Gelsolin, a Ca^{2+}-dependent Actin-binding Protein in a Hamster Insulin-secreting Cell Line

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Abstract

Using a gel overlay technique we have previously described a 90,000-mol wt actin-binding protein in a number of hormone-secreting tissues and tentatively identified this protein as gelsolin. Gelsolin is a protein that cuts or solates cross-linked actin filaments and can also serve as a nucleating site for actin polymerization. The objective of this study was to isolate this protein from a hamster insulin-secreting (HIT) cell line and compare the immunologic properties and peptide maps of purified rabbit macrophage gelsolin, human platelet gelsolin, and the HIT cell 90,000-mol wt protein. DNase I-Sepharose retained the HIT cell actin-binding proteins in 1 mM $CaCl_2$; some of the 90,000-mol wt protein could then be eluted with 1 mM EGTA. The remaining actin-binding proteins were eluted using a buffer containing SDS. The EGTA peak fractions contained two major protein bands of M_r = 90,000 and 42,000, which suggested that a 90,000-mol wt-actin complex was eluted from the DNase I-Sepharose column. Specific antibodies to the human platelet and rabbit macrophage gelsolins bound to the 90,000-mol wt bands in the eluates, but did not crossreact with other actin-binding proteins. Indirect immunofluorescence using an anti-human platelet gelsolin antibody localized the 90,000-mol wt protein to stress fibers that were also stained with phalloidin, which suggested that gelsolin is associated with actin in vivo. Tryptic peptide maps of all three radioiodinated gelsolins were virtually indistinguishable. Thus, gelsolin is a highly conserved gene product found in at least three diverse cell types, an insulin-secreting beta cell line, macrophages, and platelets, and may link a transient increase in Ca^{2+} cellular levels with changes in actin polymerization and/or the gel-sol state of these cells.

Introduction

During the course of ultrastructural studies, Orci et al. (1) identified a fine band of filaments at the periphery of the beta cell and called this region the cell web. In unstimulated cells, this area was unusually devoid of insulin secretory granules. Since cytochalasin B had been useful in establishing the involvement of similar microfilamentous structures in contraction (2), its ultrastructural and secretory effects were examined in islets. Cytochalasin B caused thinning of the beta cell web, margination of the secretory granules with the plasma membrane, and potentiated both phases of insulin secretion (1). Because the microfilaments in nonmuscle cells, like actin-rich thin filaments in muscle, bind to the myosin fragment, heavy meromyosin (3), these experiments suggested that actin is actively involved in exocytosis.

In nonmuscle cells actin exists in two forms: as a globular protein of 42,000 mol wt, G-actin, which in the presence of Mg^{2+} and ATP polymerizes to form filamentous or F-actin (4). In pancreatic islets or nonmuscle cell lines, ~60–75% of the total actin is found as G-actin (5, 6). In certain physiologic states such as fasting, there is a shift of G to F-actin (5); however, it is still not clear how actin polymerization is involved in the secretory process. Cytochalasin B inhibits polymerization by binding to the rapidly growing or barbed end of actin filaments (7), which suggests that the effects of the drug on insulin secretion may be mediated by enhanced actin depolymerization. A number of actin-binding proteins have been identified in a variety of nonmuscle cells (4). These proteins can regulate the soluble and filamentous actin pools by stabilizing G-actin, or capping, crosslinking, annealing, or cutting actin filaments. Some of these proteins have more than one function and some are Ca^{2+}-dependent actin-binding proteins.

In earlier studies using a gel overlay technique (8) we identified a number of proteins in hormone-secreting cells and other nonmuscle cells that interact specifically with actin (9). One major actin-binding protein with M_r = 90,000 mol wt demonstrated partial Ca^{2+}-sensitivity of actin binding and a pI of 6.2. This protein was tentatively identified as gelsolin, a protein previously isolated from rabbit macrophages (10) and human platelets (11) and named for its ability to solate crosslinked actin filaments (12). The object of the present study was to purify the 90,000-mol wt protein from a simian virus 40 transformed insulin-secreting cell line (13) and compare its immunologic and structural properties with purified rabbit and human gelsolins. This report shows that the gelsolins from the three species and cell types are very similar proteins.

Methods

Hamster insulin-secreting tumor (HIT)[1] *cell extract.* Monolayer cultures of HIT cells were grown in a humidified 37°C incubator with 95% air, 5% CO_2 using RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. Before harvest, the confluent cultures were rinsed with warm Puck's saline A (Gibco Laboratories) to remove any traces of serum. The cells were incubated with 1 mg/ml trypsin (Worthington Biochemical Corp., Freehold, NJ) in solution A for 5

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1. *Abbreviations used in this paper:* DIFP, diisopropyl fluorophosphate; HIT, hamster insulin-secreting tumor; MLCK, myosin light chain kinase; PMSF, phenylmethylsulfonyl fluoride.

min at 25°C and the trypsin inactivated by the addition of RPMI medium containing 10% serum. The cell suspension was centrifuged at 1,000 *g* for 10 min at 4°C and the resulting pellet was washed twice with a cold solution of 147 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 0.1 mM phenylmethylsulfonylfluoride (PMSF), pH 7.4. The washed cell pellet (10^9 cells) was transferred to a Dounce homogenizer and resuspended in 10 ml lysis buffer (90 mM KCl, 10 mM 1,4-piperazine diethanesulfonic acid, 0.5% [wt/vol] Triton X-100, 2 mM Mg acetate, 0.2 mM ATP, 0.2 mM dithiothreitol, 0.1 mM EGTA, 0.1 mM PMSF, and 1 mM NaN_3 pH 6.9 [HCl]) using five strokes of the tight fitting pestle. The resulting homogenate was centrifuged (2,900 *g*, 10 min, 4°C) and a supernatant (cell extract) and pellet (nuclei, intact cells) obtained. These two fractions were aliquoted, frozen in liquid N_2, and stored at −70°C. The protein content was assayed using bovine serum albumin as a standard in a modified Lowry procedure (14). G-actin concentrations were measured with a DNase I-immunoprecipitation assay (6) and actin-binding proteins were identified with an ^{125}I-actin gel overlay technique (8) using the buffers and wash periods detailed in reference 15.

DNase I-Sepharose. DNase I-Sepharose was synthesized using bovine pancreas DNase I type III (Sigma Chemical Co., St. Louis, MO) and the procedure described by Wang and Bryan (11). To inactivate any serine proteases in the nuclease preparation, diisopropyl fluorophosphate (DIFP) (final concentration 0.1 mM) was added to the 10 ml of coupling buffer, which was used to dissolve 100 mg of the enzyme. The protein solution was subsequently dialyzed at 4°C for 22 h with two changes of coupling buffer (1 liter) to remove excess salts and unreacted DIFP. Coupling efficiency, estimated by monitoring the protein content, was 96–100%, and 3.1 mg DNase I was coupled per milliliter of packed resin. Actin-binding capacity was tested with biologically active ^{125}I-actin, labeled using ^{125}I-Bolton-Hunter reagent (6) (Amersham Corp., Arlington Heights, IL), and rabbit skeletal muscle actin purified using the procedure of Pardee and Spudich (16).

A 1-ml DNase I-Sepharose column (0.6 × 3.5 cm) at 4°C was washed with 10 ml of Ca^{2+}-buffer (10 mM Tris–HCl, pH 7.4, 1 mM $CaCl_2$, 1 mM Mg acetate, 0.2 mM ATP, 0.05% NaN_3, and 0.1 mM PMSF) for each sample. The actin standards were dissolved in Ca^{2+}-buffer while $CaCl_2$ was added to the HIT cell supernate to a final 1 mM concentration. After incubation of the samples on the columns for 5 min at 4°C, the columns were drained and washed with Ca^{2+}-buffer (0.5-ml fractions) until there was no radioactivity or protein in the eluate. Column buffer containing 1 mM EGTA instead of 1 mM $CaCl_2$ was then used to elute the Ca^{2+}-dependent actin-binding proteins in the HIT cell extract. Finally, the column was removed from the cold and eluted with SDS buffer (10 mM Tris–HCl, pH 7.0, 1% (wt/vol) SDS, 1% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol, 2 mM EDTA, and 0.1 mM PMSF). The SDS buffer was kept at room temperature to avoid precipitation of the detergent. Because DNase I is denatured by SDS, the columns were discarded after each use.

Actin and actin-binding proteins in the column fractions. The ^{125}I-actin in the column eluates was monitored in a gamma counter while the unlabeled actin in the Ca^{2+} flow through was measured using the DNase I-immunoprecipitation assay (6). Since the actin in the SDS fractions was inactive in the DNase I assay, the actin content of these fractions was estimated by densitometric analysis of stained SDS polyacrylamide slab gels prepared according to Laemmli (17). The values obtained from the scans agreed with the total actin content minus the actin content of the Ca^{2+} eluate. The column binding capacity was 0.3 mg actin/ml resin, which indicated that ~10% of the coupled DNase I was available for actin binding. Recovery was 98–110% of the actin applied to the column. The enrichment for HIT cell actin-binding proteins in the EGTA and SDS fractions was monitored using the ^{125}I-actin gel overlay technique (15).

Antibody characterization. Gelsolin standards and anti-gelsolin antibodies were generous gifts of Drs. J. Bryan, Baylor College of Medicine, and H. Yin, Harvard Medical School. Preparation and characterization of the polyclonal rabbit anti-human gelsolin (18) and goat anti-rabbit gelsolin (19) and monoclonal mouse anti-human gelsolin (20) are detailed in the indicated references. These antibodies were used for the indirect immunofluorescence and immunoblot techniques described.

Staining of whole cells. Indirect immunofluorescent localization of gelsolin in the HIT cells was performed by the method of Brinkley et al. (21) using a specific rabbit antibody (2–5 μg/ml) (18) or harvest fluid (spent medium) from the culture of hybridomas secreting mouse IgG_1 antibodies directed against human platelet gelsolin (20). Rhodamine-conjugated anti-rabbit or fluorescein-conjugated anti-mouse antibodies were obtained from Miles Laboratories (Elkhart, IN) and diluted (1:20) with phosphate-buffered saline containing 10% (vol/vol) fetal bovine serum. Rhodamine-labeled phalloidin from Molecular Probes, Inc. (Junction City, OR) was used at a concentration of 0.7 μg/ml to stain actin filaments (22) in HIT cells.

Characterization of immunologic cross reactivity. Proteins in the eluates were electrophoresed (17) and transferred to nitrocellulose sheets (Millipore Corp., Freehold, NJ) using the procedure of Towbin (23). A transfer efficiency of 95% was estimated by comparison of duplicate gels: one stained (no transfer), one stained after transfer, and the stained nitrocellulose replica (after transfer). Immunoblots of the protein bands were obtained using mouse antiserum against human platelet gelsolin (20) and goat anti-rabbit macrophage gelsolin (19), and were visualized with the appropriate horseradish peroxidase-coupled second antibodies (Cappel Laboratories, Cochranville, PA) and 4-chloro-1-napthol (Sigma Chemical Co.) as chromogenic substrate (24).

Densitometric analysis. Densitometric analyses of the stained gels, autoradiograms from the ^{125}I-actin gel overlay procedure, and the immunoblots were performed on a Kontes Fiber Optic Scanner interfaced with a Hewlett-Packard integrator. An actin standard curve, obtained by electrophoresis and Coomassie R-250 staining of 0.5–6.0 μg rabbit skeletal muscle actin, was linear in the range tested. Comparison of ^{125}I-actin binding was performed only when samples were processed in the same gel overlay experiment and the gel strips exposed to the same sheet of film. Exposure times were chosen to give band areas in the linear range of the scanner.

Tryptic peptide maps. Autoradiographs of ^{125}I-labeled tryptic peptide maps of iodinated human platelet gelsolin, rabbit macrophage gelsolin, the HIT cell 90,000-mol wt protein in the EGTA eluate, and a nonactin-binding protein (phosphorylase B, a 94,000 mol wt standard) were obtained according to Elder et al. (25), using the increased radioactivity (1 mCi/2 μg protein/gel slice) and sequence of washes detailed by Birnbaumer et al. (26). A blank gel slice equivalent in size to the protein-containing gel pieces was also taken through the procedure to determine if the acrylamide matrix contributed any spots to the maps. (There were no such spots.) Peptide maps were obtained three times for each protein and twice for the mixture of different gelsolins.

Results

Actin-binding proteins in HIT cells. Fig. 1, lane 3, depicts the major actin-binding proteins identified in HIT cell supernate by the gel-overlay technique. While there are many proteins in the extract as shown in the Coomassie R250-stained gel (lane 2), the HIT supernate contained four major HIT actin-binding proteins with M_r = 90,000, 70,000, 50,000, and 32,000 mol wt (lane 3). ^{125}I-actin binding by these proteins have differing Ca^{2+}-sensitivities, since decreasing the Ca^{2+} concentration in the overlay solutions diminishes binding activity by different amounts. 7 μM Ca^{2+} in the overlay solution (lane 4) reduced actin binding to the 90,000-mol wt band to 47±1% (mean±SE for two experiments), to the 70,000-mol wt protein to 91.7±0.2%, and to the 50,000-mol wt band to 68±10% of that observed for the same protein in the 1 mM $CaCl_2$ control overlay, and did not affect the 32,000-mol wt binding activity. The ionized Ca^{2+} concentration was calculated using the binding constants given by Goldstein (27). Removal of Ca^{2+}

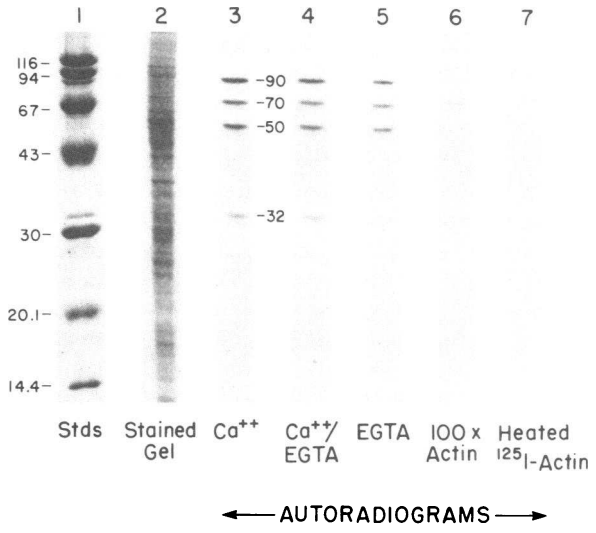

Figure 1. Actin-binding proteins in the HIT cell supernate. 25 μg HIT cell supernatant protein lane was electrophoresed on 15 lanes of 12.5% acrylamide slab gels using the discontinuous SDS-Tris-glycine buffer system of Laemmli. Molecular weight markers (*1*) are β-galactosidase (116,000 mol wt), phosphorylase B (94,000 mol wt), bovine serum albumin (67,000 mol wt), ovalbumin (43,000 mol wt), carbonic anhydrase (30,000 mol wt), soybean trypsin inhibitor (20,100 mol wt), and α-lactalbumin (14,400 mol wt). The gels containing HIT cell proteins were sliced and stained (*2*) or prepared for ^{125}I-actin overlays. All overlay solutions, unless otherwise noted, contained 1 nM ^{125}I-actin (10^6 cpm/ml) and the following reagents: 1 mM $CaCl_2$ (*3*), a mixture of 1 mM $CaCl_2$ and 1 mM EGTA resulting in 7 μM free Ca^{2+} (*4*), 1 mM EGTA (*5*), and a mixture of 1 mM $CaCl_2$ and 100 nM of unlabeled rabbit skeletal muscle actin (*6*). The gel in *7* was overlaid with 1 mM $CaCl_2$ and 10^6 cpm/ml ^{125}I-actin which had been previously heated at 100°C for 3 min. The gel strips from the various overlays were heat-vacuum dried and exposed to the same sheet of Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) for 20 h at −70°C. The figure depicts the results from one experiment. The experiment was performed twice using two different ^{125}I-actin preparations with identical results. The M_rs of the actin-binding proteins shown in *3* are the averages of M_rs calculated from 15 separate overlays.

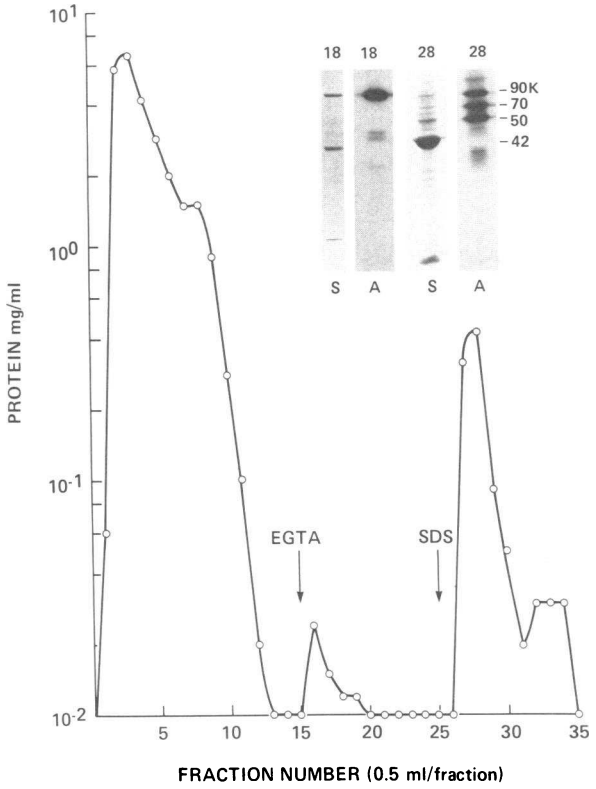

Figure 2. DNase I-Sepharose chromatography of HIT cell supernatant and ^{125}I-actin gel overlay of the column fractions. 10.5 mg of HIT cell supernatant protein containing 120 μg actin was chromatographed on 1 ml DNase I-Sepharose column with an actin-binding capacity of 300 μg. The column was washed immediately before use with 10 ml Ca^{2+}-buffer (10 mM Tris–HCl, pH 7.4, with 1 mM $CaCl_2$, 1 mM Mg acetate, 0.2 mM ATP, 0.1 mM PMSF, and 0.05% NaN_3). After incubation of the sample on the column for 10 min at 4°C the column was drained and washed with 7.5 ml Ca^{2+}-buffer. The column was then eluted with 5 ml of EGTA buffer, which was identical to the Ca^{2+}-buffer except 1 mM EGTA replaced the 1 mM $CaCl_2$. The column was transferred to room temperature and eluted with SDS buffer (10 mM Tris HCl, pH 7.0, 1% (wt/vol) SDS, 1% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol, 2 mM EDTA, and 0.1 mM PMSF). The protein content of the fractions was assayed using a modified Lowry procedure. Column fractions were electrophoresed on Laemmli slab gels (17) using 12.5% acrylamide. The inset depicts the stained gels (18, S; 28, S) and the autoradiograms (18, A; 28, A) obtained from the ^{125}I-actin overlay of fractions 18 (EGTA elution) and 28, the SDS peak fraction. Different amounts of protein were loaded, i.e., fraction 18, S = 3.4 μg, fraction 18, A = 1.5 μg, and fraction 28, S and A = 31 μg. The ^{125}I-actin overlay solution contained 1 mM $CaCl_2$. The M_rs of the actin-binding proteins and actin are indicated, e.g., 90 K equals 90,000 mol wt.

and the addition of 1 mM EGTA (lane 5) decreased binding further. In the presence of 1 mM EGTA, the 90,000-mol wt protein exhibited only 32±5%, the 70,000-mol wt band 68±8%, the 50,000-mol wt protein 40±8%, and the 32,000-mol wt band 43±5% of the actin binding measured with the 1 mM $CaCl_2$ control. Coincubation of the gel with 1 nM ^{125}I-actin and 100 nM unlabeled rabbit skeletal muscle actin (lane 6) resulted in markedly reduced ^{125}I-actin binding. Prior heating of the radiolabeled probe to 100°C for 3 min resulted in complete loss of binding (lane 7). The affinity for ^{125}I-actin must be high since the concentration of ^{125}I-actin in the overlay solution is only 1 nM.

DNase I-Sepharose chromatography. Wang and Bryan (11) and Markey et al. (28) have previously demonstrated the utility of DNase I-Sepharose affinity columns in the characterization and purification of actin-binding proteins. Fig. 2 depicts the elution profile of a 1 ml HIT cell lysate which contained 120 μg actin in 10.5 mg total protein. The Ca^{2+} flow through contained 92% of the total amount of protein added to the column and 5% of the actin. Rechromatography of these fractions on a fresh DNase I-Sepharose column resulted in the retention of 95% of the actin present in the flow through. Thus, the actin in the runoff was capable of binding to DNase I. Decreasing the total amount of actin added to the DNase I column from 120 μg to 50 or 25 μg still resulted in only ~95% of the actin being retained by the column on the first passage. Since the calculated column capacity was 300 μg of actin, the presence of actin in the eluate is not a result of overloading the DNase I binding sites in the resin. The EGTA peak comprised 0.2% of the total protein loaded on the column, while the SDS eluate represented 3.1% of the total

protein. There were two major proteins in EGTA fraction 18 as shown in the stained SDS-polyacrylamide gel of the column fraction (inset Fig. 2, 18, S) and densitometric scans of the stained gels. The major ^{125}I-actin-binding activity in this fraction was localized on the 90,000-mol wt band (18, A), which represented 37.7% of the protein in the fraction. Actin comprised 32.1% of the total protein in fraction 18. The actin in fractions 18 and 28 is not visible in the autoradiograph but is seen as a major band in the stained gels (18, S and 28, S).

When 25 μg HIT supernate and 1.5 μg fraction 18 protein (0.56 μg 90,000 mol wt) were processed in the ^{125}I-actin gel overlay procedure and ^{125}I-actin binding quantitated as integrator units per microgram protein, we obtained an estimate of 38 μg of 90,000-mol wt protein in 1 ml of HIT lysate or 0.36% of total protein. Thus, DNase I chromatography resulted in a 100-fold purification (37.7%/0.36%) of the 90,000-mol wt protein. In addition to the 90,000-mol wt band, fraction 18 also contained a few minor ^{125}I-actin binding bands which could represent 90,000-mol wt protein breakdown products (18, A). SDS fraction 28 contained three major actin-binding proteins of 90,000, 70,000, and 50,000 mol wt. 70% of the actin loaded on the column was recovered in the SDS eluate and is the major band in the stained gel (28, S). The 90,000-mol wt band, quantitated by densitometry, in fraction 28 comprised 4.8% of the protein in this fraction, representing a 13-fold purification of this protein.

It is not possible to quantitate the 70,000- and 50,000-mol wt actin-binding proteins in the HIT supernate and the SDS fractions unless one assumes that the ^{125}I-actin-binding capacity of these bands is identical to that of the 90,000-mol wt protein. As seen in Fig. 1, lane 3, these three bands are of approximately equal intensity and one can estimate the concentrations of the 70,000 and 50,000-mol wt proteins as 40 μg/ml HIT supernate. The 90,000, 70,000, and 50,000-mol wt binding proteins are visible in fraction 28, S, and each band comprises ~5% of the total protein in this fraction, representing an identical 13-fold purification of these actin-binding proteins.

A 1-ml DNase I-Sepharose column retained 95% of the actin, but only 37% of the actin-binding proteins in 1 ml HIT supernate. We have not determined if the actin-binding proteins in the actin-depleted Ca^{2+} flow through will be retained on a fresh DNase I-Sepharose column. In the typical experiment depicted in Fig. 2, the EGTA eluate (fractions 16–20) contained 19.4 μg actin or 17% of the actin bound by the DNase I-Sepharose and 3.2 μg 90,000-mol wt protein, representing 23% of that retained by the column.

Immunologic cross-reactivity of the 90,000-mol wt actin-binding proteins. Because the HIT cell 90,000-mol wt protein and the human platelet gelsolin behave identically with respect to ^{125}I-actin binding and DNase I-Sepharose chromatography, we tested the ability of a mouse monoclonal antibody directed against the 90,000-mol wt platelet gelsolin to stain HIT cell structures. The results are shown in Fig. 3, panel A. A positive immunofluorescent network of stress fibers and staining of the plasma membrane region of the cells was observed. There were ~12 HIT cells in this micrograph and filaments were visible in most but not all of the cells. We attributed this to the rounded nature of the cell clusters which grow in clumps instead of flat monolayers, resulting in incomplete lysis and the inaccessibility of the filaments to the antibodies. A control coverslip containing a similar number of cells, which was exposed only to the fluorescein-conjugated second antibody, showed no staining. Rabbit serum containing polyclonal antibodies against human platelet gelsolin stained similar filamentous arrays in HIT cells (micrographs not shown). Fig. 3, panel B depicts the stress fibers in the HIT cells visualized with rhodamine-labeled phalloidin, a cyclic peptide that specifically binds to F-actin (22). Note that the photomicrographs obtained with anti-gelsolin antibody and rhodamine-phalloidin are virtually indistinguishable.

Since Kurth et al. (18) had demonstrated that the human platelet gelsolin crossreacts with an antibody specific for rabbit macrophage gelsolin, we tested the immunologic cross-reactivity of the HIT cell proteins and partially purified human platelet and rabbit macrophage gelsolin standards using two antibody preparations. Fig. 4 demonstrates that the two antibodies identify the platelet and macrophage gelsolin standards and the HIT cell 90,000-mol wt protein. Anti-human gelsolin (left panel) detected the human platelet protein and three degradation products (lane P), bound to macrophage gelsolin (lane M) and cross-reacted weakly with the HIT cell 90,000-mol wt bands. There is cross-reactivity between anti-rabbit macrophage gelsolin antibody and the 90,000-mol wt protein from the three species (right panel). This suggests that the three gelsolins share antigenic determinants. Note that neither antibody binds to the 70,000- and 50,000-mol wt proteins or actin (42,000 mol wt) present in the HIT cell SDS fraction.

Tryptic peptide analysis of the 90K ABP's. To further compare the three actin-binding proteins, 2 μg of the purified HIT 90,000-mol wt protein in the EGTA eluate and 2 μg each of the purified human platelet and purified rabbit macrophage gelsolin standards were iodinated and digested with L-(tosylamido 2-phenyl)ethyl chloromethyl ketone-treated-trypsin as detailed in references 25 and 26. Fig. 5 presents the autoradiograms of two-dimensional analysis of the resultant ^{125}I-peptides. 16 intense spots and 19 minor spots were visible in each of the human platelet gelsolin and rabbit macrophage gelsolin peptide maps. The HIT cell autoradiographic map contained all these spots (16 major, 19 minor) in the same relative orientation. In addition, the HIT peptide map contained two minor spots not found in the other autoradiograms. Furthermore, the relative intensities of the spots (major and minor) were equivalent except for two major spots derived from the platelet and macrophage gelsolins which were present as minor spots in the HIT cell peptide map. When a mixture of ^{125}I-peptides from the digests of the three gelsolins was analyzed, essentially the same map was obtained as that for platelet or macrophage alone. This indicated that the spots co-migrate in two dimensions and were truly equivalent, and that the HIT 90,000-mol wt actin-binding protein is quite similar to gelsolins from human platelets or rabbit macrophages. The map of ^{125}I-peptides obtained with phosphorylase B (negative control) was completely different in orientation and spot intensities. The majority of the phosphorylase B ^{125}I-peptides were closer to the electrophoretic origin and migrated near the solvent front in the second dimension ascending paper chromatography (data not shown).

Discussion

This study demonstrates that a 90,000-mol wt actin-binding protein isolated from a cloned insulin-secreting hamster beta cell line is gelsolin. This conclusion is based on the actin-binding characteristics, immunologic properties, cellular localization, and tryptic peptide map of this protein. Hamster beta

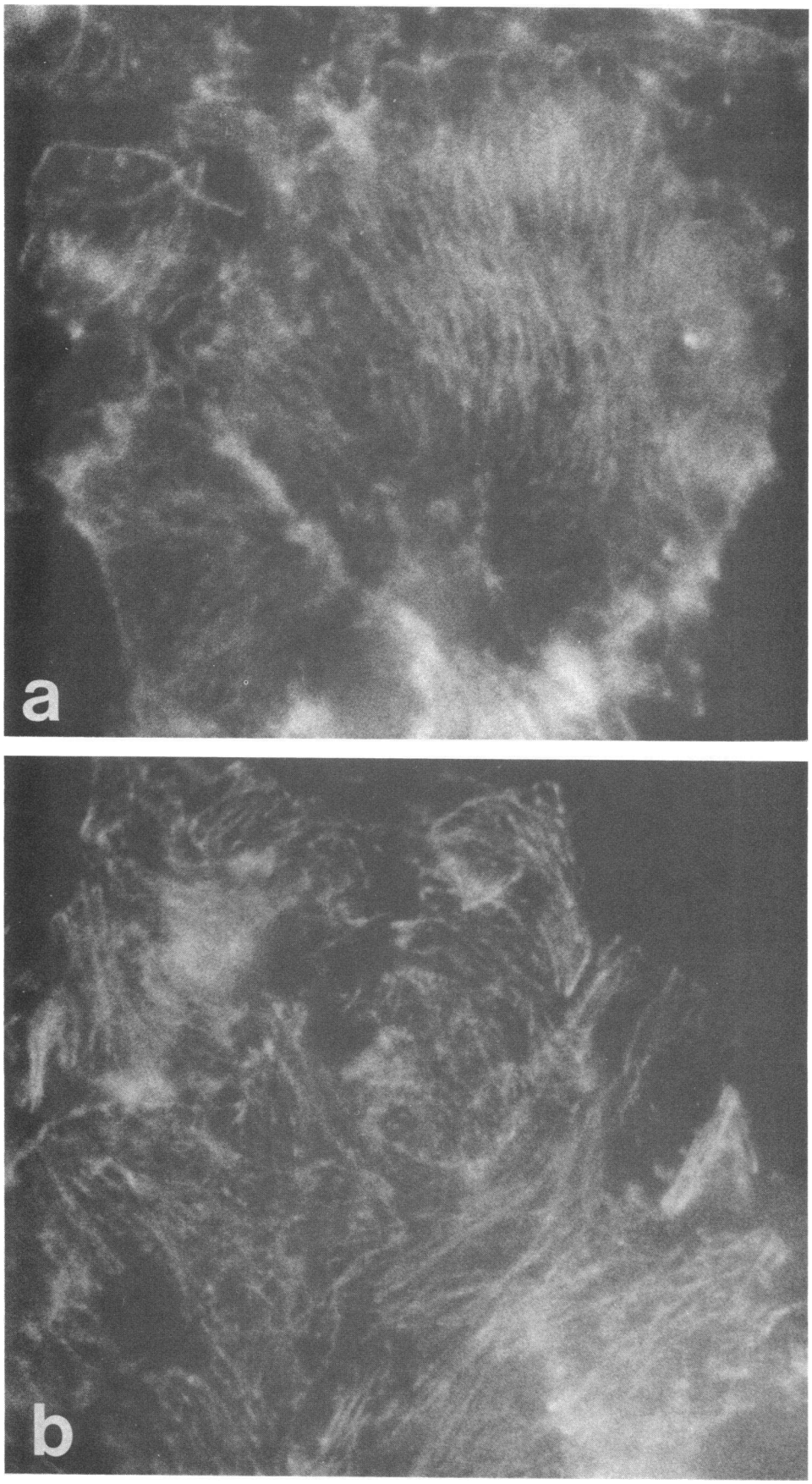

Figure 3. Fluorescent localization of the HIT cell 90,000-mol wt protein (*a*) and actin (*b*) in HIT cell monolayers. Mouse IgG monoclonal antibodies produced against human platelet gelsolin stain the periphery of the cell under the plasma membrane and decorate stress fibers of the formaldehyde-fixed HIT cells (*a*). Virtually identical structures were visualized with rhodamine-labeled phalloidin (*b*), which binds specifically to F-actin. There are ~12 cells in each micrograph. ×2,600.

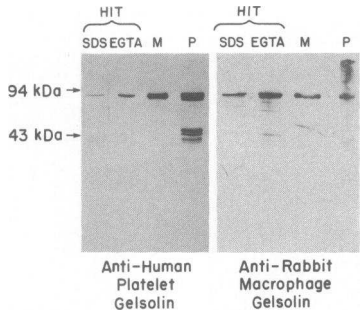

Figure 4. Immunologic cross-reactivity of HIT cell 90,000-mol wt protein, human platelet, and rabbit macrophage gelsolins with antibodies prepared to human and rabbit gelsolin. Mouse anti-human platelet gelsolin antibody detects HIT 90,000-mol wt bands and rabbit and human gelsolins (*left* panel). The goat anti-rabbit macrophage gelsolin antibody binds to HIT cell and to the rabbit and human proteins (*right* panel). The lanes contain varying amounts of the 90,000-mol wt proteins: *left* panel, HIT SDS = 0.6 μg; HIT EGTA = 1.6 μg; macrophage (M) = 0.7 μg; and platelet (P) = 1.0 μg; *right* panel, HIT SDS = 0.7 μg; HIT EGTA = 1.5 μg; macrophage (M) = 0.2 μg; and platelet (P) = 0.2 μg. The positions of the phosphorylase b (94,000) and ovalbumin (43,000) molecular weight standards are indicated. 94 kDa and 43 kDa equal 94,000 and 43,000 mol wt, respectively.

cell gelsolin is structurally and immunologically similar to human platelet and rabbit macrophage gelsolins, suggesting that gelsolin is a highly phylogenetically conserved gene product.

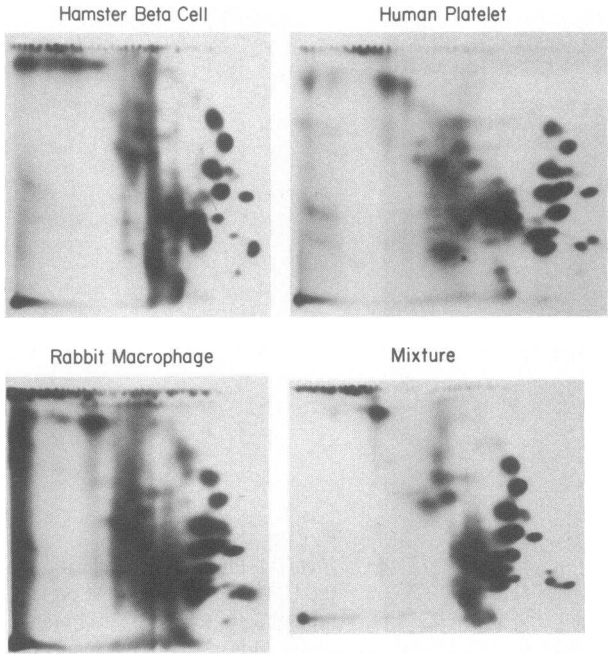

Figure 5. Maps of ^{125}I-tryptic peptides obtained from HIT cell 90,000 mol wt and human platelet, and rabbit macrophage gelsolins. Autoradiograms of the cellulose thin layer sheets after two-dimensional separation are presented. The ^{125}I-tryptic peptides were spotted on the origin, which is seen as a dark circle on the bottom left corner of each autoradiogram. Separation was achieved with electrophoresis in the horizontal direction (left to right) followed by chromatography in the vertical dimension (bottom to top). *Top left,* HIT cell 90,000-mol wt protein in the EGTA eluate; 1.5 μl of the tryptic digest was analyzed. *Top right,* human platelet gelsolin standard; 1.2 μl of the tryptic digest was analyzed. *Bottom left,* rabbit macrophage gelsolin standard; 1.6 μl tryptic digest was analyzed. *Bottom right,* equal volumes of the three tryptic digests were mixed; 1.5 μl of the mixture was analyzed.

Gelsolin has been shown to have two functions in vitro. Evidence from viscometry, flow birefringence, and electron microscopy demonstrates that in the presence of calcium, gelsolin binds to and rapidly severs actin filaments (12). Wang and Bryan (11) have also established that gelsolin nucleates the Ca^{2+}-dependent assembly of actin filaments from monomeric actin and may control actin polymerization by binding to the rapidly growing, barbed end of actin filaments. Although both these processes were thought to be reversed by decreasing the Ca^{2+} concentration, recent studies by Kurth and Bryan (18, 29) characterized Ca^{2+}-independent interactions between gelsolin and actin.

When HIT cell homogenate was applied to a DNase I-Sepharose affinity column in a Ca^{2+}-containing buffer, 25% of the gelsolin retained by the column appeared in the EGTA peak, while the remaining 75% was eluted by the SDS-buffer. This behavior can only be explained by the existence of two actin-binding sites on gelsolin with different Ca^{2+} requirements. Gelsolin forms an EGTA-stable binary complex with actin, which in the presence of Ca^{2+} can bind an additional actin molecule. Thus, a preformed actin-gelsolin complex in the HIT cell homogenate can interact with the DNase I-Sepharose resin in two ways. First, the actin in the binary complex can bind to DNase I-Sepharose. This interaction is Ca^{2+} independent (30) and is disrupted when SDS is added. Second, actin in the homogenate serves as a bridge between the gelsolin-actin complex and the resin. This second actin molecule is bound to the DNase I-resin and interacts with gelsolin via the Ca^{2+}-dependent site. When EGTA is added, the gelsolin-actin complex is eluted while the actin "bridge" remains bound to the column. The distribution of gelsolin in the eluates suggests that the DNase I molecules are in excess of "free" actin which is indeed the case. If gelsolin were present in HIT cell homogenate as a free protein, i.e., not associated with actin, it would have been retained on the DNase I column via an actin link. This gelsolin-actin interaction is Ca^{2+}-independent and all the gelsolin would have been recovered in the SDS elution.

In a series of experiments using fluorescently labeled actin to delineate the interactions between rabbit skeletal muscle G-actin and either purified human platelet gelsolin or the gelsolin-actin complex, Bryan and Kurth (31) have demonstrated that human platelet gelsolin contains two actin binding sites. One site is Ca^{2+}-dependent and the other does not require Ca^{2+} for the gelsolin-actin interaction. Their studies demonstrate that gelsolin and one molecule of platelet actin form a complex stable in EGTA and that this binary complex can be isolated using DNase I affinity chromatography (11) or standard biochemical procedures (18). Thus, the HIT cell gelsolin and platelet gelsolin may be complexed with actin in vivo.

Utilizing the ^{125}I-actin gel overlay technique, we have previously identified a 65–70,000-mol wt actin-binding protein in a variety of hormone-secreting organs including pituitary, ovary, and adrenal tissue (9). The HIT cell 70,000-mol wt actin-binding protein is probably identical to the 65–70,000-mol wt protein identified in the other secretory tissues. This protein is not eluted from DNase I-Sepharose by EGTA-containing buffers, suggesting that actin-70,000 mol wt interactions are less Ca^{2+}-dependent than actin-gelsolin binding. In this respect, the 70,000-mol wt actin-binding protein in the HIT cells resembles acumentin, a 68,000-mol wt calcium-insensitive actin-binding protein isolated from polymorphonuclear leukocytes and macrophages (32) which binds to the

pointed end of actin filaments (33). HIT cells also contain ^{125}I-actin binding activities at 50,000 and 32,000 mol wt. Additional actin-binding proteins may exist but are not detected in the ^{125}I-actin overlay assay because they do not bind G-actin, do not bind as monomers, or do not renature under the experimental conditions used.

It is not surprising that antibodies to gelsolin localize this protein to actin-containing structures in the area beneath the plasma membrane of the beta cell and to stress fibers. Stress fibers consist of bundles of microfilaments and contain, in addition to actin, many proteins which may be important for hormone transport and secretion. These proteins include myosin, filamin, tropomyosin, alpha-actinin (34), calmodulin (35), and myosin light chain kinase (MLCK) (36). A monoclonal antibody to gelsolin localizes the molecule to stress fibers and actin filaments in fibroblasts (20) and Yin et al. (19) have shown by indirect immunofluorescence that gelsolin was concentrated in the pseudopodia of macrophages or leukocytes ingesting yeast particles.

Direct evidence for the biologic function of gelsolin in any of the cells in which it has been identified is lacking. The most convincing evidence that gelsolin and Ca^{2+} regulate intracellular transport and movement is presented by Brady et al. (37), who demonstrated that gelsolin inhibited the movement of membranous organelles in isolated squid axoplasm in the presence of 10 μM $CaCl_2$, but had no effect when Ca^{2+} was absent. An understanding of the mechanisms by which gelsolin may be involved in beta cell function will require more detailed knowledge of the biochemical events relating actin and actin-binding proteins to insulin secretion. However, data from a number of laboratories allows speculation regarding a role for gelsolin in exocytosis. It is clear that calcium is intimately involved in the regulation of insulin secretion (38), and Ca^{2+} ions also control the formation of actin filaments, actin gel networks and actin-gelsolin interactions in nonmuscle cells. The transient increase in free intracellular calcium which triggers insulin secretion could cause both a localized solation of the actin filaments and a contractile event generating tension. The actin gel in the subplasma membrane region of the cell could be disrupted in the presence of calcium by gelsolin. In addition, Ca^{2+} ions independently regulate actomyosin interaction via a calmodulin-dependent MLCK (39). MLCK activity has been found in islet cell homogenates (40) and we have recently demonstrated that depolarization of the HIT cells with 40 mM K^+ is associated with the rapid phosphorylation of a 20,000-mol wt protein which on two dimensional gels has a pI of 5.3, identical to the myosin light chain (41). Therefore, a transient increase in intracellular Ca^{2+} could have localized effects on beta cell cytoplasm, causing gelsolin activation and solation of microfilaments as well as contraction of the actin-myosin system. Both these phenomena may be necessary for release of insulin from the cell, and will be investigated in future studies.

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