An immunological and biochemical comparison of 67 kDa calcimedlin and 67 kDa calelectrin

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The 67 kDa calcimedlin is a Ca^{2+} -binding protein isolated from several muscle tissues. A recent report [Morse & Moore (1988) *Biochem. J.* **251**, 171–174] indicated that the 67 kDa calcimedlin is distinct from 67 kDa calelectrin, which is purified from various non-muscle cells. In the present study we have purified the 67 kDa protein from bovine aorta (i.e. 67 kDa calcimedlin) and liver (i.e. 67 kDa calelectrin) and compared them by immunological and biochemical criteria. The aorta calcimedlin is identical with the liver calelectrin by the following criteria. (1) The calcimedlin co-electrophoresed with the calelectrin on SDS/5–15%-(w/v)-linear-gradient polyacrylamide gels. (2) The two proteins selectively cross-reacted with a chicken gizzard calcimedlin antibody. (3) An antibody raised against the bovine aorta calcimedlin also recognized the bovine liver calelectrin. (4) One-dimensional peptide maps of the two proteins revealed no significant difference. (5) The calcimedlin appeared to have an amino acid composition essentially the same as that of the liver calelectrin. (6) The amino acid sequences of the calcimedlin fragments were identical with those of the calelectrin fragments.

INTRODUCTION

Ca^{2+} has been shown to play the role as a second messenger in a large number of intracellular events. Until recently, intracellular Ca^{2+} -binding proteins were thought to belong to a structural group similar to the EF-hand proteins [1]. Recently, several groups reported a distinct family of Ca^{2+} /phospholipid-dependent proteins [2–11]. These proteins have been purified from many tissues, such as bovine adrenal medulla, bovine lung, bovine aorta, mouse mammary epithelial cells, chicken gizzard, human macrophages, human lymphocytes, human placenta and bovine liver [2–12]. Among this family, 67–68 kDa proteins were purified from different tissues and reported as 67 kDa calcimedlin, 67 kDa calelectrin, a lymphocyte membrane-associated Ca^{2+} -binding protein, a chromobindin and protein III. It has been suggested that calelectrin is very similar to the lymphocyte 68 kDa protein [13]. Smith & Dedman [14] reported that the 67 kDa calcimedlin may also be the same as these 67–68 kDa proteins. More recently, however, Morse & Moore [15] reported that the 67 kDa calcimedlin is not identical with the lymphocyte 68 kDa protein or the 67 kDa calelectrin by immunochemical criteria. By using an affinity-purified calcimedlin antibody, they showed that the 67 kDa calcimedlin only existed in muscle cells and macrophages and did not exist in liver, thymus, spleen or thymic lymphocytes. Since interpretations based solely on immunochemical reactions may be misleading, we have purified 67–68 kDa proteins from bovine aorta smooth muscle (i.e. calcimedlin) and liver (i.e. calelectrin) and directly compared each by immunological and structural criteria. Our results show that the 67–68 kDa proteins from muscle and non-muscle cells are virtually identical.

MATERIALS AND METHODS

Materials

All chemicals were reagent grade. Q-Sepharose and phenyl-Sepharose were purchased from Pharmacia. Horseradish peroxidase-conjugated anti-rabbit IgG was a product of Cappel Co. and nitrocellulose filter paper was purchased from Millipore Co. Thermolysin was a product of Boehringer, and lysyl endopeptidase was from Wako Pure Chemicals. The Novapak C_{18} column was purchased from Waters.

Protein purification and production of antibodies

The 67 kDa calelectrin was purified from bovine liver by Ca^{2+} -dependent hydrophobic chromatography and Q-Sepharose chromatography as described previously [3]. The 67 kDa calcimedlin was purified from chicken gizzard and bovine aorta as described by Mathew *et al.* [16]. Antibodies to the gizzard calcimedlin, the aorta calcimedlin and the liver calelectrin were produced by injecting 1 mg of the proteins, emulsified in complete Freund's adjuvant, intramuscularly into rabbits. Booster shots were given three times at 2-week intervals with the antigens in incomplete adjuvant. The rabbits were bled 10 days after the last injection. Antibodies to the aorta and the gizzard calcimedlins and the liver calelectrin were individually affinity-purified by the procedure of Moore [17].

Analytical procedures

One-dimensional SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) was performed as described by Laemmli [18] on 10% (w/v) gels or 5–15% linear gels. After electrophoresis, gels were stained with Coomassie

Blue R-250 or silver nitrate [19]. Proteins were transferred from SDS/polyacrylamide gels to nitrocellulose by the procedure of Towbin *et al.* [20] and detected by using horseradish peroxidase-conjugated anti-rabbit IgG and diaminobenzidine [20].

One-dimensional peptide mapping was carried out as described by Cleveland *et al.* [21], using lysyl endopeptidase. Amino acid analyses were performed with a Beckman 6300E amino acid analyser as described previously [22]. Samples were hydrolysed with 6 M-HCl in evacuated sealed tubes for 24 h at 110 °C. Limited proteolysis of the calcimedins and the calelectrin was performed as described previously [23]. Briefly, the proteins were hydrolysed at 37 °C for 2 h, in the presence of 2 mM-CaCl₂, with thermolysin [enzyme/substrate ratio of 1:40 (w/w)]. The hydrolysed peptides were fractionated by reverse-phase h.p.l.c. on a Novapak C₁₈ column. Automated amino acid sequence analyses were done with an Applied Biosystems model 477A gas-phase sequencer, and amino acid residues were identified with an Applied Biosystems model 120A on-line phenylthiohydantoin amino acid analyser, using the manufacturer's standard programs and chemicals.

RESULTS

When bovine aorta and chicken gizzard were treated by the procedure of Mathew *et al.* [16], we obtained 98 %-pure 67 kDa calcimedins as determined by SDS/PAGE and quantitative densitometry (Fig. 1). The

Fig. 1. SDS/5–15%-PAGE of the bovine aorta calcimedin and the bovine liver calelectrin

a, the aorta calcimedin; b, the liver calelectrin; c, molecular-mass standards (phosphorylase *b*, 94 kDa; bovine serum albumin, 68 kDa; ovalbumin, 42 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa).

67 kDa calelectrin was also purified from bovine liver by the method of Sudhof *et al.* [3]. Purified 67 kDa calelectrin appears to be at least 95 % homogeneous (Fig. 1). The calcimedin was purified from bovine aorta co-electrophoresed with the liver calelectrin on 10 % uniform (Fig. 2) and 5–15 % linear SDS/PAGE gels (Fig. 1).

To determine whether the two proteins share an antigenic identity, we prepared polyclonal antibodies against bovine aorta and chicken gizzard calcimedin and bovine liver calelectrin. The proteins, transferred to nitrocellulose paper from a replicate gel, were probed with the calcimedin antibody and the calelectrin antibody as indicated in Fig. 2. A polyclonal antibody to the bovine aorta calcimedin cross-reacted with the liver calelectrin. Furthermore, an antibody against the liver calelectrin cross-reacted with the aorta calelectrin. In addition, an antibody against the chicken gizzard calcimedin cross-reacted with both the aorta calcimedin and the liver calelectrin.

The technique described by Cleveland *et al.* [21] for one-dimensional peptide mapping by limited proteolysis was used to determine whether any similarities existed between the liver calelectrin and the aorta calcimedin (Fig. 3). The digestion patterns of the two proteins using lysyl endopeptidase were essentially identical, though both proteins contained small amounts of contaminating proteins, which were detected by a sensitive silver-staining procedure [19].

The amino acid compositions of the purified proteins were analysed after HCl hydrolysis and are given in Table 1, together with the amino acid composition of chicken gizzard calcimedin. The gizzard calcimedin, the bovine aorta calcimedin and the liver calelectrin had very similar compositions.

Thermolysin digestion of the aorta calcimedin and the liver calelectrin yielded two major fragments of 33 and

Fig. 2. SDS/10%-PAGE and immunoblot analysis of the aorta calcimedin (a) and the liver calelectrin (b)

A, SDS/PAGE gels stained with Coomassie Blue; B, immunoblots with anti-(gizzard calcimedin) antibody; C, immunoblots with anti-(aorta calcimedin) antibody; D, immunoblots with anti-(liver calelectrin) antibody.

Fig. 3. One-dimensional peptide mapping of the aorta calcimedin (a) and the liver calelectrin (b)

A, non-digested protein; B, digested with 2 μ g of lysyl endopeptidase/ml; C, digested with 1 μ g of lysyl endopeptidase/ml; D, digested with 0.5 μ g of lysyl endopeptidase/ml.

Table 1. Amino acid compositions of chicken gizzard calcimedin, bovine aorta calcimedin and bovine liver calelectrin

	Composition (mol/100 mol)		
	Chicken gizzard calcimedin	Bovine aorta calcimedin	Bovine liver calelectrin
Asx	10.77	10.56	10.37
Thr	4.82	4.84	4.98
Ser	6.53	6.30	6.18
Glx	11.83	11.62	11.39
Pro	3.66	3.57	3.96
Gly	7.14	7.30	7.02
Ala	9.53	9.47	9.23
Val	5.71	6.04	5.38
Cys*	0.35	0.10	0.27
Met	2.58	2.92	2.76
Ile	5.14	5.03	5.25
Leu	9.60	11.36	10.67
Tyr	3.34	2.72	3.46
Phe	3.67	3.17	3.58
Lys	7.16	7.40	7.10
His	2.03	1.81	2.09
Arg	6.14	5.79	6.31
Trp	ND†	ND	ND

* Determined as cysteic acid after performic oxidation.

† ND, not determined.

34 kDa, as described previously [23], which were fractionated by C_{18} reverse-phase chromatography (Fig. 4). The results from the sequencing experiments carried out with the thermolysin fragments are summarized in Table 2. The analyses of the thermolysin peptide sequences derived from the aorta calcimedin and

Fig. 4. Fractionation of thermolysin peptides from the aorta calcimedin (a) and the liver calelectrin (b) on reverse-phase C_{18} columns

PAGE (10% gel) of the thermolysin peptides is shown in (c). Lane 1, thermolysin 34 kDa fragment of the calcimedin; lane 2, thermolysin 33 kDa fragment of the calcimedin; lane 3, thermolysin 34 kDa fragment of the calelectrin; lane 4, thermolysin 33 kDa fragment of the calelectrin; lane 5, molecular mass standards (phosphorylase b, 94 kDa; bovine serum albumin, 68 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa).

the liver calelectrin indicated that both proteins were identical in their primary structure.

DISCUSSION

Although a previous report [14] suggested that 67 kDa calcimedin from muscle tissue is identical with the calelectrin from non-muscle tissues, Morse & Moore [15] showed the antibody raised against chicken gizzard calcimedin does not recognize non-muscle tissue calelectrin. In addition to immunohistochemical studies, they also showed that the calcimedin is not related to the calelectrin by several biochemical criteria, such as mobility on SDS/PAGE gels, amino acid composition, isoelectric point, molecular size on gel-permeation

Table 2. Sequence similarities of the thermolysin (TH) peptides obtained from the calcimedin and the calelectrin

	Peptide	Sequence
(a)	Bovine aorta calcimedin (TH ₃₃)	VARVELKGTVRPAGDFNPDA
	Bovine liver calelectrin (TH ₃₃)	VARVELKGTVRPAGDFNPDA
(b)	Bovine aorta calcimedin (TH ₃₄)	AQGAKYRGSIRDFP
	Bovine liver calelectrin (TH ₃₄)	AQGAKYRGSIRDFP

chromatography and ability to bind to hydrophobic affinity columns [9,14,15,17,25]. However, the biochemical comparisons were made with chicken calcimedin and mammalian calelectrin and based on statements in the literature.

The objective of this work was to characterize and directly compare muscle calcimedin and non-muscle calelectrin. In the present study we have purified and compared the two proteins from bovine aorta and liver, since these tissues have been shown to contain a high level of the calcimedin and the calelectrin protein respectively. In contrast with the observations of Morse & Moore [15], the calcimedin isolated from bovine aorta co-electrophoresed with liver calelectrin on SDS/5–15 % linear-PAGE gels. Since mammalian calcimedin co-electrophoresed with mammalian calelectrin, it is not possible to distinguish the two proteins on the basis of the difference of electrophoretic mobility. In addition to the identity of electrophoretic mobility, our immunological studies clearly demonstrated that the two proteins selectively cross-reacted with a chicken gizzard calcimedin antibody. An antibody raised against bovine aorta calcimedin also recognized bovine liver calelectrin. Furthermore, an antibody to liver calelectrin cross-reacted with aorta calcimedin. From the immunological cross-reactivities, we concluded that the smooth-muscle calcimedin corresponds to the non-muscle calelectrin. One-dimensional peptide maps of the two proteins revealed no significant difference, indicating the two proteins do not differ at the level of amino acid sequence. The gizzard calcimedin appeared to have an amino acid composition essentially the same as those of aorta calcimedin and liver calelectrin. Again, since amino acid compositions of the calcimedins and the calelectrin are very similar to each other, it is not possible to distinguish the calcimedin and the calelectrin by this technique.

The amino acid sequences of the aorta calcimedin fragments were identical with those of the liver calelectrin fragments. This result suggested, in accord with the one-dimensional peptide mapping, that the two proteins have the same primary structure.

In contrast with the findings of Morse & Moore [15], our biochemical and immunological results have shown that the calcimedin and the calelectrin are virtually identical. The discrepancy between their report and our results is not clear at present. However, their conclusion that the calcimedin is a distinct protein from the calelectrin rests almost solely on immunohistochemical criteria. In addition, the biochemical evidence which appears to distinguish the two proteins is almost entirely based on the comparison between avian (i.e. calcimedin) and mammalian (i.e. calelectrin) proteins [9,14,15,17,25].

These apparent differences may only reflect the species differences of the same protein.

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