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Development of a Radioimmunoassay for Quantitation of Calregulin in Bovine Tissues†

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ABSTRACT: Experimental conditions are described for a convenient and simple one-step method for radioimmunoassay (RIA) of the calcium binding protein calregulin [Waisman, D. M., Salimath, B. P., & Anderson, M. J. (1985) *J. Biol. Chem. 260*, 1652–1660]. The radioimmunoassay utilizes ^{125}I-labeled calregulin and pansorbin cells (*Staphylococcus aureus*) coated with rabbit anti-calregulin antibody. Binding equilibrium is attained in 30 min, and the total time of the assay is 1 h. The assay is sensitive to about 30 fmol of calregulin. Calregulin was quantitated in various bovine tissue extracts and was detected in all tissues except erythrocytes. It was present in particularly high amounts in pancreas (540 μg/g of tissue), liver (375 μg/g of tissue), and testis (256 μg/g of tissue). While about 80% of the total tissue calregulin is soluble, about 20% of this protein was found to be associated with particulate fractions. Unmasking of particulate calregulin required the presence of nonionic detergent. Gel permeation chromatography of bovine brain 100000*g* supernatant in the presence or absence of calcium has resolved a single peak of calregulin by RIA. In addition, the distribution of calregulin in the soluble or particulate fraction of bovine brain was unaffected by the presence or absence of calcium during homogenization, suggesting that calregulin does not form either calcium-dependent or calcium-independent association with soluble or particulate proteins. These results identify calregulin as a major tissue Ca^{2+} binding protein.

The development of techniques to allow quantitation of changes in cytosolic free Ca^{2+} concentrations with varied stimuli has catalyzed a resurgence of interest in the Ca^{2+} second messenger system. Information obtained from intracellular Ca^{2+} indicators such as aequorin (Shimomura et al., 1962) and quin 2 (Tsein, 1980) has provided solid evidence for a second messenger role of Ca^{2+} in many cellular processes. Critical to an understanding of the second messenger function of Ca^{2+} is the elucidation of changes in cytosolic free Ca^{2+} concentration into cellular activation. Considering the millimolar Mg^{2+} concentration of the cytosol (Rink et al., 1982), a prerequisite for the demonstration of a cytosolic calcium binding protein is the binding of calcium with a micromolar dissociation constant in the presence of millimolar Mg^{2+}.

We have begun a systematic analysis of the 100000*g* supernatant of several tissues for calcium binding proteins (Waisman, 1983; Waisman et al., 1983, 1985a,b). Our experimental approach has involved chromatography of tissue 100000*g* supernatant on DEAE-cellulose and analysis of resultant fractions for calcium binding activity by the chelex-100 competitive calcium binding activity assay (Waisman & Rasmussen, 1983). In bovine liver 100000*g* supernatant, we

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identified four major peaks of calcium binding activity, and recently we have purified and characterized a protein, named calregulin (calcium regulated protein), responsible for one of the calcium binding activity peaks (Waisman et al., 1985a). In the presence of 3 mM $MgCl_2$ and 150 mM KCl, calregulin bound 3.0 mol of calcium/mol of protein with an apparent K_d = 0.1 μM. Immunoblotting procedures identified calregulin in a variety of bovine tissues.

One of the difficulties in working with calregulin has been the lack of a suitable assay. Presently, the chelex-100 competitive calcium binding activity assay is the only assay available to measure this protein in tissue extracts. The poor sensitivity of this assay coupled with the interference by any substance capable of binding calcium has made measurement of calregulin impossible. It was therefore important to develop a radioimmunoassay for the quantitative measurement of calregulin. Information on its tissue and subcellular distribution could provide valuable information on the physiological function of this protein.

In the first part of this paper the specificity and validity of a rapid and sensitive radioimmunoassay for calregulin are documented. The second part summarizes the concentrations of calregulin in various bovine tissues and examines the possible Ca^{2+}-dependent interaction of calregulin with other cytosolic or membrane proteins. These results suggest that calregulin is a major tissue calcium binding protein of the cytosol and appears not to form Ca^{2+}-dependent associations with other cellular proteins.

Experimental Procedures

Materials

Bovine serum albumin, gelatin, diisopropyl fluorophosphate, soybean trypsin inhibitor, leupeptin, and Sephadex G-50 were obtained from Sigma Chemical Co. Iodine-125 (100 mCi/mL, carrier free) and L-[^{35}S]methionine (800 Ci/mmol) were purchased from Amersham. Iodobeads were from Pierce Chemical Co.; pansorbin and sansorbin cells were from Calbiochem. Tissue culture materials were obtained from GIBCO; molecular weight markers and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[1] materials were from Bio-Rad.

Bovine blood and tissues were obtained from a local slaughterhouse. Calregulin was purified from bovine liver by the method of Waisman et al. (1985a). The protein concentration of purified calregulin was determined by amino acid analysis. Polyclonal antibodies were developed in rabbits, and the purified IgG fraction was obtained as described previously (Waisman et al., 1985a). Troponin C (rabbit skeletal muscle), S-100 (bovine brain), calmodulin (bovine brain), parvalbumin (rabbit skeletal muscle), and calsequestrin (rabbit skeletal muscle) were generous gifts from Dr. J. H. Wang, The University of Calgary.

Methods

Radioiodination of Calregulin. Purified calregulin was radioiodinated by the method of Markwell (1982). In brief, four Iodobeads were washed twice with 50 mM disodium hydrogen phosphate buffer, pH 7.5, and were dried on Whatman 52 paper. Four beads were added to 20 μL of ^{125}I-labeled solution (2 mCi), diluted with 400 μL of the phosphate buffer, and left at room temperature for 5 min. To the preloaded Iodobeads was added 12 μg of calregulin, and the reaction mixture was incubated for 10 min. The products were transferred directly to a Sephadex G-50 column (1 × 40 cm) preequilibrated in 50 mM sodium phosphate, pH 7.5, containing 0.1% each of sodium azide and gelatin (assay buffer). The major peak of radioactivity in the void volume was collected and used as tracer in the RIA. Autoradiographs of sodium dodecyl sulfate–polyacrylamide gels showed that the iodinated protein comigrated with unlabeled bovine liver calregulin.

Coating of Anti-calregulin Antibodies on Pansorbin Cells. To the 10% (w/v) suspension of pansorbin cells (*Staphylococcus aureus*) was added 1 volume (10 mL) of purified IgG fraction of anti-calregulin antiserum and thoroughly mixed on an end to end shaker for 1 h at room temperature. The suspension was centrifuged at 1000*g* for 10 min, and the pellet of pansorbin cells was washed 3 times with 5 volumes of assay buffer. The washed pellet was resuspended in the assay buffer at a final concentration of 1% w/v. The suspension was stored at 0–4 °C.

Sample Preparation. Tissues to be assayed were collected on ice and homogenized at 0–4 °C in 5 volumes of extraction buffer containing 40 mM Tris-HCl (pH 7.5), 0.1 mM DFP, 1 mM EDTA, 1 mM DTT, and 5 μg/mL soybean trypsin inhibitor with a Brinkman polytron homogenizer (5 × 30 s bursts using a PT-10 generator at a setting of 5). The homogenates were centrifuged at 100000*g* for 60 min, and the supernatant were collected for total protein and calregulin quantitation. Bovine tissues were also homogenized in the extraction buffer containing 0.1% Triton X-100 (the EDTA-nonextractable fraction).

For preparation of 100000*g* supernatant of erythrocytes, fresh bovine blood was collected with EDTA (1 mg/mL) at 0–4 °C. The erythrocyte population was prepared according to the method of Kakiuchi et al. (1982). The washed erythrocyte pellet was mixed with an equal volume of extraction buffer containing 0.1% Triton X-100. The suspension was frozen and thawed 3 times and finally homogenized with a polytron as described above. The homogenate was centrifuged at 100000*g* for 60 min, and the supernatant was assayed for calregulin concentration.

Assay System. The assay system consisted of 100 μL each of diluted tracer ^{125}I-calregulin (10^4 cpm), sample or standard, and antibody-coated pansorbin cells suspended in the assay buffer. After incubation at room temperature (25 °C) for 1 h, the tubes were centrifuged at 1000*g* for 10 min. Supernatant liquids were discarded, and tubes were thoroughly wiped with tissue paper to reduce the nonspecific binding. Pellets were analyzed for ^{125}I in an LKB γ spectrophotometer. Nonspecific binding was checked either by pansorbin cells coated with preimmunized serum as above or by sansorbin cells (lack protein A) mixed with immunized serum. Nonspecific binding was always less than 100 cpm per tube. The data were analyzed by the method of Rodbard (1974) using a computer for iterative weighted linear regression analysis of logit B/B_0 vs. long calregulin concentration.

Gel Filtration of 100000g Liver Supernatants. Bovine liver was homogenized (1:2 w/v) as described previously. The homogenate was passed through a nylon sieve, and the filtrate was centrifuged at 100000*g* for 60 min. The clear supernatant was filtered through 0.2-μm Millipore filter, adjusted to 0.2 mM $CaCl_2$, and loaded on a TSK-3000 column (LKB) preequilibrated with 40 mM MOPS, pH 7.1, containing 0.5

[1] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; ICBP, vitamin D dependent intestinal calcium binding protein; DFP, diisopropyl fluorophosphate; TN-C, troponin C; RIA, radioimmunoassay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

mM DTT, 0.15 M NaCl, and 0.2 mM $CaCl_2$. Fractions (2 mL) were collected with the same buffer and were monitored for absorbance at 280 nm. Each fraction was analyzed for calregulin immunoreacivity as described previously. The peak of immunoreactivity was pooled and was analyzed by immunoblot of a SDS–polyacrylamide gel as described previously (Waisman et al., 1985a).

Cell Culture and Immunoprecipitation. Bovine fibroblasts (heart) were prepared as described in the previous report (Waisman et al., 1985a). The cells in the log phase were cultured in a methionine-deficient medium RPMI-1640 (containing 10% of the normal amount of methionine) and including 50 μCi/mL [^{35}S]methionine and 10% fetal calf serum. After 24-h incubation at 37 °C in a humidifed atmosphere of 5% CO_2, the medium was removed, and cells were washed 4 times with 10 mL each of 0.9% NaCl containing 100 μM methionine. The cells were then scraped from the flask and were extracted with 1 mL of 10 mM sodium phosphate, pH 7.5, 150 mM NaCl, 1 mM EDTA, 100 μg/mL soybean trypsin inhibitor, 100 μM methionine, and 1% each of Triton X-100 and sodium deoxycholate as described by Chafouleas et al. (1983). It was necessary to "preclear" the cell lysate by adding 20 μL of 10% pansorbin suspension and then removing them prior to adding antibodies to deplete the lysate of molecules which may bind to pansorbin cells alone; 0.2-mL aliquots of precleared cell lysates were incubated at 0–4 °C for 16 h with excess (10 μg) but equal quantities of preimmunized and immunized IgG fractions. Immune complexes were precipitated by addition of 20 μL of 10% (w/v) pansorbin suspension. After incubation for 30 min at 0–4 °C, the tubes were centrifuged at 1000*g* for 10 min, and the pellets were washed 5 times with 2 mL of 50 mM Tris-HCl, pH 8.3, containing 0.5 M NaCl and 0.5% Triton X-100 as described by Goding and Herzenber (1980). The washed pellets were disrupted with 100 μL of SDS–PAGE sample buffer and then centrifuged in microfuge tubes. The clear supernatants were analyzed on 12.5% SDS–PAGE by the method of Lammeli (1970), without the addition of 2-mercaptoethanol.

Other Methods. SDS–polyacrylamide gels were stained with Coomassie brilliant blue. Dried gels were fluorographed on Kodak X-omatic X-ray film to detect the radiolabeled proteins. Protein in the tissue extracts was estimated by the method of Bradford (1976) using bovine serum albumin as a standard.

Results

A rapid, simple, and sensitive solid-phase radioimmunoassay (RIA) is described for the estimation of a novel calcium binding protein, calregulin. *Staphylococcus aureus* bacteria bearing protein A have the ability to bind immunoglobulins through their complement binding fragment (Lind et al., 1970) and have been used successfully for the isolation of immune complexes in RIA (Kessler, 1975; Reddy et al., 1977; Khanna & Sharma, 1983). Specific binding of immune complexes to the staphylococci is extremely rapid, and the nonspecific binding of other proteins is low (Kessler, 1981). When ^{125}I-labeled calregulin (10^4 cpm) was incubated at room temperature for different time intervals with antibody-coated pansorbin cells, as described under Methods, equilibrium of binding was achieved in 30 min and remained unchanged even after 24-h incubation at room temperature (data not shown).

Specificity of the Assay System. To confirm the specificity of the anti-calregulin antibody, it was necessary to show that immunoprecipitation of a solution containing a mixture of radioactive cellular proteins yielded a single product. Therefore, immunoprecipitation was carried out on a cell lysate

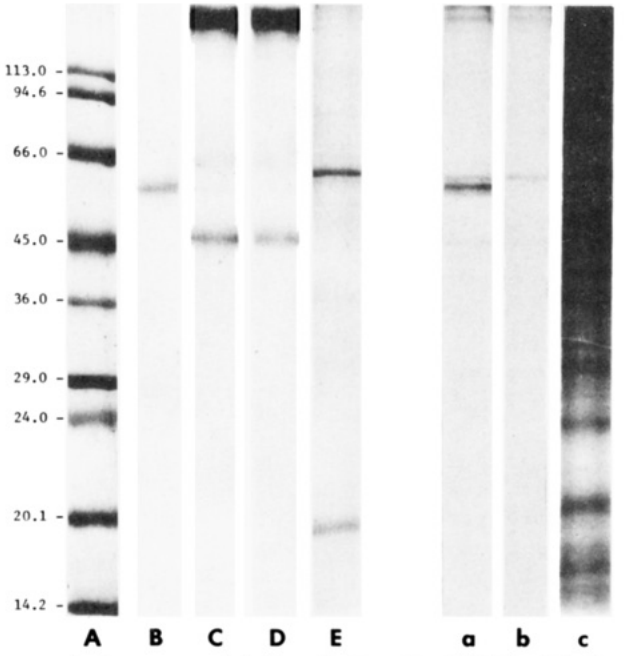

FIGURE 1: Immunoprecipitation of calregulin of [^{35}S]methionine-labeled bovine fibroblasts. 80% confluent cultures of bovine fibroblasts were incubated in methionine-deficient RPMI-1640 containing 50 μCi/mL [^{35}S]methionine as described under Methods. The cell lysate was precleared with 20 μL of 10% pansorbin cells after washing and extraction. The precleared cell lysate was incubated with immune and control IgG fractions. The immune complexes were isolated with 20 μL of 10% pansorbin cells, washed (Methods), and analyzed on 12.5% SDS–PAGE without the addition of 2% mercaptoethanol as described under Methods. Coomassie blue stained gels (lanes A–E) were fluorographed for the presence of radiolabeled proteins. Lane A, molecular weight markers; B, standard calregulin; C, immunoprecipitate with anti-calregulin antibody; D, immunoprecipitation with control IgG; E, total cell lysate of bovine fibroblasts. Lanes a, b, and c are the fluorographs of lanes C, D, and E, respectively.

of bovine fibroblast labeled metabolically with [^{35}S]methionine as described under Methods. Figure 1 describes the results of a typical experiment. Coomassie blue stained gel (lanes A–E) was fluorographed (lanes a–c) to detect the radiolabeled proteins. Lane c demonstrates the presence of many labeled proteins in the total cell lysate. Immunoprecipitation of this mixture resulted in a typical doublet pattern of calregulin (lane a). The control experiment did not show the presence of calregulin (lane b). A minor contamination observed just above the calregulin band was also observed in the control experiment (lanes a and b). Contamination by the 44 000-dalton actin polypeptide, which appears to have an affinity for immunoglobulins or immune complexes (Barber & Delovitch, 1978), was also detected in our immune complexes isolated with immunized (lane C) and preimmunized immunoglobulins (lane D).

Anti-calregulin antibody was also tested for its cross-reactivity with other known calcium binding proteins. For this, various amounts of different calcium binding proteins including troponin C (rabbit skeletal muscle), calmodulin (bovine brain), S-100 (bovine brain), paravalbumin (rabbit skeletal muscle), and calsequestrin (rabbit skeletal muscle) were allowed to compete with ^{125}I-labeled calregulin in a typical standard curve. As shown in Figure 2, none of the calcium binding proteins were able to displace the binding of ^{125}I-labeled calregulin when used at 1000-fold molar excess of calregulin. approximately 0.25 pmol of nonlabeled calregulin displaced 50% of the binding of ^{125}I-calregulin. The sensitivity of the assay is about 30 fmol. We have also demonstrated that the slope of the RIA competition curve is unchanged whether pure calregulin or

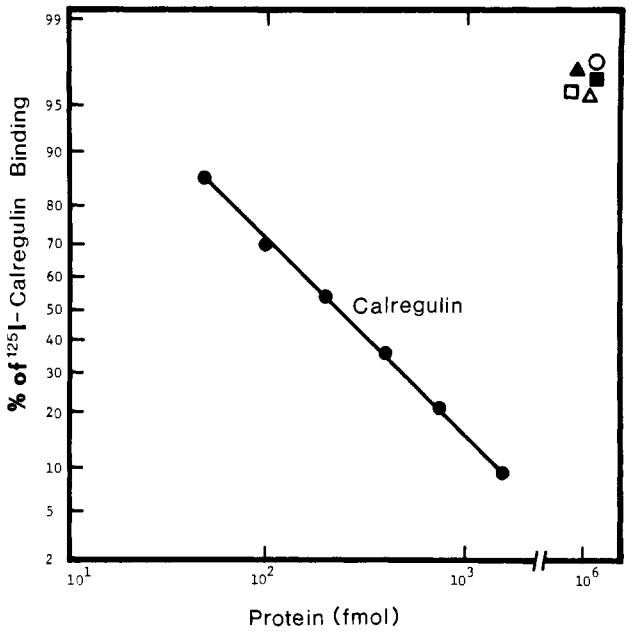

FIGURE 2: Typical logit–log curve of calregulin radioimmunoassay. Assays consisted of 100 μL each of ^{125}I-calregulin antibody-coated pansorbin cells and either test sample of the calregulin standard. After incubation for 60 min at room temperature, tubes were centrifuged at 1000*g* for 10 min, and pellets were counted for ^{125}I. Results are expressed as percentages for ^{125}I-calregulin bound to antibody-coated pansorbin cells. (○) TN-C; (△) calmodulin; (□) parvalbumin; (▲) S-100; (■) calsequestrin.

tissue 100000*g* supernatant is used as a source of antigen (data not shown).

Quantitation of Calregulin in Various Tissue and Erythrocyte Extracts. 100000*g* supernatants of various tissues and erythrocytes were prepared as described under Methods. Appropriate dilutions of tissue extracts were added to assay tubes containing 10^4 cpm of ^{125}I-labeled calregulin. The competition was initiated by the addition of 100 μL of antibody-coated pansorbin cells. After 1 h of incubation, tubes were centrifuged, and pellets were counted for ^{125}I as described under Methods. The results were analyzed by the linear regression analysis of logit B/B_0 vs. log calregulin concentration. A typical standard curve is shown in Figure 2. The typical assay has an interassay variability of 5% and an intraassay variability of 3%. As shown in Table I, calregulin immunoreactivity was detected in all the tissues tested. The pancreas, liver, lung, and testis have very high amounts (200–500 μg/g of tissue) followed by moderately high amounts (100 μg/g of tissue) in kidney, spleen, adrenals, and parathyroid. Cerebral cortex, skeletal and smooth muscles, and heart showed considerably lower amounts of calregulin (20 μg/g of tissue). We were unable to detect immunoreactivity in erythrocyte extracts.

Quantitation of Calregulin in Supernatant and Particulate Fractions of Bovine Tissues. A comparison of the distribution of calregulin in the 100000*g* supernatant and pellet of bovine liver and heart is presented in Table II. Approximately 80–85% of calregulin was measured in the supernatant fraction. A significant amount of calregulin activity remained in particulate fractions after extensive washings of the particulate material with EDTA. The particulate calregulin was unmasked by the addition of Triton X-100. These results are consistent with the data presented in Table I which suggest that the addition of Triton X-100 results in extraction of an EDTA-nonextractable form of calregulin.

Interactions of Calregulin with Particulate and Soluble Proteins. The interaction of calregulin with the particulate fraction of liver and heart is presented in Table II. The distribution of calregulin in the soluble phase is unaffected by the presence of either Ca^{2+} or EDTA in the extraction media. Furthermore, the quantity of detergent-extractable calregulin was unaffected by extensive washings with Ca^{2+}- or EDTA-containing buffers.

When the 100000*g* supernatant of liver homogenate (Methods) was analyzed by gel permeation chromatography (TSK 3000 LKB), a single peak of immunoreactivity was observed regardless of the presence of Ca^{2+} or the presence of EDTA (data not shown). The recovery of calregulin was 78%. The pooled immunoreactive material was analyzed by the immunoblot procedure. These results suggest that calregulin does not form Ca^{2+}-dependent associations with soluble or particulate proteins of the liver 100000*g* supernatant.

Table I: Calregulin Concentration in Bovine Tissues[a]

tissue	EDTA extractable μg/g of tissue	EDTA extractable μg/mg of protein	EDTA + Triton X-100 extractable μg/g of tissue	EDTA + Triton X-100 extractable μg/mg of protein
pancreas (4)[b]	420 ± 12.6[c]	5.48	540 ± 33	7.00
liver (4)	230 ± 7.36	3.12	375 ± 17	2.79
lung (3)	200 ± 9.4	3.04	197 ± 4.9	2.62
testis (2)	182	8.30	256	9.80
kidney (3)	130 ± 6.37	1.44	115 ± 3.54	1.40
spleen (3)	130 ± 9.1	1.57	100 ± 3.26	1.31
adrenals (2)	117	0.915		
parathyroids (3)	52 ± 4.5	1.89	80 ± 2.2	1.69
cerebral cortex (3)	27 ± 0.35	0.930	26 ± 0.59	0.89
skeletal muscle (2)	26	1.31	16.4	0.614
smooth muscle (2)	17	0.504	35.6	1.08
heart (4)	16.4 ± 0.27	0.38	22.8 ± 2.3	0.39
erythrocytes (2)	ND[d]		ND	

[a] Tissue 100000*g* supernatants were prepared in the presence of 1.0 mM EDTA and in the presence or absence of 0.1% Triton X-100 as outlined under Methods. [b] Number of determinations is shown in parentheses. [c] Values are mean ± SEM. [d] Not detectable.

Table II: Subcellular Distribution (Percent) of Calregulin[a]

fraction	liver +EDTA	liver +Ca^{2+}	heart +EDTA	heart +Ca^{2+}
100000*g* supernatant	65.6	71.8	61.1	57.4
first wash	17.0	15.0	13.3	13.8
second wash	4.1	3.2	5.9	6.9
Triton X-100 extracted pellet	13.3	10.1	19.7	21.9

[a] Bovine tissues (2 g) were homogenized in 8 mL of extraction buffer (40 mM Tris-HCl, pH 7.5, 0.1 mM DFP, 1 mM DTT, 5 μg/mL leupeptin, 100 μg/mL soybean trypsin inhibitor, and 0.15 M NaCl) in the presence of 1.0 mM $CaCl_2$ or 2.0 mM EDTA and centrifuged at 100000*g* for 60 min. The calregulin content of the 100000*g* supernatant was determined by RIA, the 100000*g* pellet was resuspended in 8 mL of the homogenization buffer and centrifuged at 100000*g*, and the content of calregulin in the supernatant was determined. The wash procedure was repeated, and the resultant pellet was homogenized in extraction buffer containing 0.1% Triton X-100. Values are expressed as the percent of the amount of calregulin present in the total of all the fractions and represent the results of a typical experiment.

Discussion

The chelex-100 competitive calcium binding assay (Waisman & Rasmussen, 1983) was used to identify calregulin as a major calcium binding protein of bovine liver 100000*g* supernatant (Waisman et al., 1983). Subsequently, this protein was purified and demonstrated in the immunoblots of a variety of tissue extracts (Waisman et al., 1985a). Since the physiological function of calregulin is unknown, radioimmunoassay represents the only method for the quantitation of this protein in various tissues.

The specificity of the radioimmunoassay for calregulin is clearly demonstrated. First, immunoprecipitation of metabolically labeled bovine heart fibroblast extracts resulted in a single calregulin band. Second, a 1000-fold molar excess of troponin C, calmodulin, parvalbumin, S-100, or calsequestrin failed to affect the radioimmunoassay. Third, radioimmunoassay of fractions from gel filtration chromatography of liver 100000*g* supernatant identified a single peak of calregulin. Fourth, the slope of the RIA competition curve was unchanged whether pure calregulin or tissue 100000*g* supernatant was used as a source of antigen.

Since calregulin is present at high concentrations in most tissues, it is reasonable to suggest that calregulin might be involved in a calcium-dependent process common to all tissues. The physiological importance of this protein is also suggested by its relatively high concentration in many tissues as well as by the high affinity of this protein for calcium. For example, at 1.0 μM free cytosolic calcium, we have calculated that calregulin will bind 16 μmol of Ca^{2+}/kg of liver or about 30% of the total cytosolic calcium pool (Brinley et al., 1977). Recently we have used the antibodies against calregulin for immunocytochemical localization of this protein in bovine heart fibroblasts and bovine liver slices (Waisman et al., 1985a). Antibodies for calregulin were found to decorate a system of perinuclear membranes indistinguishable from the endoplasmic reticulum. The possible regulation of endoplasmic reticular function by calregulin would be consistent with the detection of this protein by RIA in all tissues except erythrocytes, since erythrocytes are devoid of endoplasmic reticulum.

The data of Tables I and II suggest that the majority of calregulin is present in the soluble fraction. In addition, there appears to be a small but significant quantity of particulate-associated calregulin which is EDTA-nonextractable, and solubilization of this form of calregulin requires the presence of nonionic detergent (Table II). Since calregulin RIA uses antibodies raised against the soluble form of calregulin, it is likely that the particular calregulin is homologous to the soluble calregulin. Studies are currently under way to establish the identity of the particulate calregulin.

Recently, Sudhof (1984), Moore et al. (1984), and Creutz et al. (1983) have identified a heterogeneous group of Ca^{2+} binding proteins which exhibit Ca^{2+}-dependent hydrophobic interactions. Similarly, the Ca^{2+}-dependent regulatory protein calmodulin exhibits Ca^{2+}-dependent interactions with both particulate (Vandermeers, 1978) and soluble (Andreasen et al., 1981) proteins. The data presented in Table II suggests that calregulin exhibits neither Ca^{2+}-dependent or Ca^{2+}-independent interactions with particulate or soluble proteins. Therefore, it appears that calregulin does not function as a Ca^{2+}-dependent regulatory protein. It is therefore possible that calregulin might function as a calcium-regulated protein or enzyme. The development of an RIA for this protein is an important step in the elucidation of the function of this protein.

Registry No. Ca, 7440-70-2.

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