REVERSIBILITY OF GELSOLIN/ACTIN INTERACTION IN MACROPHAGES

Evidence of Ca^{2+}-dependent and Ca^{2+}-independent Pathways

By C. CHAPONNIER, H. L. YIN, AND T. P. STOSSEL

From the Hematology-Oncology Unit, Department of Medicine, Massachusetts General Hospital, and Harvard Medical School, Boston, Massachusetts 02114

Rapid and reversible assembly of an actin matrix in the peripheral cytoplasm is associated with the motile functions of mammalian phagocytes (1). Although certain agonists produce a transient rise in the intracellular Ca^{2+} concentration which precedes the onset of leukocyte actin assembly and motility (2–9), it is also apparent that leukocyte actin assembly and associated movements can also take place in some cases without a detectable increase in the cytosolic Ca^{2+} level (10–12). Therefore, besides Ca^{2+}, an additional signal is implicated for the regulation of actin assembly associated with leukocyte motility. Gelsolin, a Ca^{2+}-binding protein of mammalian leukocytes (13) and other cells (14–19), is a potent modulator of actin filament length and gelation. In the presence of micromolar Ca^{2+} concentration, purified gelsolin severs actin filaments and remains bound to two actin monomers at the fast-growing end of the severed filaments where it blocks further exchange of monomers at the end (13–19). If these effects were reversible, gelsolin could, in concert with other actin-binding proteins, regulate the state of polymerization of actin in response to changes in the cytosolic Ca^{2+} concentration. However, whereas the binding of purified gelsolin to actin is highly Ca^{2+}-dependent, it is only partially reversed in vitro by a lowering of the calcium concentration (20–23). The addition of the calcium chelator EGTA to the 2:1 actin/gelsolin complex permits only one of the two bound actins to dissociate, resulting in a 1:1 actin/gelsolin complex which is incapable of severing actin filaments even though the complex can block the fast-growing end of actin filaments. If another mechanism, not directly involving changes in Ca^{2+} concentration, was able to dissociate the EGTA-resistant actin/gelsolin complex, it would suggest that gelsolin has a central role as a regulator of the actin matrix in vivo.

Materials and Methods

Triton Extraction of Cells. Rabbit lung macrophages were isolated according to Myrvik et al. (24). 1 ml of packed cells was resuspended in 30 volumes of Krebs-Ringer phosphate

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buffer (KRP),[1] pH 7.4, containing 10^{-3} M $CaCl_2$. To prepare cytoplasmic extracts, 0.5 ml of cell suspension was mixed with an equal volume of Triton extraction buffer (TEB), containing 1.5% Triton X-100, 120 mM Pipes, 50 mM Hepes (pH 7.2), 20 mM EGTA, 4 mM $MgCl_2$, 20 μg/ml leupeptin, 156 μg/ml benzamidine, 80 μg/ml aprotinin, and 1 mM PMSF (25). After 2 min at room temperature, the samples were centrifuged at 13,800 *g* for 2 min in an Eppendorf centifuge, and the supernatants, referred to as Triton-soluble extracts, were collected.

Identification of Gelsolin by Immunoblotting. The Triton-soluble extracts and pellets were boiled in gel sample buffer and electrophoresed on 5–15% acrylamide gradient slab gels in the presence of SDS (26). Proteins on the gel were transferred to nitrocellulose paper (Schleicher & Schull, Inc., Keene, NH) and gelsolin was identified by immunoblotting according to the method of Towbin et al. (27) with an antigelsolin mAb 2C4C10G2 (28), and with horseradish peroxidase–labeled rabbit anti–mouse IgG as a second antibody.

Immunoadsorption Assay for EGTA-irreversible Actin/Gelsolin Complexes. IgG purified from ascites fluid of mice injected with antigelsolin clone 2C4C10G2 (28) was covalently linked to CNBr-activated Sepharose 4B beads. 20 μl of the antigelsolin beads were incubated for 30–60 min in a 1-ml solution containing 0.75% Triton X-100, 60 mM Pipes, 25 mM Hepes (pH 7.2), and 1 mM EGTA. After centrifugation for 2 min at 13,800 *g* in an Eppendorf centrifuge, the beads were washed twice with 1 ml of the above buffer. In some cases, an additional wash with 0.3 M $MgCl_2$ was included. Proteins associated with the beads were analyzed by polyacrylamide electrophoresis and stained with Coomassie blue dye. The intensities of the gelsolin and actin bands were determined by scanning with a densitometer (Biomed Instruments, Inc., Fullerton, CA) and their molar ratios were calculated. Coomassie blue stains gelsolin, which has twice the molecular weight of actin, twice as intensely as actin on a molar basis (17).

Results

The partitioning of gelsolin in Triton-soluble and -insoluble extracts was assessed by immunoblotting. Fig. 1 shows one of five experiments that revealed that 95 ± 5% of the gelsolin in macrophages is solubilized by the detergent. A small amount of gelsolin was found in the insoluble fraction, and even this amount may be an overestimate, because the pellets were not washed and may therefore be contaminated by soluble gelsolin. Of the cellular actin, 40% is in the Triton-soluble fraction. To determine the amount of actin bound to gelsolin in EGTA (i.e., EGTA-resistant actin/gelsolin complexes), gelsolin and associated actin were extracted with the monoclonal antigelsolin Sepharose. To validate the assay, sufficient beads to bind all gelsolin present were incubated with gelsolin and actin at a 1:2 molar ratio in the presence of 1 mM EGTA or $CaCl_2$ (Fig. 2*A*). Lane *a* shows that when the beads were incubated with actin and gelsolin in EGTA, and washed in EGTA, a gelsolin band and two more rapidly migrating bands corresponding to H (50 kD) and L chains (25 kD) of the antigelsolin antibody were associated with the beads. No band is visible for actin, confirming that actin does not bind significantly to gelsolin in EGTA and demonstrating that actin does not bind nonspecifically to the beads. In Fig. 2*A*, lane *b*, which resolves proteins on beads incubated with actin and gelsolin in $CaCl_2$ and then washed with buffer containing Ca^{2+}, the actin and gelsolin bands are very similar in intensity, and the scan of this gel reveals a 1:2 gelsolin/actin molar ratio. In lane *c*, which contains polypeptides derived from beads incubated with actin and

[1] *Abbreviations used in this paper:* KRP, Krebs-Ringer phosphate buffer; TEB, Triton extraction buffer.

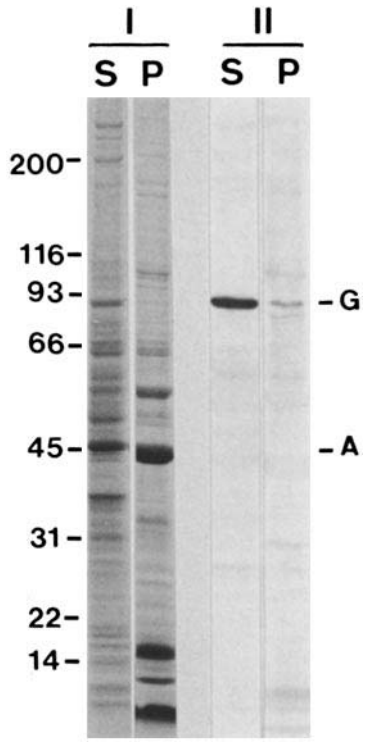

FIGURE 1. Distribution of gelsolin in the Triton-soluble and -insoluble fractions of macrophages. The supernatant (*S*, Triton-soluble extract) and pellet (*P*, Triton-insoluble pellet) were collected and analyzed by PAGE. Equivalent amounts of samples were loaded on the gel in duplicate. One part of the gel (*I*) was stained with Coomassie blue, and the other (*II*) was immunoblotted onto nitrocellulose paper with antigelsolin antibody. The M_r ($\times 10^{-5}$) of marker proteins are indicated. *A* and *G* indicate the positions of actin and gelsolin, respectively.

gelsolin in Ca^{2+} and then washed with EGTA, the actin band is half the intensity of the gelsolin band, indicating a 1:1 complex.

Fig. 2*B* shows that incubation of the beads with a Ca^{2+}-enriched Triton extract of macrophages resulted in binding of actin and gelsolin to the antigelsolin beads at a molar ratio of 4:1 (lane *a*) and that incubation with an EGTA-containing extract resulted in a 0.8:1 actin/gelsolin ratio (lane *d*). Since gelsolin has only two actin-binding sites per molecule (17, 20–23), binding of four actins per gelsolin on beads in the first case suggests that some of the actins bound to gelsolin were in an oligomeric state under these conditions. The extent of actin polymer association in vivo may in fact be higher than that detected on the antibody-coated beads, because some of the filaments may depolymerize during extensive washing of the beads. Lane *b* shows that beads incubated with extracts in $CaCl_2$ but washed with EGTA did not result in a significant decrease in the amount of actin complexed with gelsolin even after prolonged incubation in EGTA. To determine accurately the number of actin monomers bound directly to gelsolin on the beads, an additional washing step with 0.3 M $MgCl_2$ in EGTA facilitated the depolymerization of actin oligomers and therefore avoided overestimation of actin bound to gelsolin. Control experiments have shown that

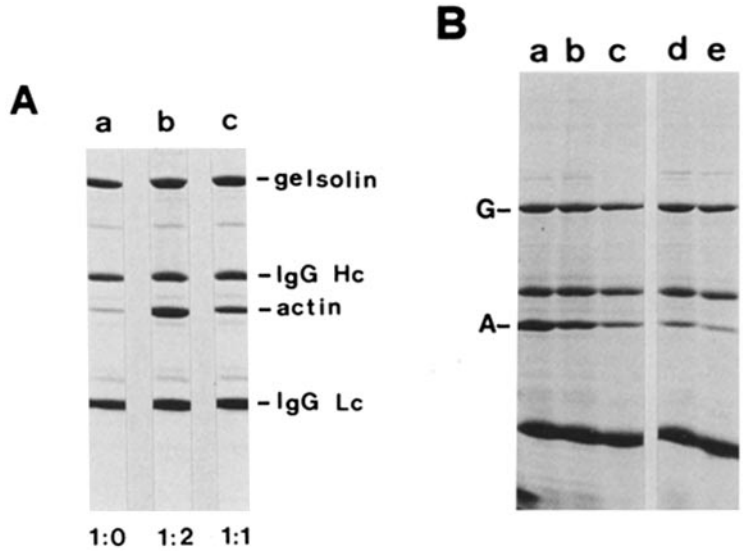

FIGURE 2. Assay for gelsolin/actin complexes. (*A*) Immunoabsorption of purified proteins. 20 μl of beads were incubated for 30 min with rabbit skeletal muscle actin and human plasma gelsolin at a 2:1 molar ratio diluted in 1 ml of a solution containing 0.75% Triton X-100, 60 mM Pipes, 25 mM Hepes (pH 7.2), and 1 mg/ml BSA in the presence of 1 mM EGTA (*a*) or 1 mM $CaCl_2$ (*b* and *c*). The beads were washed twice with 1 ml of buffer containing EGTA (lanes *a* and *c*), and $CaCl_2$ (lane *b*) and analyzed on an SDS-polyacrylamide gel. The antibody-coated beads bind gelsolin and actin/gelsolin complexes equally well. *Hc* and *Lc* represent H and L chains of the IgG from the monoclonal antigelsolin bound to the beads. The gelsolin/actin ratios are indicated at the bottom of each lane. (*B*) Immunoadsorption of gelsolin from Triton-soluble extracts. 3% suspensions of macrophages, incubated for 30 min at 37°C in KRP, were extracted with TEB containing 1 mM $CaCl_2$ and no EGTA (lanes *a–c*) or 20 mM EGTA (lanes *d* and *e*). 20 μl antigelsolin beads were added to 1 ml Triton-soluble extracts. After 1 h at 4°C, the beads in lanes *a–c* were washed three times in a solution containing 0.75% Triton X-100 and 1 mM $CaCl_2$. Those in lanes *b* and *c* were further washed three times with 150 mM NaCl, 1 mM EGTA, 10 mM Tris-HCl, pH 7.4, and the latter beads were also washed once more with the same solution containing 0.3 M $MgCl_2$. The beads in lanes *d* and *e* were washed in EGTA or 0.3 M $MgCl_2$ as in lanes *b* and *c*, respectively. The washed antigelsolin beads were prepared for PAGE electrophoresis as described. *G*, gelsolin; *A*, actin.

pyrene-iodoacetamide–labeled F-actin is depolymerized in 0.3 M $MgCl_2$ rapidly, and that the EGTA-resistant 1:1 actin/gelsolin complex is stable at $MgCl_2$ concentration as high as 3 M. As shown in Fig. 2*B*, lanes *c* and *e*, washing the beads with 0.3 M $MgCl_2$ reduced the amount of actin associated with the antibody-coated beads but did not affect gelsolin binding to them. The estimated molar ratios of actin to gelsolin in the Ca^{2+} and EGTA-treated samples are 1:1 and 0.5:1, respectively. Since under these conditions, only the free gelsolin and the 1:1 actin/gelsolin complex are expected, we infer from the above ratios that 100% of the gelsolin is complexed with actin in the first case, while 50% of gelsolin is complexed with actin in the second case.

Fig. 3 shows that incubation of macrophages in suspension leads to the formation of EGTA-resistant actin/gelsolin complexes. Cells extracted immediately after removal from the rabbit demonstrate a relatively low proportion of actin bound to gelsolin in an EGTA-resistant complex, as shown in lane *a*. Lanes *b–d* are of immunoprecipitated extracts of the same cells incubated for 10, 20,

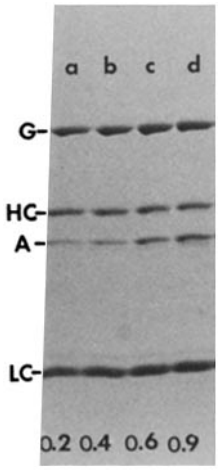

FIGURE 3. Formation of EGTA-resistant actin/gelsolin complexes in macrophages in suspension as a function of time. A 3% suspension of macrophages in KRP was gently agitated at 37°C in a 50-ml plastic tube. At 0 min (*a*), 10 min (*b*), 20 min (*c*), and 30 min (*d*) of incubation, 0.5-ml aliquots were removed and extracted with 0.5 ml of TEB solution containing 20 mM EGTA. Centrifugation, immunoadsorption with antigelsolin beads, washing of the beads with EGTA- and 0.3 M $MgCl_2$-containing solutions, electrophoresis, and gel scanning were performed as described in Materials and Methods. Indicated on the Coomassie blue–stained gels are *G*, gelsolin; *A*, actin; *HC* and *LC*, IgG H and L chains. The molar ratios of actin to gelsolin remaining on the washed beads, determined by densitometry scanning of the gel, are indicated below each gel lane.

and 30 min, respectively, in suspension at 37°C. During the 30-min incubation, the proportion of gelsolin in the EGTA-resistant complex increased from 20 to 90%. This increase with time was consistently observed in numerous experiments, although the baseline fraction of free gelsolin, the rate and extent of EGTA-resistant complex formation were variable.

The Ca^{2+} ionophore, ionomycin, at a concentration of 1 μM, in the presence of 1 mM extracellular Ca^{2+}, increased further the amount of actin complexed with gelsolin (Fig. 4). The percent of total gelsolin complexed to actin rose from 50% initially to 80% by 30 s. Therefore, ionomycin promotes EGTA-resistant actin/gelsolin complex formation in macrophages, as was previously reported for platelets (23). In contrast to the results with the Ca^{2+} ionophore, addition of 10 nM FMLP to macrophages that had been incubated in suspension in the presence of 1 mM $CaCl_2$ at 37°C for 30 min caused within 5 s an abrupt drop in the proportion of gelsolin bound to actin in an EGTA-irreversible form (Table I). A comparison of the actin and gelsolin contents in the Triton-soluble and -insoluble pellets of these samples shows that there was no detectable change in response to FMLP stimulation and that the gelsolin content remained low in the insoluble fraction. Therefore, the disappearance of actin/gelsolin complexes from the Triton-soluble fraction after FMLP stimulation could not be due to selective incorporation of the complex into the insoluble fraction, but rather reflects a dissociation of the complex.

Unlike cells in suspension that spontaneously accumulated EGTA-resistant actin/gelsolin complexes, cells plated on tissue culture dishes rapidly lost such

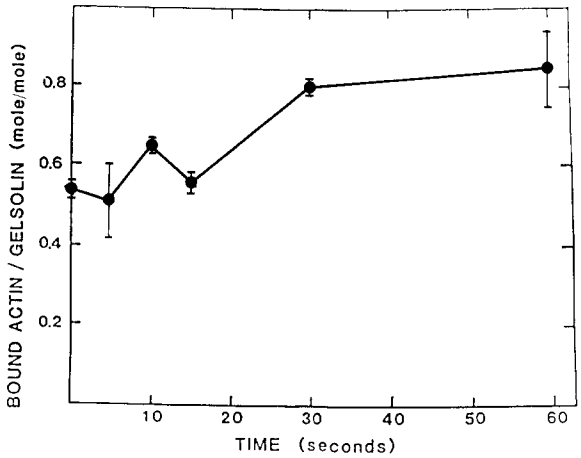

FIGURE 4. Effect of ionomycin on the proportion of EGTA-resistant actin/gelsolin complexes. Macrophages incubated in KRP for 30 min at 37°C were treated with 10^{-6} M ionomycin for the intervals indicated before extraction with an equal volume of TEB. Ionomycin was added as a 10-mM stock solution in DMSO, so that the final DMSO concentration was 0.01%. The basal level of EGTA-resistant complex did not change during the short period of the experiment, and the DMSO concentration did not alter the basal complex level in a control sample (not shown). Result was mean and range of two separate experiments.

TABLE I

Effect of FMLP on the Proportion of EGTA-resistant Actin/Gelsolin Complexes in Extracts of Suspended Macrophages

Percentage of gelsolin in an EGTA-resistant complex			
Experiment number	Additions*		
	FMLP	DMSO	None
1	23	32	43
2	24	34	40
3	25	51	51
4	26	35	47
Mean value	25	38	45
±SD	1	4	2

* Macrophages were incubated in suspension as described in Fig. 2. After 30 min, FMLP (dissolved in DMSO as a 1-mM stock solution) was added to an aliquot of the cells to a final concentration of 10^{-8} M. As a control, an equivalent amount of DMSO was added to another aliquot of cells to a final concentration of 0.01%. A third aliquot of cells had no addition. After 5 s, 0.5 ml of the cell suspensions were extracted by adding 0.5 ml of TEB for 2 min at room temperature, followed by centrifugation as described. Immunoadsorbtion of the extracts and analysis of polypeptides obtained were as described. The Student's t test shows there are significant differences between columns 2 and 4 ($p < 0.001$) and columns 2 and 3 ($p < 0.025$), but not between columns 3 and 4 ($p > 0.2$).

complexes. In the experiment depicted in Fig. 5, macrophages were suspended in a balanced salt solution for 30 min at 37°C and then plated onto petri dishes. Lane *A* shows that before plating, cells in suspension have 66% of their gelsolin in an EGTA-resistant complex with actin, whereas lane *B* shows that 10 min after plating, a stimulus known to increase actin assembly (29) and recently shown

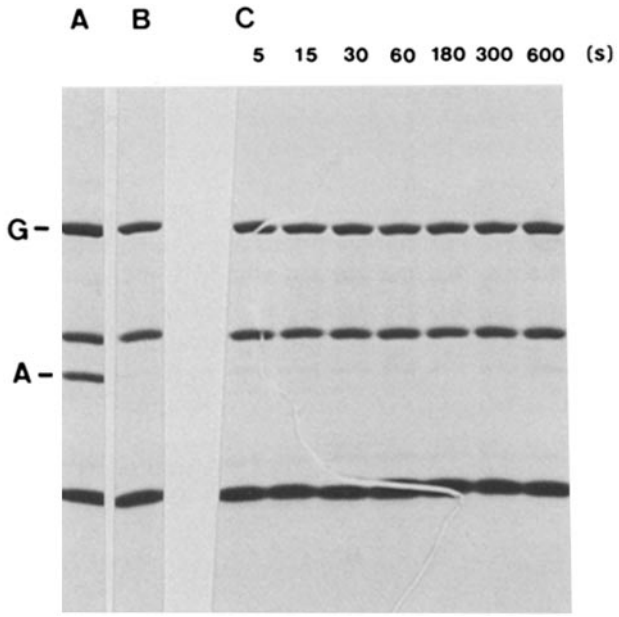

FIGURE 5. Effect of macrophage attachment and spreading on the proportion of EGTA-resistant actin/gelsolin complexes extracted. Cells were suspended at 37°C as in Fig. 3 for 30 min, at which time 0.5 ml of the cells were added to 5 ml of KRP in 6-cm diameter Petri dishes. After 5 min at 37°C, the KRP solution was replaced and the nonadherent cells were counted. The plating efficiency was consistently 90–95%, as further evidenced by the similar intensity of the gelsolin band on the SDS gels of suspended (*A*) and plated (*B* and *C*) cells. Plated cells were extracted with TEB solution diluted 1:1 with water. The extract was centrifuged for 2 min at 13,800 *g* in an Eppendorf microcentrifuge, and gelsolin was immunoadsorbed as described. Shown are Coomassie blue–stained gels after electrophoresis in SDS of extracts of suspended cells (*A*), cells plated for 10 min (*B*), and plated cells to which 10^{-6} M ionomycin was added for the times indicated (*C*).

(9) to raise transiently the intracellular Ca^{2+} concentration, actin is no longer associated with the immunoprecipitated gelsolin. Furthermore, once cells were plated (lanes in panel *C*), EGTA-resistant complexes did not form in the presence of 1 μM ionomycin and 1 mM $CaCl_2$. This result suggests either that in spite of a rise in intracellular Ca^{2+}, gelsolin in plated cells does not bind to actin or, alternatively, gelsolin does react with actin but the complexes formed under these conditions are fully reversible by EGTA and are therefore not detected in extracts containing EGTA.

Discussion

These experiments demonstrate that EGTA-resistant actin/gelsolin complexes can form in macrophages in response to a rise in intracellular Ca^{2+} concentration, indicating that the Ca^{2+}-dependent interaction of gelsolin with actin, extensively documented in vitro, and for platelets in vivo, takes place in these cells. On the other hand, in contrast to experimental results with purified proteins, macrophages can dissociate actin/gelsolin complexes in vivo. Furthermore, the chemotactic peptide FMLP and spreading of the cells on a surface may induce the dissociation of the EGTA-resistant actin/gelsolin complex. In view of the lack of

a similar dissociation of complexes made from purified gelsolin and actin in vitro, it appears likely that the mechanism for the dissociation of actin from the EGTA-stable actin-binding site on gelsolin involves an additional factor or factors, which are activated by the chemotactic peptide or cell spreading. Our findings that gelsolin's actin-modulating effects may be reversible in vivo suggest that this regulator of actin filament length and possibly of actin cytoskeletal structures is therefore under at least dual control, by Ca^{2+} and by another signal that may provide an explanation for Ca^{2+}-independent signal mechanisms in cell activation. A possible way in which these findings could relate to actin assembly in the macrophage is if monomeric actin is ordinarily prevented from polymerization by its association with macrophage profilin, but that dissociation of gelsolin from the fast-growing ends of existing actin filaments in response to stimulation of the cell provides nuclei that compete effectively with profilin for actin monomers (30). The rapid initial rise in cytosolic Ca^{2+} preceding the onset of motility in response to certain agonists might cause a population of free gelsolin molecules to sever actin filaments, increasing the number of ends eventually exposed by the mechanism for dissociating actin/gelsolin complexes. According to this interpretation, the role of Ca^{2+} is to potentiate rather than initiate actin assembly.

Summary

We have developed an immunoadsorption technique for quantitating EGTA-resistant gelsolin/actin complexes in macrophages extracted with Triton X-100. We report here that the proportion of gelsolin complexed irreversibly to actin is low in freshly harvested macrophages. The amount of the EGTA-resistant complex increases spontaneously during incubation of the cells in suspension at 37°C, or after exposure to the Ca^{2+} ionophore ionomycin. On the other hand, exposure of suspended cells to the chemotactic oligopeptide, FMLP, or plating of the cells onto tissue culture dishes causes the EGTA-resistant complex to dissociate rapidly. Plating even prevents Ca^{2+} ionomycin-treated cells with elevated intracellular Ca^{2+} from inducing this complex. Therefore, our results suggest that macrophages possess a mechanism, not directly involving Ca^{2+}, for dissociating actin/gelsolin EGTA-resistant complexes. This mechanism may be a Ca^{2+}-independent signal for leukocyte activation.

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