), stirred for 30 min at 4 °C, and centrifuged. The pellet was redissolved in PBS and reprecipitated three times. Finally, the antibodies were dissolved in PBS containing NaN_3 (0.01%) and stored at −70 °C. For immunoblots we have found that the PEG fraction gave satisfactory results.

Polyclonal antibodies to CK2 were prepared and cross-linked to Sepharose 4B beads as previously described (Goueli *et al*., 1990).

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed by the method of Laemmli (1970) with a 4% stacking gel and 12% separating gel. The gels were stained with Coomassie Blue and photographed. They were then destained and the bands were identified by silver stain.

Immunoblotting of the proteins that had been separated by SDS−PAGE was performed according to the transblotting procedures described by Towbin *et al*. (1979). An alkaline phosphatase reaction was utilized to develop the color. The indicator dye was a combination of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad, Richmond, CA).

Peptide Sequencing. The purified CBP2 and CBP3 were cleaved with cyanogen bromide by the method of LeGendre and Matsudaira (1989). In this procedure the proteins were separated by SDS−PAGE and transblotted onto PVDF paper (Immobilon-P, Millipore, Bedford, MA) in CAPS buffer (10 mM, pH 11) with 10% methanol. The proteins were stained with Ponceau S (0.5%) in 1% acetic acid. The protein bands were cut out and incubated overnight with cyanogen bromide (0.15 M) in 70 formic acid (0.6 mL). The peptides were eluted in Tris-HCl (50 mM, pH 9.5) containing SDS (2%) and Triton X-100 (0.3%). They were then separated by SDS−PAGE with a 16% gel, transferred to a PVDF membrane, stained with Coomassie Blue, and sequenced. The sequences were determined on 35−50 pmol of the peptides.

We sequenced CBP1 after it had been digested with trypsin (1 μg/50 μg of protein) in NH_4HCO_3 (100 mM) for 24 h at 37 °C. The peptides were purified by HPLC chromatography on a C_{18} column (2.1 mm × 25 cm) (Vydac, Hesperia, CA) by the method of Stone *et al*. (1989). The digest was place on the column with an aqueous elution buffer of trifluoroacetic acid (0.06%) and eluted at 0.5 mL/min in trifluoroacetic acid (0.052%) with a 0−70% acetonitrile gradient (50 mL).

We next selected several peaks which showed high absorbance when eluted from the C_{18} column and further purified them on a TSK G2500 PWxL gel-filtration column (6 μm; 7.8 mm × 30 cm) (TosoHaas, Montgomeryville, PA). In this purification we used a mobile phase of acetonitrile (45%) containing trifluoroacetic acid (0.1%) in water (54.9%) at a flow rate of 0.3 mL/min. The peaks were detected at 215 nm. The molecular masses versus the retention times were calibrated with a set of peptide standards (165−2685 Da) obtained from Sigma Chemical Co. (St. Louis, MO).

Sequences of several of the purified peptides obtained by the above procedures were determined at the Microchemical Facilities, University of Minnesota, on a Model 477A protein sequencer (Applied Biosystems, Foster City, CA). The samples contained 50−250 pmol of the purified peptides.

Calcium Binding Assays. The protein fractions were separated by PAGE on a 10.5% gel in the absence of SDS and without heating before application to the gel. If we included SDS in the system, we found that all of the proteins bound Ca^{2+}. The proteins were then transferred to a PVDF

membrane in a glycine−Tris buffer (192 mM 25 mM, pH 8.6). The membranes were washed three times with KCl−imidazole hydrochloride (2 mM 2 mM, pH 7.4 for CBP1 and CBP2 and pH 8.6 for CBP3) and then immersed for 30 min in the KCl−imidazole buffer containing $^{45}Ca^{2+}$ (50 μCi in 50 mL). The membranes were finally washed with Ca^{2+}-free buffer and dried at 80 °C. Autoradiograms of the membranes were obtained by exposing them to X-ray film (XAR-5, Eastman Kodak, Rochester, NY) for 10−20 h. The membranes were then stained for 5 min with 0.5% Amido Black in methanol−acetic acid−water (40:10:50) in order to detect the protein bands. The membranes were then washed with the methanol−acetic acid−water mixture.

Protein Kinase Assay. Protein kinase activity was determined by three different assays. In the first the samples were incubated for 5 min at 37 °C in a buffer containing imidazole hydrochloride (50 mM, pH 7.4), $MgCl_2$ (5 mM), and [γ-^{32}P]-ATP (1−8 μCi in 20 μL). The incubations were terminated by the addition of the SDS−PAGE sample buffer (SDS−glycerol−DTT−Tris: 1%/20%/25 mM/0.5 M, pH 6.8). The proteins were then separated by SDS−PAGE and transferred to PVDF membranes. The membranes were stained for 5 min with 0.5% Amido Black in methanol−acetic acid−water (40:10:50) and then washed with the methanol−acetic acid−water mixture. Finally, autoradiograms were obtained by exposing the membranes to the X-ray film for between 10 min and 3 days depending upon the enzymatic activity of the preparation and the specific activity of the [γ-^{32}P]ATP.

The protein kinase activity for the purified CBP2 was also quantitated by the method of Payne *et al*. (1983). In this procedure the buffer containing imidazole hydrochloride (50 mM, pH 7.4), $MgCl_2$ (5 mM), and [γ-^{32}P]ATP (1 μM, 0.2 μCi) in a final volume of 50 μL was incubated for several minutes at 37 °C. The reaction was initiated by the addition of the protein fraction, the samples were incubated for 5 min, and the reaction was terminated by the addition of 10 μL of a solution of bovine serum albumin in water (10 mg/mL). Fifty-five microliters of the mixture was adsorbed onto a thick filter paper (ET-31, Whatman) and the paper was placed into cold TCA (15 mL) (10%) containing potassium pyrophosphate (8%). It was then washed several times in the TCA−pyrophosphate solution, placed into a scintillation mixture (Opti-Fluor, Packard Instruments, Des Plaines, IL) (15 mL), and counted in a 1900c β-liquid scintillation counter (Packard Instruments, Des Plaines, IL).

Finally, the activity of the cell cycle-dependent protein kinase, cdc2, was determined in MOPS buffer (40 μL, 25 mM, pH 7.0) containing β-glycerophosphate (40 mM), *p*-nitrophenol phosphate (5 mM), EGTA (5 mM), $MgCl_2$ (15 mM), [γ-^{32}P]ATP (0.1 mM, 500 cpm/pmol), DTT (1 mM), sodium vanadate (0.1 mM), peptide substrate (50 μM), and the enzyme fraction. The enzyme was diluted in imidazole hydrochloride (50 mM, pH 7.3), EGTA (0.2 mM), 2-mercaptoethanol (0.1% v/v), bovine serum albumin (1 mg/mL), and glycerol (10% v/v). The reaction was initiated by the addition of the enzyme. The mixture was incubated for 5 min at 30 °C. A 20-μL aliquot was withdrawn and spotted on P-81 disks. The disks were washed 3−4 times in phosphoric acid (1% v/v) and the radioactivity was determined as above.

Thiol:Protein Disulfide Oxidoreductase Assays. The thiol:protein disulfide oxidoreductase activity was determined by a modification of the insulinase assay of Varandani (1973) as previously described (Srivastava *et al*., 1991). In this assay the samples were incubated for 5 min in a medium containing K_2HPO_4 (100 mM, pH 7.5), EDTA (5 mM), [^{125}I]-insulin (20 μM, 100 000 dpm), and GSH (0.5 mM). The incubations were terminated by the addition of a solution of bovine serum albumin in water (3 mg/mL) followed by TCA (0.3 M final concentration). The samples were then centrifuged at 10000*g* for 5 min and the radioactivity in the supernatants was determined in a 1900c β-liquid scintillation counter as described above. The enzymatic activity was taken as the difference between the radioactivity in the presence and absence of enzyme.

Other Assays. The protein concentrations were determined by the instant dye method with bovine serum albumin as the standard (Bio-Rad, Richmond, CA).

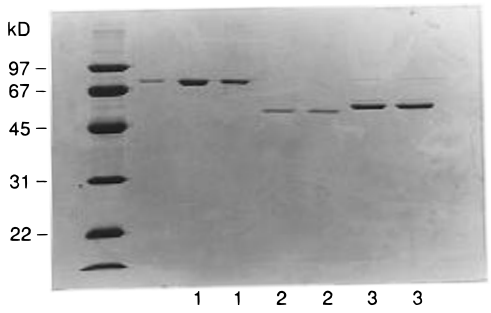


FIGURE 1: SDS−PAGE of the purified microsomal calcium-binding proteins CBP1−3. Left-hand lane, standards; lanes 1, CBP2; lanes 2, CBP1; lanes 3, CBP3. The proteins were detected with silver stain.

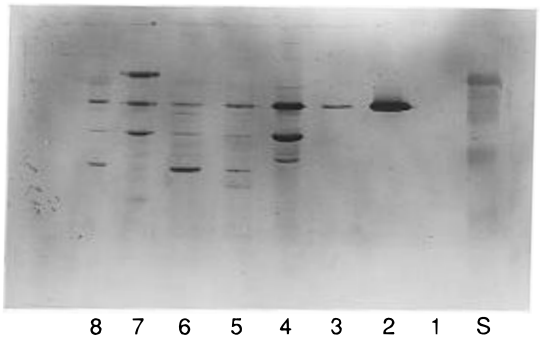


FIGURE 2: SDS−PAGE with immunoblotting against anti-CBP2 antibody to the purified microsomal CBP2. Lane S, standards; lane 1, CBP1; lane 2, CBP2; lane 3, CBP3; lane 4, liver microsomes; lane 5, brain microsomes; lane 6, heart microsomes; lane 7, kidney microsomes; lane 8, skeletal muscle.

RESULTS

Purification and Characterization of CBP1−3. With the addition of the octyl-Sepharose column and hydroxyapatite chromatography steps, we were able to obtain CBP2 as an essentially homogeneous protein as determined by SDS−PAGE with silver staining (Figure 1). In the study shown in Figure 1, about 10 μg of protein was applied to the gel. Since it is possible with silver stain to detect about 10 pg of protein/band, this would suggest that any contaminating proteins represented less than 0.1% of the concentration of the CBP2. Similarly, on immunoblotting of the purified preparation, it showed only a single band (Figure 2, lane 2). These immunoblots indicated that CBP2 was present not only in the liver but also in the brain, heart, lung, and kidneys (Figure 2). Antibodies to CBP2 cross-reacted with a number

A

Fragment 1
CBP2: -L A S K F D V D S G Y P T I K I L K K G Q A V D Y D G-
ERp72: -L A S K F D V D S G Y P T I K I L K K G Q A V D Y D G-

Fragment 2
CBP2: -D V Q G S T E A S A I K |?| Y V V K H A |?| P |??| G H R-
ERp72: -D V Q G S T E A S A I K |D| Y V V K H A |L| P |LV| G H R-

B

Fragment 1 (Identical to calmodulin residues 127 - 144)
CBP1: -E A D I D G D G Q V N Y E E F V Q M-

Fragment 2 (The first 10 residues are identical to residues 15 - 24 of calmodulin)
CBP1: -E A F S L F D K D G D G T I T T K-

Fragment 3
CBP1: -G F P T I (Y,K)-

Fragment 4
CBP1: -I F Q K G E S P V D Y D G G R-

FIGURE 3: Amino acid sequences of (A) two cyanogen bromide fragments of CBP2 compared to homologous sequences from mouse hepatic microsomal protein (ERp72) and (B) four tryptic peptides of CBP1.

of microsomal proteins but not with CBP1 or CBP3. The preparation of CBP3 shown in Figure 1 had a slight contamination by a protein with the same M_r as CBP2. But this band was not immunoreactive with antibodies against CBP2 (data not shown). Similarly, with the addition of the Nucleopac chromatography step we were able to obtain an apparently homogeneous preparation of CBP1 (Figure 1). The purified CBP1, CBP2, and CBP3 fractions showed significant Ca^{2+}-binding activities (data not shown). Furthermore, each of the purified proteins showed only a single Ca^{2+}-binding band after PAGE in the absence of SDS and without heating before application to the gel (data not shown).

Sequence Analysis of CBP1−3. We were unable to obtain an N-terminal amino acid sequence for either the intact CBP1 or CBP2, but when we cleaved CBP2 with cyanogen bromide, we obtained two fragments which were then sequenced. Together, these sequences were at least 92% homologous with the implied sequence reported by Mazzarella *et al*. (1990) for the mouse microsomal protein ERp72 (Figure 3A) (Chen *et al*., 1993). These results are in agreement with the recent report of Van *et al*. (1993) and the implied sequence as reported by Rupp *et al*. (1994).

Fullekrug *et al*. (1994) have published a partial DNA sequence (1296 bases) of CBP1. We sequenced four tryptic peptides derived from CBP1 (Figure 3B). The first peptide showed 100% homology with human calmodulin, residues 127−144. We sequenced 17 residues of the second peptide. The first 10 residues were homologous with residues 15−24 of calmodulin. Neither of these calmodulin sequences was included in the partial sequence reported by Fullekrug *et al*. (1994). We also sequenced eight residues from the third peptide and 15 from the fourth. These sequences were 100% homologous with the implied sequence of CBP1 reported by these workers. Six of the residues in the third peptide were homologous with ERp72. Finally, the fourth peptide contained 15 residues which showed partial homology with residues 105−119 of the thiol:protein disulfide oxidoreductase isoform Q-2 (Bennett *et al*., 1988; Srivastava *et al*., 1991, 1993). In all of these sequences of CBP1 we recovered 150−250 pmol in the first 3−4 cycles of the Edman degradation.

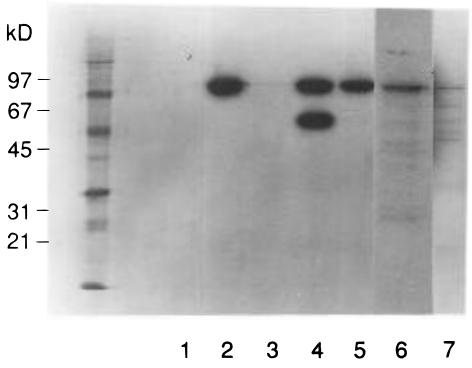


FIGURE 4: Phosphorylation of CBP1−3. Lane 1, CBP1; lane 2, CBP2; lane 3, CBP3; lane 4, CBP1 and CBP2; lane 5, CBP2 and CBP3; lane 6, hepatic microsomes; lane 7, microsomal extract.

Thiol:Protein Disulfide Oxidoreductase Activity of CBP2. ERp72 has three sets of the peptide motif -EFYAP-NCGHCK-. This sequence is thought to be a motif for thioredoxin and the thiol:protein disulfide oxidoreductases (Van *et al*., 1993). Hence, CBP2 should have thiol:protein disulfide oxidoreductase activity. Yet, when we examined our preparation for insulinase activity, we found that it had a much lower specific activity then the isozymes of thiol:protein disulfide oxidoreductase which we have previously identified and characterized (Srivastava *et al*., 1991, 1993). In our previous studies we found that the two isoforms of thiol:protein disulfide oxidoreductase, Q-2 and Q-5, had insulinase specific activities of 14.4 and 45.9 nmol min^{-1} (mg of protein)$^{-1}$, respectively (Srivastava *et al*., 1991), whereas we have found here that CBP2 had an insulinase specific activity of only 0.69 nmol min^{-1} (mg of protein)$^{-1}$, or about 5% of that which we observed for Q-2. This low specific activity could explain why, in our original report, we failed to identify this protein as a thiol:protein disulfide oxidoreductase (Srivastava *et al*., 1991). It is possible that their preparation could have had a slight contamination by either of these two previously identified oxidoreductases, since both of them are intralumenal proteins of the endoplasmic reticulum which are found in the Na_2CO_3−EDTA microsomal extract.

Phosphorylation of CBP1−3. We next examined the possibility that some of these Ca^{2+}-binding proteins could be protein kinases. This study was predicated on the hypothesis that Ca^{2+} binding might require the initial phosphorylation of the proteins. If such were the case, then this could partially explain the ATP requirement for microsomal Ca^{2+} uptake. When the purified proteins were incubated in the presence of Mg^{2+} and [γ-^{32}P]ATP and then were separated by SDS−PAGE, we found that CBP2, but not CBP1 or CBP3, was phosphorylated (Figure 4). This phosphorylation was also seen with preparations obtained after hydroxyapatite chromatography. Furthermore, when mixtures of CBP2 with CBP1 and CBP3 were incubated together, CBP1, but not CBP3, was also phosphorylated (Figure 4). These results suggested that CBP2 is either a protein kinase or was contaminated by one. The phosphorylation was linear for 15 min and for protein concentrations up to 20 μg/mL (data not shown). The stoichiometry for the phosphorylation of CBP2 appeared to be about 5 mol of phosphate/mol of protein.

It is of interest that by far the most heavily phosphorylated band in the microsomal incubation had a molecular mass of

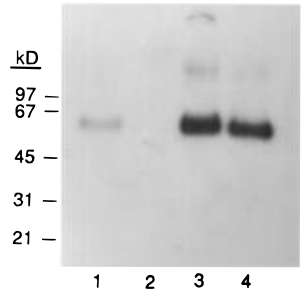


FIGURE 5: Effect of heparin and spermine on the phosphorylation of CBP2 without added CK2. Lane 1, CBP2 control; lane 2, heparin (5 μ/mL); lanes 3 and 4, spermine (1 mM).

about 72 kDa (Figure 4, lane 6). These data might suggest that CBP2 (ERp72) is either a very active protein kinase or a natural substrate for a protein kinase within the lumen of the endoplasmic reticulum.

When we sought to determine whether the kinase activity was catalyzed by CBP2 or was due to a minor contamination by a known protein kinase, we found that the activity was probably due to a very low level of contamination of the preparation by CK2. This conclusion is based on the following observations. First, besides CBP1 and CBP2, a number of lower molecular weight proteins, both in the intact microsomes (Figure 4, lane 6) and in the total microsomal extract (Figure 4, lane 7), were also phosphorylated in the presence of ATP and Mg^{2+}. These bands were not observed with the purified CBP2, even when very high specific activity [γ-^{32}P]ATP was used and 1.7×10^7 dpm/incubation was added (Figure 4, lane 2). The lower molecular weight, phosphorylated bands seen in the total extract had molecular weights in the range that would be expected for the monomers of the α and β chains of CK2 (Ou *et al*., 1992). This might suggest that the phosphorylation of CBP1 and CBP2 was catalyzed by CK2 contamination of the CBP2 preparation. Against this possibility, the purified preparation of CBP2 did not phosphorylate casein or specific peptide substrates for CK1, CK2, tyrosine kinases, or cdc2 (data not shown). Similarly, inhibitors of protein kinases A and C had no effect. Finally, immunoblotting with antibodies to CK2 showed no immunoreactive bands with the purified preparations of CBP2 (data not shown).

On the other hand, several observations strongly suggested that the phosphorylation was due to contamination of the CBP2 preparation by concentrations of CK2 which were undetectable by our standard procedures. First, the sequence of ERp72 contains a -YGIVDYMVEQSGPPSKEILTLKQVQ- motif which is 76% homologous to a specific CK2 phosphorylation site (Feng *et al*., 1993). Furthermore, the activity was totally inhibited by heparin (5 μg/mL) (Figure 5, lane 2) and markedly stimulated by spermine (1 mM) (Figure 5, lanes 3 and 4). Both of these observations are consistent with a role for CK2 in the phosphorylation of this protein (Ahmed & Tawfic, 1994). We feel that the strongest evidence suggesting that this phosphorylation was catalyzed by CK2 was the observation that the protein kinase activity could be removed from the preparation without a significant loss of protein by incubating it with antibodies to CK2 bound to Sepharose beads (Figure 6).

Since we could not detect even a minimal contamination by CK2 by our standard procedures, these data would

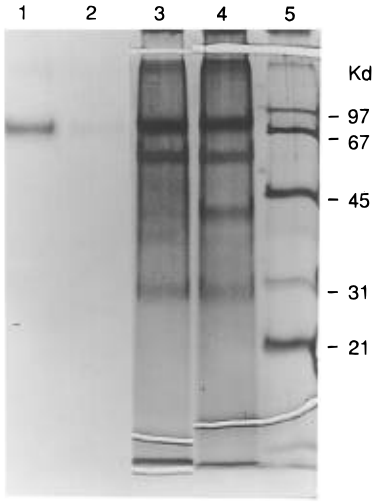


FIGURE 6: Effect of treatment with antibody to CK2 on the phosphorylation of CBP2. Lane 1, phosphorylation of CBP2 after treatment with Sepharose-bound antibody to lysozyme; lane 2, phosphorylation of CBP2 after treatment with Sepharose-bound antibody to CK2; lane 3, silver-stained preparation of CBP2 after treatment with Sepharose-bound antibody to lysozyme; lane 4, silver-stained preparation of CBP2 after treatment with Sepharose-bound antibody to CK2; lane 5, protein standards. The extra bands seen in the silver-stained lanes are due to tRNA which had been added to block nonspecific adsorption of the protein to the Sepharose.

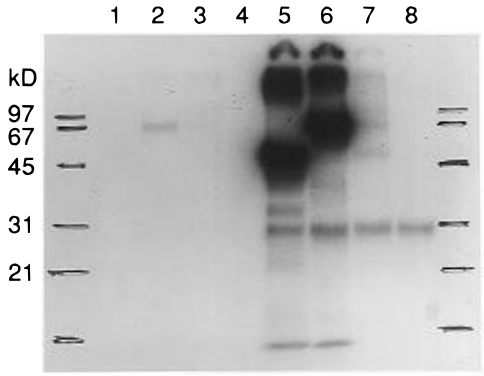


FIGURE 7: Phosphorylation of CBP1−3 by CK2. Lane 1, phosphorylation of CBP1 without added CK2; lane 2, phosphorylation of CBP2 without added CK2; lane 3, phosphorylation of CBP3 without added CK2; lane 5, phosphorylation of CBP1 with added CK2; lane 6, phosphorylation of CBP2 with added CK2; lane 7, phosphorylation of CBP3 with added CK2; lane 8, autophosphorylation of CK2 alone.

indicate that both CBP1 and CBP2 may be highly reactive substrates for this protein kinase and that these two proteins were phosphorylated by minimal amounts of the CK2 which appear to have been present in our preparations. In line with this concept, highly purified CBP1 and CBP2, but not CBP3, were readily phosphorylated by the addition of CK2 (Figure 7). These incubation included 1−5 μg of the respective CBPs and 10 ng of CK2. Furthermore, the X-ray film was exposed to the gel for only 10 min rather than the usual 1−3 days. These data would indicate that this phosphorylation was very rapid. It should be noted that even though the synthetic substrate for CK2, RRREEETEEE, was not phosphorylated by the CBP2 preparation, this does not rule out the presence of CK2 in this preparation since this peptide is a poor substrate for CK2.

Both protein kinases A and C also phosphorylated CBP2, but they were far less active than CK2 (data not shown).

Table 1: Effect of Mg^{2+}, Ca^{2+}, and EGTA on the Phosphorylation of Calcium Binding Protein 2[a]

5 mM Mg^{2+}	20 μM Ca^{2+}	0.1 mM EGTA	5 μg/mL CBP2	pmol of phosphorylation min^{-1} (μg of protein)$^{-1}$
+	+	+	−	2.3 ± 0
+	−	+	+	675.5 ± 4.2
+	−	−	+	596.9 ± 21.6
−	+	−	+	14.5 ± 0.7
−	−	−	+	8.9 ± 0.3
+	+	−	+	355.4 ± 2.4

[a] Values are the average of duplicate incubations ± SEM.

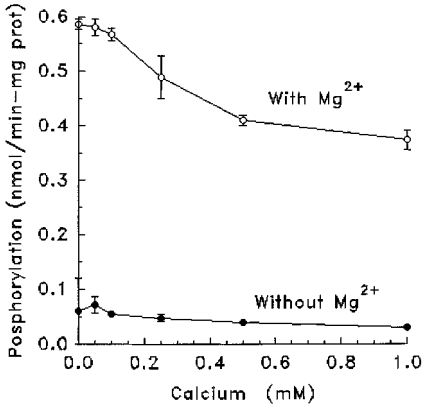


FIGURE 8: Effect of Ca^{2+} alone and with 5 mM Mg^{2+} on the phosphorylation of CBP2 without added CK2.

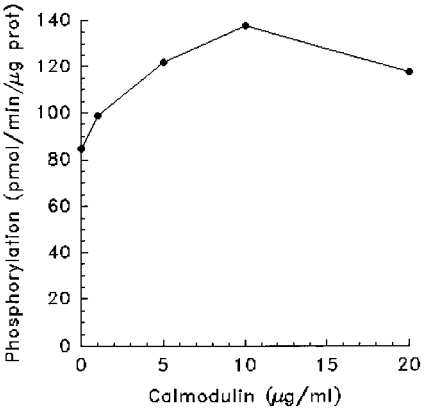


FIGURE 9: Effect of varying concentrations of calmodulin on the phosphorylation of CBP2 without added CK2. The incubation mixture contained 50 μM Ca^{2+} and 5 mM Mg^{2+}. The samples were incubated for 5 min.

Furthermore, highly active inhibitors of these two protein kinases had no effect on the phosphorylation.

The phosphorylation did not affect the Ca^{2+} binding of either CBP1 or CBP2 (data not shown). Similarly, treatment of CBP2 with alkaline phosphatase had no effect on the Ca^{2+} binding (data not shown). These data would suggest that the phosphorylation and Ca^{2+} binding are unrelated.

The phosphorylation required Mg^{2+} but was modestly inhibited by increasing concentrations of Ca^{2+} (Table 1, Figure 8). Although it is unclear what the physiological concentrations of Ca^{2+} are within the lumen of the endoplasmic reticulum, it is unlikely that they exceed those which we used in this study. Calmodulin (10 μg/mL) stimulated the phosphorylation by 63% (Figure 9). There was a small increase in phosphorylation when EGTA was added in the absence of added Ca^{2+} (Table 1). Finally, the phosphorylation was markedly inhibited by the presence of NEM ($\geq$50 μM) (data not shown), indicating that the maximal protein kinase activity requires one or more free sulfhydryl groups. GSH reversed this inhibition (data not shown). The phosphorylated CBP2 did not react with a monoclonal antibody specific for phosphotyrosine, indicating that the phosphorylation site was probably on a serine or threonine (data not shown).

DISCUSSION

These data indicate that even a highly purified preparation of CBP2 showed protein kinase activity. The inhibition of this kinase activity by heparin, its stimulation by spermine, and the ability of antibodies which are specific for CK2 to remove this activity from the preparation together strongly suggest that this protein kinase activity is due to an extremely low level of contamination by CK2. Furthermore, we found that both CBP1 and CBP2 were readily phosphorylated by this protein kinase, while calreticulin was not. Our data might suggest CK2 is found within the lumen of the endoplasmic reticulum and that CBP2 may be a physiological substrate for this protein kinase within this organelle. On the other hand, the association between these two proteins may have been fortuitous. It is possible that cytosolic CK2 could have adhered to the microsomal membranes and been washed off by the EDTA−carbonate solution used to extract the lumenal contents. The evaluation of this possibility will have to await immunolocalization studies with electron microscopy.

Our immunological studies suggesting that the protein kinase activity is catalyzed by CK2 are somewhat surprising since in these studies we observed a number of results which had suggested that CBP2 was autophosphorylated. First, on the basis of standard protein studies, our preparations appeared to be extremely pure. For example, there was no evidence of contamination as determined by silver staining of a heavily loaded SDS−polyacrylamide gel. Furthermore, we observed only a single phosphorylated band in the presence of very high specific activity [γ-^{32}P]ATP. Since CK2 autophosphorylates, it would be expected that under these conditions we should have been able to identify one or both of the subunits of this kinase on the autoradiograms. Similarly, our preparation did not phosphorylate specific substrates for any of the major proteins kinases which would have been the most likely contaminants, such as protein kinases A and C, as well as CK1, CK2, and cdc2. Furthermore, specific inhibitors of protein kinases A and C did not affect the phosphorylation. Finally, against the activity resulting from contamination by CK2, Ca^{2+}−calmodulin gave a modest stimulation of the protein kinase activity. On the other hand, it has been reported that Ca^{2+}−calmodulin does not stimulate either CK1 or CK2 activity (Meggio *et al*. 1987). These protein kinases do phosphorylate calmodulin and as a result the phosphorylated calmodulin could have stuck to the filter paper to give an apparent increase in kinase activity. Against this, CK2 is only thought to phosphorylate calmodulin in the presence of polyamines, yet these were not included in the incubations shown in Figure 9 (Meggio *et al*., 1987). Furthermore, calmodulin phosphorylation is blocked by Ca^{2+} which was included in the incubation medium in our studies (Meggio *et al*. 1987). Hence, on the basis of all of these observations, it might appear that the phosphorylation we have observed was not due to contamination by any of the common protein kinases,

including CK2. Yet it is clear that CBP1 is not a protein kinase but rather is a very excellent substrate for CK2.

Similarly, in our studies we have also found that, in spite of the presence of a motif which is supposedly characteristic of the thioredoxins and thiol:protein disulfide oxidoreductases, CBP2 has little insulinase activity. Hence, it is unlikely that its primary function is to act as a thiol:protein disulfide oxidoreductase or protein disulfide isomerase. All of these observations might raise the question as to the role of CBP1−3 in normal cellular metabolism. We feel that the body of evidence from others would suggest that the primary function of these proteins is as chaperones in the posttranslational modification of secretory and membrane proteins rather than serving as a sink for intralumenal Ca^{2+}. Furthermore, the phosphorylation by CK2 may be a critical step in this function in which it would be required to displace the nascent protein from the chaperone. Its role as a chaperone is suggested by observations from several other groups. These include the finding that the protein in humans (Hunang *et al*., 1989, 1991) and the nematode *Caenorhabditis elegans* (gene *cmo 6d3*) (Waterston *et al*., 1992) are essentially identical. It is unlikely that if Ca^{2+} binding were the primary function of this protein that the proteins would be so highly conserved. Finally, Nigam and co-workers (Nigam *et al*., 1994; Kuznetsov *et al*., 1994) and Rupp *et al*. (1994) have shown that CBP2 (ERp72) shows chaperone activity in *in vitro* assays. Our observation that CBP2 is readily phosphorylated might suggest that this activity may play an important role in the chaperone process.

Finally, several workers have examined the phosphorylation of other microsomal proteins. Ou *et al*. (1992) have reported that the chaperone calnexin is phosphorylated by GTP and that this phosphorylation can be catalyzed by CK2. These authors did not determine whether the CK2 was present in the lumen of the endoplasmic reticulum. Quemeneur *et al*. (1994) have reported that the microsomal thiol:protein disulfide oxidoreductase, most likely the isoform Q-5, also autophosphorylates. These authors did not examine the possible role of CK2 contamination of their preparation in this process. Yet, our data might suggest that this phosphorylation could also have been due to contamination of their preparation by CK2, since it would appear that essentially undetectable concentrations of this enzyme can catalyze the protein kinase activity. In light of our results, it clearly will be necessary to utilize antibody binding studies to rule out this possibility.

In summary, our data suggest that CK2 may be a major protein kinase within the lumen of the endoplasmic reticulum. It would appear that this enzyme catalyzes the phosphorylation of two intralumenal proteins, CBP1 and CBP2. It is possible that this protein kinase found in our preparation was a cytosolic contaminant of the microsomes, even though it was present in preparations which had been extensively washed before extraction of the lumenal proteins. We believe that our current study is the first to suggest that this protein kinase may be within the lumen of the endoplasmic reticulum, although we cannot definitively resolve this question without immunolocalization studies.

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