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67 k calcimedin (67 kDa) is distinct from p67 calelectrin and lymphocyte 68 kDa Ca^{2+}-binding protein

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The 67 k calcimedin is a Ca^{2+}-binding protein present in both muscle cells and peritoneal macrophages. Many tissues, including lymphoid tissues, liver and lymphocytes, have been shown to contain Ca^{2+}-binding proteins of similar molecular size, such as the p67(67 kDa) calelectrin or the 68 kDa lymphocyte protein. We have tested affinity-purified antibodies raised to the smooth-muscle 67 k calcimedin in these several tissues and here report that the 67 k calcimedin is not detectable in liver, thymus, spleen or thymic lymphocytes. These findings support recent biochemical evidence, discussed here, suggesting that the 67 k calcimedin is a protein different from calelectrin and the 68 kDa lymphocyte protein. The more limited tissue distribution of the 67 k calcimedin, which includes muscle and macrophages, suggests that the 67 k calcimedin may function in Ca^{2+}-mediated events special to these cell types. The affinity-purified antibodies to the 67 k calcimedin will be useful in obtaining information concerning the special roles of this Ca^{2+}-binding protein in these cells.

INTRODUCTION

The 67 k calcimedin is a Ca^{2+}-binding protein found in muscle tissues and peritoneal macrophages (Moore *et al.*, 1984; Moore & Morse, 1986). The protein has one high-affinity Ca^{2+}-binding site, which has higher Ca^{2+}-binding affinity than the sites in calmodulin (Moore, 1986). Binding Ca^{2+} causes the protein conformation to alter (Sohár *et al.*, 1986).

Other proteins which have been described as Ca^{2+}-binding proteins of similar size are 67 kDa (p67) calelectrin (Südhof *et al.*, 1984), found in liver, brain and many other tissues, and the lymphocyte 68 kDa Ca^{2+}-binding protein (Owens & Crumpton, 1984). It has been suggested that the calelectrin and the lymphocyte 68 kDa protein are the same (Südhof *et al.*, 1983). More recently it has been suggested that the 67 k calcimedin may also be the same as these other proteins (Smith & Dedman, 1986), and the relationships among these proteins are unresolved. However, antibodies raised to the smooth-muscle 67 k calcimedin failed to recognize the p67 calelectrin from brain (Rhoads *et al.*, 1985). We here report further evidence that the 67 k calcimedin is not identical with the lymphocyte 68 kDa protein or the p67 calelectrin. Antibodies to the 67 k calcimedin did not stain thymus or spleen, tissues which contain many lymphocytes and should be rich in the 68 kDa lymphocyte Ca^{2+}-binding protein (Owens & Crumpton, 1984), and did not stain liver, which should be rich in p67 calelectrin (Geisow *et al.*, 1984). In addition, lymphocytes isolated from suckling-mouse thymus showed no staining. By contrast, mouse peritoneal macrophages and muscle tissues treated identically with this antibody stained intensely for the 67 k calcimedin, as expected.

METHODS

Isolation of 67 k calcimedin

The 67 k calcimedin was purified and characterized as previously described (Moore *et al.*, 1984; Moore, 1986). Briefly, the isolation scheme consisted of Ca^{2+}-dependent hydrophobic chromatography of EDTA extracts from chicken gizzard on a fluphenazine–Sepharose 4B matrix, followed by DEAE-cellulose chromatography, gel-permeation chromatography and finally Ca^{2+}-dependent chromatography using phenyl-Sepharose. The extraction was performed in the presence of the proteinase inhibitors aprotinin, pepstatin, phenylmethanesulphonyl fluoride and leupeptin. The protein was stored in 0.075 M-NaCl/0.04 M-Tris/HCl/1 mM-EDTA, pH 7.3.

Isolation of 67 k calcimedin antibody

Sheep (at Bethyl Laboratories, Montgomery, TX, U.S.A.) were immunized with purified calcimedin emulsified in Freund's complete adjuvant. Immune sera were chromatographed on an affinity matrix prepared by coupling purified 67 k calcimedin to CNBr-activated Sepharose 4B (Pharmacia Product Use Booklet) (Moore, 1988). After equilibrating the column with 0.075 M-NaCl/0.04 M-Tris/HCl/1 mM-EDTA buffer, pH 7.3, 20 ml of serum was added to the column. The non-adsorbing protein eluate was collected and stored at 4 °C. The column was washed with 5–10 vol. of borate/saline buffer containing 100 mM-H_3BO_3, 25 mM-$Na_2B_4O_7$ and 75 mM-NaCl, pH 8.0, followed by 5 vol. of 0.2 M-glycine, pH 6.0. Antibody which bound to the antigen-coupled matrix was eluted with 0.2 M-glycine, pH 2.7. The pH was adjusted immediately to 7.0 with 2 M-Tris (Sigma). The pooled antibody fractions were dialysed against several changes of borate/saline buffer and stored at 4 °C. The antibody is able to recognize calcimedins from diverse species (Moore *et al.*, 1984; Moore & Morse, 1986; Moore, 1988).

Immunofluorescence staining

Hamster and mouse tissues were excised, minced, and fixed in 2.8% formaldehyde in Dulbecco's phosphate-buffered saline (DPBS) minus Ca^{2+} for 30 min. The

Abbreviations used: 67 k calcimedin, 67 kDa calcimedin; p67 calelectrin, 67 kDa calelectrin.

tissues were frozen in O.C.T. (Lab-Tek Division, Miles Laboratories, Naperville, IL, U.S.A.) cryostat medium and 4 μm sections were cut at -60 °C with an International Equipment Co. cryostat (Luna, 1968). Thymus sections were prepared as impression smears (Underwood, 1981) and fixed with 2.8% (w/v) formaldehyde in DPBS.

Thymocytes were prepared by a modification of the method of Dausset (Mishell & Shiigi, 1980). Briefly, thymuses from 5–7-day-old suckling mice (Balb/c or ICR Swiss background) were washed in Hanks buffered salt solution (HBSS), placed in 60 mm-diameter culture dishes (Corning, Corning, New York, NY, U.S.A.) with 1–2 ml of culture medium [Dulbecco's modified Eagle's medium with 10% (v/v) fetal-bovine serum], and teased apart with fine forceps. The disaggregated cell suspension was transferred to a 15 ml conical polypropylene centrifuge tube (Corning), an additional 5 ml of culture medium was added, and the epithelial debris was allowed to settle for 5–10 min. The supernatant fluid was transferred to a second centrifuge tube, and the thymocytes were pelleted at 500 ***g*** for 10 min. The pellet was washed once with HBSS and resuspended in culture medium. Cells were >90% viable on the basis of Trypan Blue dye exclusion. The thymocytes were washed twice by centrifugation in a Fisher Microfuge (model 59A) at approx. 1500 ***g***, followed by resuspension into Ca^{2+}- and Mg^{2+}-free DPBS. The pelleted cells were then resuspended in 2.8% formalin in DPBS and fixed for 30 min with occasional mixing to resuspend. The fixed cells were centrifuged and washed three times with DPBS. Indirect immunofluorescent staining was performed in suspension, using the staining protocol described below for cells on coverslips but washing the cells by centrifugation and resuspending in DPBS.

For macrophage staining, peritoneal cells from young adult mice (Charles River, Kingston, NY, U.S.A.) were recovered in Ca^{2+}- and Mg^{2+}-free HBSS by sterile peritoneal lavage, washed in Ca^{2+}- and Mg^{2+}-free HBSS, and suspended at a concentration of $(1–2) \times 10^6$/ml in complete modified Eagle medium with 10% fetal-bovine serum. To purify the macrophages, cells were plated on to sterile 22 mm-diameter coverslips (#1 thickness) (Corning) in 35 mm-diameter tissue-culture dishes (Corning) and macrophages were allowed to attach for at least 2 h at 37 °C (Moore & Morse, 1986; Morse & Moore, 1987). Coverslips were then washed three times with DPBS to remove non-adherent cells, and fixed in 2.8% formaldehyde in DPBS for 30 min. The fixed cells were washed and treated with normal goat serum for 30 min at 37 °C to block possible non-specific antibody binding to F_c receptors.

Affinity-purified 67 k-calcimedin antibody (Moore & Morse, 1986; Moore, 1988; Morse & Moore, 1987) was used to detect the 67 k calcimedin by indirect immunofluorescence. Tissues on glass slides or cells on coverslips were treated with primary anti-(67 k calcimedin) for 60 min at 37 °C. After three washes with DPBS for 20 min each, fluorescein isothiocyanate-conjugated rabbit antisheep immunoglobulin (Boehringer-Mannheim, Indianapolis, IN, U.S.A.) was added and the cells were incubated for 30 min at 37 °C. The slides or coverslips were again washed three times with DPBS and mounted, and immunofluorescence was observed under a Nikon fluorescence microscope with epifluorescence using the fluorescein isothiocyanate interference filter. Photographs were made on Kodak Tri-X film using a 20× or 40× Neofluor objective for 28 s at ASA 400.

RESULTS

Thymus from adult mice and hamsters showed no staining for 67 k calcimedin when antibody against the 67 k calcimedin was used. Spleen sections were also prepared from both species. Again, neither mouse nor hamster spleen tissue showed positive immunofluorescent staining.

In order to corroborate the findings with tissue slices, primary thymocytes, prepared as described in the Methods section, were also tested. As shown in Fig. 1(*a*), there was little if any staining above background in the thymocyte preparation. As a control, mouse macrophages were stained with the antibody preparation used in staining the other tissues, under similar conditions. Fig. 1(*b*) shows that the peritoneal macrophages stained intensely for 67 k calcimedin. Mouse macrophages were previously demonstrated to contain 67 k calcimedin (Moore & Morse, 1986; Morse & Moore, 1987).

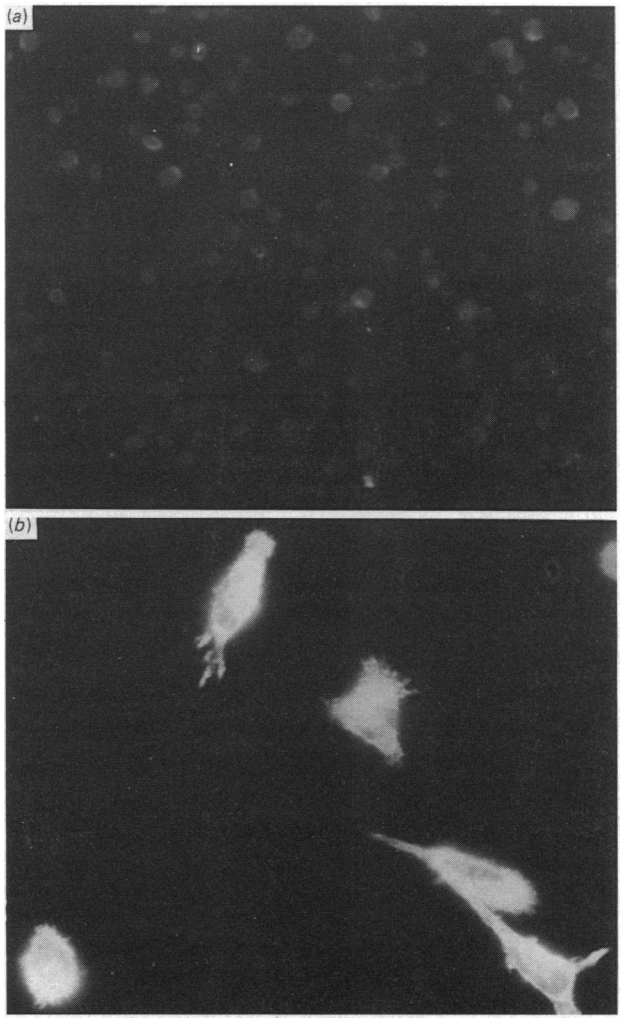

Fig. 1. Indirect immunofluorescence staining of mouse thymocytes (*a*) or mouse peritoneal macrophages (*b*) stained with anti-(67 k calcimedin) antibody

Final magnification in (*a*) is 395× and in (*b*), 470×.

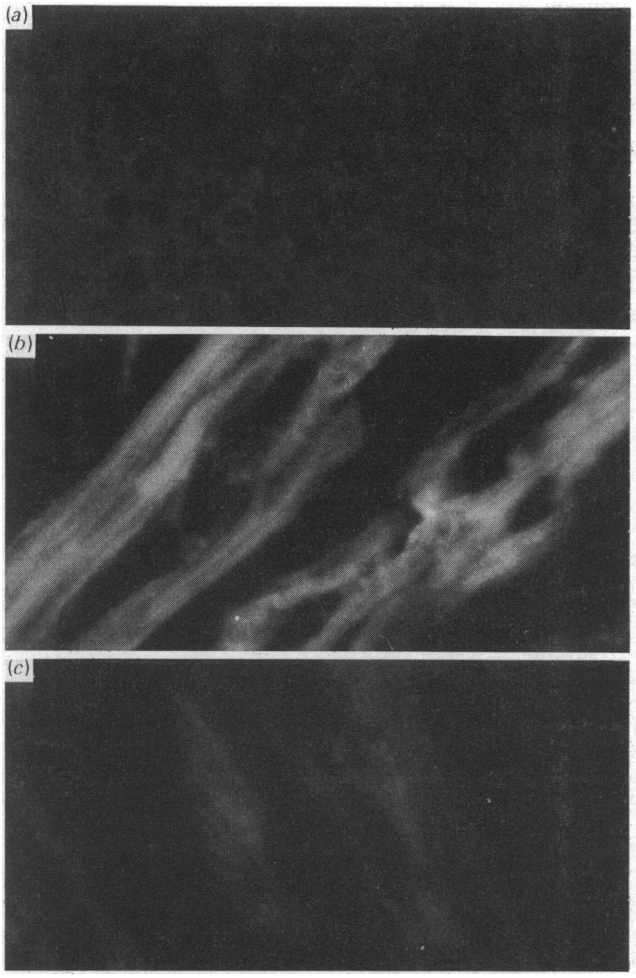

Fig. 2. Indirect immunofluorescence staining of cryostat sections of hamster liver (*a*) or hamster cardiac interventricular septum (*b*) stained with antibody to the 67 k calcimedin

Hamster cardiac septum was stained with pre-immune serum in panel C. Final magnification is 270×.

Liver tissue from hamster was also prepared and stained with anti-(67 k calcimedin) antibodies. As shown in Fig. 2(*a*), sections of liver failed to show a positive reaction for the 67 k calcimedin. Muscle tissue, shown in Fig. 2(*b*), on the other hand, stained for the 67 k calcimedin when tested under identical conditions. Background staining of muscle tissue with pre-immune serum is shown in Fig. 2(*c*) for comparison.

DISCUSSION

The 67 k calcimedin, localized with affinity-purified antibodies to the protein as isolated from smooth muscle, appears to be present as the homologous protein in mouse peritoneal macrophages (Moore & Morse, 1986; Morse & Moore, 1987), as well as in various primary muscle cells and cell lines (Moore, 1988). The affinity-purified anti-(67 k calcimedin) antibody readily detects the homologous protein in tissues and cells of various species (Figs. 1*b* and 2*b*), even though 67 k calcimedin is a low-abundance protein estimated to represent less than 0.1% of the total cellular protein by weight in muscle (Moore, 1986; Morse & Moore, 1987).

Since it has been suggested that the 67 k calcimedin (from muscle) is the same as the calelectrin from non-muscle tissues (Smith & Dedman, 1986), the purified monospecific anti-(67 k calcimedin) antibody (Moore, 1988) was tested against thymus, spleen and liver, three tissues which have been shown to contain high levels of the calelectrin protein (Owens & Crumpton, 1984; Südhof *et al.*, 1983; Geisow *et al.*, 1984), for possible cross-reactivity. As we have shown here, the antibody raised against the smooth-muscle-derived protein does not recognize any protein in these other tissues. The affinity-purified antibody to 67 k calcimedin was previously shown not to cross-react with brain calelectrin isolated by (Rhoads *et al.* (1985). The few cells in these tissues that showed any staining appeared morphologically to be macrophages. Small blood vessels, when present in the sections, also appeared stained.

In contrast with the negative results obtained with these tissues, the 67 k calcimedin antibodies readily recognized a 67 k calcimedin protein in several muscle tissues and in macrophages (Figs. 1*b* and 2*b*; Moore, 1988), as expected. If the p67 calelectrin, or the 68 kDa lymphocyte protein, were identical with the 67 k calcimedin, the 67 k-calcimedin antibody should cross-react with proteins in lymphocytes and liver, both sources which have been reported to have high levels of p67 calelectrin detectable by immunofluorescence (Südhof *et al.*, 1983; Geisow *et al.*, 1984).

Lymphocytes contain as a major membrane protein, the 68 kDa lymphocyte protein, a calcium-binding protein similar to the p67 calelectrin in molecular size and Ca^{2+}-binding affinity (Geisow *et al.*, 1984). This lymphocyte protein has been identified as a cytoskeletal protein associated with the lymphocyte plasma membrane (Owens & Crumpton, 1983; Davies & Crumpton, 1985). Owens & Crumpton (1984) have estimated that the 68 kDa lymphocyte protein represents 18% of the cytoskeletal protein complex isolated with lymphocyte membranes. Since it is likely that additional quantities of the protein are in the cytoplasm (Owens & Crumpton, 1984; Davies & Crumpton, 1985), this is probably a low estimate of the protein concentration. In view of the amount of the 68 kDa lymphocyte protein present in lymphocytes, a high level of staining associated with the plasma membranes would be expected if the 67 k-calcimedin antibody recognized this protein. The thymus comprises T-lymphocytes at various stages of maturation. The spleen contains a wide variety of lymphocytes of all classes: T, B and 'null'. Thus any major lymphocyte protein should have been well represented in these lymphoid tissues.

The various proteins (p67 calelectrin, 68 kDa lymphocyte protein, 67 k calcimedin) are isolated by slightly different purification schemes [Südhof *et al.* (1984), Owens & Crumpton (1984) and Moore (1986) respectively], so that distinct proteins with some overlapping physical properties may have been isolated in each case. Calelectrin appears to be ubiquitous in its tissue distribution (Südhof *et al.*, 1983; Geisow *et al.*, 1984), whereas the 67 k calcimedin appears to be more restricted. It is possible that some cell types (e.g. mouse macrophages) could contain both proteins, whereas others contain only the calelectrin. The recent report that a doublet of 67 kDa proteins isolated from lung tissue are two distinct proteins unrelated to each other (Fauvel *et al.*, 1987) is also consistent with the hypothesis that

both proteins may be present in some tissues, whereas only one protein is present in others. The protein isolated as 'liver 67 k calcimedin' (Mathew *et al.*, 1986) now also appears to be p67 calelectrin (Smith & Dedman, 1986), but appears by the evidence presented here to be different from the 67 k calcimedin.

The suggestion (Südhof *et al.*, 1983, 1984; Smith & Dedman, 1986) that the p67 calelectrin is identical with several other proteins rests almost entirely on immunochemical cross-reactivity. On the other hand, evidence collected on the muscle 67 k calcimedin, both immunochemical and biochemical, indicates that the 67 k calcimedin is not related to the calelectrin. The evidence which appears to distinguish these proteins from each other can be summarized as follows. (1) The muscle 67 k calcimedin does not co-electrophorese with brain calelectrin (Moore, 1988). (2) The muscle 67 k calcimedin does not appear as a doublet on electrophoresis, unlike the liver protein (Moore & Dedman, 1982; Moore, 1986; Smith & Dedman, 1986). (3) The 67 k calcimedin appears to have an amino acid composition significantly different from that of the calelctrin (Moore, 1986). (4) The calcimedin has a more acidic pI (Moore *et al.*, 1984). (5) The calcimedin is eluted from DEAE-cellulose at a lower NaCl concentration (and is easily separated from the other three calcimedins by this procedure) (Moore, 1986). (6) The chicken gizzard 67 k calcimedin is eluted from a gel-permeation column as if it had a slightly larger molecular size than the protein from two liver sources (Smith & Dedman, 1986), which agrees with our electrophoresis data comparing the two proteins (S. S. Morse & P. B. Moore, unpublished work). (7) Unlike p67 calelectrin, which loses its ability to bind phenyl-Sepharose upon purification (Südhof, 1984), the purified 67 k calcimedin does not lose its ability to bind the fluphenazine–Sepharose matrix when stored at 4 °C. (8) The 67 k calcimedin does not bind Cibacron Blue, unlike the brain protein (Rhoads *et al.*, 1985). (9) Affinity-purified antibody to 67 k calcimedin did not recognize the brain calelectrin (Rhoads *et al.*, 1985). We now add to this evidence the result reported here, which confirms and extends the results of Rhoads *et al.* (1985), that antibodies directed against the 67 k calcimedin do not stain tissues and cells reported to contain large quantities of the calelectrin. These antibodies to the 67 k calcimedin do stain tissues and cells which contain the 67 k calcimedin. The immunochemical data in the literature need to be resolved to determine if anti-calelectrin antibody preparations could contain two different populations of antibodies reacting independently with the calelectrin and the 67 k calcimedin. Pending resolution, we recommend that the terms 'calcimedin' and 'calelectrin' not be used as synonyms. The calelectrin or calelectrin-like protein may be ubiquitous. By contrast, the apparently more limited distribution of the 67 k calcimedin suggests that there may be specific roles for the 67 k calcimedin in Ca^{2+}-mediated functions unique to the cell types that contain the 67 k calcimedin. The antibodies to the 67 k calcimedin will prove useful in defining the specific roles of this new Ca^{2+}-binding protein and its relationship to other proteins.

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