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Rapid Report

Both ankyrin and band 4.1 are required to restrict the rotational mobility of band 3 in the human erythrocyte membrane

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A population of band 3 proteins in the human erythrocyte membrane is known to have restricted rotational mobility due to interaction with cytoskeletal proteins. We have further investigated the cause of this restriction by measuring the effects on band 3 rotational mobility of rebinding ankyrin and band 4.1 to ghosts stripped of these proteins as well as spectrin and actin. Rebinding either ankyrin or 4.1 alone has no detectable effect on band 3 mobility. Rebinding both these proteins together does, however, reimpose a restriction on band 3 rotation. The effect on band 3 rotational mobility of rebinding ankyrin and 4.1 are similar irrespective of whether or not band 4.2 is removed from the membrane. We suggest that ankyrin and 4.1 together promote the formation of slowly rotating clusters of band 3.

The spectrin-actin network of erythrocytes is connected to the membrane by two linker proteins, ankyrin and band 4.1 [1,2]. Ankyrin, a 215 kDa polypeptide, possesses binding sites for spectrin and the cytoplasmic domain of the integral protein, band 3 [3,4]. Band 4.1 exhibits a polyphosphoinositide-dependent association with glycophorin [5] and also binds at the spectrin-actin junction [6]. In addition, band 4.1 binds with lower affinity to band 3 [7].

Rotational diffusion of band 3 in the erythrocyte membrane may be measured by detecting transient dichroism [8,9] or phosphorescence anisotropy [10–12] of the triplet probe eosin-5-maleimide. Initial measurements revealed a slow rotational component [9] which was subsequently shown to arise from a restriction to free rotation imposed by interaction with cytoskeletal proteins [13]. This restriction may be lifted either by proteolytic cleavage of the cytoplasmic domain of band 3 or by removal of spectrin, actin, ankyrin and band 4.1 from the membrane. These biophysical studies appeared to be in accord with binding experiments with purified proteins which indicate linkage of band 3 to spectrin via ankyrin [1,2].

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Recent experiments in our laboratory, however, raise further questions concerning the molecular interactions which restrict band 3 rotational mobility [14]. An earlier result that removal of spectrin-actin alone has little effect on band 3 rotation was confirmed and it was also found that in situ proteolytic cleavage of ankyrin also had no effect on band 3 mobility. In further experiments, it was shown that spectrin remains highly flexible when attached to the erythrocyte membrane [15]. The various results can be rationalised by supposing that although band 3 is indeed connected to spectrin via ankyrin as indicated by binding studies, the spectrin-actin network is too flexible to impose any restriction to anguiar displacements of band 3 over $+/- \pi/2$.

If the above supposition is correct, it raises the question of what are the molecular interactions which slow band 3 rotation. The fact that band 3 mobility is enhanced by removal of ankyrin and band 4.1 (subsequent to spectrin-actin depletion) shows that one or both of these proteins must be somehow involved. Here we present data which indicate that both proteins are in fact required to restrict band 3 rotational mobility.

Band 3 was labeled with eosin-5-maleimide in intact erythrocytes and ghosts prepared as previously described [9]. Band 6 was removed by washing ghosts twice in PBS (pH 7.5). Removal of spectrin and actin by low salt incubation, subsequent removal of ankyrin

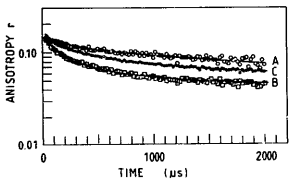

Fig. 1. Reversibility of effect of removal of ankyrin and band 4.1 on band 3 rotational mobility in erythrocyte ghosts. Curve A, membranes depleted of spectrin, actin and band 6. Curve B, as curve A but membranes additionally incubated in high salt to dissociate ankyrin and band 4.1, then centrifuged, washed and resuspended in low salt. Curve C, as curve A but membranes incubated in high salt to dissociate ankyrin and band 4.1, then dialysed into low salt without prior centrifugation and washing.

and band 4.1 by high salt incubation and removal of the band 3 cytoplasmic domain by mild proteolysis with trypsin were all performed essentially as described by Nigg and Cherry [13]. Membranes depleted of band 6, spectrin, actin, ankyrin and band 4.1 are hereafter referred to as 'stripped' ghosts. In some experiments, band 4.2 was removed concomitantly with ankyrin and band 4.1 using the method of Hargreaves et al. [16]. Band 4.1 was purified from Triton shells according to Perkrun et al. [17] and ankyrin essentially as described by Gardner and Bennett [18].

Rebinding experiments were performed in two different ways. In the first method, after incubating spectrin-actin depleted ghosts in 1 M KCl at 37°C for 45 min to dissociate ankyrin and band 4.1, half of the sample was immediately dialysed overnight against 5PB (5 mM sodium phosphate, pH 7.5) to return to low salt conditions. The remaining half of the sample was centrifuged to remove dissociated ankyrin and band 4.1 prior to dialysis to act as a control. In the second method, stripped ghosts were incubated with purified ankyrin or band 4.1 in 90 mM KCl, 0.1 mM EDTA, 0.2 mM DTT, 10% sucrose, 7.5 mM KCl, 7.5 mM sodium phosphate (pH 7.4) for 3 h at room temperature. Normally 0.5 mg/ml of band 4.1 or 0.325 mg/ml of ankyrin were present per 1 mg/ml of membrane protein. After incubation the sample was layered onto an equal volume of 100 mM KCl, 0.2 mM EDTA, 0.1 mM DTT, 20% sucrose and centrifuged for 30 min at 18000 rpm. The pellet was collected and washed twice with 5PB prior to resuspension for transient dichroism measurements. Removal and rebinding of the peripheral proteins was checked by SDS-PAGE.

Rotational diffusion of band 3 in the various membrane preparations was measured by observing transient dichroism of the eosin probe, as previously described [19]. The absorption anisotropy decay $r(t)$ was monitored at 515 nm following excitation of the eosin probe at 532 nm by a brief pulse of light (15–20 ns) from a Nd-YAG laser. Typically 512 signals were averaged and all measurements were performed at 37°C with membranes in 66% glycerol (w/w). Prior to measurement, samples were deoxygenated by a stream of argon.

Fig. 1 shows anisotropy decays of eosin-labeled band 3 in ghosts depleted of spectrin and actin (curve A) and of spectrin, actin, ankyrin and band 4.1 (curve B). In agreement with previous studies [13], removal of ankyrin and band 4.1 enhances the rotational mobility of band 3 as evidenced by the faster anisotropy decay. It has not, however, been demonstrated that this effect is reversible. To investigate reversibility, we reduced the salt concentration by dialysis without prior separation of the dissociated ankyrin and band 4.1 from the membranes. Curve C in Fig. 1 shows that the restriction on band 3 rotation is partially restored. Loss of protein during the rebinding procedure could account for the incomplete reversibility.

We next investigated whether ankyrin or band 4.1 is responsible for the restriction on band 3. Fig. 2(a) shows the results of experiments in which ankyrin was rebound to stripped ghosts. No effect on the anisotropy decay was detectable as a consequence of ankyrin

Fig. 2. Effect on the rotational mobility of band 3 of rebinding ankyrin or band 4.1 to stripped ghosts. (a) □, Stripped ghosts; ●, stripped ghosts with ankyrin rebound. (b) □, Stripped ghosts; ○, stripped ghosts with band 4.1 rebound. In both panels the lower curve has been vertically displaced for clarity, otherwise the two curves essentially superimpose.

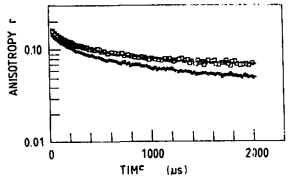

Fig. 3. Effect on the rotational mobility of band 3 of rebinding both ankyrin and band 4.1 to stripped ghosts. ○, Stripped ghosts; □, stripped ghosts with ankyrin and band 4.1 rebound.

binding. A similar result was obtained even if a large excess of ankyrin was used in the incubation. Although the rebinding was not quantified, SDS PAGE of the sample indicated that ankyrin had reassociated with the membrane in amounts comparable to that present in the native membrane. Similar results were obtained when band 4.1 was rebound to stripped ghosts. Fig. 2(b) shows that rebinding band 4.1 had no effect on the anisotropy decay.

As rebinding neither ankyrin nor band 4.1 reduced band 3 mobility, we proceeded to investigate the effect of rebinding both ankyrin and band 4.1 together. Ankyrin was first rebound to stripped ghosts and the sample subsequently incubated with band 4.1. As before, ankyrin alone had no effect but this time a reduction of band 3 mobility was detected upon subsequent rebinding of band 4.1 (Fig. 3).

To check that the loss of band 3 mobility observed upon rebinding both ankyrin and band 4.1 involved the cytoplasmic domain of band 3, we examined the effect of removing this domain by mild proteolysis with trypsin. Rebinding both ankyrin and band 4.1 to trypsin-treated membranes had no effect on the anisotropy decay of eosin-labeled band 3 in trypsin-treated samples (Fig. 4).

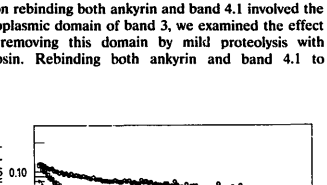

Fig. 4. Effect on the rotational mobility of band 3 of rebinding ankyrin and band 4.1 to ghosts treated with trypsin to remove the cytoplasmic domain of band 3. Curve A, control ghosts. Curve B, trypsin-treated ghosts. Curve C, trypsin-treated ghosts with ankyrin and band 4.1 rebound (curve has been vertically displaced for clarity).

Some experiments were performed with stripped ghosts which were additionally depleted of band 4.2 (data not shown). Additional removal of band 4.2 appeared to slightly increase band 3 mobility. When ankyrin and band 4.1 were rebound to stripped ghosts depleted of band 4.2, the effect on the anisotropy decay was similar to that obtained in the presence of band 4.2. We conclude that band 4.2 does not play a significant role in the interactions involving ankyrin and band 4.1 which restrict band 3 mobility.

The conclusion of these studies is that the slow rotational component of band 3 arises from interactions which require the presence of both ankyrin and band 4.1 on the membrane. Since rotational diffusion is rather sensitive to molecular size, we assume that ankyrin and band 4.1 promote the formation of small clusters of band 3 molecules. The precise nature of the interactions resposible for such clustering are largely a matter of speculation. In the absence of spectrin, ankyrin is thought to bind only to band 3. Band 4.1 on the other hand, binds both to glycophorin and, with lower affinity, to the cytoplasmic domain of band 3. Competition studies with antibodies and the purified cytoplasmic domain of band 3 indicate that in the native membrane, about 30% of band 4.1 is bound to glycophorin and 70% to band 3 [7]. Band 3 is immobilised when glycophorin A is crosslinked by specific antibodies, suggesting that these proteins are associated in the membrane [20].

None of the above interactions in themselves explain how ankyrin and band 4.1 restrict band 3 mobility. Band 4.1 has the potential to crosslink glycophorin and band 3 and thus induce clustering of band 3-glycophorin complexes. This would not, however, explain the requirement for ankyrin. Moreover, band 4.1 bound to band 3 is dissociated by incubation with the purified cytoplasmic domain of band 3 [7], indicating that it is not simultaneously bound to glycophorin. It would appear therefore, that further interactions of these linker proteins remain to be elucidated. It is possible that the binding properties of these proteins when bound to the membrane are different than when in solution. Furthermore, after proteins are brought into close proximity by binding to high affinity sites on the membrane, low affinity interactions could become of significance. Another possibility is that binding of ankyrin and band 4.1 could indirectly promote association of band 3-glycophorin A complexes, either by inducing a conformational change or possibly by neutralising charges which contribute to the repulsive force between molecules. We are currently performing further experiments with purified proteins to investigate these different possibilities.

Acknowledgements

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