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Calcimedins: Purification and Characterization From Chicken Gizzard and Rat and Bovine Livers

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A procedure for the simultaneous extraction and purification of four calcimedins from chicken gizzard, rat liver, and bovine liver is described. These proteins bind to hydrophobic resins in a calcium-dependent manner similar to calmodulin and troponin C. The four calcimedins purified had molecular weights 67,000 (67K), 35,000 (35K), 33,000 (33K), and 30,000 (30K) as determined by SDS polyacrylamide gel electrophoresis. Their ability to bind calcium was demonstrated using the Hummel-Dreyer method. Their tissue concentration ranged between 1–4 mg/100 g wet weight in the three tissues studied. During gel filtration, calcimedins 67K and 35K, had Rf (Ve-Vo/Vt-Vo) values of 0.46 and 0.74, respectively, indicating monomeric structure. However, the 33K and 30K calcimedins had Rf values of 0.26 (molecular weights > 90,000) suggesting that they occur as subunit complexes in their native state. Antibodies raised against the 67K and 35K calcimedins showed cross reactivity suggesting possible common origin. However, peptide mapping studies showed that they are independent proteins with considerable peptide homology. Antibodies to 30/33K calcimedins did not cross-react with either 67K or 35K calcimedins. Moreover, their peptide maps were strikingly different from those of 67K and 35K calcimedins indicating that they are unique. At present, the regulatory function of this group of proteins is not clear. Indirect evidences support the possibility that they are involved in membrane associated events, such as endocytosis and secretion.

Key words: calcium-binding proteins, calcium mediation, calcimedins

Measurements of intracellular Ca^{++} levels demonstrate a transient elevation of free Ca^{++} following stimulation by a variety of humoral factors [1]. The Ca^{++} flux in turn triggers responses such as contraction, secretion, phagocytosis, glycogenolysis, and mitogenesis. The regulation of intracellular Ca^{++} has been shown to be mediated through high-affinity metal selective protein receptors [2]. Striated muscle acts as a model in Ca^{++} signal discrimination. One receptor, troponin-C, binds micromolar Ca^{++} and regulates the thin filament controlled contraction while a

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second receptor, calmodulin, provides primary regulation of glycogenolysis. The question exists as to whether there are other independent mediators of the Ca^{++} signal.

Moore and Dedman [3] identified a group of proteins from vertebrate tissues (rabbit longismus dorsi and chicken gizzard) that bind to the calmodulin inhibitor fluphenazine in the presence of Ca^{++}. They later applied the term "calcimedin" to describe these proteins that functioned in a molecular fashion similar to calmodulin and troponin-C [4]. Analogous to calmodulin and troponin-C, calcimedins become hydrophobic upon binding to Ca^{++}; however, calcimedins do not take part in any calmodulin or troponin-C mediated events and differ considerably in their molecular properties [5]. Calcium-binding proteins similar to calcimedins have been independently described in other tissues, including the electric organ of *Torpedo marmorata* [5], bovine tissues [6–8], pig lymphocyte [9], and rat adrenal medulla [10].

It is unclear at this time whether there is a molecular or functional relationship between the various reported Ca^{++}-binding proteins. The present study was undertaken to purify and characterize four calcimedins and to develop antibodies to them. Knowledge of these Ca^{++}-binding proteins may lead to a greater understanding of the mediation of intracellular Ca^{++} and the mechanisms of various pathological conditions.

MATERIALS AND METHODS

All chemicals were reagent grade. DE-52 was purchased from Whatman Lab Sales, Inc. (Hilsboro, OR); phenyl-Sepharose, aprotinin, phenyl methyl sulfonyl fluoride (PMSF) and molecular weight standards were obtained from Sigma Chemical Co. (St. Louis, MO). The source of Ultrogel AcA-44 was LKB (Gaithersburg, MD) and New England Nuclear (Boston, MA) supplied $^{45}CaCl_2$.

Tissue Preparation

Fresh chicken gizzard was obtained from Pilgrim Packing Co. (Dallas, TX) and fresh bovine liver from Freeman Packing Co. (Houston, TX). All tissues were cleaned and transferred to the laboratory in ice and were stored at −70°C until used. Rat liver was collected from freshly sacrificed animals and rapidly frozen in crushed dry ice.

Purification of Proteins

Frozen tissue (100 g) was crushed, mixed with 500 ml buffer A (40 mM Tris, pH 7.5, 75 mM NaCl, 2 mM EDTA, and 0.05% sodium azide) containing 0.5 ml aprotinun and 1.0 mM PMSF, and homogenized in a Waring Blender (3 × 10 sec) with cooling on ice between each burst. All procedures unless stated otherwise were carried out at 4°C. The homogenate was centrifuged at 27,000g for 30 min in a GSA rotor. The supernatant was filtered through −20°C chilled glass wool and dialized overnight against buffer B (10 mM Tris, pH 7.5, 1 mM EDTA, 0.025% sodium azide). The dialysate was then added to 120 g of DE-52 equilibrated in buffer B. The mixture was stirred gently for 30 min, then filtered through a Buchner funnel. The resin was washed with buffer B until O.D. 280 of the filtrate was < 0.05. Further washing was carried out with buffer B containing 100 mM NaCl until O.D. 280 was

< 0.05. The slurry was then mixed with 400 ml of buffer B containing 500 mM NaCl, stirred for 20 min, and filtered to dryness.

We added $CaCl_2$ to the filtrate to make a final concentration of 2 mM. The mixture was degassed, brought to room temperature, and applied to a phenyl–Sepharose column (2.5 × 50 cm) (equilibrated at room temperature). The calcimedins, along with calmodulin, were eluted from phenyl–Sepharose using 4 mM EGTA according to the procedure of Dedman and Kaetzel [11]. The eluent was collected in 2-ml fractions. Protein peaks (O.D. 280) were pooled and dialyzed overnight against buffer C (10 mM imidazole, pH 6.2, containing 1 mM EDTA). The dialysate was loaded onto a DE-52 column (0.75 × 15 cm) equilibrated in buffer C. Following washing, calcimedins were eluted with buffer C containing 150 mM NaCl. Protein peaks were concentrated in sucrose and applied to an Ultrogel AcA-44 column (1.0 × 80 cm) equilibrated in buffer D (50 mM Tris, pH 7.5 containing 200 mM NaCl, 2 mM EDTA and 0.05% NaN_3) and eluted with the same buffer at a flow rate of 5 ml/hr. Fractions (1.0 ml) were collected and monitored for O.D. 280.

Polyacrylamide Gel Electrophoresis

Aliquots (50 μl) of individual fractions from Ultrogel and samples taken at various stages of purification were subjected to SDS polyacrylamide gel electrophoresis (PAGE) according to the procedure of Laemmli [12] and were stained with Coomassie brilliant blue.

Antibody Preparation

Calcimedins purified from rat liver were used for antibody production. Protein fractions eluted from ultrogel AcA-44 column (Fig. 3) were pooled. Fractions 39 through 41 were pooled for purified 30/33K calcimedin; fractions 46 through 54 were pooled for purified 67K calcimedin; and fractions 58 through 64 (not shown) were pooled for purified calcimedin 35K. Antibodies were developed in sheep and were affinity-purified according to the procedure of Dedman et al [13] on corresponding calcimedin–Sepharose columns. Purified antibodies were dialized against 100 mM sodium borate buffer (pH 8.4) containing 75 mM NaCl and stored in the same buffer containing 1.0 mg/ml BSA.

Calcium-Binding Determination

Calcium-binding to protein was determined by the procedure described by Hummel and Dreyer [14]. The rat liver calcimedins, rat testis calmodulin, and bovine serum albumin were extensively dialized in distilled water and lyophilized. Samples (0.5 mg) were dissolved in 0.2 ml of a 40 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl, 1 mM $MgCl_2$, and 2 μM $^{45}CaCl_2$, and then added to Sephadex G-25 column (0.75 × 30 cm) equilibrated and eluted with the same buffer. Aliquots (0.8 ml) were collected and radioactivity was determined in a scintillation counter (Beckman, Model LS-7500). Calmodulin and bovine serum albumin were used as positive and negative controls, respectively.

RESULTS

The sequence of chicken gizzard calcimedin 67K purification is shown in Fig. 1. DE-52 was used twice in the procedure, a batch method at pH 7.5, and column

elution at pH 6.2. Initial binding to DE-52 at pH 7.5 and elution using 0.5 M NaCl served as an early step to prepare the sample for phenyl–Sepharose. Using EGTA, the four calcimedins and calmodulin were eluted from phenyl–Sepharose, all of which bound again to DE-52 at pH 6.2. Partial separation of 30K and 33K calcimedins from 35K and 67K calcimedins could be accomplished by eluting the column first with 50 mM NaCl, followed by 150 mM NaCl, but for routine purification, the column was eluted directly with 150 mM NaCl and further separation of calcimedins was accomplished on Ultrogel AcA-44. Calmodulin was not eluted from DE-52 until 300 mM NaCl was applied.

Gel (Ultrogel AcA-44) filtration profiles of the calcimedins from chicken gizzard, rat liver, and bovine liver are shown in Figure 2. In general, there were three peaks of optically dense material eluting from the column with Rf (Ve-Vo/Vt-Vo) values of 0.26, 0.46 and 0.74. SDS-PAGE of individual fractions (Figs. 3a–c) showed interesting aspects of the proteins. Proteins with Rf = 0.46 and Rf = 0.74 displayed

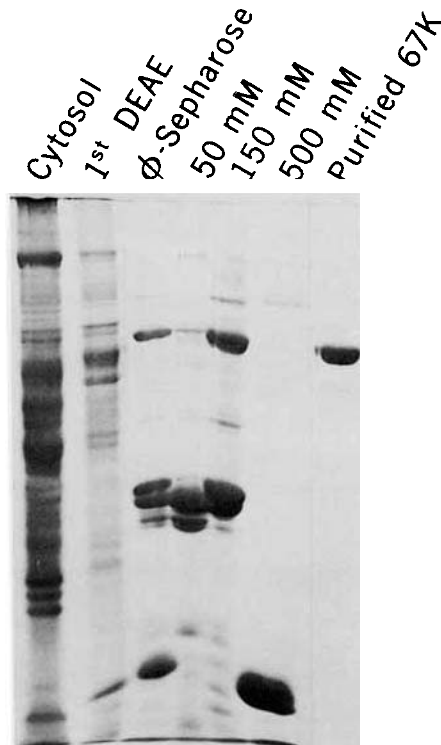

Fig. 1. SDS gel analysis of calcimedin (67K) purification from chicken gizzard. Aliquots of samples at different stages of 67K calcimedin purification were analyzed on SDS-polyacrylamide gels (12%) according to the procedure of Laemmli [12]. The fractions were: Cytosol, 27,000g supernatant from chicken gizzard homogenates; 1st DEAE, the cytosol fraction was added to DE-52 at pH 7.5 and eluted with 500 mM NaCl; ϕ-Sepharose, the DE-52 eluent was mixed with 2 mM $CaCl_2$ and applied to phenyl–Sepharose. The bound proteins were eluted with EGTA. Phenyl-Sepharose eluent was dialyzed against 10 mM imidazole buffer and added to DE-52 at pH 6.2. The bound proteins were eluted in batches with 50 mM NaCl (50 mM), 150 mM NaCl (150 mM), and 500 mM (500 mM). Purified 67K, the 150 mM eluent from DE-52 was subjected to gel filtration and the protein peak with Rf (Ve-Vo/Vt-Vo) value of 0.46 was found to calcimedin 67K.

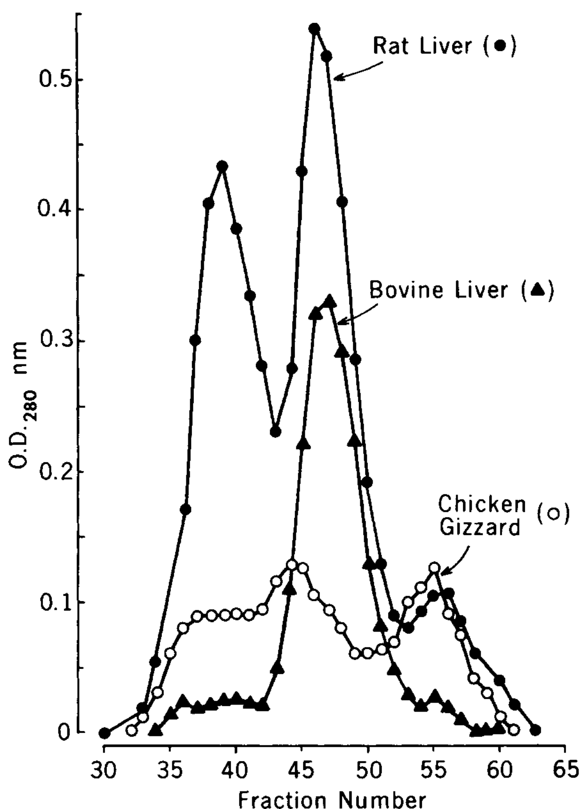

Fig. 2. Eluent profile from ultrogel AcA-44. The 150 mM NaCl eluent from De-52 was concentrated using sucrose and applied to Ultrogel AcA-44 column (0.75 × 80 cm). Proteins were eluted at a flow rate of 5 ml/hr and 1-ml fractions were collected.

the expected molecular weights of 67,000 and 35,000 on SDS gels and they were obtained with > 95% purity. However, 67K calcimedin was found to be a doublet in chicken gizzard and rat liver. The first peak of calcimedin (Rf = 0.26) had subunit molecular weights of 30,000 and 33,000 on SDS gels, indicating that they may occur as complex proteins in the native state. It is not clear if they are made of identical or nonidentical subunits. But a closer examination of this region (Fig. 3b) shows an initial high concentration of 33K with a gradual appearance of 30K proteins. This observation suggests that there are two heavy molecular weight forms, each composed of identical subunits. Support of this possibility is also evident from the differential elution pattern from DE-52 (pH 6.2) (Fig. 1). The 33K calcimedin was distributed equally between 50 and 150 mM NaCl eluents while greater than 90% of 30K calcimedin appeared in 50 mM NaCl eluent.

The profiles of the gel filtration step were consistently distinct when comparing chicken gizzard, rat liver, and bovine liver. SDS-PAGE analysis of individual fractions followed by densitometric scanning showed that each tissue possessed a unique combination of the four calcimedins. Bovine liver's profile was almost totally composed of 67K (> 93%), with 35K and 30/33K only in trace amounts. Although 67K was the predominant calcimedin in chicken gizzard (70%) and rat liver (63%), these tissues also contain 35K about (25%) and smaller amounts of the 30/33K calcimedins

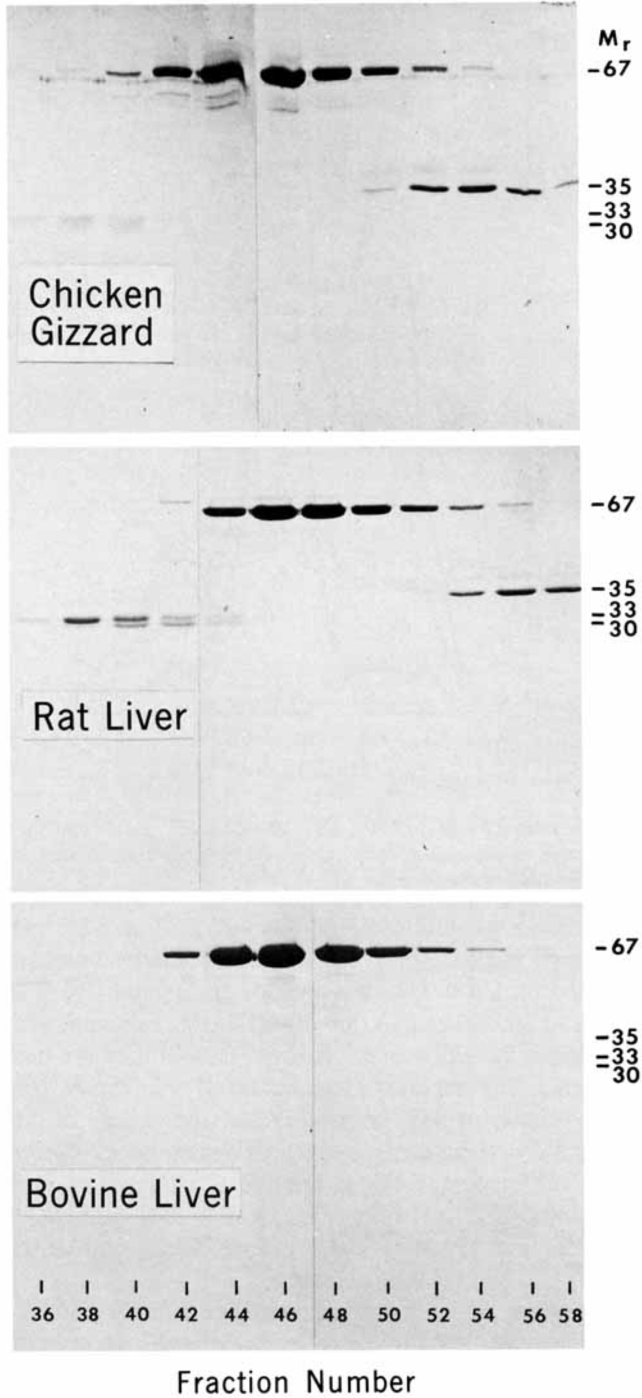

Fig. 3. SDS gel analysis of Ultrogel AcA-44 eluent fractions. Aliquots (50 μl) from individual Ultrogel (Fig. 2) fractions was subjected to SDS-PAGE (containing 12% acrylmide) according to the procedure of Laemmili [12].

TABLE I. Yields of Calcimedins From Various Tissues

	Calcimedins (mg/100 g wet wt)[a]		
Tissue	67K	35K	33/30K
Chicken gizzard	1.6	0.5	0.2
Rat liver	2.5	1.0	0.5
Bovine liver	2.8	0.1	0.1

[a]Average determined from three independent preparations with an error range of 15%.

(12%). The results of the purification of calcimedins from the three tissues are shown in Table I. The numbers shown are the averages from three separate preparations with an error bar of $\pm$ 15%.

Ca^{++} Binding

Although the purification of the calcimedins required Ca^{++}-induced binding to hydrophobic matrices, this is not a direct proof of the ability of these proteins to bind Ca^{++}. Moreover, it has been suggested that pure calcimedin cannot bind to hydrophobic columns even in the presence of Ca^{++} [23]. Therefore, experiments were designed to measure directly the ability of the calcimedins to bind Ca^{++}. The procedure selected, the Hummel–Dreyer method, is analogous to equilibrium dialysis. It involves the binding of Ca^{++} to calcimedins as they pass through a column equilibrated with $^{45}Ca^{++}$ that results in a peak of radioactivity coinciding with the protein peak. As the bound $^{45}Ca^{++}$ is removed from the solvent, there is a depletion of $^{45}Ca^{++}$ that results in a region of decreased radioactivity (trough) in the eluent. Calcimedin 67K and 35K were tested for their ability to bind Ca^{++}. Results are shown in Fig. 4. BSA did not bind to $^{45}Ca^{++}$ proving that the binding was specific for Ca^{++} binding proteins.

Immunoreaction

Aliquots from rat liver homogenates and phenyl–Sepharose purified samples were subjected to SDS-PAGE on 12% gels. The separated proteins were electrophoretically transferred to nitrocellulose and antibody affinity purified to 35K calcimedin was tested. The antibody reacted with both 67K and 35K calcimedins in phenyl–Sepharose purified samples and with two additional proteins (molecular weight = 47K and 27K) in the crude homogenate (Fig. 5). In other studies not shown, it was found that antibody affinity purified against 67K calcimedin showed a similar cross-reactivity. Antigens 67K and 35K inhibit the binding of either 67K or 35K antibodies to both 67K and 35K calcimedins. However, the 30/33K antigens blocked neither 67K or 35K antibodies and their respective antibodies did not cross-react with either 67K or 35K calcimedins. This immuno-cross-reactivity suggested that 35K and 67K calcimedin share common epitopes, whereas the 30/33K calcimedins appear to be unique.

Peptide Mapping

In order to determine whether the 35K calcimedin was a cleavage product of 67K calcimedin, peptide mapping studies were carried out on these proteins according to the procedure of Elder et al [21]. The individual protein bands from SDS gels were excised and iodinated (^{125}I) with chloramine T. The labeled proteins were exhaustively

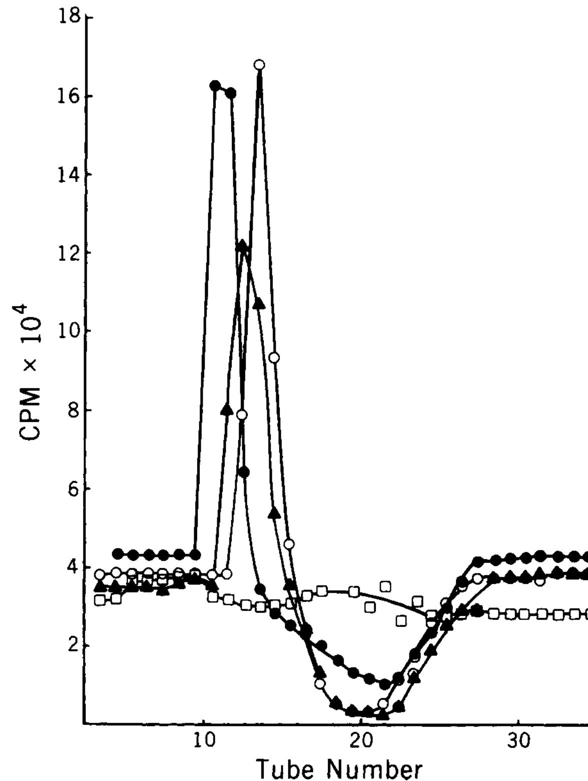

Fig. 4. Measurement of Ca^{++} binding by the Hummel-Dreyer method. Rat liver calcimedins, rat testis calmodulin, and bovine serum albumin (BSA) were extensively dialyzed against distilled water and lyophilized. Samples (0.5 mg) were dissolved in 0.5 ml of buffer containing 40 mM Tris (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$ and 2 μM $^{45}CaCl_2$ (3 $\times$ 10^4 cpm/ml) and applied to Sephadex G-25 column (0.75 $\times$ 30 cm) equilibrated and eluted with the same buffer. Aliquots (0.8 ml) were collected and measured. (●), 67K Calcimedin; (▲), calmodulin; (○), 35K calcimedin; (□), BSA.

digested with trypsin and analyzed in two dimensions on silica gel plates by electrophoresis followed by chromatography. As shown in Figure 6, the 67K and 35K calcimedins show similarities to one another sharing at least eight coincident peptides. However, the 35K calcimedin has several unique peptides not found in the 67K calcimedin maps. This finding makes it unlikely for the 35K calcimedin to have derived from the 67K calcimedin. This view is further supported by the fact that the use of several protease inhibitors other than aprotinin and PMSF in the extraction medium did not affect the ratio of 67K and 35K calcimedins. Peptide maps of 33K and 30K calcimedin were individually unique.

DISCUSSION

Recently, there have been several reports on Ca^{++} binding proteins other than calmodulin and troponin C [3,8,15,17]. These proteins were partially purified by their Ca^{++}-induced interaction with cytoplasmic membrane vesicles and subsequent solubilization with Ca^{++} chelators. The procedure described in this report is a new approach towards purification of four calcimedins. The procedure is direct, reproduc-

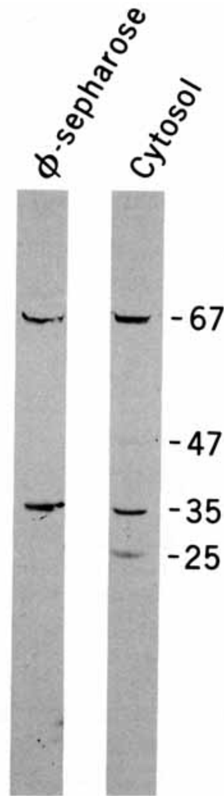

Fig. 5. Cross reactivity of the anti-35K calcimedin antibody. Aliquots from rat liver homogenates (cytosol) and from phenyl-Sepharose purified samples (ϕ-Sepharose) were subjected to SDS-PAGE on 15% gels. The separated proteins were electrophoretically transferred to nitrocellulose and affinity purified 35K calcimedin antibody was tested for its ability to bind to proteins in the two preparations.

ible, and provides good yields of all four individual proteins in a form purer than any other reported methods. Of the four calcimedins purified, two (67K and 35K) were apparently homogeneous and two (33K and 30K) were obtained as a pair. Separation of 33K-30K pair may require additional steps, such as high pressure liquid chromatography, which are currently in progress.

Molecular weights of calcimedins are comparable to those of Ca^{++} binding proteins reported by other works as judged by SDS-PAGE. These include 67K and 32.5K "calelectrins" from bovine liver, brain, and adrenal medulla [8], the 71K, 70K, 37K, and 33K "Ca^{++}-binding cytosolic proteins" from bovine adrenal medulla [15], the 68K, 33K, and 28K "Ca^{++}-dependent membrane binding proteins" identified from lymphocytes [17], and the 66K and 34K chromaffin granule binding proteins [7]. It is less likely that these proteins are related to the 63K liver calregulin [22]. However, it is premature to arrive at any conclusions before a comparative study of these proteins is made.

Gel filtration, followed by SDS-PAGE suggest that both 67K and 35K calcimedins exist as monomers in the native form. However, Rf value of 0.26 (equivalent to molecular weight of 110–130,000) of the first peak of proteins did not agree with their subunit molecular weight (33,000 and 30,000) on SDS gels. This observation suggests

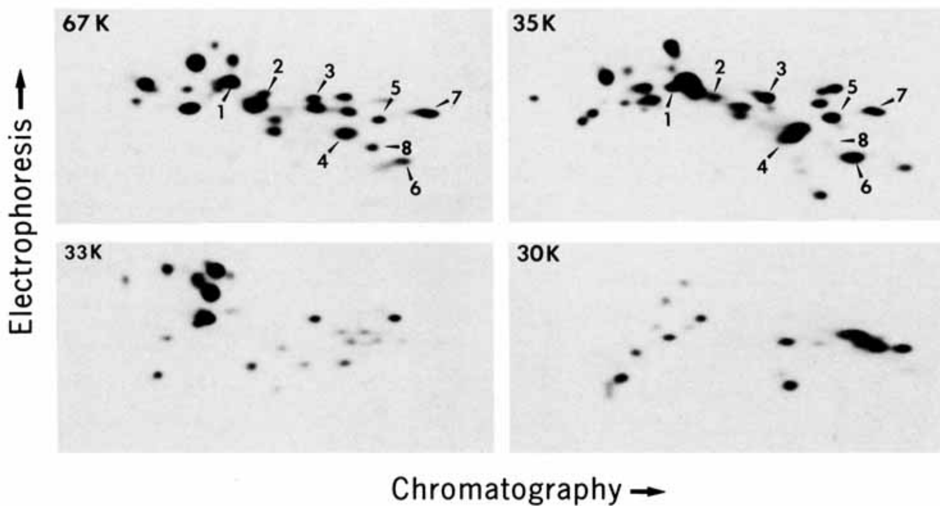

Fig. 6. Two-dimensional peptide maps. Calcimedin bands from SDS gels were excised, radioiodinated, and digested with trypsin according to the procedure described by Elder et al [21]. The digests were subjected to two-dimensional analysis on silica gel plates and analyzed by autoradiography. Of the four calcimedins, peptide maps for 67K and 35K showed several identical migrating spots that are respectively numbered.

that the 33K and 30K calcimedins occur as complex proteins in the native state. The question then arises if they are made of homologous subunits of either 33K or 30K or a combination of both. SDS gels of individual fractions show unequal distribution of these subunits (ie, initial tubes abundant in 33K with a gradual increase in 30K calcimedins. It appears from this preliminary data that two separate complex proteins composed of identical subunits of either 33K or 30K calcimedins. Further physiochemical studies, such as chemical cross-linking, are required in order to resolve the subunit composition of 33K and 30K calcimedins. Gerke and Weber [18] described a Ca^{++}-binding protein, Protein 1, with a molecular weight 85,000. It is composed of two subunits of molecular weight 36,000 and one of 10,000. Although the molecular weight of proteins with Rf value of 0.26 is very close to Protein 1, the difference in the molecular weight of subunits and the inability to detect a 10K subunit limits the possibility that they are the same proteins.

Yields of calcimedins purified by the described procedure is shown in Table I. For want of a proper assay procedure, it is difficult to measure recovery at each step of purification. Currently, calcimedins are characterized by their Ca^{++}-induced hydrophobic binding to phenyl-Sepharose columns and their behavior on SDS polyacrylamide gels. We are reanalyzing the initial concentrations and purification recoveries using immunochemical techniques.

Our results also suggest that calcimedins have a broad tissue distribution. It was present in all tissues studied including the three described in Figure 3, and others including dog liver, bovine kidney and brain, and chicken pectoralis muscle (data not shown). However, their relative amounts vary from tissue to tissue and from species to species. It is striking that for the same tissue (liver), there should exist a distinction between two species (rat and cow) in the relative ratio of the four calcimedins. Bovine liver had 93% of the total calcimedins in the 67K form, while rat liver had only 63%

of calcimedins as 67K. A study of the functional difference of livers in the two species may indirectly reveal the possible function of these proteins.

That calcimedins bind to Ca^{++} is indicated by Hummel–Dreyer method and by the Ca^{++}-dependent binding of these proteins to hydrophobic columns. Preliminary results suggest that one mole of the 67K calcimedin bind to 4–6 moles of Ca^{++} and 35K calcimedins bind to 3–4 moles of Ca^{++}. Owens and Crumpton [9] observed a single high affinity Ca^{++}-binding site (K_D 1.2 μm) for a related Ca^{++} binding protein (68K) from lymphocytes.

It is not clear at this time if these proteins are a heterogenous group in structure and function sharing a common property to bind Ca^{++} or rather if they are closely related proteins derived from gene duplication events. In fact, Geisow et al [24] have identified a consensus repeat sequence present in the calcimedins, calelectin, and lipocortin. A full answer to this issue must await a complete sequencing of all calcimedins and related proteins.

The cellular function of the calcimedins is not understood. Preliminary immunohistological studies (data not shown) show an association of these proteins with membranous surfaces of cytoplasmic vesicles and filamentous structures. Association of calcimedin-like proteins with membranes has been shown by other workers and is in fact employed as a means of purification [9,15,10]. Moreover, we observed in our comparative study of different tissues a relatively higher concentration of calcimedins in a secretory organ like the liver as compared to chicken gizzard. Based on these observations, one may hypothesize secretion as a process involving the calcimedins. The possibility also exists that the four (and possibly more) calcimedins are each specialized for particular cellular functions providing a discrete discrimination of the intracellular Ca^{++} signal.

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