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Phosphorylation of CaBP1 and CaBP2 by Protein Kinase CK2[1]

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Several proteins in the mammalian endoplasmic reticulum are substrates for protein kinases. Many unidentified phosphoproteins from this compartment are described in the literature, and this prompted us to try to identify at least the more dominant ones. When solubilized bovine and murine microsomes were phosphorylated with protein kinase CK2 and [^{32}P]ATP and separated on SDS-PAGE, the corresponding autoradiogram showed three dominant ^{32}P-labeled proteins. These three [^{32}P]phosphoproteins were identified as calcium-binding proteins (CaBP) 1, 2, and 4 after purification on a MonoQ column followed by SDS-PAGE, proteolytic cleavage and subsequent amino acid sequencing of the purified ^{32}P-labeled peptides. All three were also phosphorylated by an endogenous kinase, found by us to be of the CK2 type. This kinase phosphorylated CaBP1 N-terminally at serine 427. Of the three proteins, only CaBP4 was previously known to be a substrate of CK2. The newly identified substrates CaBP 1 and 2 are members of the thioredoxin family and have a signal tetrapeptide in the C-terminal of the protein for retention in the ER. Serines and/or threonines in the C-terminal were phosphorylated in CaBP1 when the endogenous CK2 was used as protein kinase. A protein with the same molecular mass as CaBP1 on SDS-PAGE was phosphorylated when intact hepatocytes were grown in the presence of [^{32}P]phosphate. The *in vitro* phosphorylation with protein kinase CK2 can be used as a specific and sensitive method for identification of CaBP1, 2, and 4 in microsomes.

Key words: CaBP1, CaBP2, endoplasmic reticulum, liver, protein kinase CK2.

In eukaryotic cells, the lumen of the endoplasmic reticulum (ER) is an optimal environment for correct folding and maturation of proteins that are to be secreted, inserted into membranes or directed either to the cell surface or other cellular compartments (*1, 2*). It is well known that special proteins are required for these processes. The proteins involved in folding, called chaperones, are highly conserved proteins found in prokaryotes, plants and mammals (*3*). The lumen of ER is a comparatively oxidizing milieu allowing ER proteins to be folded by disulphide bonds with, for instance, protein disulphide isomerase (PDI) as a catalyst (*4, 5*).

A general feature of this folding system is the requirement of ATP (*6*). Recent studies have shown that the ER contains ATP concentrations in the μM range (*7, 8*). It is transported from the cytosol through the ER membrane into the lumen by a specific ATP translocator (*1, 7*). The binding of ATP to many chaperones facilitates folding and its hydrolysis is coupled to the release of the folded protein (*7, 9, 10*). The binding sites for ATP have also been utilized for the purification of several chaperones (*11*).

A less well characterised post-translational modification of chaperones is phosphorylation, which is mediated through enzyme-catalysed phosphorylation and dephosphorylation of serine and threonine residues. It triggers structural changes in proteins, thereby altering their biological properties. Some chaperones ubiquitously present in ER have been found to be phosphorylated (*12-20*). Among the best known phosphorylated chaperones are calnexin, a membrane-bound chaperone (*13-16*), and GRP94/endoplasmin, which belongs to the Hsp90 family is also known as calcium-binding protein 4 (CaBP4) (*17*), both being phosphorylated by CK2.

Several investigations dealing with proteins in ER show unidentified ^{32}P-labeled proteins after phosphorylation with [^{32}P]ATP (*13-20*). This encouraged us to perform experiments to identify at least some of the more predominant ones. We found that two resident ER proteins from cow and rat liver—calcium-binding protein 1 and 2 (CaBP1 and CaBP2) belonging to the thioredoxin family (*21*)—were phosphorylated *in vitro* by rat liver CK2. We also characterized the protein kinase in the microsomal fraction responsible for the phosphorylation of these proteins.

EXPERIMENTAL PROCEDURES

Materials—Microsomes were prepared from bovine

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Abbreviations: CaBP, calcium binding proteins; CK2, protein kinase CK2 (earlier called casein kinase II); ER, endoplasmic reticulum; ERp, endoplasmic reticulum proteins; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PDI, protein disulfide isomerase; SDS, sodium dodecylsulfate; TFA, trifluoroacetic acid.

liver (supplied by the local slaughter house), from the livers of male Sprague Dawley rats and from rat hepatocytes. Petri dishes 1008 were from Falcon. Hyper film ™MP, [γ-^{32}P]ATP and [^{32}P]orthophosphate were obtained from Amersham. LysC was from WAKO. Acrylamide and low molecular weight protein standards were from Bio-Rad. SMART, FPLC, Mono Q columns, and Sephadex G 25 were from Pharmacia. Other reagents were of at least analytical grade and obtained from commercial sources.

Preparation of Protein Kinases—Protein kinase CK2 was prepared from rat liver as described by Meggio *et al.* (*22*). One unit of protein kinase activity was defined as the amount of enzyme that transferred 1 pmol of ^{32}P-labeled phosphate to casein per min under the conditions used in Ref. *22* with 2.5 mg/ml.

Preparation of Microsomes—Approximately 100 g of bovine liver was quickly excised, rinsed and transported to the laboratory in ice-cold 0.9% (w/v) NaCl solution. For the rat material, about 10 g of rat liver was quickly excised and rinsed in the homogenization buffer (see below) at 4°C. All the following procedures were performed on ice or at 4°C.

The tissues were transferred to homogenization buffer, minced with scissors and homogenized in 4 volumes of 0.1 M Tris-HCl buffer, pH 7.4, containing 0.15 M KCl, 1 mM EDTA, and 0.4 mM phenyl methyl sulphonyl fluoride using a motor-driven glass-Teflon grind homogenizer. The homogenate was centrifuged at 10,000 × *g* for 20 min and the pellet discarded. The supernatant was filtered through glass wool and centrifuged at 100,000 × *g* for 60 min. The supernatant fraction was decanted, and the pellet was suspended in 50 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 0.1 mM dithiothreitol, and 20% (w/v) glycerol and stored at −70°C until use.

Preparation of Rat Hepatocytes—Hepatocytes were isolated by the procedure of Pertoft and Smedsröd (*23*). The cells were suspended in Dulbecco's modified Eagle's medium and supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (0.1 μg/ml). Two-milliliter portions of cell suspension containing 1×10^6 cells were added to 35-mm-diameter plastic dishes precoated with 30 μg of fibronectin. Four dishes were incubated at 37°C at 5% CO_2 for 45 min, after which the medium was renewed and the incubation continued for 18 h under the same conditions.

Incubation with [^{32}P] Orthophosphate—The cells in each dish were rinsed with 1-ml portions of medium, and after the second change the incubation was continued for an additional 40 min in the presence of [^{32}P]orthophosphate (100 μCi/dish), which was added to two of the four dishes. It has been shown previously that the intracellular pool of ATP is fully equilibrated with [^{32}P]orthophosphate after this time (*24*).

The medium was removed and the cells were washed with PBS three times, after which homogenization buffer (see above) was added and the cells were scraped off with a rubber policeman. The hepatocytes were homogenized with a small Potter-Elvehjem glass homogenizer and centrifuged for 20 min at 10,000 × *g*. The resulting supernatant was centrifuged at 100,000 × *g* for 60 min and the microsomal pellet was resuspended in SDS-PAGE sample buffer (*25*) before SDS-PAGE followed by autoradiography (see below).

Solubilization of Endoplasmic Reticulum—The suspension of endoplasmic reticulum was adjusted to an absorbance of 2.0 at 280 nm in 100 mM Tris-HCl buffer, pH 7.7, containing 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.4 mM phenyl methyl sulphonyl fluoride, and 0.8% (w/v) Lubrol PX and stirred for 30 min at 4°C (*26*). Insoluble material was removed by centrifugation at 100,000 × *g* for 60 min at 4°C. The cleared supernatant was applied to a Sephadex G 25 column, equilibrated, and eluted with FPLC-buffer A (see below).

Chromatography on Mono Q—Solubilized proteins from the endoplasmic reticulum (15–20 mg of protein) of bovine or rat liver in FPLC-buffer A (10 mM Tris-HCl buffer, pH 7.7 containing 20% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.2% Lubrol PX) were fractionated on a Mono Q HR 5/5 column using a total volume of 80 ml of a linear NaCl gradient from buffer A to buffer B (buffer A plus 1 M NaCl) (*26*). The fractions (1 ml) were analyzed for protein by UV monitoring at 280 nm and thereafter stored at −20°C for further analysis.

Gel Electrophoresis—SDS-PAGE (10% gel) was performed using a discontinuous buffer system according to Laemmli (*25*) as modified by O'Farrell (*27*). A mixture of reference proteins was applied to each gel. After electrophoresis the proteins were visualized by staining with Coomassie Brilliant Blue (R 250) and the gel was subjected to autoradiography at −70°C using intensifying screens.

Phosphorylation Assay—Phosphorylation of 25 μl of the solubilized microsomal fraction or partially purified microsomal proteins by either CK2 (100 U) or the endogenously present microsomal kinase was carried out in a total volume of 60 μl of Tris-HCl buffer, pH 7.4, containing (final concentrations) 75 mM Tris, 100 mM NaCl, 50 mM KCl, 15 mM $MgCl_2$, 5 mM dithiothreitol, 0.08% Lubrol PX, and 8% glycerol. The reactions were started by the addition of 10 μl of 37 mM [^{32}P]ATP (200 cpm/pmol) and after incubation at 34°C for 30 min, terminated by the addition of 30 μl of a 3-times concentrated SDS-PAGE buffer and boiled for 3 min. Proteins in 50 μl of this mixture were analyzed for [^{32}P]phosphate incorporation by SDS-PAGE followed by autoradiography.

Phosphorylation (total volume 14.4 ml) of 6 ml of solubilized bovine liver microsomes was performed under the conditions described above in the absence of an exogenous kinase. This incubation was terminated by filtration through a Sephadex G 25 column equilibrated with FPLC-buffer A. The void fraction was chromatographed on a Mono Q column as before. Fractions were analyzed by SDS-PAGE followed by autoradiography. The ^{32}P-labeled bands were excised and subjected to peptide mapping and the peptide containing the highest amount of [^{32}P]phosphate was sequenced.

The kinase activity in the fractions from Mono Q was assayed under the conditions given above, unless otherwise stated, with casein (1.5 mg/ml) and CaBP1 and 2 as substrates. The reaction was interrupted by the addition of 50 μl of the mixture to a Whatman 3 MM paper which was immediately transferred to a solution of cold 10% trichloroacetic acid. The filter papers were washed three times with fresh acid, dried in ethanol and the radioactivity was measured as Cerenkov radiation (*28*).

Procedure for Peptide Sequencing—Proteins were cut out from the gel and digested as described by Rosenfeld *et al.* (*29*) with trypsin or LysC. In the latter case the buffer was

100 mM Tris-HCl, pH 8.5. Extracted peptides were separated on a SMART chromatography system equipped with a μRPC C2/C18 SC 2.1/10 column. Peptides were eluted with a gradient of 0 to 50% acetonitrile both in water and 0.1% TFA over 75 min with a flow rate of 100 μl/min and their radioactivity was measured. Unless otherwise stated, [^{32}P]phosphopeptides were sequenced either on an Applied Biosystem model 470A gas-phase sequencer with an online PTH analyzer 120A or on model 476A according to the manufacturer's instructions.

Determination of Ca^{2+}-Binding—The binding of Ca^{2+} to proteins was determined using the radiochemical assay described by Hoffman and Celis (*30*).

RESULTS

Solubilized proteins from endoplasmic reticulum from bovine liver were phosphorylated with purified CK2 followed by SDS-PAGE and autoradiography. One of the predominant ^{32}P-labeled proteins (Fig. 1, lane 1) in the autoradiogram was a protein with a molecular mass of 50 kDa. The two other labeled bands had molecular masses of 70 and 90 kDa (Fig. 1, lane 1). Less phosphorylation was obtained when unsolubilized microsomes were incubated with [^{32}P]ATP (data not shown).

Solubilized bovine microsomal proteins were also incubated with [^{32}P]ATP in the absence of CK2. The predominance of the [^{32}P]phosphorylated 50-kDa protein on the autoradiogram in this case indicated the presence of a protein kinase in the microsomal preparation (Fig. 1, lane 2). The result was similar when proteins from rat liver microsomes were used in identical phosphorylation experiments (Fig. 1, lanes 3, 4, and 5). Figure 1, lane 5 also shows that heparin, a well-known inhibitor of CK2 (*31, 32*), inhibited this endogenous phosphorylation.

Therefore, solubilized microsomal proteins from bovine and rat liver were separated on a Mono Q column and eluted fractions phosphorylated with purified rat CK2 and [^{32}P]-ATP and analyzed by SDS-PAGE for ^{32}P-labeled proteins (Fig. 2). The phosphorylatable 50-kDa protein band that eluted at 350 mM NaCl from the Mono Q column was excised from the subsequent polyacrylamide gel (Fig. 2) and subjected to proteolytic digestion followed by amino acid sequencing. Two ^{32}P-labeled peptides (peptide 1 and 2 in Table I) from the 50-kDa protein from bovine liver were sequenced. The table shows that peptide 1 from bovine liver matched the amino acid sequence of both rat CaBP1 and hamster P5 in 6 of 8 residues. Peptide 2 matched the amino acid sequence of rat CaBP1 in 14 of 18 residues and P5 from hamster in 15 of 18. Peptide 3 from the rat liver microsomal 50-kDa protein was 100% homologous with CaBP1 from rat liver and matched the hamster P5 sequence in 20 of 21 residues. Thus the identity of the phosphorylated protein of 50 kDa as CaBP1—homologous with protein P5—was established.

The phosphorylatable amino acids in these experiments were located in the N- and C-terminals: one to three serines

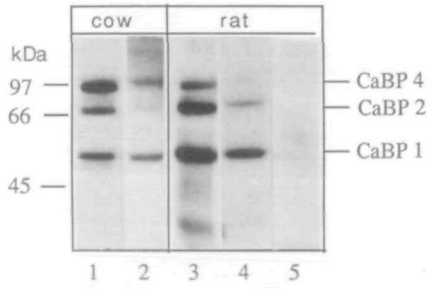

Fig. 1. ***In vitro* phosphorylation of solubilized microsomal proteins from cow and rat.** Phosphorylation of microsomal proteins was carried out in the absence (lanes 2, 4, and 5) or presence (lanes 1 and 3) of exogenous CK2 with [^{32}P]ATP. Heparin was included in the incubation mixture (lane 5). Approximately 10 μg of protein was loaded onto each lane on a polyacrylamide gel. The corresponding autoradiogram (exogenous kinase, exposure 14 h; and endogenous kinase, 48 h) shows ^{32}P-labeled proteins. The migration of molecular weight markers is indicated on the left and the migration of CaBP1, 2, and 4 is shown on the right.

TABLE I. **Comparison of sequenced [^{32}P]phosphopeptides with rat CaBP1 and protein P5 from hamster.** –, could not be determined. Microsomal proteins were phosphorylated with purified CK2 from rat liver. The ^{32}P-labeled 50-kDa protein thus obtained was digested with trypsin and the resulting [^{32}P]phosphopeptides were separated and analyzed as described in the "EXPERIMENTAL PROCEDURES." Peptides 1 and 2 were from bovine liver microsomes, whereas peptide 3 was from rat liver microsomes. Peptide 3 was also formed when CK2 purified from rat microsomes was used as kinase. The initial yield was 1-18 pmol for peptide 3 and 1 -5 pmol for peptides 1 and 2. The amino acid sequence of CaBP1 (length 431) and protein P5 were from Ref. *33*.

Substrate	Peptide sequence
CaBP1	^{20}LYS S SDDV^{27}
Peptide 1	LY- S - DDV
Protein P5	^{20}LYS S SDDV^{27}
CaBP1	^{391}RGS TAPVGGGSFPNITPR^{408}
Peptide 2	RGS TAPVGGGAFPT IS - R
Protein P5	^{391}RAS TAPVGGGSFPA ITAR^{408}
CaBP1	^{408}REPWDGKDGELPVEDD IDLSD^{428}
Peptide 3	REPWDGKDGELPVEDD IDLSD
Protein P5	^{408}REPWDGRDGELPVEDD IDLSD^{428}

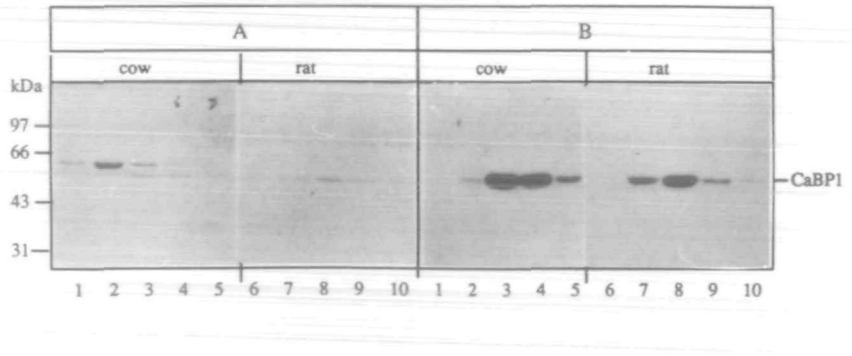

Fig. 2. **Identification of CaBP1 in fractions eluted from Mono Q by *in vitro* phosphorylation with rat CK2.** Mono Q fractions obtained from liver microsomes were *in vitro* phosphorylated by rat CK2 and [^{32}P]ATP. Panel A: Coomassie-stained proteins after SDS-PAGE. Approximately 3 μg of protein was loaded onto lanes 1-5 and 5 μg onto lanes 6-10. Panel B: The corresponding autoradiogram (exposure 7 h). The migration of molecular weight markers is indicated on the left and the migration of CaBP1 is shown on the right. For details see "EXPERIMENTAL PROCEDURES."

corresponding to position 22-24 (peptide 1), serines 393, 406 and threonine 394, 404 (peptide 2), and serine 427 (peptide 3).

In another experiment solubilized bovine liver microsomal proteins were phosphorylated with [^{32}P]ATP in the absence of exogenous kinase before their separation on the Mono Q column. One ^{32}P-labeled peptide from the ^{32}P-labeled 50-kDa protein which eluted at 350 mM NaCl was sequenced. This peptide, ^{391}RGSTAP^{396}, was identical to a part of peptide 2 in Table I. The presence in the microsomes of a protein kinase capable of phosphorylating CaBP1 was thus confirmed.

The Mono Q chromatograms were analyzed for protein kinase activity using casein as substrate. Two peaks of kinase activity were found eluting at 80 and 400 mM NaCl. The fractions with highest activity were incubated with CaBP1 from the same chromatogram and [^{32}P]ATP followed by SDS-PAGE and autoradiography. The casein kinase that eluted ahead of CaBP1 did not phosphorylate CaBP1 and was probably of the CK1 type, since its phosphorylation of casein and CaBP1 was not inhibited by heparin (data not shown). The endogenous protein kinase in the second peak phosphorylated CaBP1 (Fig. 3, lane 3) and was eluted after CaBP1 with 400 mM NaCl, in the same fraction as the 70-kDa ^{32}P-labeled protein (see Fig. 3). CaBP1 and the 70-kDa protein competed for the protein kinase, as the phosphorylation of the 70-kDa protein decreased in the presence of CaBP1 (Fig. 3, lane 3). The incorporation of phosphate was 0.52 and 0.14 mol [^{32}P]-phosphate/mol CaBP1 and 2, respectively, after incubation with this kinase for 2 h, and the reactions were still linear after this time. Serine 427 in CaBP1 was the phosphate-accepting amino acid, determined as described in "EXPERIMENTAL PROCEDURES." The [^{32}P]phosphate incorporation was in the same order when exogenous protein kinase CK2 was used. The protein kinase was of the CK2 type, as the phosphorylation of rat CaBP1 was decreased to approximately 30% in the presence of heparin (Fig. 3, lane 5). Similar results were obtained when proteins from bovine liver microsomes were used in identical experiments (data not given). When unlabeled GTP, which is a substrate for CK2, was added to the phosphorylation mixture, the incorporation of [^{32}P]phosphate into CaBP1 decreased (Fig. 3, lane 4), also supporting the theory that it was a protein kinase of CK2 type.

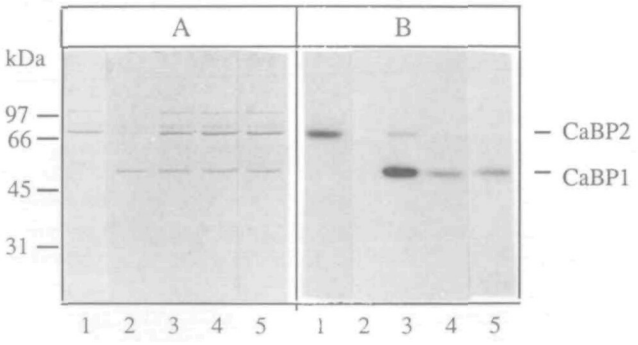

Fig. 3. **Characterization of the kinase responsible for the phosphorylation of CaBP1.** Panel A: Coomassie-stained proteins (about 2 μg) after SDS-PAGE. Panel B: The corresponding autoradiogram (exposure 6 h). Solubilized proteins from rat microsomes were separated on a Mono Q column as described in "EXPERIMENTAL PROCEDURES." Lane 1, the endogenous phosphorylation of the fraction containing both CaBP2 and CK2. Lane 2, the CaBP1-containing fraction phosphorylated in the absence, and lane 3, in the presence of the CK2-containing fraction. Lane 4, the phosphorylation of CaBP1 with CK2 in the presence of 0.75 mM GTP, and lane 5, in the presence of 230 nM heparin. The migration of molecular weight markers is indicated on the left and that of CaBP1 and CaBP2 is shown on the right. For details, see "EXPERIMENTAL PROCEDURES."

TABLE II. **Comparison of sequenced peptides from rat liver microsomal 70-kDa protein with CaBP2 from rat.** The unlabeled 70-kDa protein from rat microsomes was digested with trypsin and the resulting peptides were separated and analyzed as described in "EXPERIMENTAL PROCEDURES." Peptides 1 and 3 were from one peak and peptides 4 and 5 from another peak in the chromatogram obtained from the HPLC column. Sequence 2 was obtained after LysC cleavage of the 70-kDa protein. The initial yield was 18-30 pmol for peptides 1, 3, 4, and 5 and 2 pmol for peptide 2. The amino acid sequence of CaBP2 from rat was from Ref. *34*.

Substrate	Peptide sequence
CaBP2	^{205}KLAPEYEK^{213}
Peptide 1	KLAPEYEK
CaBP2	^{333}KFHHTFSTEIAK^{344}
Peptide 2	KFHHTFSTEIAK
Ca BP2	^{344}KFLK^{347}
Peptide 3	KFLK
CaBP2	^{507}KLKPVIK^{513}
Peptide 4	KLKPVIK
CaBP2	^{614}KFEGGNR^{620}
Peptide 5	KFEGGNR

The phosphoprotein of molecular mass 70 kDa (originating from rat microsomes) was a substrate for the kinase present in the same fraction (Fig. 3, lane 1). Trypsin and LysC fragments from the 70-kDa protein (unlabeled) were sequenced and found to be fully homologous with CaBP2/ERp 72 (see Table II). The 90-kDa protein that eluted with 450 mM NaCl was also phosphorylated with the endogenously present protein kinase (see Fig. 1 and data not given) and was identified by sequences from two unlabeled peptides (RFQNVAK and KAQAYQTGK) as CaBP4/endoplasmin, a known substrate of CK2 (*17, 35*).

To investigate whether phosphorylation of CaBP1 could

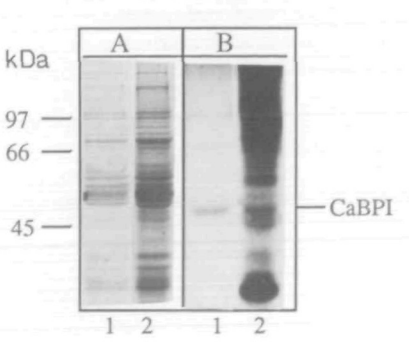

Fig. 4. **Phosphorylation of CaBP1 in hepatocytes *in vitro* and *in vivo*.** Microsomes from hepatocytes (2×10^6) were solubilized and the proteins phosphorylated by endogenously present protein kinase and [^{32}P]ATP before separation on polyacrylamide gel in SDS (lane 1). Hepatocytes (2×10^6) were incubated with [^{32}P]orthophosphate, after which microsomal proteins were subjected to SDS-PAGE followed by autoradiography (lane 2). Panel A: Coomassie-stained polyacrylamide gel. Panel B: The corresponding autoradiogram (exposure about 10 h). For details see "EXPERIMENTAL PROCEDURES."

be detected in intact cells, isolated hepatocytes were used. First the presence of phosphorylatable CaBP1 from hepatocytes was confirmed (Fig. 4, lane 1). Then hepatocytes were incubated with [^{32}P]orthophosphate, after which microsomes were prepared and subjected to SDS-PAGE and autoradiography (Fig. 4, lane 2). A [^{32}P]phosphoprotein was found at the same distance in the gel as *in vitro* phosphorylated CaBP1 from microsomes from rat hepatocytes (Fig. 4, lanes 1 and 2).

DISCUSSION

One of the strongest ^{32}P-labeled bands on the autoradiogram following the SDS-PAGE of ER proteins from cow and rat and phosphorylation with CK2 from rat liver (Fig. 1) was the 50-kDa species. After amino acid sequencing of three tryptic [^{32}P]phosphopeptides, this protein was found from comparison with known protein or cDNA sequences to be a member of the thioredoxin family and to correspond to CaBP1 in rat. It was also nearly identical to protein P5 in hamster (*33, 36*), which is probably the CaBP1 analogue in this species (see Table I). The two other proteins of molecular mass 70 and 90 kDa in the rat microsomal fraction phosphorylated with CK2 from rat were identified as CaBP2 and CaBP4, respectively. We confirmed that they all bound Ca^{2+} in an assay in which the proteins from SDS-PAGE were transferred to a nitrocellulose filter, which was incubated with $^{45}Ca^{2+}$ and autoradiographed (data not given) (*37, 38*). The designation of these substrates derives from the order in which they elute from a Mono Q anion exchange column (*37*). So far the only known properties common to all three are that they are present in the ER-lumen, bind to calcium, have a KDEL/KEEL C-terminal tail (*17, 33, 34, 36*) and are induced in cell cultures upon stress (*2, 3, 35, 36*).

The protein kinase that phosphorylated CaBP1, CaBP2, and CaBP4 was present in the ER fraction since these proteins were phosphorylated (in that order) when solubilized microsomes were incubated with ^{32}P-labeled ATP (Fig. 1, lanes 2 and 4). For maximal phosphorylation, the proteins had to be solubilized prior to phosphorylation (data not shown), which accords with the results of Cala and Jones (*17*), who also used detergent to increase the CK2 activity (*17*). Since we detected less phosphorylation of unsolubilized microsomal proteins in the presence of added [^{32}P]ATP (data not given), we conclude that the nucleotide was not translocated over the membranes under the conditions used.

The kinase in the microsomal fraction that phosphorylated CaBP1, 2, and 4 was identified as a protein kinase of the CK2 type, for the following reasons. (1) It was eluted on chromatography of solubilized ER proteins on a Mono Q column with 400 mM NaCl, which is typical of the elution of CK2 from a Mono Q column, as shown by others (*39-41*). (2) The endogenous phosphorylation of CaBP1, CaBP2, CaBP4 (Fig. 1, lane 5, Fig. 3, lane 5) was inhibited by heparin. (3) The ^{32}P-labeling of CaBP1 was decreased when GTP was added to the phosphorylation mixture (Fig. 3, lane 4). (4) CaBP4, a protein known to be phosphorylated both *in vivo* and *in vitro* by CK2 (*17, 35*), was phosphorylated by the same microsomal kinase as CaBP1 and 2 (Fig. 1 and data not given). (5) Peptide 3 in Table I, the dominant [^{32}P]phosphopeptide when rat CaBP1 was phosphorylated with purified protein kinase from the same source, was phosphorylated on a serine (427) in a consensus sequence for protein kinase of the CK2 type.

In an experiment with intact hepatocytes, a ^{32}P-labeled protein was seen with the same mobility as CaBP1 after PAGE and autoradiography (Fig. 4, lane 2). Protein kinase CK2 could be one of the kinases that is responsible for a possible phosphorylation of CaBP1 *in vivo*. Cala and Jones (*17*) suggest the same for the phosphorylation of CaBP4 with CK2, and this was confirmed by Shi *et al.* (*35*), who demonstrated the *in vivo* phosphorylation of CaBP4 after simultaneous expression of CK2 and CaBP4 in *Escherichia coli*.

CaBP1 and CaBP2 are known to be calcium-binding proteins belonging to the thioredoxin family (*21*) with PDI activity (*33, 42, 43*), and CaBP2 has been reported to have additional functions (*44-47*). Until the physiological functions of the proteins are fully understood, it will not be possible to theorize about the physiological implications of the phosphorylations beyond the effect of the phosphorylation on the C-terminal tail of CaBP1. CaBP1 is present in the lumen of ER and has a KDEL C-terminal, which is a signal sequence for ER retention/recycling to Golgi by a receptor for this tetrapeptide (*48*). The phosphorylation of CaBP1 by the endogenously present and purified CK2 from the same source resulted in [^{32}P]phosphate incorporation (peptide 2 and 3, Table I) in the C-terminal part of CaBP1. It is generally accepted that a modification by a phosphate group at the N- and/or C-terminal ends of a protein influences the conformation and, thereby, the function of the protein. Thus, it can be hypothesized that this phosphorylation might effect the function of CaBP1, the study of which will be the aim of continued investigations. However, the *in vitro* phosphorylation with CK2 demonstrated here could be used as a sensitive tool to identify CaBP1, 2, and 4 in microsomes.

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