Macrophage Caldesmon Is an Actin Bundling Protein†

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ABSTRACT: A rapid purification procedure was developed for the isolation of caldesmon (CaD) from rabbit alveolar macrophage. The purified protein migrated with an apparent M_r of 74 000 ± 4000 on SDS−PAGE and cross-reacted with anti-gizzard CaD antibodies. A higher M_r isoform was isolated from chicken gizzard. Their actin-binding parameters and effects on actomyosin−ATPase activity were investigated under identical experimental conditions. Electron microscope studies revealed that macrophage CaD was able to cross-link actin filaments into both networks and bundles. Compact F-actin bundles were predominantly or exclusively seen at cross-linker to actin molar ratios in the 1:20 to 1:10 range. Apparent K_a at extrapolated saturation of the CaD-binding sites on F-actin was 1.2×10^6 M^{-1} for macrophage CaD and 1.6×10^6 M^{-1} for chicken gizzard CaD. CaD from either source was able to stimulate the actin-activated ATPase activity of macrophage myosin. Unexpectedly, chicken gizzard CaD also increased the ATPase activity of gizzard myosin. The degree of stimulation was approximately doubled in the presence of a large excess of Ca^{2+}-calmodulin but was unaffected by the presence of macrophage tropomyosin. However, macrophage CaD did not behave as a Ca^{2+}- and calmodulin-regulated actin-binding protein. These results, together with published data on other well-characterized actin bundling proteins, suggest that nonmuscle CaD could be essentially involved in the formation and organization of actin bundles at adhesion sites and cell surface projections. However, they afforded no evidence that the macrophage isoform might play a specific role in the Ca^{2+}-dependent regulation of actin and myosin II interactions.

Variations in intracellular Ca^{2+} concentrations play an important regulatory role in smooth muscle contraction and cell contractile activities. Ca^{2+} can activate myosin II by binding CaM[1] and activating myosin light chain kinase (MLCK) (*1*). Activated MLCK phosphorylates the regulatory light chain of myosin II, leading to the formation of contractile myosin II filaments (*2*). Little is known, however, on the molecular events that underlie the structural reorganization of actin and its productive interactions with myosin filaments. These events are likely to be of importance since activated motile cells and cell extracts undergo Ca^{2+}-dependent actin-based gel-sol conversions that precede contraction (*3*). Studies on the gelation properties of macrophage cell extracts have led to the identification of proteins that regulate the length of actin filaments (gelsolin) and their reversible cross-linking (L-plastin) in a Ca^{2+}-dependent manner (*4*−*6*). The ubiquitous actin-binding protein CaD, first identified in smooth muscle by Sobue et al. (*7*), is also thought to be involved in the thin filament regulation of contraction. Such a function is believed to be related to the ability of CaD to inhibit the TM-stimulated actin-activated ATPase activity of myosin II, which can be attenuated by Ca^{2+}-CaM or other Ca^{2+}-binding proteins (reviewed in refs *8* and *9*). Nevertheless, the mechanisms underlying these in vitro observations are still debated. On the basis of primary sequence similarities, a similar regulatory function has been assumed for nonmuscle CaD.

Chicken gizzard CaD is a 756 residue polypeptide chain forming an elongated molecule with two compact N- and C-terminal domains that bind to myosin and actin, respectively (*10*, *11*). These two domains are connected by a long α-helical linker region (*12*) that is missing in the smaller nonmuscle isoform (*13*). The functional consequences of this deletion are still unknown. CaD has unusual structural features: it contains 50% charged residues and possesses two cysteine residues at opposite ends of the polyeptidic sequence, which can form both intra- and intermolecular disulfide bridges. Its native-like elongated conformation is dependent on the integrity of these two residues (*14*, *15*).

The full-length smooth muscle CaD is able to interact with a number of proteins in vitro, including CaM (*7*, *16*) and S100 proteins (reviewed in ref *17*), TM (*18*), and myosin but with a weaker binding affinity than for actin (*10*). Nonmuscle CaD has been very poorly characterized. Different groups of investigators have, however, suggested its involvement in mitosis (*19*), vesicular transport (*20*), and platelet activation (*21*). This report describes the purification

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[1] Abbreviations: kDa, kilodaltons; DTT, dithiothreitol; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-3-propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; ATP, adenosine 5′-triphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TM, tropomyosin; CaD, caldesmon; CaM, calmodulin; ATPase, adenosinetriphosphatase.

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of macrophage CaD and some of its functional properties. Studies on the purified protein were carried out (1) to assess the mode of interaction of cellular CaD with actin filaments, (2) to examine the effect of Ca^{2+}-CaM on its binding to F-actin, and (3) to investigate whether it behaves as a protein that might play a regulatory role in actin and myosin II interactions. The significance of the results is discussed in comparison with those reported for other well-characterized actin-binding proteins.

EXPERIMENTAL PROCEDURES

Macrophages. New Zealand white rabbits were intravenously injected with Freund's complete adjuvant. Alveolar macrophages were harvested 10−14 days later by tracheal lavage and washed as previously described (*22*).

Cytosolic Extracts. These were prepared as described by Pacaud and Harricane (*6*). Washed macrophages were disrupted in a glass/Teflon homogenizer with 2.5 volumes of extraction buffer (15 mM HEPES, 0.34 M sucrose, 5 mM EGTA, 2 mM EDTA, 2 mM NaN_3, and 1 mM DTT, pH 7.2) supplemented with a cocktail of protease inhibitors (1 mM diisopropyl fluorophosphate, 2 μg/mL pepstatin, 2 μg/mL leupeptin, 1 μg/mL chymostatin, 0.1 mM tosyl-L-lysine chloromethyl ketone, 20 μg/mL soybean trypsin inhibitor, and 5 μg/mL α_2-macroglobulin). The cell lystate was centrifuged at 120000*g* for 1 h at 4 °C. The supernatant was collected and the pH readjusted to 7.2 by addition of 0.1 M NaOH.

Purification of Macrophage Caldesmon. The protein was purified using a modification of the procedure described for the isolation of smooth muscle CaD (*23*). The cytosolic extract was brought to a final concentration of 0.6 M KCl by the addition of solid salt. After 30 min of stirring at 4 °C, it was fractionated by ammonium sulfate precipitation with 35−50% saturation at pH 7.2. The pellet was dissolved, dialyzed, and submitted to ion-exchange chromatography on a DEAE-Trisacryl column (2.5 × 3 cm) equilibrated in a buffer containing 10 mM Tris-HCl, 10% glycerol, 50 mM KCl, 1 mM EGTA, 0.5 mM DTT, and 0.02% NaN_3, pH 7.6 (supplemented with protease inhibitors 0.1 mM diisopropyl fluorophosphate, 2 μg/mL leupeptin, and 5 μg/mL soybean trypsin inhibitor). The column was washed with 3 volumes of this buffer and then eluted with a linear 50−300 mM KCl gradient at 30 mL/h. Fractions containing CaD were pooled and dialyzed overnight against 100 mM KCl, 0.1 mM EGTA, 0.5 mM DTT, and 10 mM Tris-HCl, pH 7.5, in the presence of protease inhibitors. The calcium concentration was adjusted to 0.2 mM prior to affinity chromatography on a 4 mL CaM−Sepharose column equilibrated in the same buffer containing calpain inhibitors I and II (3 μg/mL). Bound CaD was eluted with EGTA as described in the legend for Figure 1. Purified protein preparations were dialyzed for 4 h against a buffer containing 15 mM HEPES, 10% glycerol, 0.1 mM EGTA, 60 mM KCl, 0.2 mM DTT, 2 mM NaN_3, and protease inhibitors, pH 7.0. They were then immediately divided into aliquots and used directly for biochemical investigations or were frozen in liquid nitrogen and stored at −80 °C. They were generally used over the next 1−2 weeks with no evidence of self-aggregation or loss of functional activity. All buffer solutions were freshly prepared and purged with nitrogen gas to prevent oxidation.

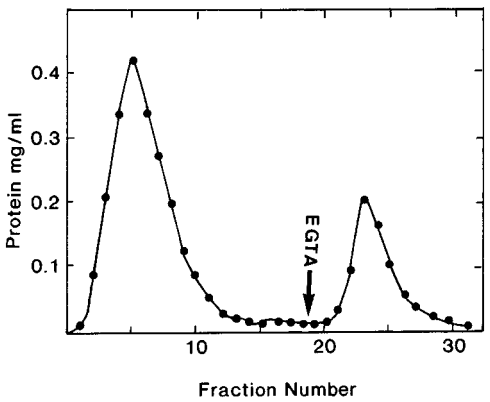

FIGURE 1: Separation of macrophage caldesmon on calmodulin−Sepharose. Fractions cross-reacting with anti-CaD antibodies were collected from a DEAE-Trisacryl column and dialyzed in 10 mM Tris-HCl, 100 mM KCl, 0.1 mM EGTA, and 0.5 mM DTT, pH 7.5. The pooled fractions were loaded onto a 4 mL CaM−Sepharose 4B affinity column after the addition of 0.3 mM $CaCl_2$. The column was first washed with the dialysis buffer containing 0.2 mM $CaCl_2$ instead of 0.1 mM EGTA, and the bound protein was then eluted at the point indicated by the arrow using the same buffer containing 2 mM EGTA.

CaD in various protein fractions was identified by Western immunoblotting using specific polyclonal antibodies directed against the 35 kDa C-terminal fragment of gizzard CaD (*24*).

Purification of Other Proteins. Actin was prepared from rabbit skeletal muscle using a modification of the procedure of Spudich and Watt (*25*), with one cycle of polymerization and depolymerization with 0.8 M KCl. Monomeric actin was further purified by gel filtration on a Sephacryl S-200 column equilibrated in buffer G (2 mM Tris-HCl, 0.1 mM DTT, 0.1 mM $CaCl_2$, 0.2 mM ATP, and 0.02% NaN_3, pH 7.8). After repolymerization, actin prepared by this method gave rise to the formation of a large majority of single filaments. CaM from ram testes was purified as described by Autric et al. (*26*). CaM−Sepharose was obtained from Pharmacia. Macrophage TM was collected at the end of the KCl elution gradient via the DEAE-Trisacryl chromatography used for CaD preparation. It was further purified by chromatography on an AcA-34 column. Macrophage actomyosin was prepared using the procedure of Trotter and Adelstein (*27*) with minor modifications. Myosin was further isolated by gel filtration on an Ultrogel AcA-34 column (95 × 1.6 cm) using the KI−Mg^{2+}-ATP and 0.6 M KCl discontinuous buffer system of Pollard et al. (*28*). Chicken gizzard CaD and smooth muscle myosin were kindly provided by D. Mornet and prepared as previously described (*29*).

Actin-Binding Assays. After addition of 0.1 mM EGTA (for Ca^{2+} chelation), monomeric skeletal muscle actin was mixed with increasing amounts of purified CaD in standard actin polymerization buffer (10 mM HEPES, 2 mM $MgCl_2$, 80 mM KCl, 0.2 mM EGTA, 0.2 mM ATP, and 0.2 mM DTT, pH 7.2). The samples were held at 25 °C for 1 h and then centrifuged in a Beckman TL 100 at 100000*g* for 20 min. Supernatants and pellets were analyzed by SDS−PAGE according to Laemmli (*30*). The quantity of CaD and actin present in individual fractions was determined by scanning the Coomassie blue stained gels with a CS-930 densitometer (Shimatzu Corp., Tokyo, Japan). Standard samples of CaD and actin were examined to relate the densitometric scans

to the quantity of protein present and to demonstrate linearity with protein concentrations.

Western Blotting. Proteins were resolved by SDS−PAGE and analyzed by western blotting on nitrocellulose as described (*31*). Transferred proteins were visualized using horseradish peroxidase-coupled goat anti-rabbit IgG.

Protein Concentrations. Protein concentrations were determined by the method of Lowry et al. (*32*), using bovine serum albumin as a standard. The concentration of the following protein solutions was determined using the indicated values for the absorbance of a 1% solution with a path length of 1 cm: actin, 6.3 at 290 nm; myosin, 4.5 at 280 nm (*33*); CaM, 1.95 at 277 nm (*34*). The molecular weights used for calculating protein concentrations were 43 000 for actin, 16 500 for CaM, 60 000 for macrophage TM, 88 000 for chicken gizzard CaD, and 59 000 for macrophage CaD. These molecular weights for smooth muscle and nonmuscle CaD are those assigned by the cDNA sequencing of each protein isoform (*35*).

ATPase Assays. In standard assays with CaD, F-actin−TM was mixed with CaD−CaM and kept at 30 °C for 2 min to initiate the cross-linking of actin filaments. Myosin was then added with gentle stirring. Protein mixtures were incubated at 30 °C for 10 min (with gentle stirring) prior to the addition of ATP. Aliquots of the reaction medium were quenched in an equal volume of 6% trichloroacetic acid. The released phosphate was measured using the Fiske−Subbarow method (*36*). The rate of phosphate release was approximately linear over the time course of the assay (5 min). Similar but less reproducible results were obtained in duplicate samples when all proteins were mixed at the same time. However, no stimulation or even a small inhibition of the actomyosin−ATPase was observed when CaD was added after ATP or when ATP was added immediately to protein mixtures (i.e., without any preincubation period). On the other hand, the inclusion of protease inhibitors (mainly calpain inhibitors) was found to be necessary to protect CaD from cleavage by trace amounts of Ca^{2+}-activated proteases during the incubation period. Detailed conditions are given in the legends to figures.

Electron Microscopy. Samples of CaD−actin complexes were applied to Formvar/carbon-coated grids and negatively stained with 1% uranyl acetate as indicated earlier (*6*). The grids were viewed under a JEOL 200 CX electron microscope.

RESULTS

Purification of Macrophage Caldesmon. Earlier attempts to purify the Ca^{2+}-regulated 70 kDa component from macrophage cytosolic extracts, a protein further identified as L-plastin (*22*, *31*), had led to the detection of another actin-binding protein that migrated with an apparent size in the range of 74 ± 4 kDa on SDS−PAGE. Under appropriate conditions both proteins were retained on DEAE-Trisacryl columns and independently recovered after elution with a KCl gradient. Further investigations had revealed that the partially purified 74 kDa polypeptide was heat-stable and capable of cross-reacting with polyclonal antibodies directed against chicken gizzard CaD. On the basis of these criteria, it was assumed to be a low M_r isoform of smooth muscle CaD. The cross-reactive protein was purified under native conditions by a method involving two ammonium sulfate precipitations at high ionic strength, followed by two chromatographic separations on DEAE-Trisacryl and CaM−Sepharose (see Experimental Procedures). The elution profile on the CaM−Sepharose column is illustrated in Figure 1. Bound CaD was selectively eluted with 1 mM EGTA. However, contaminants and most of the protein could also be eluted in the presence of $CaCl_2$ simply by increasing the KCl concentration from 100 to 250 mM, indicating low association constants between CaD and CaM. This new purification procedure was developed because heat treatment of macrophage cytosolic extracts or partially purified fractions had caused significant losses (30−40%) of the cross-reactive component by proteolysis. The purification results are summarized in Table 1. In a typical preparation, 0.7−0.8 mg of macrophage CaD can be obtained from 1000 mg of cytosolic proteins. SDS−PAGE analysis of the fractions collected after each purification step is shown in Figure 2. The final protein was more than 95% pure (Figure 2, lanes

Table 1: Purification of Macrophage Caldesmon[a]

fraction	total protein (mg)	CaD (mg)	yield (%)
cytosolic extract	1000	3.60	100
40−55% ammonium sulfate	198	3.20	88.8
DEAE-Trisacryl	7.5	1.70	45.9
CaM−Sepharose	0.75	0.75	20.8

[a] The CaD content of each fraction was determined by quantitative densitometry of Coomassic blue stained gels as described in Experimental Procedures. CaD in cytosolic extracts and ammonium sulfate fractions was identified by immunoblotting using monospecific anti-gizzard CaD antibodies. The data represent the average of three separate preparations from 30 to 35 mL of packed cells.

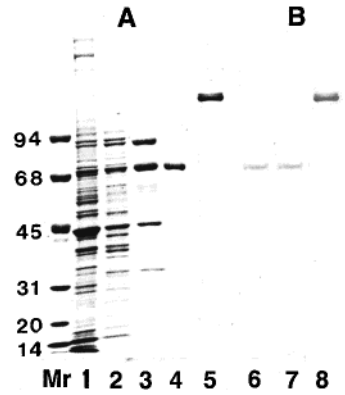

FIGURE 2: SDS−polyacrylamide gels showing various steps in the purification of macrophage caldesmon and its cross-reactivity with anti-chicken gizzard caldesmon antibodies. The initial soluble extract and pooled fractions were electrophoresed on either 5−20% polyacrylamide gels for Coomassie blue staining (panel A) or transfer to nitrocellulose paper (panel B). Bound antibodies were detected with peroxidase-labeled goat anti-rabbit IgG. Protein fractions shown are as follows: macrophage cytosolic extract (lane 1), protein recovered after ammonium sulfate precipitation (lane 2), fractions collected from the DEAE-Trisacryl column (lane 3), the purified protein eluted from the CaM−Sepharose column shown in Figure 1 (lane 4), and purified gizzard CaD (lane 5). The immunoblot in panel B comes from the 35−50% ammonium sulfate fraction (lane 6) and the purified CaD (lane 7) and gizzard CaD (lane 8). The molecular mass markers (M_r) are indicated on the left in kilodaltons. The mixture used was as follows: phosphorylase *b*, 94 kDa; bovine serum albumin, 68 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; chymotrypsin inhibitor, 21 kDa; and α-lactalbumin, 14 kDa.

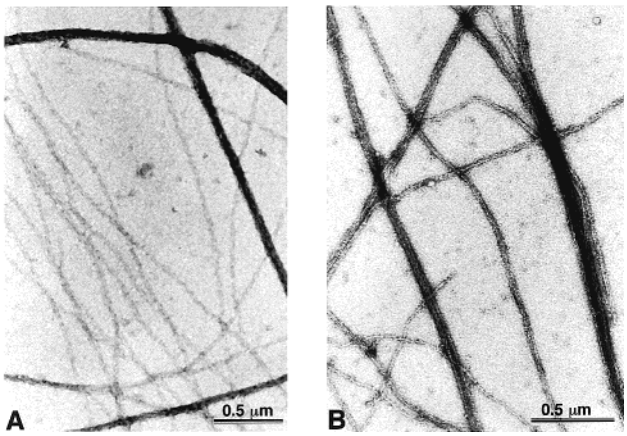

FIGURE 3: Electron micrographs of negatively stained preparations of F-actin cross-linked by macrophage caldesmon. Monomeric skeletal actin (5 μM) was mixed with increasing concentrations of CaD (from 0.15 to 0.75 μM) in polymerization buffer (10 mM HEPES, 80 mM KCl, 2 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM DTT, 0.2 mM NaN_3, and 0.2 mM ATP, pH 7.2). After incubation for 1 h at 25 °C, the samples were applied to grids and visualized by negative staining with 1% uranyl acetate. (A) Actin + CaD incubated at a molar ratio of 1:20. (B) Actin + CaD incubated at a molar ratio of 1:10. Note the presence of small actin bundles and cross-linked filaments in (A) and the complete assembly of actin filaments into compact bundles in (B).

4 and 7). It migrated with an apparent size in the range expected for nonmuscle CaD isoforms (70−83 kDa). Comparatively, gizzard CaD behaved as a polypeptide having an apparent molecular mass of 130 ± 8 kDa (Figure 2, lanes 5 and 8). The large and small CaD isoforms are known to migrate anomalously on SDS−PAGE. In fact, they have calculated molecular masses of 88 and 58−63 kDa, respectively (*37*) (see Experimental Procedures).

Electron Microscopy of the Structures Formed by Macrophage Caldesmon and F-Actin. To investigate how CaD modifies the structural organization of actin filaments, different amounts of CaD and monomeric actin were mixed under polymerizing conditions and negatively stained for electron microscopy. Loose and irregular networks of actin filaments were mainly observed for molar ratios of CaD to actin lower than 1:30, whereas arrays of laterally aligned actin filaments were easily detectable at molar ratios ≥1:20. The influence of CaD concentrations on the spatial organization of actin filaments is illustrated in Figure 3. In time course experiments compact F-actin bundles appeared approximately 10 min after CaD and actin were mixed at room temperature, provided protein ratios and concentrations were favorable for bundle formation. It is noteworthy that these structures are analogous to those previously observed with chicken gizzard CaD by Martin et al. (*14*) in our laboratory and typical actin bundling proteins such as α-actinin (*38*).

Comparative F-Actin-Binding Properties of Macrophage and Gizzard Caldesmon. The amounts of CaD bound to skeletal muscle F-actin were evaluated by high-speed sedimentation assays. The results of the binding experiments are illustrated in Figure 4. The molar ratios of pelleted CaD (bound) to actin were plotted as a function of the total CaD concentration. The binding curves of the two protein isoforms are similar, but they level off at different CaD to actin molar ratios. Under the conditions used, the maximum binding

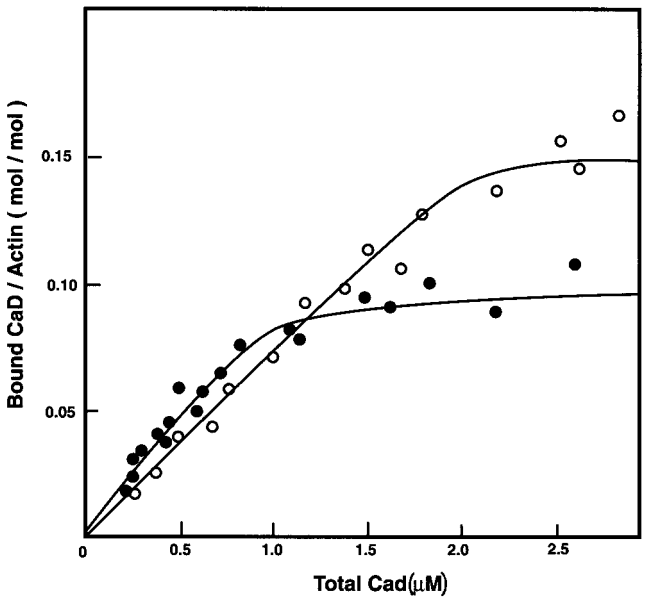

FIGURE 4: Quantitative analysis of the binding of the low and high M_r caldesmon isoforms to F-actin at 25 °C and pH 7.2. Binding of CaD to F-actin was assessed by sedimentation assay as indicated in Experimental Procedures. Skeletal muscle actin (9.2 μM) was polymerized in the presence of various concentrations of CaD under the incubation conditions described in the legend for Figure 3. The experimental points for macrophage CaD (●) and gizzard CaD (○) represent the average of two different experiments.

Table 2: Caldesmon- to F-Actin-Binding Parameters

caldesmon source	ref	binding sites (class number)	app $K_a \times 10^6$ M^{-1}	B_{max} (CaD/actin)
macrophage[a]	this study	one	1.2 ± 0.25	1:12 ± 1
chicken gizzard[a]	this study	one	1.6 ± 0.3	1:7 ± 1.4
chicken gizzard	*40*	one	1.5	1:7−1:50
sheep aorta	*41*	two, A	2−40	1:21
		two, B	10−30	1:4−1:6
rat cells	*42*	nd[b]	3−8	1:4
human platelets	*43*	nd	nd	1:14
bovine adrenal medulla	*44*	nd	nd	1:2.5−3

[a] These actin-binding parameters were determined at pH 7.2 and 25 °C. They were derived from the curves shown in Figure 5. Published binding stoichiometries were corrected to a M_r of 88 000 for smooth muscle CaD and 59 000 for nonmuscle CaD. Binding of chicken gizzard CaD to F-actin was studied by Velaz et al. in 9.6 mM imidazole hydrochloride, 4.8 mM $MgCl_2$, 0.25 mM EGTA, 42 mM NaCl, and 0.5 mM DTT at 25 °C (pH not indicated). Binding of sheep aorta CaD to F-actin was determined by Smith et al. in 10 mM Tris−MES, 10 mM NaN_3, 5 mM $MgCl_2$, 60 mM KCl, and 1 mM DTT, pH 7.0, at 25 °C. Binding assays for CaD from rat cultured cells were carried out in 20 mM imidazole, 100 mM KCl, and 0.5 mM DTT, pH 7.0, at room temperature. [b] nd: not determined.

(B_{max}) reached one molecule of macrophage CaD per 12 ± 1 actin monomers and one molecule of gizzard CaD per 7 ± 1.4 actin monomers. The data, analyzed with the computer curve-fitting program ALL-FIT (*39*), could be accounted for by a major class of binding sites with apparent association constants near 1.2×10^6 M^{-1} for macrophage CaD and 1.6×10^6 M^{-1} for chicken gizzard CaD. The actin-binding parameters obtained in this study are given in Table 2, in comparison with those reported by other investigators. Estimates for the high M_r smooth muscle isoform are in close agreement with those of Velaz et al. (*40*). CaD from rat cultured cells is the only low M_r isoform for which the actin-

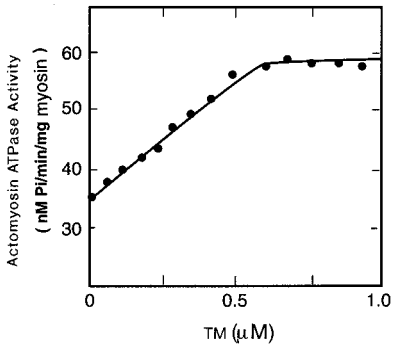

FIGURE 5: Stimulation of the actin-activated ATPase activity of macrophage myosin by tropomyosin isolated from the same source. Macrophage myosin (0.5 μM) was mixed with skeletal muscle actin (7 μM) and incubated at 30 °C with increasing concentrations of TM in ATPase buffer (30 mM MES, 75 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, and 0.2 mM DTT, pH 7.2). ATPase reactions were initiated 10−12 min later by the addition of ATP at a final concentration of 2 mM. ATP hydrolysis was measured as indicated in Experimental Procedures. The data are means from two separate determinations.

binding affinity has been reported. However, measurements were made in the absence of ATP-Mg^{2+} (that stabilize F-actin) and with partially purified CaD preparations (*42*).

Does Ca^{2+}-Calmodulin Affect the Binding of Macrophage Caldesmon to F-Actin? Since CaM interacts with CaD in vitro, the influence of this Ca^{2+} target protein has been explored on both the formation and dissociation of CaD−F-actin complexes. The amounts of CaD that cosedimented with F-actin were observed to be significantly decreased only under extreme conditions, i.e., when Ca^{2+}-CaM concentrations were increased to a 6−8-fold molar excess relative to CaD. Under ATPase reaction conditions and with 6 μM CaM, a 15% loss of CaD was thus observed with concentrations of 9.8 μM actin and 0.7 μM CaD. Furthermore, CaD could not be dissociated from F-actin when the pelleted CaD−F-actin complexes were resuspended in actin polymerization buffer containing 0.2 mM $CaCl_2$ (instead of EGTA) with or without 10 μM CaM (data not shown). Macrophage CaD thus has a much lower affinity for CaM than for F-actin.

Effects of Tropomyosin and Macrophage Caldesmon on the Actin-Activated ATPase Activity of Macrophage Myosin. Unlike muscle TMs, low M_r TMs from nonmuscle cells have not been thoroughly studied. It was therefore of importance to define the optimum conditions under which macrophage TM binds to F-actin. At pH 7.2−7.4 and in the presence of 100 mM KCl, maximum amounts of macrophage TM cosedimented with skeletal F-actin at Mg^{2+} concentrations between 1 and 4 mM (data not shown). Under such conditions, TM was observed to increase the ATPase activity of macrophage myosin by a factor of 1.6−1.7 (Figure 5). Maximum stimulation was reached at a molar ratio of 1 TM molecule to 6 actin monomers, i.e.,when actin filaments were saturated with TM. These results are analogous to those reported for low M_r TM isoforms (reviewed in ref *45*). They differ from other published data, however, indicating that a mixture of fibroblast TM-4 and TM-5 binds to F-actin with a stoichiometry of 1:29 in the absence of fibroblast CaD and of 1:6 in its presence (*42*). Such low binding stoichiometries were very likely determined using TM preparations that had partially lost their functional integrity.

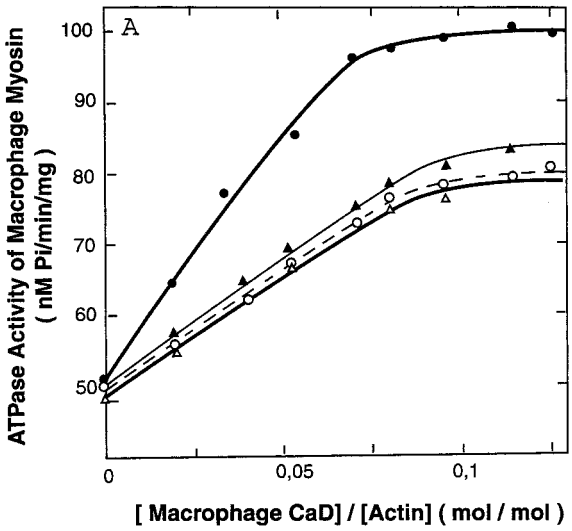

FIGURE 6: Effects of macrophage caldesmon on the actin-activated Mg^{2+}-ATPase activity of macrophage myosin in the presence or absence of calmodulin and with or without Ca^{2+}. Skeletal muscle F-actin (10 μM) and macrophage TM (0.9 μM) were mixed at room temperature and left to stand for 10 min. Increasing concentrations of macrophage CaD (preincubated at room temperature for 15 min with a 6-fold molar excess of CaM) were then added. Macrophage myosin (1.2 μM) was added 2 min later. The solution's other components were identical to those indicated in the legend for Figure 5, except in determinations where EGTA was replaced by 0.2 mM $CaCl_2$ and added with calpain inhibitors I and II (2 μg/mL). Protein mixtures with or without CaM were left to stand at 30 °C for 10 min prior addition of ATP. Plots: samples containing 1 mM EGTA (△) or 0.2 mM $CaCl_2$ (○); samples containing CaM plus 1 mM EGTA (▲) or CaM plus 0.2 mM $CaCl_2$ (●). Symbols indicate data from two separate experiments.

Due to limiting amounts of purified macrophage TM, ATPase assays with CaD were performed at a TM to actin molar ratio of 1:11 (about half-saturation). It is important to emphasize, however, that this TM to actin ratio is closed to that found in macrophage cell extracts (unpublished data). The results illustrated in Figure 6 show that macrophage CaD was capable of potentiating the TM enhancement of the actin-activated ATPase activity of macrophage myosin in a concentration-dependent manner, and this even in the absence of CaM. In this case, the CaD-induced stimulations of myosin−ATPase rates were approximately the same in Ca^{2+} as those in EGTA. However, when CaD was preincubated with CaM, the stimulation levels in Ca^{2+} were higher than those in EGTA. The maximum stimulatory effect of macrophage CaD, in the presence of a 6-fold molar excess of CaM, was 1.5−1.6-fold with 1 mM EGTA and 2-fold with 0.2 mM $CaCl_2$. Similar levels of stimulation were observed in experiments conducted in the absence of TM. In this case, the ATPase activity of actomyosin was increased with 0.8 μM macrophage CaD from 34 to 43 nmol of P_i/(mg of myosin·min) in EGTA−CaM and from 32 to 51 nmol of P_i/(mg of myosin·min) in Ca^{2+}-CaM.

Effects of Chicken Gizzard Caldesmon on the Actin-Activated ATPase Activity of Gizzard and Macrophage Myosin. Although smooth muscle and nonmuscle myosins II have some common structural features, they display some functional, differences (*27*, *46*). Other series of ATPase assays were therefore performed with gizzard CaD to examine whether the stimulatory effect of macrophage CaD (described in Figure 6) could be depending on myosin's

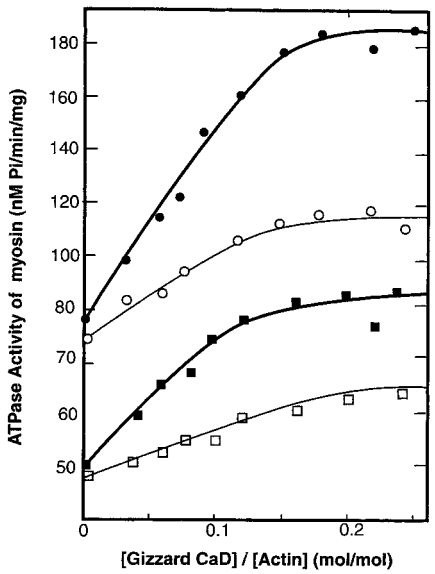

FIGURE 7: Effects of gizzard caldesmon on the actin-activated Mg^{2+}-ATPase activity of macrophage and gizzard myosin in the presence of calmodulin with or without Ca^{2+}. Experimental conditions were as described in the legend for Figure 6. Top: samples containing gizzard myosin with CaM plus EGTA (○) or CaM plus Ca^{2+} (●). Bottom: samples containing macrophage myosin with CaM plus EGTA (□) or CaM plus Ca^{2+} (■).

origin and/or related to the low M_r CaD isoforms. The results illustrated in Figure 7 indicate clearly that gizzard CaD was also able to enhance the actin-activated ATPase activities of both macrophage and gizzard myosin, and CaM had a similar potentiating effect in the presence of Ca^{2+}. A greater degree of stimulation was obtained, however, with smooth muscle myosin than with macrophage myosin, 1.5 vs 1.3 times greater in EGTA−CaM and 2.1 vs 1.7 times greater in Ca^{2+}-CaM, suggesting some myosin specificity.

In every situation examined (Figures 6 and 7) the rates of ATP hydrolysis by TM and actin-activated myosin were maximally stimulated at a cross-linker to actin molar ratio of 1:12 with macrophage CaD and 1:6−1:7 with gizzard CaD. These stoichiometries coincided with the actin-binding stoichiometry that was estimated for each protein isoform. The stimulatory effect might thereby be a direct consequence of CaD binding to actin filaments, with concomitant changes in actomyosin interactions and ATPase activities. Gizzard CaD or macrophage CaD either alone or in the presence of F-actin had no detectable ATPase activity. Moreover, F-actin alone did not activate the Mg^{2+}-ATPase of macrophage myosin without CaD, thus confirming that myosin was essentially dephosphorylated (*46*, *47*). This was also suggested from the linear dependence of ATPase activities on CaD concentrations.

It should be emphasized that the stimulatory property of either CaD was reproducibly observed if conditions in ATPase assays were such as to favor specific and complete interactions between the five protein components (see Experimental Procedures). Usual assays of actomyosin−ATPase activities involve either no preincubation period or an incubation of reaction components (in the presence of Ca^{2+}-CaM only) for 2 min at 25−30 °C prior to the addition of ATP. The results illustrated in Figures 6 and 7 were, however, obtained from ATPase assays in which CaD (preincubated with Ca^{2+} plus CaM or with EGTA plus CaM) was mixed with F-actin−TM and myosin and left to stand at 30 °C for 10 min prior to the addition of ATP. This incubation time coincided approximately with that required for the formation of stable F-actin bundles by CaD under similar experimental conditions.

DISCUSSION

A nonmuscle CaD isoform with an apparent molecular mass of 74 ± 4 kDa was isolated from macrophage cytosolic extracts. The purification procedure described here differs from those conventionally used for the preparation of muscle and nonmuscle CaD essentially by the omission of a chromatographic step and initial boiling of cell extracts (*23*, *42*, *43*). The final protein was generally recovered with a 20% yield.

Comparison of the F-Actin-Binding Properties of Macrophage Caldesmon with Those Reported for Caldesmon from Other Cells and Tissues. Electron microscopic observations revealed clearly that, by increasing CaD concentrations, actin networks shift from a structure characterized predominantly by single filaments to a structure characterized predominantly by clusters of longitudinally aligned filaments. As previously demonstrated for typical actin bundling proteins such as α-actinin (*38*, *48*, *49*) and L-plastin (*31*), the formation of cross-linked F-actin bundles is critically dependent on the molar ratio of bundling protein to actin. In the present study this ratio was found to be ≥1:20. Such structures were generated in the presence of reducing agents and with perfectly soluble CaD preparations. They cannot therefore be attributed to the formation of S−S oligomers, as previously proposed for smooth muscle CaD (*50*) and nonmuscle CaD (*42*). These results support the view that macrophage CaD is an actin cross-linking and bundling protein. The failure by other investigators to detect any actin cross-linking ability of low M_r CaD might have arisen from the use of excessively heated (100 °C for 10 min), i.e., partially inactivated, CaD preparations (*43*) and/or inappropriate experimental conditions. It is, indeed, important to emphasize that the functional properties of CaD from adrenal medulla and rat cultured cells have all been investigated without Mg^{2+}(*44*) or ATP-Mg^{2+}(*42*). Yet it is well established that ATP-Mg^{2+} is required for the stabilization of actin filaments and prevention of their depolymerization (*51*, *52*). The presence of both ATP and Mg^{2+} is therefore of an absolute necessity for the formation of physiologically relevant F-actin−CaD bundles.

Since the mechanism by which CaD induces the lateral assembly of actin filaments is not known, the binding data obtained from sedimentation assays were analyzed in the simplest way, i.e., on the basis of noninteracting binding sites. The data obtained with each protein isoform suggested the existence of one class of binding sites with similar apparent affinity constants [of the order of $(1.2-1.6) \times 10^6\ M^{-1}$] but different actin-binding stoichiometry. The B_{max} values determined for chicken gizzard CaD were approximately half those for macrophage CaD (1:6−7 vs 1:12−13). Velaz et al. (*40*) have identified a single class of sites for chicken gizzard CaD with estimated K_a and B_{max} values identical to those determined in the present investigation (Table 2). Different B_{max} values have been published for nonmuscle CaD. Yamashiro-Matsmura and Matsumura (*42*) reported a

B_{max} of one CaD molecule per four actin monomers (corrected to a M_r of 59 000 for CaD) and an apparent $K_a = (3-8) \times 10^6$ M^{-1}, i.e., perceptibly weaker than the values estimated for macrophage CaD.

Is Macrophage Caldesmon a Ca^{2+}-Calmodulin Regulated Actin-Binding Protein? Several attempts to dissociate CaD from F-actin by addition of Ca^{2+}-CaM were unsuccessful. We also observed that Ca^{2+}-CaM has no physiologically significant effect on the binding of purified CaD to F-actin. These results argue against the hypothesis whereby the low M_r CaD can bind alternatively to CaM or F-actin depending on the Ca^{2+} concentration, the so-called "flip-flop binding" (*7*, *44*). It should be, however, pointed out that this widely accepted model was initially constructed from sedimentation assays carried out under very special conditions: (1) the three proteins CaM, CaD, and actin were incubated at molar ratios of 7:1:30, i.e., with a large excess of CaM and without Mg^{2+}, and (2) the electrophoretic analysis of samples was performed with limiting amounts of CaD (as shown on Figure 8 in ref *44*) with no further quantitative evaluation of the CaM inhibitory effect.

Stimulation of the Actomyosin−ATPase Activity by Caldesmon. It is generally believed that CaD acts as a negative regulator of actomyosin interactions (*8*, *9*, *53*). However, we observed in this study that CaD purified from rabbit alveolar macrophage or chicken gizzard causes a significant enhancement of the actin-activated Mg^{2+}-ATPase activity of macrophage myosin by 50% and 25%, respectively. These stimulation levels were unaffected by the presence of macrophage TM, but they were approximately doubled in the presence of Ca^{2+}-CaM. The stimulatory property of chicken gizzard CaD was further confirmed with myosin isolated from the same source. Regardless of the presence of CaM, maximal stimulations by each isoform occurred at threshold CaD concentrations corresponding to those required to saturate their respective binding sites on actin filaments. This strict correlation and the fact that CaD did not change the rate of ATP hydrolysis by myosin in the absence of actin lead to suggest that the stimulatory property of CaD might be essentially related to its ability of cross-linking and bundling actin filaments. Interestingly, Ishikawa et al. (*54*, *55*) characterized a 210 kDa actin bundling protein from *Physarum* which cross-reacted with anti-gizzard CaD antibodies and was capable of enhancing the actin-activated ATPase activity of phosphorylated *Physarum* myosin 2−3-fold. They also observed that this activity could be stimulated 1.5−2-fold by chicken gizzard CaD.

The fact that macrophage CaD was able to enhance the TM and actin-activated ATPase rates of myosin even in the presence or absence of Ca^{2+} without CaM indicates that this effect was not strictly CaM-dependent. Moreover, since CaM did not affect the stimulatory effect of CaD at stoichiometric concentrations, a physiological interaction between the two proteins seems, once again, unlikely. The double heads of myosin II contain two IQ motifs in their neck domain which are thought to function as binding sites for CaM or other members of the CaM EF-hand family (*56*). This raises the possibility that at high concentrations CaM might well interact directly with macrophage myosin II.

Interestingly, Ishikawa et al. (*54*) and Haeberle et al. (*57*) demonstrated that, under certain conditions, chicken gizzard CaD enhances the sliding velocity of actin filaments over a surface coated with phosphorylated smooth muscle myosin. In this study, the stimulatory property of either CaD isoform was also observed to be dependent on the experimental conditions used for ATPase assays (as already mentioned in the Results section). It is obvious that the formation and composition of a stable quaternary complex between actin, myosin, CaD, and TM are related to the incubation time and the concentration, binding affinity, and stoichiometry of each protein for F-actin. Finally, although the two CaD isoforms displayed a similar behavior in vitro, it cannot be concluded that they are functionally identical. In light of the information published on CaM (*59*), which possesses a 26-residue helical central linker (*60*), it is tempting to suggest that the 240−250-residue linker in the central region of smooth muscle CaD might unwind to different extents (*61*) to accommodate the binding of distinct muscle target proteins. Alternatively, this linker region might well be required for CaD interactions with actin structures that are stiffer, more stable, and larger than those present in motile cells.

Functional Significance of Caldesmon in Macrophages. One of the most obvious features of macrophage CaD revealed in this study is its ability to assemble actin filaments into bundles. According to our estimates, there is roughly 1 molecule of CaD per 37 actin monomers in macrophage cytosolic extracts, whereas a maximum of 1 molecule of CaD can bind 12 actin monomers in a filamentous form. α-Actinin, which is a well-characterized actin bundling protein, is present at a lower molar concentration than CaD. Our previous data indicated that there is approximately 1 molecule of α-actinin per 55 actin monomers in all the soluble proteins (*38*). As α-actinin binds F-actin with a stoichiometry very close to that of CaD, the latter may have significant actin bundling activity within motile cells. This interpretation agrees with the phenotypic changes that have been observed in fibroblasts overexpressing the full-length CaD (*62*). It is also consistent with the preferential localization of this protein in stress fibers and membrane ruffles of cultured cells (*63*). The other important finding that macrophage CaD does not inhibit but instead stimulates the actin-activated ATPase activity of myosin II should be interpreted in the light of the results reported for other actin cross-linking proteins. Most of them, including filamin (*64*), α-actinin (*65*), fodrin, a nonerythroid spectrin (*66*), and villin (*67*), were capable of enhancing the actin-activated Mg^{2+}-ATPase activity of myosin II in vitro. Although none of these proteins has been reported to interact directly with myosin, *Dictyostelium* mutants lacking both α-actinin and ABP-120 (a filamin-like protein) were defective in growth rate, cell shape changes, phagocytosis, and ameboid locomotion (*68*). Thus, the stimulatory effect of CaD on the actin-activated ATPase activity of myosin described here may well have some physiological relevance in nonmuscle cells. Consistent with this, Walker et al. (*69*) have produced evidence to show that CaD is involved in an actomyosin-mediated event required for receptor capping in lymphoma cells. Hegmann et al. (20) observed, moreover, that inactivation of CaD by microinjection of monoclonal antibodies into cultured fibroblasts caused the inhibition of intracytoplasmic granule movement.

In conclusion, the results reported here rule out the possibility that CaD is a Ca^{2+}- and CaM-regulated inhibitor of myosin II-dependent contractility in nonmuscle cells. However, they support the idea that CaD may act in concert

with other actin cross-linking proteins in the maintenance and/or generation of various actin assemblies involved in cell motility and morphogenesis. In view of the weak interactions between CaD and CaM in vitro, the question arises of whether the binding affinity of CaD for F-actin is regulated and, if so, how. Conceivably, the differential regulation and distribution of actin cross-linking proteins within cells might dictate where, when, and how microfilaments have to interact with individual members of single- or double-head myosin motors (reviewed in ref *70*).

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