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Association of Spectrin With Its Membrane Attachment Site Restricts Lateral Mobility of Human Erythrocyte Integral Membrane Proteins

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Interactions between spectrin and the inner surface of the human erythrocyte membrane have been implicated in the control of lateral mobility of the integral membrane proteins. We report here that incubation of "leaky" erythrocytes with a water-soluble proteolytic fragment containing the membrane attachment site for spectrin achieves a selective and controlled dissociation of spectrin from the membrane, and increases the rate of lateral mobility of fluorescein isothiocyanate-labeled integral membrane proteins ($>$ 70% of label in band 3 and PAS-1). Mobility of membrane proteins is measured as an increase in the percentage of uniformly fluorescent cells with time after fusion of fluorescent with nonfluorescent erythrocytes by Sendai virus. The cells are permeable to macromolecules since virus-fused erythrocytes lose most of their hemoglobin. The membrane attachment site for spectrin has been solubilized by limited proteolysis of inside-out erythrocyte vesicles and has been purified (V. Bennett, J Biol Chem 253:2292 (1978). This 72,000-dalton fragment binds to spectrin in solution, competitively inhibits association of ^{32}P-spectrin with inside-out vesicles with a K_i of 10^{-7}M, and causes rapid dissociation of ^{32}P-spectrin from vesicles. Both acid-treated 72,000-dalton fragment and the 45,000 dalton-cytoplasmic portion of band 3, which also was isolated from the proteolytic digest, have no effect on spectrin binding, release, or membrane protein mobility. The enhancement of membrane protein lateral mobility by the same polypeptide that inhibits binding of spectrin to inverted vesicles and displaces spectrin from these vesicles provides direct evidence that the interaction of spectrin with protein components in the membrane restricts the lateral mobility of integral membrane proteins in the erythrocyte.

Key words: spectrin, erythrocyte membrane, membrane attachment site, membrane protein mobility

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Regulation of membrane protein mobility by transmembrane interactions with cytoplasmic structural proteins has been described for many eukaryotic cells [1, 2]. To study these phenomena at the molecular level it is desirable to have a system in which perturbations of membrane protein mobility can be directly correlated with changes in specific protein-protein associations in the membrane. Fused, hemolyzed human erythrocytes provide a convenient model system to measure membrane protein mobility [3], in which the cytoplasmic side of the membrane is readily accessible to molecular manipulation. In addition, the associations between proteins in the red cell membrane have been characterized in some detail [4, 5] (see below).

Lateral diffusion of erythrocyte integral membrane proteins is relatively restricted compared to other types of cells [3, 6]. Diffusion constants estimated for fresh cells at 37°C range from 4×10^{-11} cm^2/sec for the slowest cells to 10^{-10} cm^2/sec for the fastest [3]. The rates are highly temperature-dependent, and significantly faster in fresh than in ATP-depleted cells [3]. These characteristics suggested that various cellular processes, such as cell aging and metabolism, may regulate the lateral mobility of the membrane proteins in the erythrocyte.

Spectrin, the major peripheral membrane protein in erythrocytes, has been demonstrated by elegant morphological experiments to be associated in some manner with intramembrane particles [7–10], and receptors for colloidal iron hydroxide [11], which structures are thought to be composed of the major integral membrane proteins [9, 12–16]. A high-affinity ($K_d = 10^{-7}$M) interaction between spectrin and a protein component localized on the inner surface of the plasma membrane has been measured in studies of reassociation of pure ^{32}P-spectrin with inside-out vesicles depleted of spectrin and actin [17, 18]. A water-soluble 72,000-dalton proteolytic fragment from spectrin-depleted inverted vesicles has been purified which competitively inhibits binding of ^{32}P-spectrin to vesicles with a K_i of about 10^{-7}M [19], which is similar to the K_d for association of ^{32}P-spectrin with membranes. Both inhibitory activity of the fragment and spectrin-binding activity of vesicles are destroyed by either dilute acetic acid or N-ethyl maleimide [19]. The 72,000-dalton fragment comigrates with inhibitory activity on gel filtration with an effective Stokes radius of 39Å, and binds to spectrin in solution with a stoichiometry of 1 mole of fragment per mole of spectrin dimer [19]. Isoelectric focusing of the purified 72,000-dalton fragment partially resolves two peaks of protein and closely associated inhibitory activity with apparent isoelectric points of 4 and 4.4 [19]. The fragment constitutes a minimum of 2.4% of the membrane protein, which is close to the value predicted if spectrin binds in a 1:1 ratio to a 72,000-dalton protein.

It was concluded from these properties that the 72,000-dalton proteolytic fragment represents the membrane attachment site for spectrin [19]. Other polypeptides in the digest, including a 45,000-dalton fragment which has been attributed to the cytoplasmic portion of band 3 [20], do not compete for ^{32}P-spectrin binding to membranes [19]. Moreover, this fragment is released in equal amounts from vesicles prepared from untreated and α-chymotrypsin-digested erythrocytes, even though such external cleavage degrades band 3 to a 60,000-dalton fragment [19]. The 72,000-dalton fragment therefore cannot originate from band 3, and is localized exclusively on the inner surface of the membrane. Glycophorin and the other sialoglycoproteins can also be excluded as a source of this polypeptide, since the periodic acid-Schiff staining profile of vesicles is unchanged by digestion which abolishes binding of ^{32}P-spectrin and releases maximal quantities of the fragment [19].

If an interaction between spectrin and its membrane attachment site constrains the lateral distribution and mobility of the erythrocyte integral membrane proteins, then

treatments which displace spectrin from the membrane should correspondingly influence membrane protein mobility. Experimental verification of modulation of diffusional rates of membrane proteins by spectrin has not been heretofore possible, because extraction of spectrin by conventional methods elutes other proteins, including actin, and causes erythrocyte ghosts to disintegrate into small vesicles [7, 13]. Furthermore, in addition to simply dissociating spectrin from the membrane, these methods also destroy proposed spectrin-actin interactions in the membrane [21–25]. We report here that incubation with the 72,000-dalton fragment achieves a selective and controlled dissociation of spectrin from membranes of inside-out vesicles and increases the rate of lateral diffusion of fluoroscein isothiocyanate-labeled integral membrane proteins in fused, hemolyzed human erythrocytes.

METHODS

Human blood stored at 4°C in acid-citrate-dextrose was used within 10 days of drawing. Intact cells were labeled with fluorescein isothiocyanate (FITC; Sigma, Lot No. 114C-5018) and fused to unlabeled cells by Sendai virus as described in Fowler and Branton [3], except that 10 mM Tris, 145 mM NaCl, pH 7.5 Tris-buffered saline (TBS) was used as the fusion buffer. The specific experimental conditions for the incubations are given in the figure legend. Aliquots were withdrawn periodically for observation and counting as described previously [3].

^{32}P-spectrin was prepared and purified as previously described [17, 18]. The 72,000-dalton and the 45,000-dalton fragments were solubilized by limited chymotrypsin digestion of spectrin-actin-depleted inside-out erythrocyte vesicles, and purified as described in Bennett [19]. Measurement of ^{32}P-spectrin reassociation with spectrin-depleted inside-out vesicles was performed as previously described [17–19].

RESULTS

Mobility of membrane proteins was measured as an increase with time in the percentage of uniformly fluorescent cells after fusion of FITC-labeled with unlabeled erythrocytes by Sendai virus [3]. The labeling of erythrocytes with FITC has been shown to be restricted to covalent linkage of the dye with integral membrane proteins (> 70% of the total label is in band 3 and PAS-1) [3]. The fused cells lose most of their hemoglobin and are therefore permeable to macromolecules. Fused erythrocytes preincubated for 2 h at 24°C with 4×10^{-6} M of the 72,000-dalton solubilized attachment site exhibit a marked increase in the rate of redistribution of labeled proteins following elevation of the temperature to 30°C as compared to fused cells incubated in buffer alone (Fig 1). Qualitatively similar results were obtained in several other experiments at the same temperature, at 24°C, or at 37°C. The enhancement of membrane protein mobility is selective, since the 45,000-dalton proteolytic fragment which has been attributed previously to the cytoplasmic portion of band 3 [20] has no effect (Fig 1). Furthermore, no change in rates of mobility is observed following exposure of the 72,000-dalton fragment to dilute acid, a treatment which also abolishes the capacity of the fragment to compete for binding of ^{32}P-spectrin [19].

The solubilized attachment site causes a rapid and complete dissociation of ^{32}P-spectrin from inside-out vesicles (Fig 2) under conditions similar to those under which this polypeptide accelerates lateral mobility of membrane proteins in fused, hemolyzed erythrocytes (Fig 1). The 45,000-dalton fragment of band 3 at equivalent concentrations

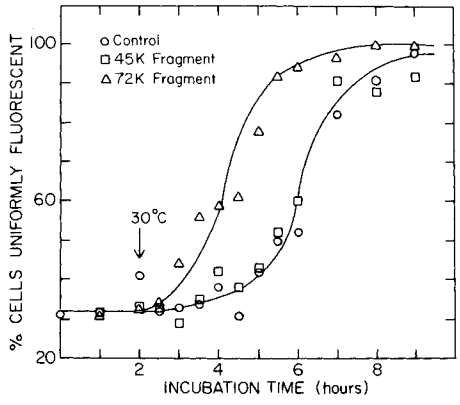

Fig 1. Redistribution of FITC-labeled integral membrane proteins of fused, hemolyzed erythrocytes incubated with 72,000-dalton fragment (△), 45,000-dalton fragment (□), or buffer alone (○), where uniformly fluorescent cells are expressed as a percentage of the total number of fused, hemolyzed cells that are at least partially fluorescent. After fusion, the fused cells were diluted directly into four volumes of TBS, or into TBS containing either the 72,000-dalton (4×10^{-6} M final concentration) or the 45,000-dalton fragment at the same final concentration. After preincubation for 2 h at 24°C, the fused cells were transferred to 30°C as indicated by the arrow.

displaces only a small portion of the reassociated spectrin (Fig 2), and this activity may be due to contamination with the 72,000-dalton fragment [19]. However, incubation of the 72,000-dalton fragment (or the 45,000-dalton fragment) with ghosts or fused cells under conditions similar to those of Figure 1 does not release spectrin or other proteins into the supernatant (as ascertained by polyacrylamide SDS gel electrophoresis; data not shown). This cannot be ascribed simply to trapping of spectrin polypeptides inside the ghosts, since brief sonication does not influence the results. A likely explanation for the difference in displacement of native and reassociated spectrin is that in ghosts additional interactions occur of spectrin with itself or with other proteins (eg, actin; see Refs 21–25) which have not been reconstituted in the binding assay. It is of interest in this regard that the rate of dissociation of spectrin from reconstitued vesicles is greatly reduced if the incubation of spectrin with vesicles is allowed to continue for 90 min before addition of fragment (not shown). It may be implied that spectrin can dissociate from its anchorage site on the membrane, and yet remain bound by secondary interactions. In this event, the initial, rapid rate of dissociation (Fig 2) reflects the true residence time of spectrin with the membrane attachment site.

Incubation of erythrocyte ghosts or fused, hemolyzed cells with the 72,000-dalton fragment under these conditions has no discernable effect on their morphology as determined by phase contrast microscopy. Furthermore, Triton X-100 residues of ghosts [25] are similarly unaffected by incubation with this fragment (not shown). It is therefore reasonable to propose that the 72,000-dalton polypeptide has no major effect on associations of spectrin with other peripheral membrane proteins, and that the increase in lateral motion of membrane proteins is indeed due to perturbation of the association of spectrin with the membrane.

DISCUSSION

The idea that lateral movements of erythrocyte integral membrane proteins are constrained by interactions with spectrin and possibly actin was suggested following

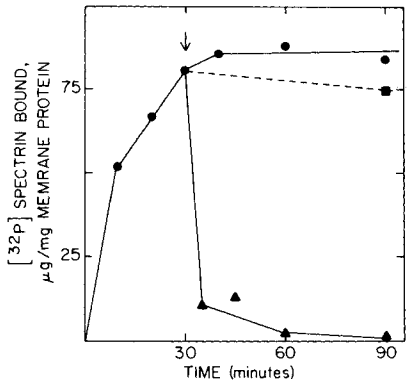

Fig 2. Time course of dissociation of ^{32}P-spectrin from inverted erythrocyte vesicles after addition of 72,000-dalton fragment (▲), 45,000-dalton fragment (■), or no additions (●). ^{32}P-spectrin (40 μg/ml, 1,400 cpm/μg) was incubated at 30°C with inside-out vesicles (100 μg/ml membrane protein) in a buffer containing sodium phosphate (2.5 mM), KCl (30 mM), $MgCl_2$ (0.6 mM), dithiothreitol (0.6 mM), and sucrose (2.4% w/v). At the time indicated by the arrow 72,000-dalton fragment and 45,000 dalton fragment were added at final concentrations of 92 and 99 μg/ml respectively. Aliquots (0.2 ml) were removed from the samples at various times and the membrane-bound ^{32}P-spectrin was determined. The values are corrected for nonspecific binding by subtracting the binding obtained at various times with heat-denatured (5 min, 60°C) ^{32}P-spectrin [17].

observations of factors controlling the aggregatability of intramembrane particles and various surface receptors in the plane of the membrane [7, 8, 10, 11]. Normally, the intramembrane particles are randomly distributed in the plane of the membrane [7, 26]. However, after pretreatment of ghosts under conditions that dissociate spectrin and actin, manipulation of pH or ionic strength induces rearrangement of the particles to form clusters [7, 8]. Also, clustering of colloidal iron hydroxide binding sites (localized primarily on glycophorin) follows the sequestration of antispectrin antibodies inside erythrocyte ghosts [11]. A direct association of spectrin with the intramembrane particles has been proposed on the basis of the results of these antibody experiments [11] and the observation that conditions which promote precipitation of spectrin and actin (low pH, Ca^{2+}, Mg^{2+}, basic proteins) also favor aggregation of particles in membranes partially depleted of spectrin [8]. This hypothesis is supported by the demonstration that the intramembrane particles coaggregate with residual spectrin molecules visualized using ferritin-conjugated antispectrin antibodies [10].

While these sorts of experiments do suggest that under certain conditions close structural associations may occur between spectrin and the integral membrane proteins, studies of protein aggregation are not adequate to analyze the dynamic behavior of membrane proteins, as has been pointed out by Elgsaeter and Branton [7]. In addition, the nonphysiological treatments used to induce particle aggregation may cause nonspecific coprecipitation of proteins in the membrane and may also alter the bulk properties of the membrane lipids. For example, although coaggregation of intramembrane particles with receptors for various lectins [12, 13, 16, 27], influenza virus receptors [12], A antigenic sites [15], anionic sites [28], and surface proteins labeled with a nonspecific hapten, p-diazoniumphenyl-β-D-lactoside [10] invariably occurs in the erythrocyte ghost, anti-concanavalin A antibodies can induce a temperature-dependent redistribution of concanavalin A bound to the cell surface without any effect on the random distribution of

the intramembrane particles in intact erythrocytes [29]. An alternative explanation for the restraint of intramembrane particles, which is also consistent with previous data, is that the cytoplasmic portions of these structures are simply trapped within a postulated spectrin-actin meshwork without formation of specific protein-protein associations [5, 7, 8].

The initial observation that FITC-labeled proteins are mobile in the erythrocyte membrane under conditions where spectrin remains associated with the membrane and particle aggregation does not occur [3] suggested to us that if spectrin controls membrane protein mobility, it must do so by a dynamic and regulatable mechanism. Incubation of ghosts with a water-soluble fragment of the membrane anchorage site for spectrin is a mild and selective method for displacing spectrin from the membrane without perturbing interactions of other proteins with spectrin. The specific enhancement of lateral movement of fluorescent-labeled membrane proteins in fused, hemolyzed erythrocytes by the solubilized attachment site for spectrin, together with the displacement of spectrin from inside-out vesicle membranes, indicates that association of spectrin with its membrane anchorage site does indeed limit the lateral mobility of integral membrane protiens. A minimum diffusion constant for lateral motion (estimated as described in Fowler and Branton [3]) is increased from 10^{-11} cm^2/sec to 2×10^{-11} cm^2/sec in the experiment presented in Figure 1. This value is still low relative to diffusion constants determined for plasma membrane proteins in other cells [30–32], which suggests that erythrocyte integral membrane proteins are only partially freed from constraints by incubation with the solubilized attachment site. This is perhaps not surprising in view of the high local concentration of spectrin in ghost membranes, although the possibility cannot be excluded that other factors (such as possibly a spectrin-actin complex) may regulate membrane protein mobility.

The 72,000-dalton proteolytic fragment does not originate from the major band 3 protein or from PAS-1 [19], whose mobility is monitored in these experiments; moreover, the 45,000-dalton fragment of band 3 is without effect on the lateral movement of these proteins (Fig 1). The anchorage protein for spectrin may itself interact with band 3 and glycophorin and thus could be a heretofore unrecognized component of the intramembrane particles. It is also conceivable, however, that spectrin or the anchorage protein may modify protein mobility through an indirect mechanism, such as altering the physical state of membrane lipids.

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