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M. Fechheimer\*

H. M. Ingalls†

R. Furukawa‡

Elizabeth J. Luna\*\*

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‡

\*\*University of Massachusetts Medical School, Elizabeth.Luna@umassmed.edu

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## Association of the *Dictyostelium* 30 kDa actin bundling protein with contact regions

Marcus Fechheimer<sup>1,\*</sup>, Hilary M. Ingalls<sup>2,†</sup>, Ruth Furukawa<sup>1</sup> and Elizabeth J. Luna<sup>2,3</sup>

<sup>1</sup>Department of Zoology, University of Georgia, Athens, GA, USA

<sup>2</sup>Department of Biology, Princeton University, Princeton, NJ, USA

<sup>3</sup>Worcester Foundation for Experimental Biology, Shrewsbury, MA, USA

\*Author for correspondence

†Present address: Biosis, Inc., E and R Bldg, Room 112, 401 Haddon Avenue, Camden, NJ 08103, USA

### SUMMARY

'Contact regions' are plasma membrane domains derived from areas of intercellular contact between aggregating *Dictyostelium* amoebae (H. M. Ingalls et al. (1986). *Proc. Nat. Acad. Sci. USA* 83, 4779). Purified contact regions contain a prominent actin-binding protein with an  $M_r$  of 34,000. Immunoblotting with monoclonal antibodies identifies this polypeptide as a 34,000  $M_r$  actin-bundling protein (known as 30 kDa protein), previously shown to be enriched in filopodia (M. Fechheimer (1987). *J. Cell Biol.* 104, 1539). About four times more 30 kDa protein by mass is associated with contact regions than is found in total plasma membranes isolated from aggregating cells. In agreement with these observations, immunostaining of the 30 kDa protein in aggregating cells reveals a prominent localization along the plasma membrane at sites of intercellular contact. By contrast, alpha-actinin does not appear to be significantly enriched at sites of cell to cell contact. Binding experiments using purified plasma membranes, actin and 30 kDa protein indicate that the 30 kDa protein is associ-

ated with the plasma membrane primarily through interactions with actin filaments. Calcium ions are known to decrease the interaction of actin with 30 kDa protein in solution. Surprisingly, membrane-associated complexes of actin and the 30 kDa protein are much less sensitive to dissociation by micromolar levels of free calcium ions than are complexes in solutions lacking membranes. These results suggest that the interaction of the 30 kDa protein with F-actin at regions of cell-cell contact may be less sensitive to disruption by free calcium ions than elsewhere in the cell cortex. The positively cooperative assembly of stable complexes of actin and the 30 kDa protein at contact regions may be an important factor in the organization of both the cortex and these membrane domains that are specialized for intercellular adhesion.

Key words: cell adhesion, plasma membrane, cytoskeleton, actin cross-linking protein, *Dictyostelium*

### INTRODUCTION

*Dictyostelium discoideum* amoebae grow vegetatively as single cells on a rich food supply. Upon starvation, these cells initiate a process of multicellular development that leads to formation of mature fruiting bodies composed of spore and stalk cells. Interactions of the cytoskeleton with the plasma membrane are thought to be involved in motile processes, including phagocytosis and cytokinesis, during vegetative growth, and in chemotaxis and cell-cell interactions during development (Luna et al., 1990). The cell adhesion protein gp80 (Gerisch, 1986; Siu et al., 1987) and actin are concentrated in both filopodia and contact regions of adherent cells (Choi and Siu, 1987). These contact regions have been proposed to arise through rearrangement of the proteins present in the filopodia that initiate the intercellular interactions.

Interactions between actin filaments and the *Dictyostelium* plasma membrane have been extensively characterized (Luna et al., 1990). Most of the interactions in vegetative cells are

mediated by ponticulin, a transmembrane protein that promotes the assembly and binding of actin onto isolated plasma membranes (Wuestehube and Luna, 1987; Shariff et al., 1990; Chia et al., 1991, 1993). A number of other actin-binding proteins, many of which are known to be membrane associated, have also been identified and characterized in some detail (reviewed by Luna and Condeelis, 1990; Schleicher and Noegel, 1992). The membrane-associated actin-binding proteins in *Dictyostelium* include hisactophilin (Scheel et al., 1989), comitin (p24; Stratford and Brown, 1985; Weiner et al., 1993), myosins I (Côté et al., 1985; Fukui et al., 1989; Jung et al., 1989, 1993; Titus et al., 1989, 1993), and a spectrin-like protein (Bennett and Condeelis, 1988).

The *Dictyostelium discoideum* 30 kDa actin-binding protein (30 kDa protein) cross-links actin and promotes formation of actin bundles in the presence of low, but not high (micromolar), concentrations of free calcium ions (Fechheimer and Taylor, 1984; Fechheimer, 1987; Fechheimer and Furukawa, 1993). The 30 kDa protein is located in filopodia, cortical

cytoplasm, phagocytic cups and cleavage furrows of *Dictyostelium* (Fechheimer, 1987; Johns et al., 1988; Furukawa et al., 1992; Furukawa and Fechheimer, 1994). A 27 kDa fragment of this protein retains calcium-regulated actin cross-linking, but not bundling, activity indicating that the terminal region(s) of the polypeptide are required to direct assembly of highly polarized actin bundles (Fechheimer and Furukawa, 1993). Inhibition of actin filament depolymerization by the 30 kDa protein suggests a role not only in formation of cross-linked actin arrays, but also in selective stabilization of filaments in cells (Zigmond et al., 1992). The deduced amino acid sequences of 30 kDa proteins from *Dictyostelium* and *Physarum* show little homology to the sequences of other actin-binding proteins (Fechheimer et al., 1991; St Pierre et al., 1993), though antigenic homologs have been identified in *Drosophila*, *Schistosoma* and mammalian cells (Johns et al., 1988; Furukawa and Fechheimer, 1990). Transformed fibroblasts were reported to contain higher concentrations of the 30 kDa protein than non-transformed controls (Johns et al., 1988). Most recently, a 30 kDa protein homolog has been reported in Lewis lung adenocarcinoma, in the cortical actin cytoskeleton of migratory sub-confluent MDCK cells, and at intercellular junctions during the early stages of monolayer formation (Huh et al., 1993). Thus, the 30 kDa protein may be characteristic of motile or transformed cells.

In the present study, we report the presence of the 30 kDa actin-bundling protein in plasma membranes purified from developing cells, its concentration in regions of cell-to-cell contact, and actin-mediated binding of the 30 kDa protein to contact regions purified from *Dictyostelium*. In addition, the interaction between the 30 kDa protein and actin bound to contact regions appears to be less sensitive to disruption by calcium ions than is binding of the 30 kDa protein to actin in solution. These results suggest that the 30 kDa protein may stabilize the membrane-associated actin-based cytoskeleton and support the model of Siu and colleagues suggesting that filopodial interdigitation is an important early step in the formation of cell to cell contacts (Choi and Siu, 1987).

## MATERIALS AND METHODS

### Cells

*Dictyostelium discoideum*, strain AX3K (a gift from Dr R. Kessin, Columbia University), were grown axenically in HL-5 culture broth at 20°C as described previously (Loomis, 1971). *Dictyostelium discoideum*, strain NC-4, were cultured on 2% nutrient agar in Petri dishes in the presence of *Escherichia coli* B/r as described previously (Bonner, 1947). Aggregating amoebae were developed either in suspension for 12 hours with cAMP pulses every 6 minutes as described (Ingalls et al., 1986) for membrane purification, or on non-nutrient agar for 5 hours in preparation for immunofluorescence microscopy.

### Membranes

Plasma membranes were purified from vegetative and aggregating amoebae by three different methods. First, 'Renografin membranes' were purified from filter-lysed cells by sequential centrifugation on gradients of sucrose and Renografin-76 (Squibb Diagnostics, New Brunswick, NJ) as previously described (Ingalls et al., 1989). Renografin membranes are sealed vesicles of mixed orientation that contain variable amounts of trapped cytoplasmic protein (Goodloe-Holland and Luna, 1987; Ingalls et al., 1989). 'Triton/ConA membranes' were prepared from cells stabilized with concanavalin A,

lysed in 0.2% Triton X-100, and fractionated by sucrose density shift centrifugation (Luna et al., 1984; Goodloe-Holland and Luna, 1987). These very highly purified membranes are in the form of unsealed sheets and large vesicles. Finally, 'contact regions', plasma membranes derived from sites of cell-to-cell association in developing amoebae, were purified from suspension-developed cells after lysis with 0.2% Triton X-100 (Ingalls et al., 1986). Apparently stabilized by their extracellular adhesive interactions, contact regions resist detergent solubilization even in the absence of concanavalin A and also consist of large multilamellar sheets and vesicles with some filamentous material apparently trapped within the inner lumens of the largest structures (Ingalls et al., 1986). Membranes were iodinated (Luna et al., 1984) with <sup>125</sup>I-labeled Bolton-Hunter reagent (New England Nuclear Dupont Products, Boston, MA) before solubilization with 3% octyl glucoside, clarification by ultracentrifugation, and fractionation on F-actin and control columns as described previously (Wuestehube and Luna, 1987; Wuestehube et al., 1991). Quantitative analyses of alpha-actinin and the 30 kDa protein employed two to four different preparations of each type of membrane and the stated number of independent immunoblot analyses.

### Proteins

Actin was prepared from rabbit skeletal muscle (Spudich and Watt, 1971), and chromatographed on Sephadex G-150 (MacLean-Fletcher and Pollard, 1980). Gelsolin was purified from rabbit plasma and stored frozen in small portions at -70°C (Cooper et al., 1987). The *Dictyostelium* 30 kDa calcium-regulated actin-bundling protein (30 kDa protein) was purified as previously described (Fechheimer and Furukawa, 1991). Rabbit polyclonal and murine monoclonal (B2C) antibodies reactive with the 30 kDa protein have been described (Fechheimer, 1987; Furukawa et al., 1992), as has the adsorbed rabbit polyclonal antibody recognizing ponticulin (Wuestehube and Luna, 1987), and monoclonal antibody 2A1 specific for *Dictyostelium* alpha-actinin (Furukawa and Fechheimer, 1994). An antibody specific for cell-cell adhesion protein, gp80, was prepared by immunizing rabbits with keyhole limpet hemocyanin conjugated (Green et al., 1982) to a peptide corresponding to Lys240 through Ile259 in the gp80 sequence (Noegel et al., 1986). A high-affinity antibody specific for gp80 on immunoblots (Ingalls, 1989) was obtained by affinity purification of immune sera on an AffiGel-10 column containing the same peptide conjugated to lysozyme. After elution of non-specifically bound and low-affinity antibodies with 1 M guanidine, 50 mM Tris-HCl, pH 7.4, high-affinity anti-gp80 antibodies were eluted with 4.5 M MgCl<sub>2</sub> (Pfeffer et al., 1983), dialyzed into 50% glycerol, 150 mM NaCl, 10 mM sodium phosphate, pH 7.2, and stored at -20°C.

### Analytical methods

Protein concentration was determined by the method of Lowry et al. (1951). F-actin affinity chromatography was carried out as described previously (Wuestehube et al., 1991). Polyacrylamide gel electrophoresis in the presence of SDS was performed using the discontinuous buffer system described by Laemmli (1970) in gradient gels of 6% to 16% polyacrylamide. Electrophoretic blotting to nitrocellulose, probing with specific rabbit polyclonal antibodies, and visualization with <sup>125</sup>I-labeled Protein A (New England Nuclear Dupont Products) were performed as previously described (Ingalls et al., 1986; Towbin et al., 1979). Monoclonal antibodies were visualized using 5 µg/ml Fc-specific rabbit anti-mouse secondary antibody (Pierce Chemical Co., Rockford, IL) and 10 µCi/ml <sup>125</sup>I-labeled Protein A. Exposed film was scanned with a densitometer (PDI, Huntington Station, NY). Labeled bands were analyzed with Discovery Series™ software, Version 2.0, and the areas under the peaks were determined automatically.

### Radioiodination

Actin was iodinated with <sup>125</sup>I-labeled Bolton-Hunter reagent and stored in dialysis at 0°C for no longer than 2 weeks with buffer

changes every 2 days (Schwartz and Luna, 1986). The 30 kDa protein (1 mg) was iodinated by incubation at 0°C for 2-4 hours with 250  $\mu$ Ci of  $^{125}$ I-labeled Bolton-Hunter reagent in 10 mM sodium phosphate, pH 8.5 (1 ml). Radiolabeled 30 kDa protein was separated from unincorporated label by chromatography on Sephadex G-25, and further purified and concentrated by binding to and elution from hydroxylapatite (Fechheimer and Furukawa, 1991). In a typical preparation, 0.5 mg of 30 kDa protein was recovered with a specific activity of 400,000 cpm/ $\mu$ g (13,600 cpm/pmol).

### Immunofluorescence microscopy

Cells (NC-4 or AX3K) were harvested during vegetative growth, developed for 5 hours, and then fixed using the agar overlay method (Fukui et al., 1987; Yumura et al., 1984), and stained with monoclonal antibody B2C reactive with the 30 kDa protein. In some experiments, the 30 kDa protein was localized using polyclonal rabbit antibody followed by rhodamine-labeled goat anti-rabbit antibody, and alpha-actinin was co-localized using mouse monoclonal antibody 2A1 that was directly labeled with fluorescein isothiocyanate.

### Binding assays

F-actin columns were prepared and used for F-actin affinity chromatography as described (Luna et al., 1982; Wuestehube and Luna, 1987; Wuestehube et al., 1991). Affinity columns were composed of 0.1 ml of IgG-Sepharose S-1000 beads, with or without bound F-actin. Columns were equilibrated in 50 mM KCl, 1 mM MgCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, 0.5  $\mu$ M phalloidin, 20 mM Tris-acetate, pH 7.0, at 20°C (column buffer), and blocked with 4% BSA in column buffer.

Co-sedimentation of the 30 kDa protein with actin filaments in the presence and absence of micromolar concentrations of free calcium ions was performed essentially as described (Fechheimer and Furukawa, 1993). In initial experiments, results were analyzed by densitometry of both Coomassie Blue-stained SDS-PAGE gels and autoradiographs. Results from the two analyses were not significantly different, indicating that modification with Bolton-Hunter reagent does not detectably alter the actin-binding activity and calcium sensitivity of the 30 kDa protein.

Binding of actin and the 30 kDa protein to isolated contact region membranes was assessed using methods described by Schwartz and Luna (1986). Briefly, gelsolin and actin at the specified mole ratios were co-assembled overnight (>13 hours) at room temperature in polymerization buffer (50 mM KCl, 2 mM MgCl<sub>2</sub>, 20 mM PIPES, pH 6.8, 0.2 mM DTT, 1 mg/ml ovalbumin; Worthington Biochemical Corp., Freehold, NJ) containing 25  $\mu$ M phalloidin, ~80  $\mu$ M CaCl<sub>2</sub> and ~80  $\mu$ M Na<sub>2</sub>ATP. Assay mixtures contained 50  $\mu$ g/ml protein in

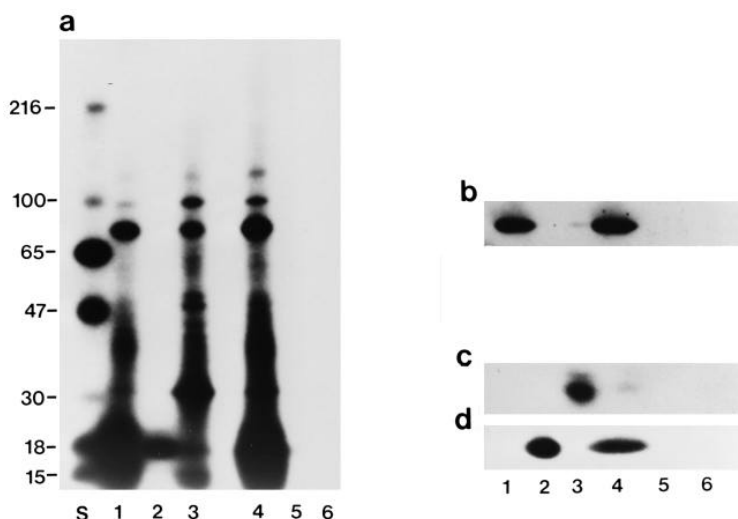
contact regions and indicated concentrations of actin and 30 kDa protein in polymerization buffer plus 0.2 mM ATP, 5  $\mu$ M phalloidin, and 2 mM EGTA  $\pm$  1.8 mM CaCl<sub>2</sub>. The calculated free calcium ion concentrations of the solutions in the absence and presence of the 1.8 mM CaCl<sub>2</sub> were <0.03  $\mu$ M and ~5.2  $\mu$ M, respectively. Some error in the actual free calcium ion concentration is expected because EGTA is not a good buffer at high ratios of calcium to EGTA and because the concentration of calcium was not independently investigated by atomic absorption analysis. Mixtures were held for 1 hour at room temperature and sedimented for 30 minutes at 27,000 g through 5% sucrose in 20 mM PIPES, 50 mM KCl, 2 mM MgCl<sub>2</sub>, 2 mM EGTA, pH 6.8. Tubes were frozen and cut for analysis of pellet and supernatant fractions.

## RESULTS

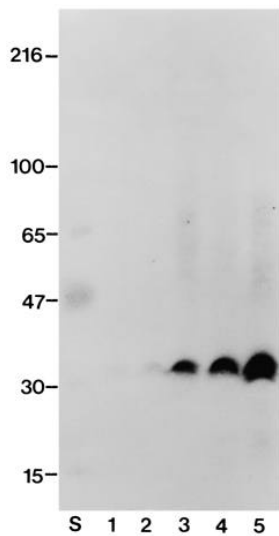
### The 30 kDa actin bundling protein is enriched in membranes derived from regions of cell to cell contact

The presence of the 30 kDa actin-bundling protein in *Dicystostelium* membranes was first noted during affinity chromatography of solubilized contact regions on F-actin affinity columns. A major 30 kDa protein bound to F-actin columns (Fig. 1a, lane 3), but not to control columns (Fig. 1a, lanes 5-6). This 30 kDa protein bound very tightly, resisting salt conditions that removed bound ponticulin (Fig. 1a, lane 2; Fig. 1d), the major actin-binding protein in plasma membranes from vegetatively growing cells (Luna et al., 1990). At least a portion of this material was the 30 kDa actin-bundling protein, as shown by reactivity of unlabeled fractions stained with specific antibody on Western blots (Fig. 1c). By contrast, very little of the adhesion protein, gp80, bound to either F-actin or control columns (Fig. 1b).

Because the interaction of this protein with actin in co-sedimentation and viscosity assays is very sensitive to micromolar concentrations of free calcium ions (Fechheimer and Taylor, 1984; Fechheimer, 1987; Fechheimer and Furukawa, 1993), the binding of essentially all of the 30 kDa actin-bundling protein to F-actin affinity columns was not anticipated. Although no calcium was added to the column buffer used for affinity chromatography, trace calcium contamination



**Fig. 1.** The 30 kDa actin-bundling protein is present in contact regions. (a) Iodinated, solubilized contact regions were fractionated by affinity chromatography on F-actin (lanes 1-3) or control (lanes 4-6) columns. Unbound proteins (lanes 1,4), proteins eluted with 2 M NaCl in column buffer (lanes 2,5), and proteins eluted with 1% SDS (lanes 3,6) were resolved on a 6% to 16% gel. Iodinated molecular mass standards (lane S), in kDa, are shown on the left. The autoradiogram of the dried gel is shown. (b-d) Unlabeled contact regions were fractionated as described, on F-actin (lanes 1-3) and control (lanes 4-6) columns, transferred to nitrocellulose, and probed with antibodies against the adhesion protein, gp80 (b), the 30 kDa protein (c), and ponticulin (d). The loading of lanes 1-3 and 4-6 was adjusted to apply a constant fraction of the total sample applied to each column, with the exception of Fig. 1c, lane 4, which was loaded on the basis of constant sample volume. Thus, the band is faint even though virtually all of the 30 kDa protein applied to the control column was eluted in the unbound fraction.



**Fig. 2.** Enrichment of the 30 kDa protein in contact regions and in plasma membranes from aggregating cells. Proteins in plasma membranes from vegetative (lanes 1, 2) and aggregating cells (lanes 3, 4) and in contact regions (lane 5) were resolved by SDS-PAGE (30  $\mu$ g per lane), transferred to nitrocellulose, and probed with antibody against the 30 kDa protein. Membranes were purified using either the sucrose/Renografin gradient (lanes 1, 3) or Triton/ConA (lanes 2, 4) methods.

of other buffer components was expected to raise the total free calcium ion concentration into the micromolar range. To determine whether this behavior was peculiar to the 30 kDa protein present in the contact regions, 30 kDa protein purified from the soluble fraction of amoebae was chromatographed on F-actin and control columns in a buffer containing 2.0 mM EGTA and 1.8 mM  $\text{CaCl}_2$ , with an estimated free calcium ion concentration of  $\sim 5.2 \mu\text{M}$ . Most of the purified 30 kDa protein also bound to the phalloidin-stabilized F-actin on the affinity column, resisted elution with 2 M NaCl in column buffer, and was removed with 1% SDS (data not shown). No binding to control columns was observed, indicating a strong interaction between purified 30 kDa protein and F-actin on affinity columns, even in the presence of micromolar calcium ions.

The amount of 30 kDa protein isolated in association with plasma membranes increased with development and was most enriched in cell-cell contact regions (Fig. 2). The amount of membrane-associated 30 kDa protein increased about 7-fold during development (Table 1; Fig. 2, lanes 3 and 4 versus lanes 1 and 2). Some of this increase may be due to entrapment of cell cortex in plasma membranes purified from developing cells, because a 3- to 6-fold increase in alpha-actinin, another cortical actin-binding protein, also was observed in these membranes (Table 1). However, specific enrichment for the 30 kDa protein may be inferred, since the quantity of the 30 kDa protein decreases 2-fold during development (Furukawa and Fechheimer, 1990), while alpha-actinin increases in abundance as development proceeds (Witke et al., 1986). Moreover, there is an additional 4-fold increase in the amount of 30 kDa protein associated with purified contact regions (Fig. 2, lane 5 versus lanes 3 and 4; Table 1), as compared to total plasma membrane from aggregating cells. By contrast, no significant difference between the amount of alpha-actinin in contact regions and that in total plasma membranes from aggregated cells is observed (Table 1).

The specific enrichment of the 30 kDa protein at sites of cell-cell contact also was observed by immunofluorescence microscopy (Fig. 3B and E). This enrichment was observed for both NC4 (Fig. 3) and AX3K cells (not shown), indicating that the result is not dependent on the strain used. By contrast,

**Table 1.** Development- and cell contact-induced changes in the amounts of membrane-associated 30 kDa protein and  $\alpha$ -actinin

Type of membrane	Relative amounts* of 30 kDa protein	Relative amounts* of $\alpha$ -actinin
Log-phase (Renografin)	1.2 $\pm$ 0.5 (n=2)	4.7 $\pm$ 3.1 (n=6)
Log-phase (Triton/ConA)	1.0 $\pm$ 0.3 (n=6)	1.0 $\pm$ 0.2 (n=7)
12-h Developed (Renografin)	5.5 $\pm$ 4.8 (n=12)	14.3 $\pm$ 5.3 (n=5)
12-h Developed (Triton/ConA)	8.6 $\pm$ 7.1 (n=12)	5.8 $\pm$ 2.0 (n=5)
12-h Developed contact regions	26.0 $\pm$ 14.5 (n=12) <sup>†</sup>	4.1 $\pm$ 1.3 (n=8) <sup>‡</sup>

Relative changes in apparent density of antigen after visualization with primary antibody and  $^{125}\text{I}$ -labeled Protein A, as described in Materials and Methods. Only values within each column can be compared.

\*Normalized relative to the average number obtained for log-phase Triton/ConA membranes. Values are means  $\pm$  s.e.m.

<sup>†</sup>Using an unpaired Mann-Whitney nonparametric test, the amount of 30 kDa protein in contact regions is significantly larger than the amount in either of the other membranes from developed cells ( $P < 0.002$ ).

<sup>‡</sup>There is no significant difference between the amount of alpha-actinin in contact regions and the amount in 12-hour Triton/ConA membranes. Both 12-hour Triton/ConA membranes and contact regions contain more associated alpha-actinin than do log-phase Triton/ConA membranes ( $P < 0.0006$ ). The large variations in the amounts of alpha-actinin trapped in the Renografin membrane vesicles complicate the use of these values in statistical comparisons.

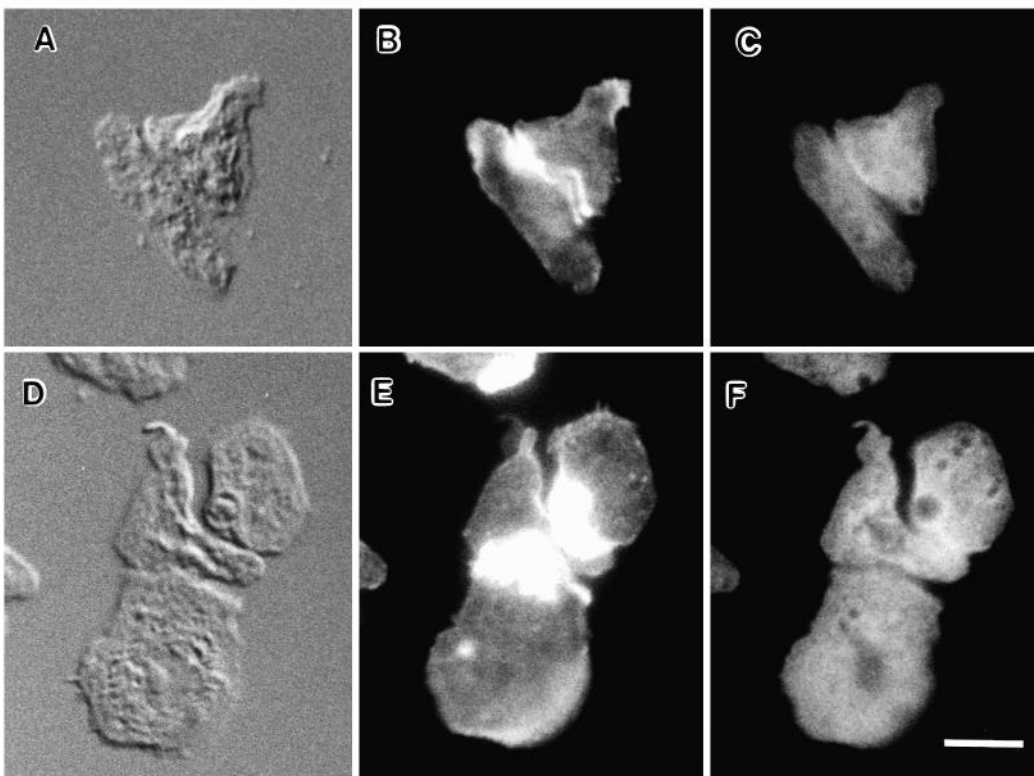
alpha-actinin was not significantly concentrated at cell-cell contact sites (Fig. 3C and F).

### The 30 kDa protein binds to contact regions, but only in the presence of membrane-bound actin

To investigate the basis for association of the 30 kDa protein with plasma membranes, reciprocal sedimentation binding studies were performed with purified membranes and  $^{125}\text{I}$ -labeled 30 kDa protein (Figs 4, 5) or  $^{125}\text{I}$ -labeled actin (Fig. 6). The results showed that the 30 kDa protein binds to *Dictyostelium* membranes, but only in the presence of actin. In the absence of actin, only background levels of the 30 kDa protein co-sedimented either with contact regions (Figs 4, 5) or with Triton/ConA membranes from vegetative cells (data not shown). The small amount of the 30 kDa protein sedimenting under these conditions was not diminished by adding a large excess of unlabeled 30 kDa protein (Fig. 4), a further indication of the non-specific nature of this low level of sedimentation.

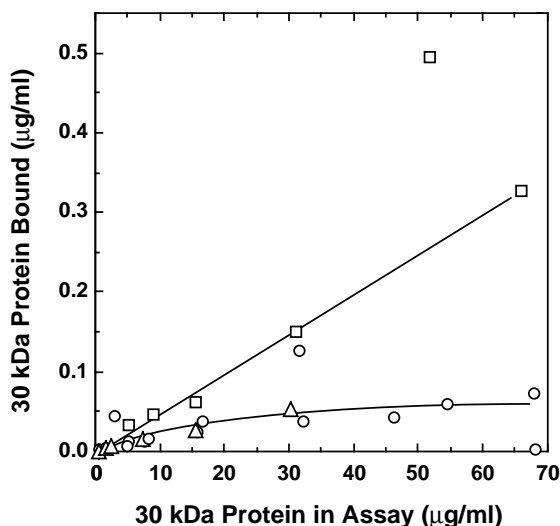
Binding of the 30 kDa protein to contact regions was increased in the presence of actin (Figs 4, 5), and at higher concentrations of the 30 kDa protein (Fig. 4). Membrane-associated 30 kDa protein, defined as the difference ( $\Delta$ ) between sedimentation with and without contact regions, was higher in the presence of low concentrations of actin (5  $\mu\text{g/ml}$ ) than in the presence of higher actin concentrations (50  $\mu\text{g/ml}$ ; Fig. 5A). For instance, in the absence of calcium ions,  $\sim 0.18 \mu\text{g/ml}$  of the 30 kDa protein bound specifically to contact regions in the presence of 5  $\mu\text{g/ml}$  actin, whereas only  $\sim 0.06 \mu\text{g/ml}$  bound in the presence of 50  $\mu\text{g/ml}$  actin (Fig. 5A).

The reduced binding of the 30 kDa protein to contact regions at the higher actin concentration may be explained by consid-



**Fig. 3.** The 30 kDa protein is concentrated at regions of cell-to-cell contact. Aggregating NC4 amoebae were fixed and stained with polyclonal antibody reactive with the 30 kDa protein (B and E), and with monoclonal antibody 2A1 reactive with *Dictyostelium* alpha-actinin (C and F). The 30 kDa protein is present in high concentration at sites of cell to cell contact, while alpha-actinin shows a diffuse localization without obvious enrichment at regions of intercellular association.

ering the relative amounts of actin in solution versus actin bound to the membrane at the different actin concentrations (Fig. 6). In the absence of the 30 kDa protein (Fig. 6A),  $^{125}\text{I}$ -labeled actin bound saturably to contact regions with the half-maximal binding of  $\sim 2 \mu\text{g/ml}$  actin observed at  $5 \pm 2 \mu\text{g/ml}$  total

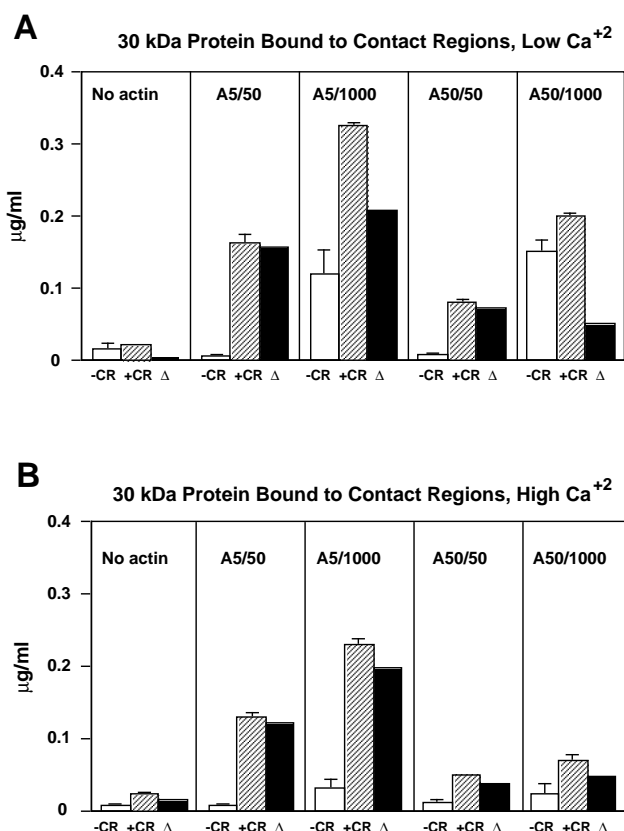


**Fig. 4.** Actin mediates binding of the 30 kDa protein to contact regions. Sedimentation of  $^{125}\text{I}$ -labeled 30 kDa protein with contact regions ( $50 \mu\text{g/ml}$ ) in mixtures containing: no additions (○),  $141 \mu\text{g/ml}$  unlabeled 30 kDa protein (△), or  $10 \mu\text{g/ml}$  actin (□). The small amount of  $^{125}\text{I}$ -labeled 30 kDa protein sedimenting in the absence of contact regions has been subtracted. Gelsolin was present during polymerization of the actin prior to inclusion in the assay at a molar ratio of 1:250 of gelsolin to actin.

actin. Nearly saturable levels of binding were observed at  $50 \mu\text{g/ml}$  total actin (Fig. 6A and data not shown). Therefore, at  $5 \mu\text{g/ml}$  actin, about half of the actin is in solution and half is bound to the membrane. By contrast, at  $50 \mu\text{g/ml}$  actin,  $\sim 90\%$  of the actin is in solution and only  $\sim 10\%$  is bound to the membrane. Thus, the actin concentration dependence of the 30 kDa protein sedimenting without (–CR) and with (+CR) contact regions (Fig. 5A) suggests that the 30 kDa protein binds both to actin in solution and to actin associated with membranes.

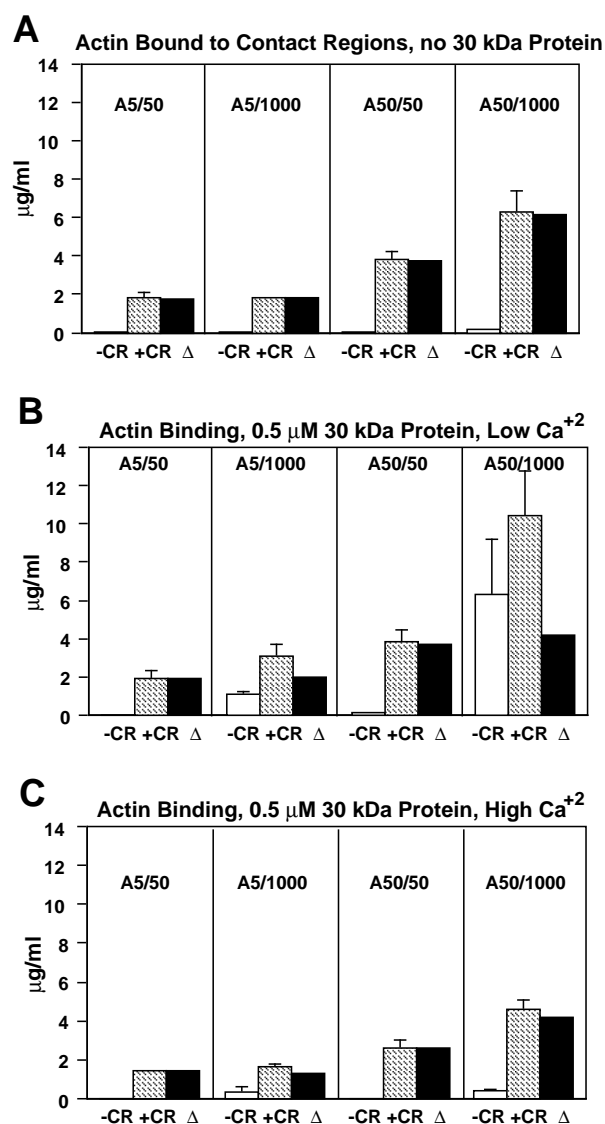
In contrast to the requirement for actin seen in experiments with  $^{125}\text{I}$ -labeled 30 kDa protein (Fig. 5), the specific binding ( $\Delta$ ) of  $^{125}\text{I}$ -labeled actin to contact regions was essentially unaffected by the addition of  $0.5 \mu\text{M}$  30 kDa protein (Fig. 6B versus A). Although the 30 kDa protein increased the sedimentation of longer actin filaments (A5/1000 and A50/1000, see legend to Fig. 5), this increased sedimentability was observed in the absence, as well as in the presence, of contact regions, and probably represents the formation of large crosslinked structures. Higher concentrations of the 30 kDa protein further increased the amounts of sedimentable 30 kDa protein and actin, presumably for the same reason (data not shown). Thus, depending upon actin concentration and filament length, the amount of sedimentable radioactivity reflected either the extent of crosslinked structures formed in solution, binding of protein to the highly sedimentable contact regions, or a combination of both activities.

Although more of the 30 kDa protein sedimented with actin filaments containing about 1000 monomers than with filaments with only  $\sim 50$  actin monomers, the amount of membrane-associated 30 kDa protein ( $\Delta$ ) was not strongly dependent on actin filament length (Fig. 5A, B). This observation is explainable by considering the relative amounts of actin associated with the membrane under these different conditions (Fig. 6). As



**Fig. 5.** Effects of actin, contact regions and calcium ions on the sedimentation of  $^{125}\text{I}$ -labeled 30 kDa protein. Sedimentation of  $^{125}\text{I}$ -labeled 30 kDa protein in (A) low concentrations of calcium ions ( $<0.03 \mu\text{M}$ ); and (B) high concentrations of free calcium ions ( $\sim 5.2 \mu\text{M}$ ).  $^{125}\text{I}$ -labeled 30 kDa protein ( $0.5 \mu\text{M}$ ) was incubated without (-CR) and with (+CR) contact regions ( $50 \mu\text{g/ml}$ ) and with 5 (A5) or 50 (A50)  $\mu\text{g/ml}$  of polymerized actin for 1 hour at room temperature. These concentrations correspond to approximately half-maximal binding and saturable actin binding to the membrane, respectively. Actin filament length was regulated by including rabbit plasma gelsolin at a molar ratio of 1:50 (/50) or 1:1000 (/1000) to actin. Labels denote the amount and length of actin filaments, e.g. A5/50 =  $5 \mu\text{g/ml}$  actin with an average filament length of 50 monomers. Values shown represent the mean  $\pm$  s.e.m. of triplicate determinations. The difference between the mean value of the 30 kDa protein sedimenting with membranes (+CR) and the mean value sedimenting without membranes (-CR) has been calculated for each condition ( $\Delta$ ).

reported previously (Schwartz and Luna, 1986), actin filament length had little effect on total binding of actin at low concentrations where filaments can bind along their lengths to the membrane surface (Fig. 6A, B). At saturating actin concentrations,  $\sim 50\%$  more of the longer filaments were attached to the membranes, presumably through smaller numbers of individual filament-membrane interactions (Fig. 6A, B). However, any increased binding of the 30 kDa protein to this increased amount of membrane-bound actin was obscured by the greatly increased sedimentability of the complexes formed from the 30 kDa protein and the much larger number of long actin filaments in solution (-CR data in Figs 5A and 6B). Thus, sedimentation of the 30 kDa protein in association with membrane-bound



**Fig. 6.** Effects of the 30 kDa protein, contact regions and calcium ions on the sedimentation of  $^{125}\text{I}$ -labeled actin. Sedimentation in (A, B) low concentrations of calcium ions ( $<0.03 \mu\text{M}$ ) and (C) high concentrations of free calcium ions ( $\sim 5.2 \mu\text{M}$ ).  $^{125}\text{I}$ -labeled actin (5 or  $50 \mu\text{g/ml}$ ) was incubated without (-CR) or with (+CR) contact regions ( $50 \mu\text{g/ml}$ ) and without (A) or with (B, C) the 30 kDa protein ( $0.5 \mu\text{M}$ ) for 1 hour at room temperature. Actin filament length was regulated by addition of rabbit plasma gelsolin at a molar ratio of 1:50 or 1:1000 to actin. Labels denote the amount and length of actin filaments, as described in the legend to Fig. 5. Values shown in A and B represent the mean  $\pm$  s.e.m. of triplicate (A, -CR) or pentuplicate (A, +CR; B) determinations; values shown in C are the average  $\pm$  range of duplicate measurements. The difference between the mean value of actin sedimenting with membranes (+CR) and the mean value sedimenting without membranes (-CR) has been calculated for each condition ( $\Delta$ ).

actin is optimal at lower actin concentrations ( $\sim 5 \mu\text{g/ml}$ ), whereas formation of actin filament aggregates in solution is enhanced at higher actin concentrations ( $50 \mu\text{g/ml}$ ) and longer filament lengths ( $\sim 1000$  monomers).

### 30 kDa protein-actin complexes on contact regions are less sensitive to calcium ions

We compared the effects of micromolar concentrations of free calcium ions on the interactions of the 30 kDa protein and actin either in solution or in association with contact regions (Figs 5B, 6C). Micromolar levels of free calcium ions greatly reduced the amounts of sedimentable solution complexes of 30 kDa protein and actin, as assessed with both  $^{125}\text{I}$ -labeled 30 kDa protein (Fig. 5B, -CR) and  $^{125}\text{I}$ -labeled actin (Fig. 6C, -CR). This effect was especially apparent in mixtures containing longer actin filaments (A5/1000 and A50/1000), in which the average amount of sedimentable protein was 3- to 16-fold lower in the presence of micromolar free calcium ions. These results were consistent with the 5-fold decreases in the amount of sedimentable complexes of actin and 30 kDa protein in the presence of calcium reported previously for samples without membranes (Fechheimer and Furukawa, 1993).

By contrast, micromolar calcium ions had relatively little effect on the binding of the 30 kDa protein to actin on contact regions ( $\Delta$ , Fig. 5). Similarly, the specific binding of  $^{125}\text{I}$ -labeled actin to membranes ( $\Delta$ , Fig. 6) was essentially insensitive to calcium levels in both the absence (Schwartz and Luna, 1986) and presence (Fig. 6C) of the 30 kDa protein. Thus, the interaction between actin and the 30 kDa protein appeared to be much more sensitive to calcium ions in solution than in the presence of contact regions. These results indicate that membrane-associated complexes of actin and the 30 kDa protein are less affected by micromolar levels of calcium ions than are complexes of these proteins in solution, suggesting that contact regions may stabilize or potentiate the interaction between actin and the 30 kDa actin-bundling protein.

## DISCUSSION

We report here that a well-characterized filopodial 30 kDa actin-bundling protein associates with actin at cell contact regions in aggregating *Dictyostelium* amoebae (Figs 1, 2). These results are confirmed by localization of the 30 kDa protein at sites of cell-cell contact in immunofluorescence micrographs (Fig. 3). The association of the 30 kDa protein with contact regions apparently does not arise solely from entrapment or non-selective binding to membrane-associated actin because alpha-actinin, another calcium-regulated actin cross-linking protein, is not similarly enriched (Table 1; Fig. 3). The co-isolation of the 30 kDa protein with contact regions is particularly striking because the total quantity of the 30 kDa protein decreases approximately 2-fold as development proceeds (Furukawa and Fechheimer, 1990). Thus, the increased association of the 30 kDa protein with contact regions reflects a true 'recruitment', or an increase in the fraction of the total 30 kDa protein associated with these plasma membrane domains. Part of the increase in membrane-associated 30 kDa protein may be attributed to the 2- to 3-fold increases in total cell actin (Alton and Lodish, 1977), membrane-associated actin (Ingalls et al., 1986) and ponticulin (Ingalls et al., 1989) observed in developing cells. However, the lack of a similar enrichment for alpha-actinin in contact sites suggests that specific mechanisms must also be involved (Table 1).

Our first hypothesis, that a specific, high-affinity receptor for

the 30 kDa protein might recruit it to the plasma membrane of developing amoebae, was not supported by any of the experimental results. No specific binding of the 30 kDa protein to contact regions was observed except in the presence of exogenously added actin (Figs 4, 5). In contrast, actin binds to these membranes in the absence, as well as in the presence, of the 30 kDa protein (Fig. 6). Thus, the 30 kDa protein appears to be peripherally associated with the membrane through binding to F-actin. The 30 kDa protein also forms bundles with actin filaments, suggesting that this protein could promote incremental growth of the cortical actin cytoskeleton from the plasma membrane. Such a positively cooperative interaction between actin and the 30 kDa protein may contribute to the striking co-localizations of these proteins in contact sites (this study), phagocytic cups (Furukawa et al., 1992; Furukawa and Fechheimer, 1994), and cleavage furrows (Furukawa and Fechheimer, 1994). The selective stabilization of actin filaments by the 30 kDa protein (Zigmond et al., 1992) may also contribute to the formation and/or stability of associated membrane domains.

Although the 30 kDa protein apparently binds to contact regions through an association with membrane-bound actin, this interaction, and/or its regulation, may differ from that observed in solution. For instance, micromolar calcium ions exert a much greater influence on the sedimentability of 30 kDa protein-actin complexes in solution than on the sedimentability of these proteins with contact regions (Figs 5, 6). One possibility is that the 30 kDa protein binds to membrane-associated actin through a calcium-insensitive actin-binding site. Alternatively, the calcium-sensitive actin-binding site(s) on membrane-associated 30 kDa protein may simply be less sensitive to disruption by calcium ions. For instance, high local actin concentrations at the membrane surface might promote extensive binding even in the presence of micromolar calcium. This interpretation is consistent with the observed tight binding of the 30 kDa protein, even in the presence of calcium, to high local concentrations of F-actin in the bead pores of affinity columns (Goodloe-Holland and Luna, 1984; Luna et al., 1982). This result also agrees with the observation that  $^{125}\text{I}$ -labeled F-actin binds to the 30 kDa protein in blot overlay assays performed in the presence of calcium ions (Chia et al., 1991; Fechheimer and Furukawa, 1993). The stronger than expected binding in the presence of calcium also might be associated with a reduction in dimensionality (Adam and Delbrück, 1968; Mosior and Epan, 1993) due to the orientation of actin filaments either along the membrane surface, or on the solid supports (i.e. bead pores or nitrocellulose filters) used in binding assays.

Our results indicate that the 30 kDa actin-bundling protein and alpha-actinin, both calcium-sensitive actin-binding proteins, segregate differently in the cell. These observations are consistent with and extend reports of spatial and/or temporal differences in localization of alpha-actinin and the 30 kDa protein in the cleavage furrow, phagocytic cup and contractile vacuole (Furukawa and Fechheimer, 1994). Such a segregation may arise stochastically (Dufort and Lumsden, 1993) or could be stimulated by recruitment or selective stability due to binding to membrane-associated actin filaments. Although the idea that membrane-associated actin filaments serve as a specific targeting or sorting signal for cytoskeletal proteins is an exciting and testable hypothesis, we cannot exclude the pos-

sibility of a more direct interaction between the 30 kDa protein and the plasma membrane. For instance, interactions important in vivo could be missed for any of a number of reasons in vitro, including loss of activity during protein or membrane purification, or perturbation of a membrane-binding site by iodination. For similar reasons we cannot exclude the possibility of a role for alpha-actinin, or any other cytoplasmic actin-binding protein, in the membrane skeleton at cell-cell contact sites. The evidence suggesting a contribution of alpha-actinin to the membrane skeleton of mammalian cells (Otey et al., 1990; Carpén et al., 1992) is in no way invalidated by the lack of obvious enrichment for alpha-actinin in contact regions in *Dictyostelium*.

Finally, our results support a previous model for the formation of cell-to-cell contacts in which filopodia mediate the initial recognition and formation of intercellular adhesions. Interdigitation of these filopodia is proposed to be followed by their retraction into the cell and by the formation of smooth stable contact surfaces (Choi and Siu, 1987). Both actin and gp80, a major adhesion protein in developing cells, have been localized in filopodia as well as at sites of cell-cell contact. The finding that the 30 kDa protein is also enriched in both filopodia and cell-to-cell contact sites provides additional support for this model. The greater resistance to disruption by calcium of actin/30 kDa protein interactions at the membrane surface could provide selective stability to filaments associated with contact sites, while other actin filaments participate in the dynamic flux accompanying cell movement and morphogenesis. The selective stability of the underlying cortex might, in turn, help stabilize the structure and/or function of these specialized membrane domains. The structural conservation of the 30 kDa actin-bundling protein from *Dictyostelium* to mammals (Johns et al., 1988; Huh et al., 1993) suggests that its function(s) at sites of intercellular contact also may have been similarly conserved.

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