Identification of Gelsolin, a Ca\(^{2+}\)-dependent Regulatory Protein of Actin Gel-sol Transformation, and Its Intracellular Distribution in a Variety of Cells and Tissues

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ABSTRACT
Antiserum prepared against gelsolin, a major Ca\(^{2+}\)-dependent regulatory protein of actin gel-sol transformation in rabbit lung macrophages, was used to detect the presence of proteins immunologically related to gelsolin in a variety of cells and tissues. Cell extracts were electrophoresed on polyacrylamide gels, and replicas of the gels on cellulose nitrate paper were stained by an indirect immunohistochemical technique. A single band of crossreactive material which comigrates with macrophage gelsolin is found in at least nine different kinds of cells and tissues derived from rabbits and humans and in four lines of cultured cells from humans and rats. Gelsolin was also identified in human serum and plasma, raising the possibility that it may contribute to the clearance of actin from the circulatory system.

Using this antiserum, we demonstrated, by indirect immunofluorescent staining of acetone-fixed macrophages and polymorphonuclear leukocytes, that gelsolin resides in the cortical cytoplasm and that during phagocytosis it is concentrated in pseudopodia engulfing particles to be ingested, an area of the cytoplasm actively engaged in movement. In longitudinal cryostat sections of contracted rabbit skeletal muscle, antigelsolin staining was associated with the I-band of the myofibril, suggesting that it may be involved, by an as yet undefined mechanism, in skeletal muscle function. In rabbit intestinal epithelial cells, gelsolin was associated with the cytoplasm and the terminal web region of the brush border, a localization distinct from that previously reported for villin, a structurally and functionally similar protein isolated from the brush borders of chicken intestinal epithelial cells.

In conclusion, our findings support the idea that gelsolin is involved in the regulation of movement and suggest that gelsolin-mediated Ca\(^{2+}\)-regulation of actin cytoskeletal structure, first characterized in macrophages, may be of general importance.
gelsolin) was injected into a male piggy goat at multiple intradermal sites on two occasions, 1 wk apart. Antibodies against macrophage gelsolin were detected with double immunodiffusion and immune polyacrylamide gel replica techniques described below. The goat was bled at monthly intervals for the subsequent 4 months, with a booster injection of 0.6 mg of gelsolin in 0.15 M NaCl given subcutaneously a week before bleeding.

IgG was prepared by ammonium sulfate precipitation and ion exchange chromatography as described in reference 6. Affinity-purified antителgolin IgG was prepared by passing IgG through a column of Sepharose 4B beads (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) covalently coupled to gelsolin (6).

Cells

Lung macrophages were obtained by tracheal lavage of rabbits previously injected intravenously with complete Freund’s adjuvant (7). Human and rabbit polymorphonuclear (PMN) leucocytes were isolated from peripheral blood by density gradient centrifugation (8) and used without further purification for immunofluorescence studies. For analysis on polyacrylamide gels, the neutrophils were further purified by centrifugation through a Ficoll-diatrizoate gradient (9). 95% of the cells collected from the bottom of the gradient were PMN leucocytes, as determined from their morphology on smears stained with Wright stain. Lymphocytes isolated from minced rabbit spleens were purified by Ficoll-diatrizoate gradient separation and further enriched by permitting contaminating monocytes to adhere to tissue culture dishes. Intestinal epithelial cells were obtained from rabbit small intestines, using a solution of EDTA to release epithelial cells from the underlying tissues (9). Serum and plasma were prepared from freshly killed rabbits. Brain, thyroid, skeletal muscle, heart, bladder, and gravid uterus of Dr. L. Levitt (Massachusetts General Hospital, Boston, Mass.) . Human fibroblasts lines 297 and W18-VA2 and rat fibroblast line W256 were grown in monolayer cultures and a gift of Dr. L. Levitt (Massachusetts General Hospital, Boston, Mass.).

Preparation of Cell and Tissue Extracts

Macrophage and neutrophil extracts were prepared as previously described (7, 8). Briefly, the cells were treated with 5 mM diisopropylfluorophosphate to inactivate serine proteases (10) and homogenized in a Dounce homogenizer in a solution containing 0.34 M sucrose, 5 mM EGTA, 10 mM dithiothreitol, 0.5 mM ATP, 2 mM phenylmethyl sulfonylfluoride, 20 mM imidazole-HCl, pH 7.0 (Buffer A). The homogenate was clarified by centrifugation at 10,000 g for 30 min. The supernatant was washed by centrifugation and dialyzed against 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, and used as crude extracts (lane d), a single polypeptide, which comigrated with purified gelsolin on the polyacrylamide gel (lane c), reacted with the immune serum. No other crossreactive polypeptide was detected, even when a large amount of macrophage pro-

Indirect Immunofluorescent Labeling

Rabbit macrophages and PMN leucocytes, isolated as described above, were resuspended in Krebs-Ringer phosphate buffer with 5 mM glucose and 20% fresh autologous serum. The cells were plated on cover slips coated with heat-fixed yeast and allowed to phagocytize as described in (15, 16). Neutrophils were fixed with 2.5% paraformaldehyde in PBS for 20 min at room temperature and subsequently with acetone for 7 min at −20°C. Macrophages were fixed with acetone alone. The fixed cells were labeled by indirect immunofluorescent staining technique as described by Stendahl et al. (15). The primary antibody used was either goat antibilina (diluted to one-twenty-fifth with PBS), IgG purified from the immune serum (800 µg/ml), or affinity-purified antителgolin IgG (270 µg/ml). The secondary antibody used was rhodamine-conjugated rabbit antibilina IgG (50 µg/ml) purchased from N. L. Cappel Laboratories.

Rabbit skeletal muscle (latissimus dorsi) was frozen rapidly in Tissue-Tek II. O.C.T. compound (Miles Laboratory, Naperville, Ill.), and longitudinal sections (4 µm thick) were cut at −20°C. Sections were collected on glass slides, air-dried, and stained with antibilina IgG absorbed with an equal amount of F-actin by weight, and secondary antibody (300 µg/ml) absorbed with muscle sections before use. Rabbit intestinal epithelial cells were fixed with sequentially 3.7% paraformaldehyde and acetone and smeared on cover slips as described by (17). The cells were stained with antibilina IgG (600 µg/ml) absorbed against actin and secondary antibody absorbed against the same cells.

RESULTS AND DISCUSSION

Specificity of the Antiserum

In the double immunodiffusion assay, the serum from a goat immunized against purified rabbit macrophage gelsolin formed a single precipitin line against gelsolin, indicating that a precipitating antibody was present (Fig. 1). The specificity of the immune serum to gelsolin was also confirmed by an immunohistochemical gel replica method. As shown in Fig. 2, purified macrophage gelsolin reacted positively with the immune serum to form a reaction band which is a faithful replica of the Coomassie Blue-stained polypeptide, confirming that the antiserum contained an IgG-type antibody against gelsolin. The immunohistochemical detection is extremely sensitive when calibrated against purified macrophage gelsolin. The intensity of the band of reaction product decreases with the amount of protein applied. 4 ng of gelsolin, below the threshold for the detection of polypeptide by Coomassie Blue, was sufficient for a positive reaction (Fig. 2, lane a).

The specificity of the antiserum against gelsolin is demonstrated by testing it against gel replicates of total macrophage proteins. In replicates of either solubilized macrophages (Fig. 3, lanes a and b) or a high-speed supernatant of macrophage extracts (lane d), a single polypeptide, which comigrated with purified gelsolin on the polyacrylamide gel (lane c), reacted with the immune serum. No other crossreactive polypeptide was detected, even when a large amount of macrophage proteins (500 µg) was loaded on the gel to ensure detection of minor reactive species. This result confirmed that the antiserum is highly specific for gelsolin and that the gel replica technique can be used to identify gelsolin in polyacrylamide gels of total cell proteins.
Fig. 1 Double immunodiffusion analysis of antiserum against rabbit macrophage gelsolin. Gelsolin (10 μg) was placed in the center well and antiserum from a goat was placed in two of the outer wells.

Fig. 2 Sensitivity of immune gel replica assay for the detection of macrophage gelsolin. The Coomassie Blue-stained gel of increasing amounts of gelsolin (left) and the corresponding immunohistochemically stained replica (right) are presented. Amounts of proteins applied were as follows: (a) 4 ng; (b) 20 ng; (c) 40 ng; (d) 220 ng; (e) 430 ng; (f) 2.2 μg.

Fig. 3 Specificity of the antiserum against gelsolin. Rabbit macrophage gelsolin, homogenate of macrophages and an extract of macrophage proteins obtained after high-speed centrifugation of the homogenate were electrophoresed in a polyacrylamide gel in the presence of SDS, and a replica of the gel was tested against goat antigelosin serum. The Coomassie Blue-stained gel is shown on the left, and the corresponding immunohistochemically stained gel replica on the right. (a) homogenate, 500 μg; (b) homogenate, 100 μg; (c) macrophage gelsolin, 2 μg; (d) extract, 200 μg.

Fig. 4 shows that, besides macrophages, human PMN leukocytes (lane a) and rabbit splenic lymphocytes (lane b) contained a crossreactive polypeptide which comigrated with purified rabbit macrophage gelsolin on polyacrylamide gels. On the basis of the similarity in molecular weight and immunologic crossreactivity of the polypeptides from these cells with macrophage gelsolin, we conclude that gelsolin is present in lymphocytes and PMN leukocytes. The intensities of the bands of immunohistochemical reaction product in neutrophils and lymphocytes were similar to that obtained when an equal amount of macrophage protein was analyzed. On the assumption that gelsolins from these sources are equally antigenic, our result would imply that lymphocytes, PMN leukocytes, and macrophages have comparable amounts of gelsolin. A 91,000-dalton polypeptide crossreactive with the immune serum was also detected in human promyelocytic leukemic HL-60 cells both before and after they had been induced to differentiate by the addition of dimethyl sulfoxide (lanes c and d; [18] into neutrophilike cells. From this, we infer that gelsolin is present in immature myeloid cells as well as terminally differentiated white blood cells. We have reported that gelsolin is present in human platelets and that it is functionally and structurally similar to rabbit macrophage gelsolin (19).

Fig. 4, lane e, shows that human serum contains a polypeptide which crossreacts with the immune serum, consistent with the finding by Norberg et al. (20) that human serum contains a 91,000-dalton protein which causes Ca$^{2+}$-dependent decrease in actin viscosity. Plasma which has been obtained carefully to avoid activation of platelets has a comparable amount of a
crossreactive polypeptide (not shown), suggesting that platelet lysis is not likely to account for the bulk of gelsolin in serum and that gelsolin is present normally in the extracellular fluid component of blood. Since blood contains millimolar concentrations of $\text{Ca}^{2+}$, gelsolin should be able to maintain any actin released into the blood stream as a result of cell lysis as short filaments, possibly preventing blockage of the microcirculation.

Besides being present in blood plasma and cells, gelsolin is also identifiable in a variety of other cells and tissues. In each of the extracts shown in Fig. 5, a single 91,000-dalton polypeptide which comigrated with purified rabbit macrophage gelsolin reacted positively with the antigelsolin serum. The single cells examined were epithelial cells from rabbit small intestine, established lines of rat and human fibroblasts, both "normal" and "transformed." The tissues examined were all from rabbits: brain, uterus, bladder, heart, skeletal muscle, and thyroid. Among these tissues, the uterus and bladder reacted most strongly with the immune serum, suggesting that they were very rich in gelsolin content. Since these tissues may contain a heterogeneous population of cells, it is not possible with this technique to determine whether gelsolin is associated with one or more types of cells in the tissue. However, a small amount of tissue protein (200 $\mu$g) was sufficient to demonstrate crossreactive material; it is therefore likely that most of the gelsolin is attributable to the major cell types in each tissue, even though the minor species may also contain gelsolin. As will be shown below, in the case of skeletal muscle, gelsolin was associated with the muscle cell. The identification of a gelsolin-like protein in these cells raises the possibility that the $\text{Ca}^{2+}$-dependent regulation of actin network structure by gelsolin first characterized in macrophages may be applicable in general to a large variety of cells.

**Immunofluorescent Localization of Gelsolin**

Our studies of gelsolin in vitro indicate that it may be the physiological $\text{Ca}^{2+}$-dependent regulator of actin gel to sol transformation in the macrophage. We examined the intracellular distribution of gelsolin in cells during phagocytosis, a process which appears to involve gel-sol transformation of the cortical cytoplasm (21), by indirect immunofluorescent staining with the specific antigelsolin IgG characterized above. Macrophages which were fixed shortly after plating on glass cover slips were round and stained by antigelsolin primarily around the peripheral cytoplasm, with no fluorescence in the nuclear region (Fig. 6A). When the macrophages come into contact with opsonized yeast particles on the slide, they extend pseudopodia to engulf the particles. Fluorescence in these cells was concentrated in the pseudopodia and relatively depleted from the rest of the cortical cytoplasm (Fig. 6B and C). In the pseudopod, fluorescence was particularly concentrated in areas that appear to have come into contact most recently with the phagocytic particle: it is brighter in the advancing tip of the pseudopod than in the base, the cup-shaped hollow where initial contact between cell and particle took place. Also, once the pseudopodia encircled the yeast, fluorescence was strongest in the distal portion of the pseudopodia, forming bright crescents around the outer edge of the yeast particles (Fig. 6 D, E, and F). No fluorescence was observed in the cytoplasm surrounding a phagocytic vacuole in the cell center (Fig. 6 F, see arrow), suggesting that gelsolin which was concentrated around the particle earlier was again redistributed after completion of phagocytosis. A similar pattern of redistribution of gelsolin is observed in PMN leukocytes. During phagocytosis, the PMN leukocyte assumed a polarized morphology with a broad anterior pseudopod and a knoblike tail, and fluorescence was concentrated in the pseudopodia encircling the yeast (Fig. 7).

The specificity of antigelsolin staining observed in these cells is supported by the following control experiments. First, when preimmune serum was used in place of antigelsolin serum, fluorescence was barely detectable in macrophages (data not shown). Second, when antigelsolin IgG was absorbed with purified macrophage gelsolin, the fluorescence was markedly
FIGURE 6 Immunofluorescent localization of gelsolin in rabbit lung macrophages. Acetone-fixed cells were stained with goat antigelsolin serum (except in E, which was stained with antigelsolin IgG) and rhodamine-labeled antigoat IgG. (A) Fluorescence photomicrograph of resting macrophages. (B and C) Fluorescence micrograph of macrophages simultaneously ingesting two yeast particles. (D and D'; E and E') Phase-contrast and fluorescence micrographs of macrophages at different stages of ingestion. (F and F') Arrow indicates ingested particle in the cell center. X 1,400.

FIGURE 7 Distribution of gelsolin in rabbit PMN leukocytes. Cells were stained with affinity-purified goat antigelsolin Ig and rhodamine-labeled antigoat IgG. Phase-contrast and fluorescence micrographs of a PMN leukocyte extending pseudopodia to engulf a yeast particle. X 1,400.

reduced compared with that observed with immune IgG used without absorption (data not shown). Third, the specificity of the antibody against gelsolin has already been established by the immune gel replica technique.

The bright antigelsolin fluorescence in the pseudopodia of cells undergoing phagocytosis suggested that gelsolin was present at a high concentration in those regions in a form which resisted extraction by acetone. On the other hand, the relative lack of gelsolin staining in the rest of the cytoplasm reflected either a depletion of gelsolin during phagocytosis or its extraction during preparation for immunofluorescent staining. The distribution of gelsolin in the resting and phagocytosing cells is very similar to that reported previously in these cells for actin (22), myosin, and actin-binding protein (15, 16), proteins presumed to be important components of the contractile machinery of the phagocytes. Therefore, our data are compatible with the idea that gelsolin is an integral part of the motile apparatus of phagocytic cells and that it regulates cell movement by changing the consistency of the cytoplasm.

We have also used the immunofluorescent staining technique to localize gelsolin in some of the cells shown to contain gelsolin by the immune gel replica technique. Longitudinal cryostat sections of contracted rabbit skeletal muscle stained with antigelsolin antibody displayed a striated pattern of fluorescence associated with the muscle fiber (Fig. 8 A'). Only very light and diffuse staining was observed with preimmune IgG (not shown). Superimposition of fluorescent and phase images revealed that the fluorescent bands were coincident with the I-band of the myofibrils (Fig. 8 A and A'). Although it was not possible to determine with this technique whether gelsolin was located within the myofibril itself or in a compartment sur-
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rounding the myofibril (e.g., sarcoplasmic reticulum or T-tubules), it was clear that gelsolin was associated with the muscle cell. This finding raises the possibility that gelsolin may be involved in muscle contraction. Antigelsolin antibody stained the cytoplasm but not the nucleus of rabbit intestinal epithelial cells (Fig. 8 B - D). In the brush-border region, fluorescence was observed in the terminal web but not in the microvilli. The specificity of antigelsolin staining is confirmed by the very low level of staining with preimmune serum (not shown). This pattern of gelsolin distribution is different from that reported for villin, another Ca"-sensitive protein isolated from brush borders of chicken intestinal epithelial cells which also shortens actin filaments. According to Bretscher and Weber (23), villin is found exclusively in microvilli but not terminal web of intestinal epithelial cells, and a few other specialized cells containing brush borders. The differential localization of gelsolin and villin within the same type of cell emphasizes that, although the two proteins are structurally and functionally similar, they are not identical. Additional differences between these proteins include: (a) a small difference in molecular weights (91,000 vs. 95,000 daltons); (b) lack of immunological crossreactivity when villin was reacted with goat anti-rabbit gelsolin antibody and gelsolin with rabbit anti-chicken villin antibody (a gift of Dr. S. Craig, Johns Hopkins University; Yin, H. L., unpublished data); and (c) villin crosslinks actin in the presence of EGTA (24) while gelsolin does not (Yin, H. L., unpublished data). Since gelsolin is present in a wide variety of cells whilst it is restricted in its distribution to cells containing brush borders, it is possible that villin represent a specialization of the Ca"-dependent regulator of actin gel-sol transformation, of which gelsolin is a prototype for mammalian cells.

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FIGURE 8 Distribution of gelsolin in rabbit skeletal muscle and intestinal epithelial cells. (A and A') Phase-contrast and fluorescence micrographs of a longitudinal cryostat section of contracted latissimus dorsi muscle stained with antigelsolin IgG (B - D, B' - D'). Phase-contrast and fluorescence micrographs of intestinal epithelial cells (arrows indicate same positions in each pair of micrographs), stained with antigelsolin IgG. × 3,400.