Calmodulin and Calmodulin-binding Proteins in Liver Cell Nuclei*

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Oriol Bachs and Ernesto Carafoli
From the Laboratory of Biochemistry, Swiss Federal Institute of Technology (ETH), CH-8092 Zurich, Switzerland

Three nuclear subfractions were prepared from isolated hepatocytes nuclei. The calmodulin content in whole nuclei was 79 ng/mg of protein. The soluble fraction obtained after digestion of the nuclei with DNase I and RNase A (S1 fraction) contained 252 ng of calmodulin/mg of protein. The pellet obtained after the digestion with nucleases was treated with 1.6 M NaCl, and the soluble fraction and the residual structures obtained after the treatment were called S2 fraction and nuclear matrix, respectively. The calmodulin contents of the S2 fraction and of the nuclear matrix were 68 and 190 ng/mg of protein, respectively. If nuclei were digested only with DNase I, the calmodulin content in the soluble fraction increased to 703 ng/mg of protein, indicating that part of the nuclear calmodulin is associated with active DNA.

Five nuclear calmodulin-binding proteins were identified. Two, having apparent molecular masses of 240 and 150 kDa were only found in the nuclear matrix, whereas the other three, having molecular masses of 120, 65, and 40 kDa were found in different proportions in all nuclear subfractions.

A calmodulin-dependent inhibition of protein phosphorylation in the S1 fraction was discovered. Purification attempts on the calmodulin-binding proteins of the S1 subfraction by calmodulin affinity chromatography yielded four major polypeptides with apparent molecular masses of about 41, 46, and 120 (two products) kDa. These polypeptides retained the ability to inhibit protein phosphorylation but not the sensitivity to calmodulin.

Calmodulin is the major Ca2+-binding protein in non-muscle cells, and is now recognized to play a central role in the processing of the Ca2+ signal (1, 2). Two major mechanisms of calmodulin action involve either a Ca2+-dependent, reversible association of calmodulin with a target protein, or the stimulation of a specific kinase which then phosphorylates target proteins. In both cases, changes in the activity of the protein depend on the modulation of the Ca2+ signal and nuclear matrix, respectively. The calmodulin contents of the S2 subfraction and of the nuclear matrix were 68 and 190 ng/mg of protein, respectively. If nuclei were digested only with DNase I, the calmodulin content in the soluble fraction increased to 703 ng/mg of protein, indicating that part of the nuclear calmodulin is associated with active DNA.

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EXPERIMENTAL PROCEDURES

Materials—All reagents used were analytical grade. Calmodulin was purified from bovine brain according to Guerin et al. (18) and iodinated as described in Ref. 19 using Enzymobeads from Bio-Rad.

Isolation of Nuclei and Subnuclear Fractions from Hepatocytes—Male Wistar rats weighing 200–250 g were killed by decapitation and the livers immediately removed and perfused with buffer A (250 mM sucrose, 5 mM MgSO4, 1 mM phenylmethylsulfonyl fluoride and 50 mM Tris-HCl, pH 7.4 at 4 °C). Nuclei were isolated according to the procedure described by Kaufmann and Shaper (20) and resuspended in buffer A containing 250 μg/ml DNase I and 250 μg/ml RNase A. After 1 h incubation, the nuclei were sedimented at 800 × g for 10 min. The supernatant was collected and named S1 fraction (or subfraction). The pellet was resuspended in 10 mM Tris-HCl, pH 7.4 (at 4 °C), 0.2 mM MgSO4, and 1 mM phenylmethylsulfonyl fluoride (buffer B). Buffer B containing 2 M NaCl was slowly added with gentle stirring to a final NaCl concentration of 1.6 M. After 15 min incubation the residual structures were sedimented at 5000 × g for 20 min. The supernatant was collected and called S2 fraction (or subfraction). The sediment was resuspended with buffer B and the last step was repeated once. In this case the supernatant was discarded and the pellet was resuspended with buffer B. This fraction was named nuclear matrix. All the steps were performed at 0–4 °C.

Assay for Calmodulin—To determine calmodulin in the nuclei and in the different subnuclear fractions, the samples were diluted in 3 volumes of 80 mM Tris-HCl, pH 7.8, containing 80 mM imidazole, 6 mM MgCl2, and 0.2 mM CaCl2. The samples were then heated at 95 °C for 5 min and rapidly cooled on ice. After centrifugation at 10,000 × g for 10 min, the supernatant was assayed for calmodulin using the activation of the calmodulin-deficient brain cyclic nucleotide phosphodiesterase as the test. The method used was that of Sharma and Wang (21).

Isolation of Plasma Membranes and Determination of the Na+/K+-ATPase Activity—Total plasma membranes were prepared from the
liver homogenates by the procedure of Bachmann et al. (22). The Na+/K+-ATPase was measured according to Sulakhe et al. (23).

**Induction of Nuclear Protein Blots with 125I-Calmodulin**—Proteins from the three nuclear subfractions were separated on Laemmli type (24) SDS-polyacrylamide slab gels (5–15% linear polyacrylamide gradient). Typically, 150 μg of protein were added to each lane. After electrophoresis the proteins were transferred electrophoretically from gels to nitrocellulose sheets at 30 V for 3 h. Nitrocellulose sheets containing the transferred proteins were preincubated in phosphate-buffered saline (140 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0) containing 2% defatted milk powder for 1 h at room temperature or overnight at 4°C in order to saturate nonspecific sites. The nitrocellulose sheets were then incubated at room temperature for 4 h in phosphate-buffered saline, 0.1% bovine serum albumin, and 125I-calmodulin (1 nM) containing 0.5 mM CaCl2 or 2 mM K-EGTA. After incubation the sheets were washed three times for 10 min in the same buffer but without calmodulin and once in phosphate-buffered saline. The nitrocellulose strips were then dried and exposed to x-ray films at −80°C with an intensifying screen.

**Affinity Chromatography Using a Calmodulin-Sepharose 4B Column**—The coupling of calmodulin to Sepharose 4B was carried out as previously described (25). A calmodulin-Sepharose column of 2 ml bed volume was equilibrated at 4°C with 10 mM Tris-HCl, pH 7.2, containing 50 μM CaCl2. CaCl2 was added to the samples to a final concentration of 50 μM. The sample was then applied to the column which was washed using 5 column volumes of 10 mM Tris-HCl, pH 7.2, containing 50 μM CaCl2. The calmodulin-binding proteins were then eluted with the same buffer containing 0.3 mM K-EGTA instead of CaCl2. The eluted proteins were lyophilized and resuspended in 500 μl of distilled water.

**Protein Phosphorylation Experiments**—Samples of nuclear subfractions (50 μg) were preincubated for 1 min at 37°C in a reaction mixture (final volume 100 μl) containing 50 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 10 mM MgCl2, and 0.2 mM K-EGTA. In some experiments 0.5 mM CaCl2 or/and 5 μM calmodulin were also added to the incubation medium. The reaction was started by adding 12 μM (final concentration) of [γ-32P]ATP. After 7 min incubation the reaction was terminated by adding 50 μl of electrophoresis sample buffer. The samples were left at room temperature for 20 min and then subjected to SDS-polyacrylamide gel electrophoresis (12% gels). The dried gels were exposed to x-ray films at −80°C. For the thio-phorylation experiments, the same procedure was used but with adenosine 5'-[γ-32P]thiotriphosphate instead of [γ-32P]ATP.

**Other Procedures**—The protein content of the fractions was measured by the method of Lowry et al. (26).

**RESULTS**

**Calmodulin Content of Nuclear Subfractions**—Purified nuclei were subfractionated as described under "Experimental Procedures," and calmodulin levels were determined in the subfractions using the stimulation of the cyclic AMP phosphodiesterase as a test system. Since calmodulin-binding proteins are present particularly in plasma membranes, particular care was taken to eliminate, or at least to minimize, cross-contamination by plasma membrane fragments. A series of three experiments in which the distribution of the classical plasma membrane marker Na+/K+-ATPase was studied gave the following results: homogenate = 24 ± 5, plasma membranes = 110 ± 9, nuclei = 2 ± 1 nmol of phosphate produced per mg of protein per min. It can thus be assumed that the nuclear membrane preparation used in the present work was essentially free of plasma membrane contamination.

As shown in Table 1, calmodulin was detected in all subfractions. The highest calmodulin content was found in the fraction obtained by DNase I and RNase A digestion (S1 fraction) (252 ng/mg of protein), whereas the lowest content was found in the fraction extracted with high salt concentrations (S2 fraction, 68 ng/mg of protein). Intermediate levels were found in the nuclear matrix (190 ng/mg of protein). The calmodulin content of whole nuclei was 79 ng/mg of protein.

To establish whether the calmodulin detected in the S1 fraction was selectively released from nuclei by one of the two nucleases used for the digestion, intact purified liver nuclei were treated with DNase I or RNase A. After DNase I digestion the calmodulin concentration was 703 ng/mg of protein. When referred to the total protein released this amount was 3 times higher than that found when both nucleases were used. This apparently paradoxical observation can be rationalized with the release of higher amounts of calmodulin-unrelated proteins by RNase A. Indeed, when RNase A was used, the concentration of calmodulin in the released medium was approximately similar to that observed using both nucleases (270 ng/mg of protein). These results suggest that nuclear calmodulin may be associated, either directly or through calmodulin-binding proteins, to active DNA. Direct binding was ruled out by applying purified DNA to a calmodulin-Sepharose 4B column: no binding of DNA was observed (data not shown). Thus, it appears likely that calmodulin is associated to DNA through calmodulin-binding proteins.

**Electrophoretic Pattern of the Proteins in the Nuclear Subfractions**—Fig. 1 shows SDS-polyacrylamide gel electrophoresis experiments on the proteins of the three nuclear subfractions. The S1 fraction did not contain histones but contained a number of proteins of molecular masses between 30 and 130 kDa. The S2 fraction was composed essentially of histones, and the main bands of the nuclear matrix, the lamins A, B, and C, migrated between 60 and 70 kDa.

**Identification of Nuclear Calmodulin-binding Proteins**—

![Fig. 1. SDS-polyacrylamide gel electrophoresis pattern of liver nuclear subfractions.](https://example.com/fig1)

**TABLE 1**

<table>
<thead>
<tr>
<th>Nuclei</th>
<th>S1 (DNase)</th>
<th>S1 (RNase)</th>
<th>S1 (RNase)</th>
<th>S2 Nuclear matrix</th>
</tr>
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<tr>
<td></td>
<td>79</td>
<td>252</td>
<td>703</td>
<td>270</td>
</tr>
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**Calmodulin levels in nuclear subfractions**

The isolation of the fractions and the conditions for the assay of calmodulin are described under "Experimental Procedures." The calmodulin concentrations are expressed in ng/mg of total protein of the fraction. Each value is the mean of three or more experiments.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; EGTA, (ethylene bis(oxyethylhienitrito)tetraacetic acid.
The binding of $^{125}$I-calmodulin to proteins of the nuclear subfractions was studied as indicated under "Experimental Procedures" in the presence of Ca$^{2+}$ or EGTA. Fig. 2 shows five nuclear proteins that bound $^{125}$I-calmodulin with high affinity in a Ca$^{2+}$-dependent way. One, of about 120 kDa, was clearly visible in the S1 fraction. Although in the particular experiment illustrated in the figure only the 120-kDa band was visible, in other experiments, additional and fainter bands of about 65 and 40 kDa could also be detected. They were apparently also present in the S2 fraction, which contained also the 120-kDa protein, although in lower amounts, in the S2 fraction and in the nuclear matrix. Two proteins, of about 240 and 150 kDa, were only detected in the nuclear matrix fraction. A number of proteins with molecular mass values corresponding to that of histones also bound $^{125}$I-calmodulin, but in this case the binding was not Ca$^{2+}$-dependent.

**Partial Purification of Nuclear Calmodulin-binding Proteins**—In order to purify the calmodulin-binding proteins present in the S1 fraction, samples were applied to a calmodulin-Sepharose column which was washed with excess Ca$^{2+}$ and eluted with an EGTA-containing buffer. Fig. 3 shows the SDS-polyacrylamide gel pattern of the S1 subfraction and of the fraction eluted from the affinity column with the EGTA-containing buffer (P fraction). Four major polypeptides were present in the latter, two of about 120 kDa and two of about 46 and about 41 kDa.

**Phosphorylation of Proteins in the Nuclear Subfractions**—Since calmodulin is frequently involved in protein phosphorylation and dephosphorylation processes, protein phosphorylation experiments were performed. In a first series of experiments, the autophosphorylation of proteins, here defined operationally as the phosphorylation observed in the absence of added protein kinases, in the different nuclear subfractions and the effects of Ca$^{2+}$ and Mg$^{2+}$ were studied. It was found that Ca$^{2+}$ concentrations higher than 2 mM inhibited protein phosphorylation in all three subfractions. It was also found that Mg$^{2+}$ was apparently necessary for the phosphorylation reactions, since no phosphorylation was detected in the presence of EDTA. On the basis of these results (not shown), all subsequent experiments were carried out in the absence of Ca$^{2+}$ (0.2 mM K-EGTA) and in the presence of 10 mM Mg$^{2+}$. Under these conditions (Fig. 4), six major protein bands, of about 138, 78, 65, 41, 37, and 31 kDa, became phosphorylated in the S1 subfraction. At least nine major phosphorylated protein bands, of approximately 140, 110, 72, 63, 53, 51, 41, 37, and 30 kDa, were detected in the S2 subfraction. Only two major bands, of approximately 225 and 140 kDa, became phosphorylated in the nuclear matrix. In all
Calmodulin dependence of protein phosphorylation in the S1 fraction. Details are described under “Experimental Procedures.” The phosphorylation was carried out in the presence of 0.2 mM K-EGTA (A), 0.2 mM K-EGTA and 0.5 mM Ca²⁺ (B), or 0.2 mM K-EGTA, 0.5 mM Ca²⁺, and 5 μM calmodulin (C).

Fig. 6. Effect of the purified calmodulin-binding proteins from the S1 fraction on the phosphorylation of nuclear matrix proteins. Full experimental details are described under “Experimental Procedures.” The phosphorylation was carried out in media containing 0.2 mM K-EGTA (A), 0.2 mM K-EGTA and 0.5 mM Ca²⁺ (B), or 0.2 mM K-EGTA, 0.5 mM Ca²⁺, and 5 μM calmodulin (C) in the presence (2) or absence (1) of proteins from the P fraction.

cases, the addition of the cyclic AMP-dependent protein kinase inhibitor to the incubation medium had no effect on the phosphorylation pattern (data not shown), indicating that the endogenous protein kinases responsible for the protein phosphorylation in the different subnuclear fractions were not of the cyclic AMP-dependent type.

The effect of calmodulin on the phosphorylation of the nuclear proteins was tested by adding 1 mM CaCl₂ and several concentrations of calmodulin to the incubation medium. A general inhibition of the protein phosphorylation in the S1 subfraction was observed at calmodulin concentrations higher than 0.3 μM. Fig. 5 shows the inhibition observed at 5 μM calmodulin. By contrast, no effects were observed in the S2 and nuclear matrix subfractions (data not shown).

To test whether the inhibitory effect of calmodulin on the phosphorylation of proteins in the S1 subfraction was due to the presence of a calmodulin-activated protein phosphatase or to the inhibition of the endogenous protein kinase(s) of the subfraction, phosphorylation experiments were performed on the purified calmodulin-binding proteins (P fraction). None of the four proteins became phosphorylated (data not shown). This indicates that no protein kinases were present in this fraction or, alternatively, that one (or more) of the four proteins had protein phosphatase or inhibitory activity. The role of the P fraction was then tested on the phosphorylation of nuclear matrix proteins. Fig. 6 shows that the fraction strongly inhibited the phosphorylation of the matrix proteins. The inhibition, however, was calmodulin-independent since it was observed in the presence as well as in the absence of calmodulin. When the last experiment was repeated using γ-35S-ATP, which can function as a substrate for protein kinases forming thiophosphoesters which cannot be hydrolyzed by protein phosphatases, a similar inhibition was observed (data not shown). These results indicate that the inhibitory effect produced by the P fraction was not due to a protein phosphatase.

**DISCUSSION**

Calmodulin-related research has boomed in the last few years. The role played by calmodulin in the regulation of reactions located in the cytosol, in the plasma membrane, and in the sarcoplasmic reticulum has been investigated in detail. The nucleus, by contrast, has been almost completely neglected, despite the promising indications offered by the few studies that have appeared (13–15). The presence and the role of calmodulin in the nucleus of liver cells have been the subject of the present work. When compared to the total protein, the calmodulin levels measured in whole liver nuclei were very low (79 ng/mg of protein) as compared to those in liver cytosol (890 ng/mg of protein) (27) and liver plasma membrane (1.86 μg/mg of protein) (28). However, the levels in the nuclear matrix and the fraction released by digestion with DNase I and RNase A (S1 fraction) were much higher (190 and 252 ng/mg of protein, respectively). The nuclear matrix consists mainly of the nuclear envelope, as deduced from the presence of the lamins A, B, and C as major components. The lamins are the main components of the lamina, a structure associated with the nuclear envelope at the internal side (20). Thus, it seems possible that the calmodulin present in the matrix fraction is associated to the nuclear envelope, to play a role similar to that in plasma membrane. This possibility is in line with the detection in the nuclear matrix fraction of two calmodulin-binding proteins of 240 and 150 kDa. These molecular masses correspond to those of two calmodulin-binding proteins in the plasma membrane of hepatocytes and other eukaryotic cells (7, 12, 28–30). At least two types of calmodulin-binding proteins have been detected in plasma membranes. One is the group of spectrin-like proteins, which contains two distinct high molecular mass polypeptides and appears to be involved in the binding of actin. One of the two polypeptides, of 240 kDa, binds calmodulin (31, 32). Another group is that of the 240-kDa desmocalmin-like proteins, which are associated with desmosomes and involved in the regulation of cytoskeleton organization. In
addition to calmodulin they also bind tonofilaments (11, 28). Since cytoskeleton filaments bind also to the nuclear envelope, the possibility arises that calmodulin and the calmodulin-binding proteins in the nuclear matrix are related to the binding of cytoskeleton to the cytoplasmic side of the nuclear envelope.

When purified liver nuclei were treated with RNase A, a fraction containing 270 ng of calmodulin/mg of protein was released. This amount is similar to that released when using both RNase A and DNase I (Table I) and indicates that calmodulin could be associated to nuclear ribonucleoprotein particles. However, a much higher amount of calmodulin (703 ag/mg of protein) was released when purified nuclei were digested with only DNase I (evidently, the DNase I treatment is less effective in the release of calmodulin-unrelated proteins). Since DNase I specifically digests relaxed DNA, two nuclear functions: DNA repair (16, 17) and gene expression, depict the 120-kDa components revealed by the two methods are the same. A correlation also seems probable between the binding proteins involved in these activities in the fraction detected in this fraction.

point, as well as the possible role of the nuclear calmodulin-binding proteins in DNA repair or in gene expression, directly bound to DNA since no binding of purified DNA to calmodulin-Sepharose columns was observed.

Calmodulin has been proposed to be involved in at least two nuclear functions: DNA repair (16, 33, 34) and gene expression (15, 35). Since these functions occur on decondensed chromatin, one must expect to recover the calmodulin-binding proteins involved in these activities in the fraction released by nucleases digestion (S1 fraction). Indeed, three calmodulin-binding proteins of 120, 65, and 40 kDa were detected in this fraction.

The calmodulin-binding proteins present in the S1 fraction have been partially purified by calmodulin affinity chromatography. Two of the four major polypeptides obtained migrate very close to each other in the gels (molecular mass about 120 kDa). The other two are 46 and 41 kDa. On comparing the molecular mass obtained for the calmodulin-binding proteins in the S1 fraction by the gel overlay method and by calmodulin affinity chromatography it seems likely that the 120-kDa components revealed by the two methods are the same. A correlation also seems probable between the polypeptides in the region of 40 kDa. However, no apparent correlation can be suggested between the 65-kDa polypeptide detected by the gel overlay method and the 46-kDa polypeptide obtained by affinity chromatography. This particular point, as well as the possible role of the nuclear calmodulin-binding proteins in DNA repair or in gene expression, demands as further study.

The results presented here indicate that calmodulin inhibits the phosphorylation of proteins in the S1 fraction, but not in the other nuclear subfractions. The experiments on the purified calmodulin-binding proteins of the S1 fraction, while confirming the inhibition of the phosphorylation of the nuclear matrix proteins, have revealed that the inhibition is not due to a protein phosphatase but to the presence of an inhibitor of the protein kinase(s). Moreover, under these conditions, the inhibition is not calmodulin-dependent. One possible explanation for this unexpected observation is the loss of a putative calmodulin-dependent inhibitor, or of the sensitivity to calmodulin of this putative inhibitor, during the purification steps.

REFERENCES