

# Spectrin and protein 4.1 as an actin filament capping complex

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Spectrin and protein 4.1, when added to G- or F-actin, cause the formation of short filaments, as judged by the appearance of powerful nucleating activity for G-actin polymerisation. F-Actin filaments are rapidly fragmented under physiological solvent conditions. The effect of cytochalasin E on the polymerisation reaction and the extent of reduction in the critical monomer concentration of actin when spectrin and 4.1 are added suggest that these proteins form a capping system for the more slowly growing, or 'pointed' ends of actin filaments. The interaction is not affected by calcium or by 4.9, the remaining constituent of the purified red cell membrane cytoskeleton.

<i>Erythrocyte</i>	<i>Cytoskeleton</i>	<i>Spectrin</i>	<i>Protein 4.1</i>	<i>Actin</i>	<i>Capping</i>
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## 1. INTRODUCTION

The properties of the red cell membrane are controlled by a complex of proteins, the membrane cytoskeleton, comprising spectrin, actin, and proteins 4.1 and 4.9. The actin in this complex is in the form of short filaments, which are linked to one another by spectrin molecules, largely in the form of symmetrical tetramers, about 200 nm in length. The linking of spectrin to actin is weak in the absence of 4.1 but strong in its presence, the association constants for the binary and ternary complexes being of the order of  $10^3 \text{ M}^{-1}$  and  $10^{12} \text{ M}^{-2}$ , respectively (to be published). The question arises of how the short actin filaments in red cells, variously estimated to be 12–30 subunits long [1,2], are stabilised and prevented from disproportionating to form long filaments. Even though spectrin and 4.1 will bind along the length of F-actin filaments [3,4], it has been found [5] that when G-actin is polymerised in the presence of appropriate proportions of spectrin and 4.1, complexes apparently containing short actin filaments are generated. These may be conjectured to resemble the branch points of the cytoskeletal network. We show here that spectrin and 4.1, in addition to binding to actin subunits along the fila-

ment, have a high affinity for filament ends, and form a capping system. This affords a rational basis for the existence of short actin filaments in the cytoskeletal complex in the cell and in vitro.

## 2. MATERIALS AND METHODS

Spectrin dimers were extracted from human red cells and purified as in [3]. Actin from rabbit muscle was prepared as in [6] and 4.1 was obtained by dissociation of cytoskeletons of human red cells in concentrated Tris, followed by column chromatography [7]. This procedure also yields protein 4.9 [8]. Actin containing a coupled pyrene fluorophore was prepared by reaction with pyrene iodoacetamide [9].

Activity with respect to nucleation of actin polymerisation was assayed by adding a preincubated mixture of spectrin dimer, 4.1 and actin to pyrene-labelled G-actin, and observing polymerisation by way of the large increase in quantum yield [9], using a perkin Elmer MPF 3L spectrofluorimeter. The solvent for these assays was 0.1 M NaCl, 12 mM Tris, 1.2 mM  $\text{MgCl}_2$ , 0.1 mM EGTA, 1 mM  $\text{NaN}_3$ , 0.2 mM ATP, 0.2 mM dithiothreitol (pH 8.0). Critical monomer concentrations were measured in the same buffer, but for the ATP con-

centration, which was increased to 5 mM. Polymerisation was allowed to reach completion at 3.5°C for 6 days and the fluorescence was measured at each actin concentration. Proteins were screened by SDS-gel electrophoresis for any degradation at the end of each experiment.  $\text{CaCl}_2$  (0.2 mM, omitting the EGTA) was included when required, as also were protein 4.9 (2  $\mu\text{g}/\text{ml}$ ) and cytochalasin E at 20 nM to 20  $\mu\text{M}$ . Protein concentrations were determined spectrophotometrically [10].

### 3. RESULTS

Nucleation of actin polymerisation by spectrin-actin-4.1 complexes was observed in terms of the increase in fluorescence of pyrene-labelled actin at a concentration at which the unseeded polymerisation was slow (fig.1). Nucleating complexes could be generated by adding spectrin and 4.1 to G-actin or F-actin. After 16 h for equilibration, both systems caused greatly enhanced nucleation relative to F-actin alone at the same concentration. It follows that the number of nucleating centres, i.e., filament ends, is very much greater than the number of ends of F-actin alone. Thus in the ternary system G-actin polymerises to much shorter filaments than it would by itself, and conversely F-actin is fragmented. The same equilibrium average filament length is evidently attained, starting from G- and F-actin. Spectrin or 4.1 alone exercise no perceptible effect of this nature. That fragmentation of F-actin indeed occurs is shown in fig.1 (inset), which demonstrates that additional filament ends appear within a few minutes of the addition of spectrin and 4.1. The amount of nucleating activity generated increases monotonically with the concentration of the added proteins.

The formation of nucleating activity is unaffected by added  $\text{Ca}^{2+}$  (0.2 mM), although the rate of polymerisation of G-actin is reduced [11,12]. Addition of protein 4.9 (2  $\mu\text{g}/\text{ml}$ ) also fails to perturb the system, and has no significant nucleating activity of its own at this concentration.

We next wished to determine whether the implied fragmenting effect of spectrin and 4.1 is associated with an affinity for the 'barbed', or rapid-growth end of the actin filament, or for the 'pointed', slow-growing end. Under our experimental conditions, the ratio of the rates of elongation of the two ends is about 4 [13,14]. At a G-actin

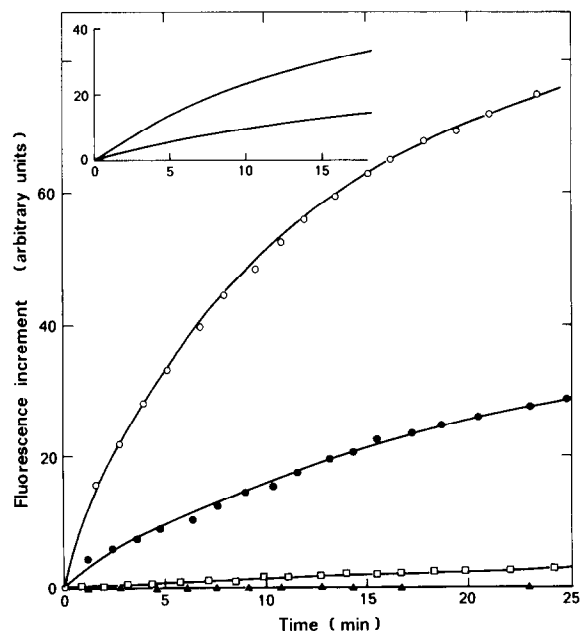


Fig.1. Nucleation of actin polymerisation by actin spectrin-4.1 complexes. G-Actin was polymerised in the absence ( $\square$ ) or presence of spectrin and 4.1, at molar ratios of 1:0.125:0.125 ( $\bullet$ ) and 1:0.25:0.25 ( $\circ$ ). The actin concentration was 20  $\mu\text{g}/\text{ml}$ . To this complex was added 0.13 vol. of pyrene-labelled G-actin at 160  $\mu\text{g}/\text{ml}$  and the increase in fluorescence was observed at 30°C. G-Actin alone at the same concentration ( $\blacktriangle$ ) shows no polymerisation over this period. The buffer (see text) contained 1.2 mM  $\text{MgCl}_2$  and 0.1 mM EGTA. When the nucleating complexes were prepared by incubating F-actin with spectrin and 4.1 essentially the same activities were obtained. Spectrin or 4.1 alone, separately incubated with actin, caused no enhancement of the nucleation given by F-actin. (Inset) Scission of F-actin by spectrin and 4.1. To F-actin were added spectrin and 4.1 to give a molar ratio of 1:0.7:0.3 and a final actin concentration of 65  $\mu\text{g}/\text{ml}$ . After 5 min at 35°C the mixture was added to 11 vols fluorescent actin at 11  $\mu\text{g}/\text{ml}$  and 30°C and the polymerisation was followed (upper curve); F-actin, preincubated at the same concentration without the other proteins, gave markedly less nucleation (lower curve).

concentration well above critical, the rate of polymerisation when the barbed filament end is blocked by a cytochalasin [15,16] will reflect the elongation of the pointed end. We have shown [10] that when red cell ghosts are added to excess G-actin in the presence of saturating concentrations of cytochalasin E, no growth occurs; by inference the pointed

ends of the actin filaments in the cytoskeleton are unavailable. The same could be concluded in regard to the complexes formed in vitro from F-actin, spectrin and 4.1 (fig.2).

Blocking of the pointed end would be predicted to cause a small reduction in the critical monomer concentration, for the affinity of the pointed end for free monomer is much lower than that of the barbed end [14,17]. We accordingly measured the critical concentration of fluorescent actin in the presence and absence of spectrin and 4.1 together and separately, and of 4.9. In the presence of spectrin and 4.1 together, the critical concentration fell from  $18.1 (\pm 0.3)$  to  $14.0 (\pm 0.9) \mu\text{g/ml}$  under the conditions of fig.3. The time required to reach equilibrium was also reduced. Spectrin alone diminished the critical concentration only marginally

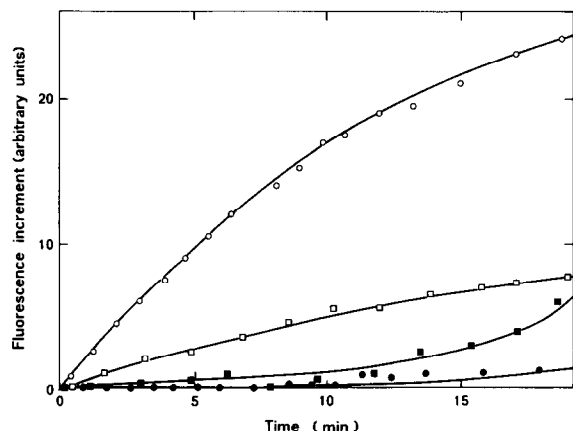


Fig.2. Filament capping effect of spectrin-4.1 system. Nucleating complexes of actin spectrin and 4.1 (1:0.125:0.125), as in fig.1, were incubated with or without cytochalasin E for 2 h to allow for re-equilibration to an increased critical concentration; the solution was added to 0.14 vol. pyrene-labelled G-actin at  $125 \mu\text{g/ml}$  at  $30^\circ\text{C}$ . The buffer (see text) contained 5 mM  $\text{MgCl}_2$  and 0.1 mM EGTA, and the final cytochalasin E concentration was  $20 \mu\text{M}$ . The polymerisation was observed in the presence (●) and absence (○) of cytochalasin E. For comparison the effect of the same concentration of F-actin without spectrin and 4.1 is shown with (■) and without (□) cytochalasin E. Note that nucleation by the complex is almost entirely inhibited when the cytochalasin is present, whereas that by F-actin is less affected. The pyrene-labelled G-actin alone undergoes no detectable polymerisation during the time of this experiment in the presence or absence of  $20 \mu\text{M}$  cytochalasin E.

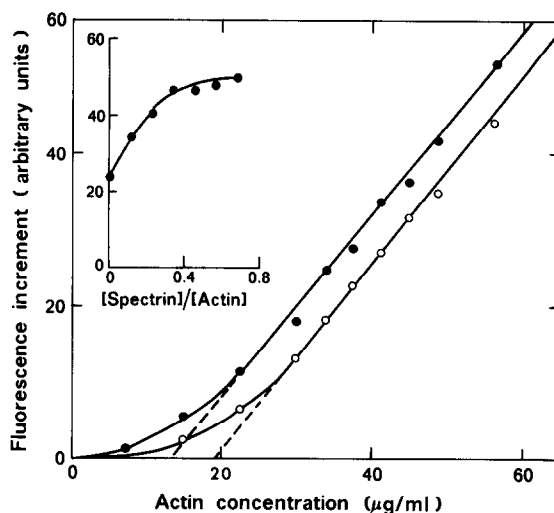


Fig.3. Critical concentration of actin, measured by fluorescence of the pyrene derivative in the presence (●) and absence (○) of spectrin and 4.1 (molar ratio 1:0.067:0.067). The solvent contained 5 mM ATP (see text) and the temperature was  $3.5^\circ\text{C}$ . Full equilibrium was reached at 5 days. The critical concentration of the pyrene derivative is identical with that of native actin [23]. (Inset) Increase in equilibrium extent of polymerisation of actin by added spectrin and 4.1. Polymerisation is measured by fluorescence of pyrene-labelled actin. The actin concentration was  $44 \mu\text{g/ml}$  and the temperature was  $4^\circ\text{C}$ . The molar ratio of 4.1 to spectrin was fixed at 1.25:1.

nally (though reproducibly), whereas neither 4.1 nor 4.9 alone at the concentrations used had any perceptible effect. The increase in polymerisation at equilibrium could also be demonstrated by the rise in fluorescence after incubation of G-actin at a concentration not far above critical with spectrin and 4.1 (fig.3, Inset).

#### 4. DISCUSSION

Our results indicate that spectrin and 4.1 constitute a filament-servicing and capping system, similar in its effect to that of many actin binding proteins [18]. It differs from the other proteins so far characterised in being additionally capable of interaction with intrafilamentous actin subunits, and thus exercising two distinct functions in the membrane cytoskeleton in red cells and presumably elsewhere [19,20]. The majority of capping proteins are specific for the barbed ends of actin

filaments. Spectrin and 4.1, on the other hand, bind to the pointed end, in common with only one capping protein so far discovered, namely acumen-tin, isolated from macrophages [21]. The partition of the complex between filament ends and the remainder of the filament, and the degree of severing activity must be expected to depend on the relative affinities for the two types of site and the concentrations of the participating proteins, as well as the solvent conditions. Protein 4.9 is evidently not implicated in capping, nor is the formation of any of the complexes calcium-dependent.

The observed reduction in critical concentration is consistent with quantitative expectation [17]. It appears to be in conflict with the observations in [22]. We cannot account for the discrepancy, but there are considerable differences in the design of the experiments. Thus authors in [22] used spectrin tetramer, which cross-links actin filaments, and also much lower 4.1 concentrations than ours, added in the form of mixtures with ankyrin; they employed an excitation transfer technique to follow polymerisation and the critical concentration was determined by pelleting, 90 min after initiation of polymerisation.

## ACKNOWLEDGEMENT

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## REFERENCES

- [1] Lin, D.C. and Lin, S. (1979) *Proc. Natl. Acad. Sci. USA* 76, 2345–2349.
- [2] Atkinson, M.A.L., Morrow, J.S. and Marchesi, V.T. (1982) *J. Cell Biol.* 18, 493–505.
- [3] Ungewickell, E., Bennett, P.M., Calvert, R., Ohanian, V. and Gratzer, W.B. (1979) *Nature* 280, 811–814.
- [4] Shotton, D.M. (1983) in: *Electron Microscopy of Proteins*, vol. 4 (Harris, J.R. ed.) pp. 205–330, Academic Press, London.
- [5] Cohen, C.M., Tyler, J.M. and Branton, D. (1980) *Cell* 21, 875–883.
- [6] Spudich, J.A. and Watt, S. (1971) *J. Biol. Chem.* 246, 4866–4871.
- [7] Burns, N.R., Ohanian, V. and Gratzer, W.B. (1983) *FEBS Lett.* 153, 165–168.
- [8] Seigel, D.L. and Branton, D. (1983) *J. Cell Biol.* 97, 279a.
- [9] Kouyama, T. and Mihashi, K. (1981) *Eur. J. Biochem.* 114, 33–38.
- [10] Pinder, J.C. and Gratzer, W.B. (1983) *J. Cell Biol.* 96, 768–775.
- [11] Borejdo, J., Muhrad, A., Leibovich, S.J. and Oplatka, A. (1981) *Biochim. Biophys. Acta* 667, 118–131.
- [12] Maruyama, K. (1980) *Biochim. Biophys. Acta* 667, 139–142.
- [13] Pollard, T.D. and Mooseker, M.S. (1981) *J. Cell Biol.* 88, 654–659.
- [14] Bonder, E.M., Fishkind, D.J. and Mooseker, M.S. (1983) *Cell* 34, 491–501.
- [15] Flanagan, M.D. and Lin, S. (1980) *J. Biol. Chem.* 255, 835–838.
- [16] Brenner, S.L. and Korn, E.D. (1980) *J. Biol. Chem.* 255, 841–844.
- [17] Wegner, A. and Isenberg, G. (1983) *Proc. Natl. Acad. Sci. USA* 80, 4922–4925.
- [18] Weeds, A.G. (1982) *Nature* 296, 811–816.
- [19] Baines, A.J. (1983) *Nature* 301, 377–378.
- [20] Lazarides, E. and Nelson, W.J. (1983) *Cell* 31, 505–508.
- [21] Southwick, F.S., and Tatsumi, N. and Stossel, T.P. (1982) *Biochemistry* 21, 6321–6326.
- [22] Husain, A., Sawyer, W.H. and Howlett, G.J. (1983) *Biochem. Biophys. Res. Commun.* 111, 360–365.
- [23] Pinder, J.C. and Gratzer, W.B. (1982) *Biochemistry* 21, 4886–4890.