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The Spectrin-Actin Complex and Erythrocyte Shape

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The effects of phosphorylation of spectrin on the properties of the cytoskeletal network of the human erythrocyte have been studied. A suspension of the cytoskeletal residues obtained after extraction of the ghosts with the nonionic detergent Triton X-100 forms a gel on addition of membrane kinase and ATP. Phosphorylation has no effect on the association state of purified spectrin. No species higher than a tetramer of polypeptide chains is formed in vitro; in the absence of divalent cations, this tetramer is an entity liberated from and evidently present in the membrane. It has not so far proved possible to detect any F-actin in the cytoskeleton before or after phosphorylation. It is suggested that the consequence of phosphorylation is formation of additional interactions between spectrin and monomeric actin molecules. This view is supported by the formation, after phosphorylation of the Triton-extracted cytoskéleton, of an insoluble mass of protein on treatment with a cross-linking reagent. In the absence of divalent cations, a series of oligomeric species is progressively liberated from the cytoskeleton on extraction with solutions of low ionic strength. These oligomers contain actin as well as spectrin, and are thought to result from disruption of the network by random denaturation of the monomeric actin in the absence of divalent metal ions. A schematic view of the effects of phosphorylation on the structure of the cytoskeleton is presented.

Key words: actin, spectrin, spectrin phosphorylation, spectrin-actin association, gelation

It has long been known [1] that the human erythrocyte requires ATP for the maintenance of its discoid shape and that distinct morphologic changes accompany depletion of intracellular adenosine triphosphate (ATP). Recently, Birchmeier and Singer [2] have shown that shape changes in erythrocyte ghosts, which appear to parallel those of intact cells, are related to the phosphorylation of the smaller subunit of spectrin. They postulate that in vivo, spectrin is maintained at an intermediate degree or "steady state" of phosphorylation by the opposing activities of an endogenous kinase and phosphatase, and that this determines the association state of the spectrin network. This raises the possibility that spectrin, alone, is responsible for the maintenance of red blood cell shape.

The identification of the erythrocyte protein band 5 as actin [3], with its familiar role in muscle and nonmuscle contractile systems, indicates that it might be involved in

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erythrocyte shape regulation. The additional observation that erythrocyte actin is extracted and copurifies with spectrin [4] suggests a functional relationship between these two proteins and this led us to search for an interaction in vitro. In our hands [4], spectrin isolated from fresh human red blood cells by conventional methods caused polymerisation of muscle G-actin in solvents which normally depolymerise F-actin, whereas Tilney and Detmers [3] have reported a different manifestation, indicative of an interaction between the same proteins. Subsequently, it became clear that actin-polymerising activity is mediated by phosphorylation of the spectrin [5]. The dramatic nature of the interaction in a model system has been demonstrated [5]: When ghosts are extracted with the non-ionic detergent Triton X-100, each leaves behind an insoluble cytoskeletal residue [6, 7], which contains predominantly spectrin and actin, with traces of other proteins and a small amount of phospholipid. These residual shells, when dispersed in buffer by homogenisation, retain their integrity, and on addition of Mg^{2+}, ATP, and a preparation [8] of a cAMP-independent erythrocyte membrane kinase, gelation of the suspension occurs. This suggests that new intermolecular interactions result from the phosphorylation. The inhibition of the gelation process by DNAase I, which is known to sequester G-actin [9], implies that the changes involve an actin response to spectrin phosphorylation. Experiments described here give evidence that in isolation phosphorylation of spectrin does not influence its association state and cannot alone account for the ATP changes in erythrocyte shape.

SPECTRIN PHOSPHORYLATION AND THE CYTOSKELETON

Spectrin with its associated actin may be seen as forming a two-dimensional network, or probably a rather irregular lattice, on the cytoplasmic surface of the membrane [7]. On phosphorylation of the smaller subunit, the pattern of protein-protein contacts is evidently changed [5]. If no minor protein constituents participate in this process, it must involve changes in spectrin-spectrin, actin-actin, or spectrin-actin interactions, or combinations of these.

Direct evidence that neither the conformation nor the association state of spectrin is affected by phosphorylation has been obtained (Ungewickell and Gratzer, Eur J Biochem, in press). In brief, we find that spectrin in solution is an equilibrium mixture of two association states – a (presumed) heterodimer, which is not readily dissociated into single chains, and a tetramer. Interconversion between dimer and tetramer is slow at room temperature and below, and if spectrin is prepared by extraction of ghosts at low ionic strength in the cold, the tetrameric species is predominantly recovered. This, however, represents a metastable state, for at 37°C the equilibrium strongly favours the dimer (Fig 1). It therefore follows that the tetramer must be stable in situ, and thus represents a fundamental unit of the network. Studies on spectrin phosphorylated in solution with membrane kinase, and on preparations dephosphorylated with bacterial alkaline phosphatase, show that the dimer-tetramer equilibrium is unperturbed by changes in the phosphorylation state. Moreover, no higher association states are generated in solution at any accessible protein concentration, except at high concentrations (in the millimolar range) of divalent cations. This effect again is independent of the degree of phosphorylation. We thus arrive at a view of the spectrin in situ, involving the attachment of tetramers to specific sites in the membrane, containing presumably a transmembrane spectrin-binding protein [10]; the tetramers may be in contact with one another (as indicated by cross-linking experiments [11]), and the stability of the system may also depend on the rather high cytoplasmic magnesium concentration.

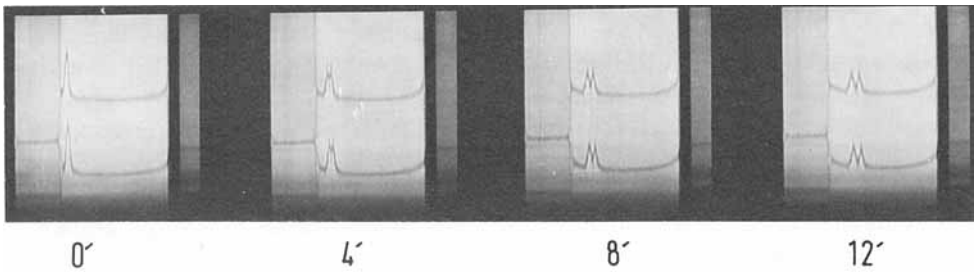

Fig 1. Sedimentation velocity of equilibrium mixture of spectrin dimer and tetramer, phosphorylated (wedge cell) and dephosphorylated (plane cell). The solvent is 0.10 M sodium chloride, 0.05 M Tris, pH 7.6. Velocity, 60,000 rpm.

We cannot entirely rule out the formation of new actin-actin associations, contingent on phosphorylation. Certainly in solutions containing spectrin and muscle actin, phosphorylation of the spectrin provokes polymerisation of the actin [5]. We have been unable so far to establish the mechanism of this process; possibly the spectrin provides a template on which actin monomers can be immobilised, and thus affords an entropically driven stabilisation of the oligomeric nuclei [12] for the polymerisation reaction. In the Triton-extracted ghost, in which the proteins are disposed in a filamentous network, gelation does not appear to be accompanied by polymerisation of the actin. At all events the dispersed gel is not birefringent and does not show actin-like filaments in negative stained preparations examined in the electron microscope (unpublished observations). Any mechanism of cell shape control based on a G→F-actin transformation would place stringent restrictions on the disposition of actin in the membrane. Given the slow diffusion of proteins in the plane of the erythrocyte membrane [13], the formation of nuclei for polymerisation (a reaction of high order) and the propagation of the association reaction would be much too slow to be useful.

The most likely consequence of phosphorylation would seem to be a change in the spectrin-actin interaction, without polymerisation, but with the formation of new intermolecular contacts. We have some evidence in favour of such a view from cross-linking experiments. It is well known that bifunctional reagents bring about extensive cross-linking of proteins in the erythrocyte membrane (see for example, Wang and Richards [14]), with the formation of many complex species. We have worked instead on the simpler system afforded by the Triton-extracted ghosts. After exposure to reagents in the series of bifunctional imidoesters [15] – dimethylsuccinimidate, dimethyladipimidate and dimethylsuberimidate, containing respectively methylene chains of two, four, and six carbon atoms – cross-linked species are recognised on extraction of the network with sodium dodecyl sulphate and gel electrophoresis. These species comprise oligomers of spectrin, as found in whole ghosts [11], and the extent of reaction is independent of phosphorylation state. At a critical chain length, however, viz eight carbons (dimethylsebacimidate), a striking effect of phosphorylation sets in: treatment of cytoskeletons from phosphorylated cells with the reagent leads to the formation of a product, insoluble in boiling sodium dodecyl sulphate, with a white fibrous appearance. No such insoluble material is formed from cytoskeletons of phosphorus-depleted cells. Estimation of the recovery of soluble proteins from the former material by solubilisation in hot sodium dodecyl sulphate shows that some 60% of the total protein is rendered insoluble after exposure to dimethylsebacimidate at 1 mg/ml. There is some uncertainty in estimating the distance spanned in practice by a cross-linking reagent containing a flexible chain of

methylene groups. If the chain were fully extended, the distance between the functional groups would be about 11 Å in dimethylsuberimidate and 14 Å in dimethylsebacimidate. However, the mean end-to-end distance is probably a better measure of the effective span, and this can be estimated [16] as 5.4 and 6.2 Å for the two reagents, respectively. These distances are relatively small in comparison with the principal dimensions of the molecules making up the network. It thus appears that phosphorylation establishes a new type of intermolecular contact in the cytoskeleton, which is repeated throughout the network, and allows extensive cross-linking to occur.

ATTEMPTS AT IDENTIFICATION OF THE REPEATING UNIT

As suggested above, the spectrin tetramer is a recognisable entity in the membrane. Attempts were made to solubilise the Triton-extracted cytoskeletal ghost network in aqueous solutions of low ionic strength, such as those used for extraction of spectrin from intact membranes. In the absence of EDTA, no significant quantities of protein are liberated over a period of several days in the cold. When EDTA is added, however, protein progressively appears in the supernatant, and the dissolution of the cytoskeleton is accompanied also by the progressive appearance of a precipitate identified by gel electrophoresis as actin. The solubilised fraction can be analysed by agarose electrophoresis in nondenaturing conditions and by sedimentation velocity. A typical schlieren pattern is shown in Figure 2. A series of boundaries can be seen, which clearly correspond to protein oligomers, consisting predominantly of spectrin. These components are poorly resolved in preparative sucrose gradient sedimentation, but samples taken across the distribution reveal that all

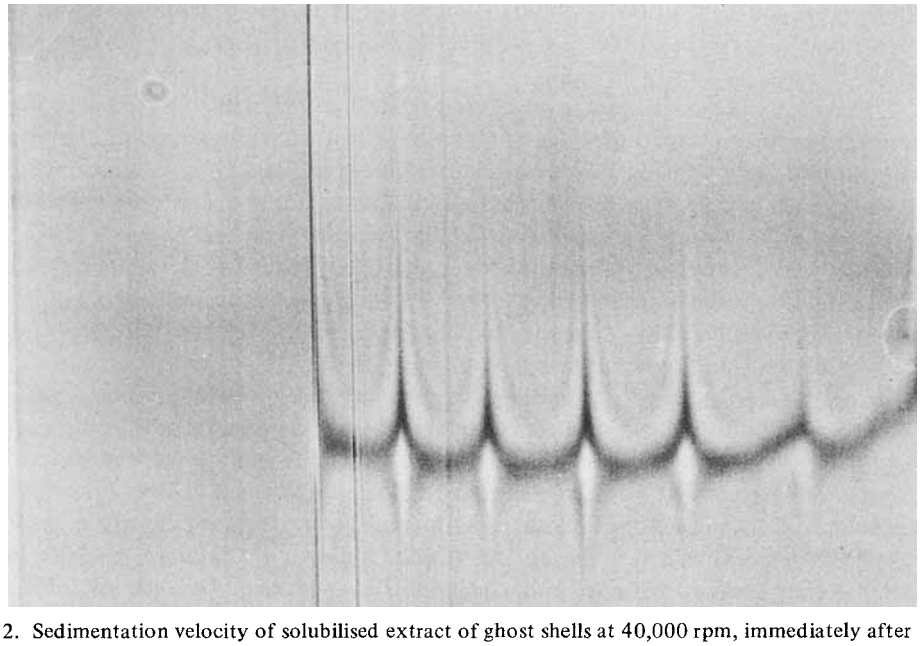

Fig 2. Sedimentation velocity of solubilised extract of ghost shells at 40,000 rpm, immediately after reaching full speed. Extraction was by dialysis against 10 mM sodium chloride, 0.1 mM EDTA, 1mM Tris, pH 7.5, for 12 h, followed by distilled water for 24 h. The salt concentration was brought to 0.1 M before sedimentation analysis.

contain actin. The mole ratio of actin to spectrin (dimer) is in the range 1.1–1.7. Since spectrin itself forms no associated species in solution beyond the tetramer, it would seem to follow that the spectrin tetramers in the oligomeric components are linked by unpolymerised actin. Because G-actin is conformationally unstable in the absence of divalent cations [17], one may infer that the progressive denaturation of actin monomers allows disruption of the network, so that parts of it are progressively liberated.

NATURE OF CYTOSKELETAL PROTEINS

Recently evidence has accrued [18] which indicates that the metabolic exhaustion of the intracellular ATP is followed only after a considerable interval by the progressive loss of the discoid character. This argues against an ATPase-linked mechanism of shape control, such as might be required by an actomyosin-like contractile element in the membrane. Additionally, the view propounded by earlier authors (see, for example, Guidotti [19] and Brandon [20]) that spectrin resembles, and may be regarded as a form of myosin, cannot be sustained in the light of recent studies of its physical properties (see, for example, Kam et al [21]). It is evidently a molecule of relatively low asymmetry, and bears no physical resemblance to myosin. The erythrocyte actin has been found (Fig 3) to be of the β type (in disagreement with another report [22] stating it to be a mixture of the

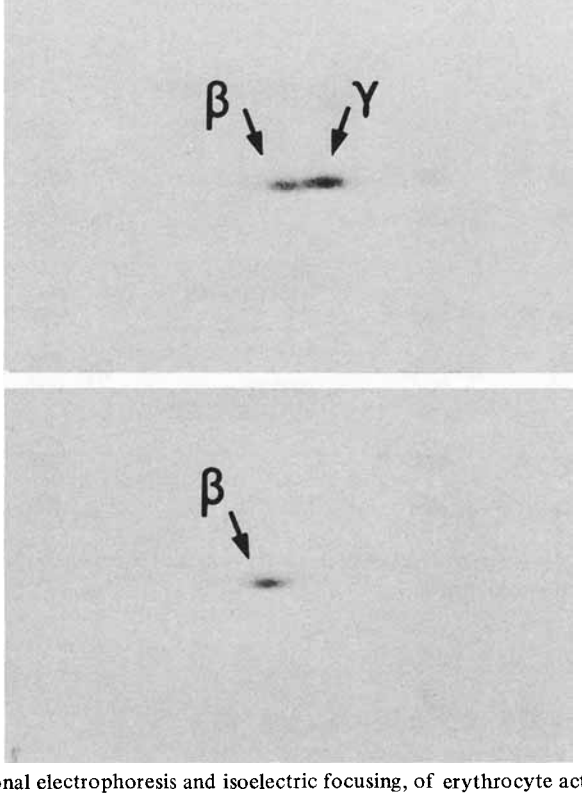

Fig 3. Two-dimensional electrophoresis and isoelectric focusing, of erythrocyte actin (lower panel), stained with Coomassie Brilliant Blue G. The upper panel shows for comparison an autoradiogram of the same gel, which contained ^{35}S-labelled actin from chicken neurones. Both β and γ actin species are present [23] (provided by Q.L. Choo).

β and γ variants); this is not the species present in either striated or smooth muscle, which contain α and γ actin respectively. Other membrane proteins (bands 4.1, 4.2, 4.9, and 3′) are also relatively prominent in gel electrophoresis patterns of the cytoskeletal residue; however, none of these is thought to play any direct role in the in vitro spectrin-actin interaction. A comparison with skeletal myofibril proteins shows that none of the minor proteins can be identified with actinin.

CONCLUSIONS

The arguments we have outlined suggest that the ATP-dependent mechanism of erythrocyte shape control is a function primarily of the cytoskeletal network of spectrin and actin. The balance of the evidence so far indicates that the actin, though capable of polymerising in vitro, is unpolymerised in the membrane, and that it functions by a mechanism quite different from that in known contractile systems. The spectrin is present as a series of tetramers, linked to each other either directly or by way of other cytoskeletal protein constitutents, but also to monomers of actin. On spectrin phosphorylation an additional type of affinity is generated, and a new spectrin-actin bond is formed; in the cytoskeletal extract this is accompanied by gelation. In Figure 4 we give a schematic and tentative representation of the structure of the cytoskeleton as a function of spectrin phosphorylation.

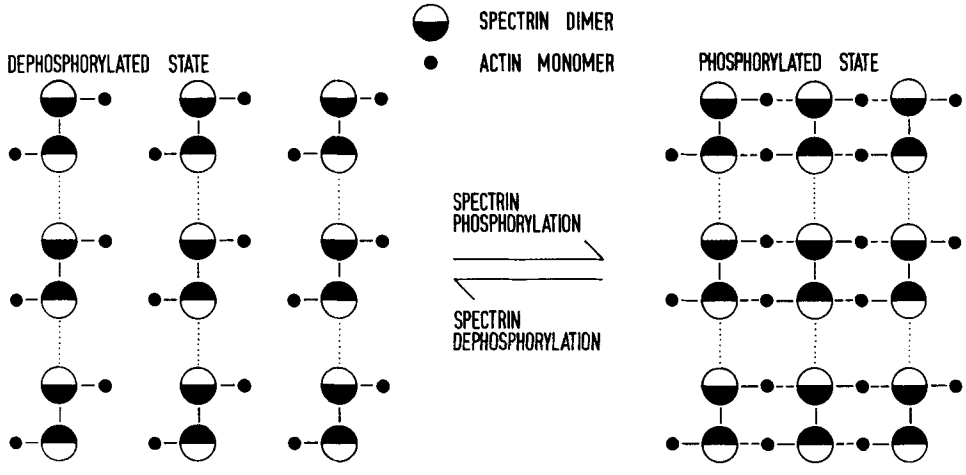

Fig 4. Schematic representation of one possible mechanism for the action of phosphorylation on the structure of the spectrin-actin matrix.

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