Purification and Further Characterization of Macrophage 70-kDa Protein, a Calcium-Regulated, Actin-Binding Protein Identical to L-Plastin†

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ABSTRACT: We have previously identified a macrophage 70-kDa, actin-bundling protein as a constituent of actin-based cytoplasmic gel and showed that its association with or dissociation from cytoplasmic gels was remarkably affected by submicromolar calcium. In this study, we purified the 70-kDa protein from soluble cytosolic extracts and carried out a more detailed characterization. The amino acid sequences of four peptidic fragments, obtained from the purified protein by enzymatic or chemical cleavage, were completely or nearly identical to those of L-plastin, a protein initially identified in transformed cells from solid tumors (Goldstein & Leavitt, 1985). By Western blot analysis of normal cells and tissues using specific anti-70-kDa protein antibodies, the 70-kDa molecule was detected only in hematopoietic cells. The 70-kDa protein bound to actin with apparent K_d values of 1.8 and 5.5 μM in the absence and presence of 20 μM free calcium, respectively. Cross-linking activity measured by falling-ball viscosimetry was optimal at free calcium lower than 0.15 μM but was progressively inhibited at higher calcium concentrations, within the physiological range. Half-maximal inhibition occurred at 1.6 μM free calcium. No severing of actin filaments by the 70-kDa protein was observed in any of these assays or previously (Pacaud & Harricane, 1987). Major conformational changes of the protein, as measured by the fluorescence emission intensity of tyrosine residues, occurred at free calcium concentration ranging between 0.15 and 1.5 μM. Magnesium did not mimic the calcium effect. The results suggest that the 70-kDa protein possesses both high-affinity sites and selectivity for calcium. It is concluded that reversible cross-linking of actin filaments by L-plastin may contribute to the regulation of cytoplasmic consistency and cell motile activities.

The actin cytoskeleton is involved in a number of cell motile activities including cell migration, cell adhesion, phagocytosis, and morphological changes induced by agonists. Calcium plays the role of a second messenger in regulating the organizational state of actin filaments (Howard & Oresajo, 1985; Sklar et al., 1985; Di Virgilio et al., 1988). It has also been demonstrated that free cytosolic Ca^{2+} increases before and oscillates during spreading in macrophages (Kruskal & Maxfield, 1987). The significance of these alterations is not clear, but they might be related to cyclic gel–sol actin transformations which are essential for cell motile functions (Taylor & Condeelis, 1979). The viscoelastic properties of cytoplasm are thought to be at least partially mediated by Ca^{2+}-induced variations in the association of actin with actin-binding proteins. Cytosolic extracts from motile cells form viscoelastic gels which contract on addition of calcium (Condeelis & Taylor, 1977), and the gelation process is inhibited in the presence of micromolar Ca^{2+} concentrations (Hartwig & Stossel, 1975; Hellewel & Taylor, 1979). Cytoplasmic gels may therefore be used for the identification of proteins whose interactions with actin are modified by calcium.

We previously identified and purified the constituents of cytoplasmic gels from alveolar macrophages. In addition to actin and filamin (Hartwig & Stossel, 1975, 1981), other major components of these gels include two actin-bundling proteins, α-actinin and a new 70-kDa[1] protein, and two unidentified molecules of 55 kDa and 17 kDa (Pacaud, 1986). We were subsequently able to demonstrate that a rise in the free calcium concentration of cytosolic extracts, within the physiological range, could trigger sequential changes in the protein composition of actin gels (Pacaud & Molla, 1987). Further studies on isolated cytoplasmic gels and purified 70-kDA protein led us to suggest that Ca^{2+} control of the interactions between this protein and F-actin may exert a dominant influence in regulating the state of microfilament organization (Pacaud & Harricane, 1987). Despite the established view that non-muscle α-actinin is a calcium-modulated protein (for a review see Pollard and Cooper (1986), calcium had no effect on the association of α-actinin with F-actin in cytoplasmic gels (Pacaud & Molla, 1987).

The aim of the present investigation was to further characterize the 70-kDa actin-bundling protein. A purification procedure was developed from soluble cytosolic extracts. Some structural, functional, and calcium-binding properties were studied. The results indicated that the protein differs from any previously characterized actin-bundling proteins.

MATERIALS AND METHODS

All chemicals were reagent grade or the purest commercially available. All buffers were made with ultrapure water (Milli-Q instrument from Millipore Corp.). Molecular weight markers (Pharmacia) for SDS–PAGE were as follows: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

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[1] Abbreviations: kDa, kilodaltons; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-3-propanesulfonic acid; p70, 70-kDa protein; DFP, diisopropyl fluorophosphate; TLCK, tosyl-L-lysine chloromethyl ketone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PIPES, piperazine-1,4-bis-(2-ethanesulfonic acid).

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Macrophage actin was purified from cytoplasmic gels as reported earlier (Pacaud, 1986). Antibodies against purified 70-kDa protein were elicited in guinea pigs (Pacaud, 1986).

Cell and Tissue Preparations. Alveolar macrophages were harvested from New Zealand white rabbits after intravenous injection of Freund's complete adjuvant as previously described (Pacaud, 1986). Neutrophils and lymphocytes were isolated from rabbit blood by two centrifugation steps on Ficoll-Paque (Pharmacia) gradient, as recommended by the supplier. Cells were washed twice with phosphate-buffered saline and centrifuged at low speed to remove contaminating platelets. Mononuclear cells were resuspended in RPMI culture medium at 5×10^6 cells/mL and allowed to adhere for 2 h to plastic Petri dishes. Lymphocytes were separated from monocytes by collecting the nonadherent cells, and then they were washed twice with phosphate-buffered saline.

Brush borders were isolated from the highest part of rabbit intestines by the method of Mooseker et al. (1978) with minor modifications. Cytoskeletal proteins from purified brush borders were then extracted with 0.2 mM $CaCl_2$ as described by Bretscher (1986). Thymocytes were dissociated from rabbit thymus. Human endothelial cells were kindly provided by Dr. Gabbiani.

Brain, stomach, and skeletal muscles were collected from rabbits. Cytoskeletal proteins from these tissues were prepared according to the procedure described for fodrin (Cheney et al., 1986) except that the extraction buffer contained 15 mM HEPES, 400 mM NaCl, 0.2 mM $CaCl_2$, and 0.1 mM DTT, pH 7.4.

Purification of 70-kDa Protein. All purification steps were performed at 4 °C. Packed macrophages were resuspended in 1.5 vol of buffer A (15 mM HEPES, 0.34 M sucrose, 6 mM EGTA, 1 mM EDTA, 2 mM NaN_3, 0.5 mM dithiothreitol, pH 7.2) supplemented with 1 mM DFP, 0.02 mM TLCK, pepstatin A (0.2 $\mu g\ mL^{-1}$), and soybean trypsin inhibitor (0.5 $\mu g\ mL^{-1}$). The cells were broken in a glass/Teflon homogenizer with a motor-driven pestle. The cell lysate was centrifuged at 12000*g* for 15 min at 4 °C, and the supernatant was collected. The pellet was resuspended, as described above, in a volume of extraction buffer equal to the volume of the initial cell pellet and centrifuged again. The two supernatants were combined and clarified by centrifugation at 120000*g* for 1 h at 4 °C. The clear cytosolic extract was brought to a final concentration of 0.6 M KCl by adding solid salt and stirred for 2 h.

Ammonium Sulfate Fractionation. The 0.6 M KCl extract was mixed with solid ammonium sulfate to 55% saturation, and the pH was adjusted to 7.2 with 0.5 M NaOH. The suspension was stirred for 1 h and centrifuged at 27000*g* for 15 min. The pellet was dissolved in a volume of buffer A (containing 0.6 M KCl) equal to the volume of the initial cytosolic extract. The turbid protein solution was centrifuged at 27000*g* for 20 min, after which the supernatant was brought to 42% saturation by adding solid ammonium sulfate and adjusted to pH 7.2. The supernatant, collected after centrifugation, was added with ammonium sulfate to 55% saturation. The precipitate, collected by centrifugation, was dissolved in buffer B ($^1/_4$ vol of the cytosolic extract) containing 10 mM HEPES, 0.25 M sucrose, 50 mM KCl, 0.2 mM dithiothreitol, and 2 mM NaN_3, pH 7.2, supplemented with protease inhibitors as indicated above. The suspension was dialyzed against 200 vol of buffer B for 2 h and clarified by centrifugation. The supernatant was diluted 4-fold with buffer B and adjusted to 0.6 M KCl and 55% ammonium sulfate saturation. The precipitate, collected by centrifugation, was dissolved in a minimum volume of buffer C (10 mM Tris-HCl, 2 mM NaN_3, 30 mM KCl, 0.2 mM dithiothreitol, 10% glycerol, pH 7.6) supplemented with protease inhibitors. The protein solution was dialyzed against 200 vol of buffer C for 12 h with one change of buffer. The final precipitate that formed during dialysis was removed by centrifugation of 120000*g* for 2 h.

DEAE–Trisacryl Chromatography. The clear supernatant was applied to a DEAE–Trisacryl (LKB) column equilibrated with buffer C. The column was washed with this buffer until the absorbance at 280 nm became constant and was then eluted with a linear gradient of KCl from 30 to 180 mM in buffer D (10 mM Tris-HCl, 1 mM EGTA, 0.2 mM dithiothreitol, 10% glycerol, pH 7.6). p70 emerged at about 120 mM KCl. The pooled fractions were supplemented with protease inhibitors (0.1 mM DFP, 0.05 mM TLCK, and 0.5 $\mu g\ mL^{-1}$ soybean trypsin inhibitor) and dialyzed for 3 h against 50 vol of buffer D containing 30 mM KCl. The dialyzed solution was then concentrated by chromatography through a small column of DEAE–Trisacryl (1.5 mL) equilibrated with the dialysis buffer. After the column was washed with 3 vol of this buffer, adherent proteins were eluted in a sharp peak by increasing the KCl concentration stepwise to 200 mM.

Size Exclusion Chromatography. The concentrated protein solution was dialyzed against buffer E (15 mM HEPES, 0.8 M KCl, 0.1 mM dithiothreitol, 2 mM NaN_3, pH 7.2) for 3 h. The dialyzed solution was supplemented with the three protease inhibitors indicated above and centrifuged at 120000*g* for 2 h. The clear supernatant was applied to a column of Ultrogel AcA-34 (1.6 × 95 cm) previously equilibrated with buffer E. Using the same buffer as eluant, 1.8-mL fractions were collected at a flow rate of 16 mL/h. The fractions containing p70 were pooled and concentrated over DEAE–Trisacryl as described above. The eluate was then dialyzed overnight against 10 mM HEPES, 10% glycerol, 50 mM KCl, and 0.1 mM dithiothreitol, pH 7, divided into aliquots, and stored at −80 °C.

Generation and Purification of Peptides. Purified p70 at 1 mg mL^{-1} was diluted 3-fold with either 15 mM HEPES buffer, pH 7.3, containing 1 mM $CaCl_2$ for thermolysin cleavage or 20 mM Tris-HCl, pH 7.8, containing 1 mM $CaCl_2$ for chymotrypsin cleavage. Each protease was added at a final concentration of 6.5 $\mu g\ mL^{-1}$. After incubation at 30 °C for 1 h, the cleaved peptides were separated on a 25 × 0.46 cm Aquapore C4 reversed-phase column (Applied Biosystems) equilibrated with 0.1% trifluoroacetic acid. The peptides were eluted with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid.

Purified p70 was also cleaved with CNBr in 70% formic acid (Gross & Witkop, 1962) for 16 h at room temperature. Peptides were purified as described above on an Aquapore C8 reversed-phase column.

Amino Acid Analysis and Sequence Determination. After extensive dialysis against 0.15% ammonium carbonate, an aliquot of purified p70 was lyophilized, hydrolyzed with 6 N HCl at 110 °C for 30 h, and then analyzed on a Beckman 120 C analyzer. The amino acid sequences of purified peptides were determined with a 470 A gas-phase sequencer on-line coupled to a 120 A PTH analyzer, both operated according to the manufacturer's recommendations (Applied Biosystems Inc., Foster City, CA).

Viscosimetry. Apparent viscosity was measured at low shear rates by the falling-ball technique described by MacLean-Fletcher and Pollard (1980). Viscosity was measured in polymerization buffer (10 mM PIPES, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.1 mM dithiothreitol, 0.1 mM ATP,

pH 7.0) containing increasing concentrations of Ca^{2+}. Macrophage G-actin was mixed with purified p70, drawn into 100-μL glass capillaries, and held at 25 °C for 2 h before measurements. The falling-ball technique yields a semiquantitative characterization of these non-Newtonian solutions which is referred to in the text as apparent viscosity. Apparent viscosity was estimated by use of calibration curves established from glycerol solutions of known viscosity.

Binding of 70-kDa Protein to Actin. Proteins were mixed and held for 2 h at 30 °C in polymerization buffer with and without $CaCl_2$. The reaction mixtures were then centrifuged at 100000*g* for 20 min at 4 °C. Supernatants and pellet fractions were analyzed by SDS–PAGE. The quantity of p70 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 p70 and actin were examined to correlate the densitometric scans with the quantity of protein present and to demonstrate linearity with protein concentrations.

Fluorescence Measurements. Fluorescence spectra were obtained with a Perkin-Elmer LS50 spectrofluorometer. Spectra were recorded after successive additions of 4–20-μL aliquots of $CaCl_2$ solutions and corrected for dilutions. Excitation was performed at 270 nm and fluorescence observed at 310 nm.

Removal of Contaminating Metal. The buffer solution used for calcium-binding studies was 20 mM HEPES/NaOH buffer, pH 7.0, containing 5% glycerol and 100 mM KCl. All buffer and reagent solutions were passed through a Chelex-100 column (Bio-Rad) and stored in plasticware. Cuvettes for spectrophotometric measurements and plasticware were soaked for 2–4 h with 50% nitric acid and extensively washed with ultrapure water. Dialysis tubing was boiled in 5% $NaHCO_3$ and 0.1 mM EDTA and rinsed extensively. p70 was freed from Ca^{2+} by dialysis, at 0–4 °C for 18 h, against 100 vol of the buffer used for the fluorescence measurements containing 1% Chelex equilibrated with the same buffer. The buffer and Chelex were changed once after 12 h of dialysis.

Immunoblotting. Cells and tissue extracts for Western blotting analysis were mixed with SDS sample buffer (20 mM Tris-HCl, pH 6.8, 2.5 M urea, 2.5% SDS, 0.1 mM DTT) at room temperature. Proteins in denatured samples were resolved by electrophoresis on 10% polyacrylamide gels in the presence of 0.1% SDS (SDS–PAGE), as described by Laemmli (1970), and transferred to nitrocellulose membranes by the method of Towbin et al. (1979). The antigen–antibody complexes on immunoblots and dot blots were detected as previously indicated (Pacaud & Molla, 1987).

Other Techniques. The concentration of monomeric actin was determined from the optical density at 290 nm using an extinction coefficient of 0.65 for a 1 mg mL^{-1} actin solution. All other protein concentrations were measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

The free calcium concentration was estimated using a K_{app} for Ca^{2+}/EGTA buffers of 2.2×10^7 M^{-1} at pH 7.0 as previously reported (Haiech et al., 1979). Calculations of free Ca^{2+} present in solutions containing 1 mM EGTA, 2 mM $MgCl_2$, calcium, and 0.1 mM ATP were kindly provided by Jacques Haiech (LCB-CNRS, Marseille, France).

The protein homology analyses were performed using the Fasta software program of Pearson and Lipman (1988), and the data bank was from Protein Identification Resources (National Biomedical Research Foundation, Georgetown University, Washington, DC).

RESULTS

Purification of Macrophage 70-kDa Protein. The new procedure for the purification of p70 from macrophage cytosolic extracts, outlined in Materials and Methods, was devised (a) to increase the yield of this protein and (b) to insure maximum dissociation from actin. Using fractionation by ammonium sulfate, more than 90% of the p70 was recovered in the 42–55% saturation fraction. Most of the protein thus obtained was eluted at a 110–120 mM KCl concentration range from a DEAE–Trisacryl column (Figure 1A). p70 was finally purified, by molecular sieve chromatography on Ultrogel AcA34, to homogeneity as determined by SDS–PAGE (Figure 1 B,C). This procedure yielded 2.5-fold more homogeneous p70 than the method previously developed for the separation of cytoplasmic gel constituents (Pacaud, 1986). Approximately 1.6–1.8 mg of purified p70 was obtained from 630 mg of cytosolic proteins. Generally each preparation started with alveolar macrophages harvested from 12–15 rabbits.

Purified p70 displayed the same isoelectric point and ability to assemble actin filaments into bundles as the protein previously isolated from insoluble cytoplasmic gels (results not shown). Proteolytic cleavage of the purified protein with enzymes of different specificity such as chymotrypsin, trypsin, papain, and thermolysin generated two major polypeptide fragments with apparent molecular masses of about 55 and 11–12 kDa. Proteolysis with chymotrypsin and thermolysin is illustrated in Figure 2. The results suggest that p70 is essentially composed of two structural domains.

Partial Amino Acid Sequencing of Macrophage 70-kDa Protein. p70 was resistant to Edman degradation, thus indicating that its NH_2-terminal end was blocked. p70 was cleaved either with chymotrypsin or thermolysin or chemically with CNBr. The resulting fragments were separated by reversed-phase HPLC (results not shown) as indicated in Materials and Methods. As in p70, the 11-kDa proteolytic fragment contained a blocked NH_2-terminus and was therefore identified as the NH_2-terminally located peptide. The chymotrypsin and thermolysin peptides of 55 kDa were partially sequenced along with two purified CNBr fragments. Approximately 16% of the total protein sequence was identified in these studies. As illustrated in Figure 3, the partial sequence of p70 was found to be almost completely identical to that of human L-plastin (Lin et al., 1988; Su et al., 1990a).

Cellular Distribution of the 70-kDa Protein. The distribution of p70 in different cell types and rabbit tissues was examined by immunoblotting with polyclonal antibodies against the purified protein (Pacaud, 1986). Figure 4 shows that p70 was only present in neutrophils, lymphocytes, and macrophages. The whole lysates from these cells also contained a weak immunoreactive protein, of higher M_r than p70, which was not detected in their corresponding cytosolic fractions. As this protein was also seen on immunoblots incubated with preimmune serum (not shown), it was a nonspecific staining protein. Proteins extracted from brain, thymus, skeletal, and smooth muscles did not give any positive reaction even after fractionation by ammonium sulfate precipitation between 42 and 55% saturation, a first step in p70 purification. The fact that thymic cells do not contain detectable amounts of p70 suggests that the expression of this protein might be related to the differentiation state of cell lineages. In agreement with these results, L-plastin was identified in a variety of human transformed cell lines, including monocytes and T and B lymphocytes. However, this protein was not found in melanoma cells, hela cells, and

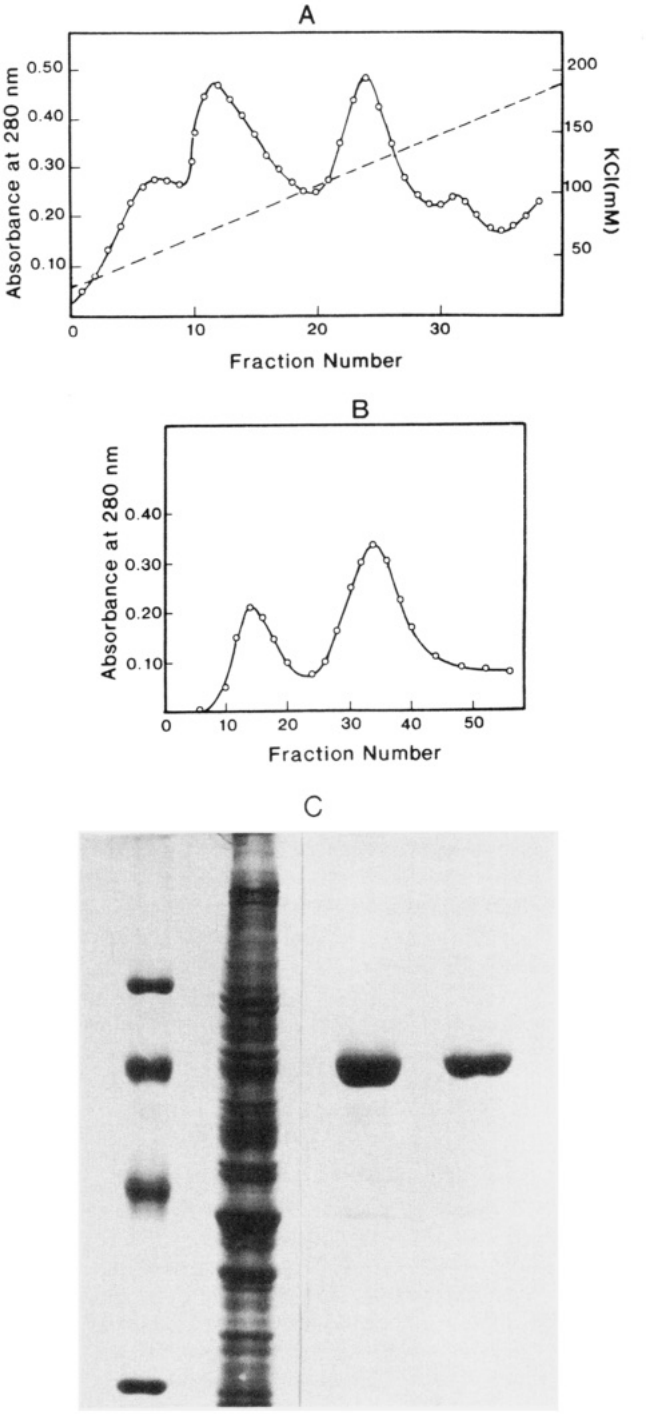

FIGURE 1: Purification of macrophage 70-kDa protein. (A) Elution profile of DEAE–Trisacryl column. Proteins precipitated from 42 to 55% ammonium sulfate saturation were applied to a DEAE–Trisacryl column (4.5 × 2.5 cm). p70 was eluted with a linear KCl gradient at about 120 mM KCl. Fractions of 4.5 mL each were analyzed by SDS–PAGE and tested for their ability to bind to F-actin by cosedimentation at high speed. (B) Elution profile of an Ultrogel AcA 34 column. Fractions 22–27 from the DEAE–Trisacryl column were pooled, concentrated, and subjected to chromatography on a 1.6 × 95 cm Ultrogel column. p70 was eluted in the second protein peak. (C) SDS–PAGE of the fractions collected after the first and last steps of purification: lane 1, ammonium sulfate fraction; lanes 2 and 3, 20 and 10 μg of purified 70-kDa protein, respectively. M_r, molecular mass markers are indicated in kilodaltons.

normal fibroblasts (Matsushima et al., 1988). Thus p70 (or L-plastin) has a much more restricted distribution than α-actinin, which is a widespread actin-bundling protein.

The p70 concentration in macrophage cytosolic extracts was determined by a quantitative dot blot procedure, using anti-p70 antibodies and the purified protein as a standard. The results indicated that the protein constitutes approximately 1.8% of all protein in cytosolic extracts (mean of two separate determinations). Comparatively, their actin and α-actinin contents were 8–9% (Pacaud, 1986) and 0.7–0.8% (Pacaud & Harricane, 1992), respectively. Thus, if the cytoskeletal proteins were uniformly distributed in the cytoplasm, the molar ratio of p70 to actin would be around 1:7–8, whereas that of α-actinin to actin would be close to 1:40. Although these determinations were approximate, they were in agreement with our previous observation that the relative molar abundance of p70 in macrophage cytoplasmic gels was 5-fold higher than that of α-actinin (Pacaud, 1986).

Influence of Calcium on the Interactions of the 70-kDa Protein with Macrophage F-Actin. The effect of increasing concentrations of Ca^{2+} on the bundling activity of p70 was measured by falling-ball viscosimetry. These experiments were performed at saturating concentrations of p70 in order to minimize heterogeneity in the size and degree of cross-linking of actin filament structures generated by the protein. Half-maximal inhibition was observed at 1.5 μM free Ca^{2+}. At the highest Ca^{2+} concentrations tested, the apparent viscosity of p70–F-actin mixtures remained at a level similar to that of actin filaments alone (Figure 5). Actin-severing proteins, such as gelsolin and villin, are known to reduce the viscosity and length of actin filaments in vitro in the presence of micromolar Ca^{2+} (Kwiatkowsky et al., 1989; Mooseker et al., 1980; Bretscher & Weber, 1980a). This observation thus confirms our previous suggestion that p70 is not capable of fragmenting actin filaments (Pacaud & Harricane, 1987).

The binding of p70 to macrophage F-actin was assessed by cosedimentation at 100000*g* for 30 min. Results of the cosedimentation experiments were analyzed using Scatchard plots (Figure 6). p70 was found to have an apparent K_d of 1.8 μM in the absence of Ca^{2+}. In the presence of 20 μM free Ca^{2+}, the apparent K_d dropped more than 3-fold to 5.5 μM.

Calcium-Binding Properties of the Macrophage 70-kDa Protein. Calcium-binding studies were restricted to the effects of Ca^{2+} ions on the p70 conformation due to the limited amounts of purified protein. As L-plastin contains a Tyr residue within the calcium-binding loop of its first EF-hand motif (Lin et al., 1990a; Zu et al., 1990), the metal-binding properties of p70 were examined by spectrofluorometry. The influence of saturating amounts of Mg^{2+} and Ca^{2+} on the fluorescence emission spectrum of Tyr residues is shown in Figure 7. In the presence of 5% glycerol, Ca^{2+} or Mg^{2+} induced a decrease in fluorescence intensity at the maximum emission wavelength with no detectable shift. This decrease was greater with Ca^{2+} than with Mg^{2+}.

Ca^{2+} also gave rise to an increase in tyrosine fluorescence intensity at wavelengths higher than 372 nm, while no change was observed on Mg^{2+} binding. Moreover, when Ca^{2+} was added to the Mg^{2+}-liganded protein, the emission spectrum did not significantly differ from that obtained with Ca^{2+} alone. These data indicate that p70 binds Ca^{2+} with a higher affinity than Mg^{2+}.

The extent of the decrease in fluorescence emission intensity was determined as a function of Ca^{2+} concentration (Figure 8). Most of the spectral changes occurred between 0.15 and 1.5 μM free Ca^{2+}, suggesting that the Ca^{2+}-binding sites of p70 might have an apparent K_d in this concentration range. The effects of Ca^{2+} at concentrations lower than 0.15 μM were not examined due to the higher rate of errors in the determinations of free Ca^{2+}. The presence of glycerol was required to obtain reproducible quantitative results. However, in the absence of glycerol an increase, instead of a decrease, was observed in the intensity of the fluorescence emission (at

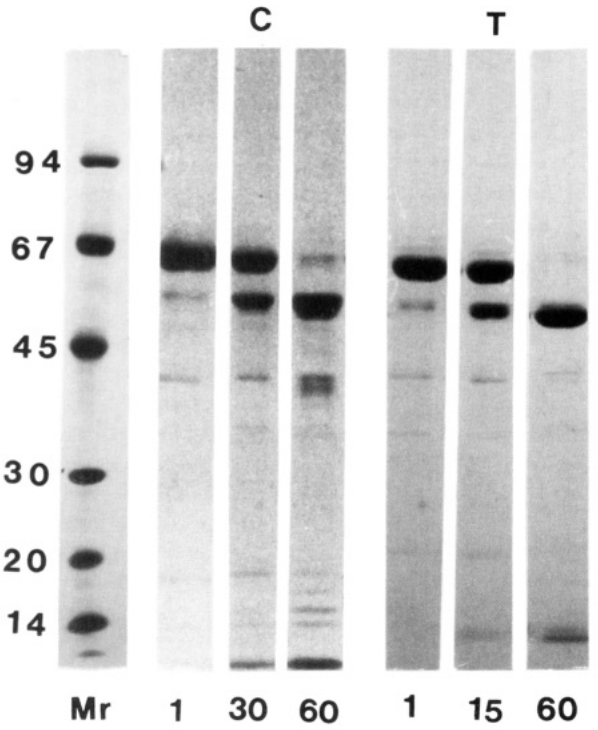

FIGURE 2: Proteolysis of 70-kDA protein by chymotrypsin (C) and thermolysin (T). Each enzyme was added to p70 at a ratio of 1:50 (w/w) under the conditions described in Materials and Methods. Aliquots were withdrawn at regular intervals, boiled in SDS sample buffer, and analyzed by SDS–PAGE on 6–20% polyacrylamide gradient gels. The two major polypeptidic fragments obtained after a 1-h incubation were separated by HPLC and used for sequence determinations. The molecular mass markers (M_r) are given in kilodaltons.

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                          <--EF HAND-->
  1  MARGSVSDEEMMELREAFAKVDTDGNGYISFNELNDLFKAACLPLPGYRVREITENLMAT

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 61  GDLDQDGRISFDEFIKIFHGLKSTDVAKTFRKAINKKEGICAIGGTSEQSSVGTQHSYSE
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                                     55 K C              55 K T

121  EEKYAFVNWINKALENDPDCRHVIPMNPNTNDLFNAVGDGIVLCKMINLSVPDTIDERTI..//
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481  QDLNEGNRTLTLALIWQLMRRYTLNILEEIGGGQKVNDDIIVNWVNETLREAEKSSSISS
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                                    CNBR I

541  FKDPKISTSLPVLDLIDAIQPGSINYDLLKTENLNDDEKLNNAKYAISMARKIGARVYAL
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                                                             CNBR II

601  PEDLVEVNPKMVMTVFACLMGKGMKRV
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FIGURE 3: Alignment of the partial amino acid sequence of the 70-kDa protein with the sequence of plastin I (taken from Zu et al., 1990b). The sequences determined from four peptidic fragments derived from p70 are aligned on the complete sequence of plastin. Identical residues are indicated with an hyphen. The two peptides of 55 kDa were isolated after chymotrypsin (C) and thermolysin (T) cleavage of p70; the other two located on the C-terminal side were obtained by CNBr cleavage. The consensus sequence of the two EF-hand Ca^{2+}-binding motifs in plastin I is also indicated.

335 nm) upon Ca^{2+} and Mg^{2+} binding. But glycerol did not seem to shift the threshold concentration of Ca^{2+} that caused a maximum change in the emission spectra.

DISCUSSION

The purification and further characterization of p70, a Ca^{2+} target protein, are part of our continuing effort to understand how Ca^{2+} regulates the general organization of microfilaments in motile cells. p70 was isolated from cytosolic protein by ammonium sulfate fractionation, DEAE–Trisacryl chromatography, and gel filgration. It was obtained with a purification yield which was about 2.5-fold higher than that obtained with a previously described procedure from insoluble actin networks (Pacaud, 1986). On SDS–PAGE, the purified protein migrated as a single major band with an apparent molecular mass of 70 kDa (Figure 1C).

P70: Identical to L-*Plastin.* Comparison of the partial amino acid sequence of p70 with those of other known proteins revealed that p70 was identical to L-plastin. Although the

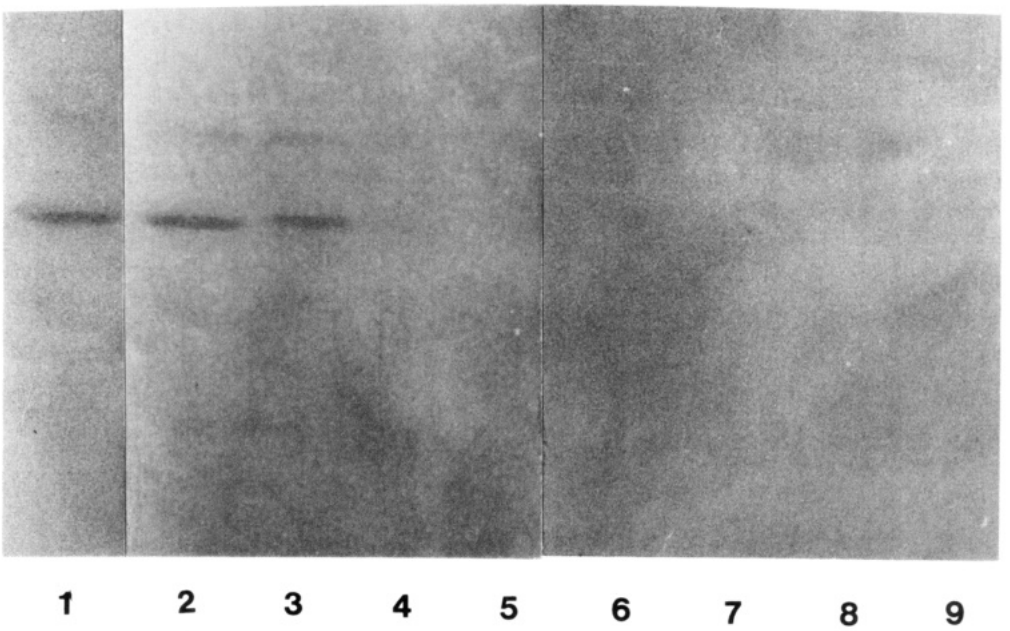

FIGURE 4: Immunoblot analysis of different cell types and tissues using polyclonal anti-70-kDa protein antibodies. Cell lysates and tissue extracts were electrophoresed under denaturing conditions and transferred onto a nitrocellulose membrane. They were reacted sequentially with anti-p70 antibodies (1:50), peroxidase-conjugated anti-guinea pig IgG antibodies (1:1000), and 4-chloro-1-naphthol. Each lane represents 80 μg of proteins from (1) alveolar macrophages, (2) neutrophils, (3) lymphocytes, (4) thymic cells, (5) endothelial cells, (6) epithelial cells from intestinal brush border, (7) brain, (8) smooth muscle from stomach, and (9) skeletal muscle. p70 was detected in the first three samples only. It should be noted that the antiserum did not give any detectable positive reaction with proteins from rabbit brush border, although a Coomassie blue stainable polypeptide of 68 kDa was present in these samples.

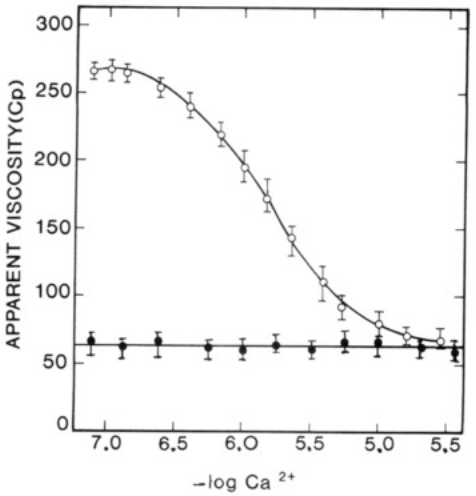

FIGURE 5: Effect of the free calcium ion concentration on the apparent viscosity of mixtures of the 70-kDa protein with F-actin. Apparent viscosity was determined by the falling-ball technique. Samples contained 0.5 mg mL^{-1} of macrophage monomeric actin and 0.25 mg mL^{-1} of p70 in the presence of 10 mM PIPES, 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM ATP, 1 mM EGTA, pH 7.0, and the free calcium ion concentration indicated. The experimental points represent the average of triplicate determinations. Actin + p70: O. Actin alone: ●.

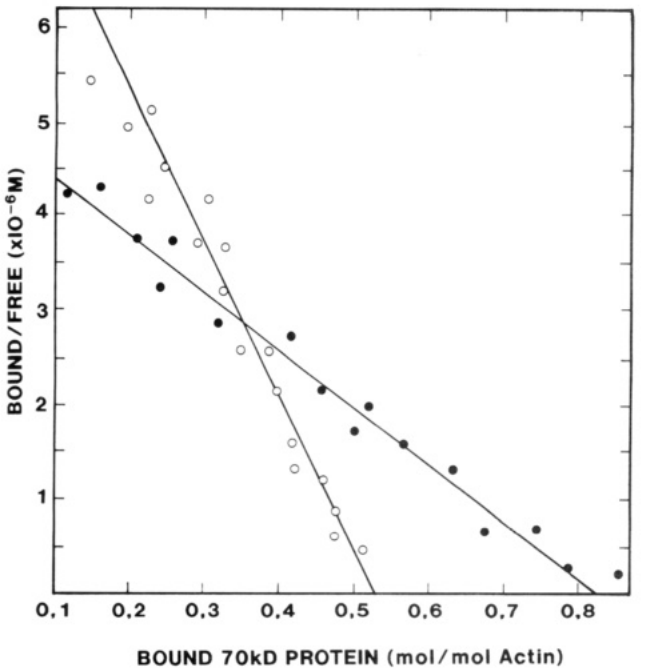

FIGURE 6: Quantitative analysis of the binding of the 70-kDa protein to F-actin at 30 °C. Binding of p70 to actin was assessed by cosedimentation as described in Materials and Methods. Various amounts of p70 were mixed with macrophage monomeric actin at 0.4 mg mL^{-1} for measurements in the presence of 20 μM free Ca^{2+} (●) and 0.3 mg mL^{-1} for measurements in the absence of Ca^{2+} (O). The incubation conditions were those indicated in the legend of Figure 5. The experimental points represent the average of two different experiments.

two proteolytic and two CNBr peptides derived from rabbit p70 covered 109 residues, their sequence showed only five conservative amino acid substitutions with human L-plastin (Figure 3). Plastin was originally identified as a 65-kDa phosphorylated polypeptide in chemically transformed neoplastic human fibroblasts (Goldstein & Leavitt, 1985). This protein was isolated in small amounts from human blood mononuclear cells (Matsushima et al., 1988) and human T cells (Zu et al., 1990a) using procedures which differed from that described here. The function of plastin was unknown until its cDNA was cloned and sequenced. The deduced amino acid sequence revealed the existence of two helix–loop–helix calcium-binding motifs (EF-hands) and two actin-binding sites on the same polypeptide chain (Lin et al., 1988, 1990; Zu et al., 1990b). On the basis of these findings, Lin et al., suggested that L-plastin might be identical to macrophage p70 (Pacaud, 1986; Pacaud & Molla, 1987), whereas Zu et al. (1990b) considered it might be acumentin, a protein which caps actin filaments at their pointed ends (Southwick & Stossel, 1981). However, their postulation was essentially based on the fact

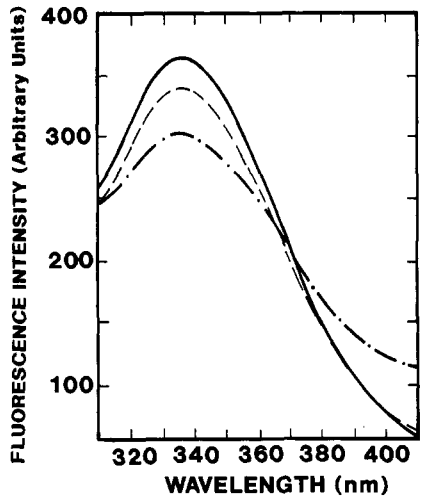

FIGURE 7: Effect of calcium and magnesium ions on the fluorescence emission spectrum of the 70-kDa protein. Spectra were recorded with 0.56 μM p70 in 20 mM HEPES, 100 mM KCl 0.2 mM dithiothreitol, and 5% glycerol, pH 7, at 20 °C containing 1 mM EDTA (—) and after the addition of Mg^{2+} (- - -) or Ca^{2+} (-··-) to a final concentration of 0.1 mM. Tyrosine excitation was performed at 270 nm with a 5-nm band width.

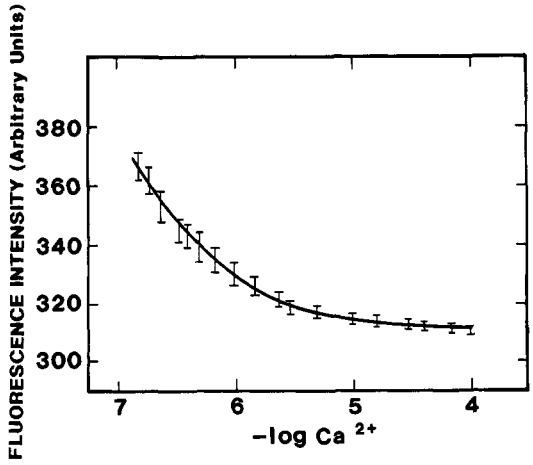

FIGURE 8: Quantitative analysis of the calcium-induced changes in fluorescence intensity of the p70 emission spectrum. The experimental conditions were identical to those of Figure 7 except that increasing concentrations of Ca^{2+} were used. Fluorescence emission was measured at 336 nm. The experimental points represent the average of two different experiments.

that L-plastin and acumentin have a similar amino acid composition. In addition, it has been erroneously reported that plastin was the same protein as fimbrin because of their sequence homology: 70% identity in 627 amino acid residues. Polyclonal antibodies against fimbrin also cross-reacted with plastin (De Arruda et al., 1990). However, our anti-plastin antiserum did not recognize the fimbrin present in intestinal cell extracts (Figure 4). Despite their structural analogy, these two proteins also differed by several functional properties.

Influence of Calcium on the Functional Properties of Macrophage L-*Plastin.* Formation of actin filament bundles in negatively stained samples of F-actin and L-plastin mixtures was readily demonstrated, as previously described (Pacaud & Harricane, 1988). The inhibitory effect of Ca^{2+} on the assembly of actin filaments by plastin was examined by low shear viscosity and high-speed sedimentation. The cross-linking activity of plastin was gradually inhibited at free Ca^{2+} concentrations varying from 0.3 to 3 μM, i.e., in the significant physiological range. Since conformational changes in the protein were also induced by the addition of micromolar Ca^{2+}, the loss in plastin activity was a direct consequence of Ca^{2+} binding.

We did not obtain any evidence that L-plastin was able to fragment actin filaments in the presence of Ca^{2+}. L-Plastin thus functionally differs from villin, a protein present in intestinal epithelial cells and which associates actin filaments into bundles in the absence of Ca^{2+} and severs them in the presence of micromolar Ca^{2+} (Janmey & Matsudaira, 1988). However, the sedimentation experiments indicated that Ca^{2+} causes a 3-fold decrease in the apparent K_d of plastin for F-actin (Figure 6). This observation suggests that the inhibition of cross-linking activity by Ca^{2+} results from a loss in protein binding to F-actin rather than breaking of cross-bridges between actin filaments. Such an interpretation is also supported by previous observations which indicated that the ability of plastin to associate with actin gel networks in macrophage cytosolic extracts was completely inhibited at submicromolar concentrations of free Ca^{2+} (Pacaud & Molla, 1987). The partial inhibitory effect of Ca^{2+} on the affinity binding of the purified protein for F-actin probably reflects difficulties in separating different types of actin structures by centrifugation. Plastin–F-actin bundles could not be separated from actin filaments at low centrifugal forces. We were also unable to obtain conditions to allow collection of plastin–F-actin bundles separate from loosely cross-linked networks.

Binding of Calcium to Macrophage L-*Plastin.* Fluorescence studies indicated that the environment of Tyr residues was affected by Ca^{2+} and Mg^{2+} binding to metal-free plastin (Figure 7). The fact that Mg^{2+} produced spectral changes of smaller amplitude than Ca^{2+} and only partially mimicked the effect of Ca^{2+} suggests ion binding selectivity for Ca^{2+}. Major changes in fluorescence emission intensity were observed at free Ca^{2+} concentrations varying from 0.1 to 1.5 μM, in the absence of Mg^{2+}, and with 100 mM KCl. Although Mg^{2+} might slightly decrease the affinity of plastin for Ca^{2+}, these data are consistent with the presence of strong Ca^{2+}-binding sites. Very likely, they reflect the functional state of the two EF-hand motifs present in the protein. These motifs are located within the first 80 amino acid residues of plastin, while the two actin-binding sites are contained in its middle and C-terminal parts (Lin et al., 1990; Zu et al., 1990b). Thus, Ca^{2+} binding induced a global conformational change in the molecule which inactivates the actin-binding sites, probably through a loss of accessibility to F-actin.

L-Plastin is composed of two structural domains linked by a protease-sensitive segment. Very likely the 11-kDa peptidic fragment contains the two-Ca^{2+}-binding sites.

Comparison of Macrophage Plastin with Other Actin-Bundling Proteins of Mammalian Origin. Plastin displays several features that distinguish it from other known actin-bundling proteins such as fimbrin and α-actinin. Fimbrin was isolated and characterized from epithelial cells of intestinal brush border (Bretscher, 1981; Glenney et al., 1981) and identified in fibroblasts and other cells (Bretscher & Weber, 1980b). Like plastin, it is a 68-kDa monomeric protein which binds to F-actin with a stoichiometry of one molecule of fimbrin per three actin monomers. Comparatively, α-actinin is a rod-shaped dimer of 205 kDa, which binds to F-actin with a stoichiometry of 1:12 (Wallraff et al., 1986; Witke et al., 1986). In contrast to α-actinin and fimbrin, L-plastin has a limited distribution, occurring only in cells of hematopoietic origin. A consensus sequence which is analogous to the Ca^{2+}-binding sites of calmodulin (de Arruda et al., 1990; Lin et al., 1990; Zu et al., 1990b) has been identified in the three proteins. However, L-plastin is the only one which possess all of the conserved amino acid residues known to be essential for Ca^{2+}

ligation in proteins of the calmodulin superfamily (Strynadka & James, 1989).

L-Plastin, α-actinin, and fimbrin also differ in their sensitivity to divalent cations and ability to bind calcium. The studies presented here indicated that plastin–F-actin interactions are inhibited by micromolar Ca^{2+} and unaffected by Mg^{2+} up to 3 mM. Furthermore, they showed that the protein binds Ca^{2+} selectively in a micromolar range of concentrations. Although α-actinin was long thought to be a Ca^{2+}-regulated protein, we have recently demonstrated that the interactions between macrophage α-actinin and F-actin are weakly inhibited by Ca^{2+} at concentrations higher than 100 mM. We have also provided evidence that the Ca^{2+}-binding sites of α-actinin have a K_d higher than 80–100 μM (Pacaud & Harricane, 1992). In contrast to those present in L-plastin, the Ca^{2+}-binding sites of α-actinin and fimbrin can be competed by Mg^{2+} (Bretscher & Weber, 1980). Moreover, the binding of fimbrin to F-actin is unaffected by Ca^{2+} (up to 5 mM) and completely inhibited by Mg^{2+}, at concentrations higher than 0.5 mM (Bretscher, 1981). Since the intracellular concentration of Mg^{2+} is 1000–10000-fold higher than that of Ca^{2+}, the metal-binding sites of fimbrin and α-actinin should be saturated by Mg^{2+} within cells. Whether such sites are involved in the maintenance of protein conformational stability through Mg^{2+} binding or are only vestiges of a common ancestral Ca^{2+}-binding protein remains to be investigated.

Functional Significance of L-*Plastin.* According to our estimates, there is approximately one molecule of L-plastin per seven or eight actin monomers in macrophage cytosolic fractions, whereas a maximum of three molecules of plastin can bind to three actin monomers in a filamentous form (Pacaud & Harricane, 1987). Thus, a large fraction of this protein might well be associated with F-actin in resting macrophages. It seems likely, therefore, that L-plastin is a major F-actin-binding protein in highly motile cells.

The selective and quantitative release of L-plastin, from macrophage-insoluble cytoplasmic gels, by submicromolar concentrations of free Ca^{2+} was previously reported (Pacaud & Harricane, 1987). The data presented here indicated that Ca^{2+} binding to the purified protein, in the physiologically important concentrations, leads to a loss in its ability to associate with actin filaments. L-Plastin thus has the expected properties of a Ca^{2+}-regulated protein which might play an important role in the regulation of cytoplasmic viscosity and, therefore, of cell shape and motility. Studies of other investigators have revealed that L-plastin is rapidly phosphorylated at serine residues in human neutrophils exposed to interleukin I (Matsushima et al., 1990) and T lymphocytes stimulated with interleukin II (Zu et al., 1990b). This suggests that the function and/or cellular localization of this protein is also controlled through phosphorylation and dephosphorylation reactions.

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