Modulation of Erythrocyte Band 4.1 Binding by Volume Expansion

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ABSTRACT Erythrocyte band 4.1 is an important protein in the control and maintenance of the cytoskeleton. Skate erythrocyte band 3, the anion exchanger, appears to play a pivotal role in the regulation of volume-stimulated solute efflux during volume expansion. Because band 4.1 interacts with band 3, we tested whether their interaction might change during volume expansion. Skate red blood cells were volume-expanded in either hypotonic media (one-half osmolarity) or were swollen under isoosmotic conditions by inclusion of ethylene glycol or ammonium chloride in the medium. Microsomal membranes isolated from red cells under volume expanded conditions demonstrated a significant decrease in the amount of band 4.1 bound to band 3. In unstimulated cells, approximately one third of the binding of band 4.1 occurred to band 3. This binding was characterized as being sensitive to competition by the peptide IRRRY. The majority of band 4.1 is bound to glycoporphin (as demonstrated in other species), and this binding does not change during volume expansion. The alteration in band 4.1:band 3 interaction occurs within 5 min after volume expansion and is transient, returning to near normal interaction within 60 min. Two drugs that promote band 3 oligomerization, pyridoxal-5'-phosphate and DIDS, also decreased band 4.1 interaction with band 3. Band 4.1 and ankyrin binding to band 3 may be reciprocally related as high-affinity ankyrin binding sites to band 3 observed under volume-expanded conditions are decreased by inclusion of band 4.1 in the binding reactions. J. Exp. Zool. 289:177–183, 2001. © 2001 Wiley-Liss, Inc.

The cytoskeleton of the erythrocyte is a complex network of a number of proteins including spectrin, actin, ankyrin, adducin, band 4.2, and band 4.1, among many others (for reviews see Gilligan and Bennett, '91; Liu and Derick, '92). One cytoskeletal protein that has received notable attention is band 4.1, since it may play an important role in the organization of the cytoskeletal network (Gilligan and Bennett, '91). Erythroid band 4.1 is an approximately 80-kDa protein that appears to be pivotal in the polymerization of spectrin with actin into a two-dimensional network due to the interaction of band 4.1 with β-spectrin, thereby forming a calmodulin-dependent binding site for actin (Tanaka et al., '91). Erythrocyte band 4.1 is a member of a gene family encoding for proteins in many tissues of the body (as examples Lamb et al., '98; Rettig et al., '99). Erythrocyte band 4.1 may exist in a number of states, and it has been speculated that alterations in band 4.1 may represent a marker of erythrocyte aging, although no causative link has been identified (Allosio et al., '85). Band 4.1 has been demonstrated to interact with a wide variety of erythrocyte proteins including glycoporphins C and D (Allosio et al., '85; Reid et al., '90; Hemming et al., '94; Hemming et al., '95), possibly glycoporphin A (Lovrien and Anderson, '80), p55 (Hemming et al., '94), and band 3 (Pasternak et al., '85; Jons and Drenckhahn '92; Lombardo et al., '92) as well as interacting with the lipid bilayer (Sato and Ohnishi, '83; Cohen et al., '88). Band 4.1 interaction with glycoporphin C has been determined to be important in the anchoring of the spectrin–actin skeleton (Workman and Low, '98). Of the total interaction of band 4.1 with the membrane, a large majority is to the glycoporphins with lesser percentages to p55 and band 3 (Hemming et al., '94).

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Considerable data therefore exist supporting the importance of the interaction of band 4.1 with glycophorins and band 3 in regulating the mechanical properties of the erythrocyte membrane. However, less is understood about the regulatory role that band 4.1:band 3 binding may play in regulation of band 3 transport functions.

Band 3 activity is pivotal not only in its normal function as an anion exchanger but also under certain conditions whereby specialized activity of band 3 may be required. Skate erythrocytes, like many other cell types, demonstrate a dramatic response to volume expansion. After the cells are swollen, they lose solutes and therefore the accompanying requisite water through a process called the volume regulatory decrease (RVD) (Garcia-Romeu et al., '91; Goldstein and Brill, '91; Joyner and Kirk, '94). One of the most important solutes utilized in this process is the β-amino acid taurine. This non-metabolized amino acid accumulates in many cells and upon volume expansion, it is rapidly lost. Data from the skate, as well as the trout erythrocyte, suggest that in these cells that the band 3 protein is either the transporter for this volume-stimulated efflux of taurine or that the band 3 is a pivotal component of the transport pathway (Garcia-Romeu et al., '91; Goldstein and Brill, '91; Brill et al., '92; Joyner and Kirk, '94; Fievet et al., '95; Fievet et al., '98).

A number of biochemical events occur rapidly upon volume expansion in the skate erythrocyte including band 3 oligomerization (Musch et al., '94) and tyrosine phosphorylation (Musch et al., '98, '99) and increased affinity ankyrin binding to band 3 (Musch and Goldstein, '96). Because it is known that ankyrin as well as band 4.1 interacts with band 3, the present studies were undertaken to determine if volume expansion alters the interaction of the skate erythrocyte band 4.1 protein with band 3.

**MATERIALS AND METHODS**

**Isolation of cells**

Little skates (*Raja erinacea*) were caught off Frenchman’s Bay, ME, or Woods Hole, MA, and kept in running seawater. Blood was removed from a tail vessel into a heparinized syringe. Cells were pelleted (400g for 2 min at room temperature), and the plasma and buffy coat removed by aspiration. Erythrocytes were resuspended in 5 volumes of isotonic (940 msomol/liter) elasmobranch incubation medium (940 EIM) (composition in mmol/l: 300 NaCl, 5.2 KCl, 2.7 MgSO4, 5 CaCl2, 370 urea, 15 Tris, pH 7.4), washed twice, and resuspended at 50% hematocrit in 940 EIM. To volume-expand the cells, erythrocytes were diluted 1:10 into 460 EIM (NaCl was reduced to 100 mM and urea to 250 mM). At varying times, 200 µl of incubation medium (100 µl cell equivalent) was removed and immediately pelleted for 10 sec into a microcentrifuge, and the cell pellet was snap frozen.

**Isolation and labeling of band 4.1 and ankyrin and binding to erythrocyte membranes**

Membranes were prepared from the snap-frozen pellets as previously described (Musch and Goldstein '96). Briefly, cell pellets were thawed on ice into lysis buffer (10 mM Tris, pH 7.4, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, with 10 µg/ml each leupeptin, aprotinin, and pepstatin). Ghosts were isolated by centrifugation (10,000g for 15 sec at 4°C). Lysis was repeated three times when no hemoglobin was present. Inside-out vesicles were isolated by incubation for 5 min in spectrin-stripping buffer (0.2 mM EDTA, pH 7.4, with protease inhibitors as above). Spectrin removal resulted in >80% inside-out vesicles. These vesicles were pelleted (50,000g for 20 min at 4°C) and resuspended in 5 ml of KI-stripping buffer (1000 mM KI, 7.5 NaH2PO4, 1 mM EDTA. pH 7.4, with protease inhibitors as above). The vesicles were pelleted (50,000g for 20 min at 4°C) and resuspended in binding buffer (5 mM NaH2PO4, 1 mM EDTA, 150 mM NaCl, 5% w/v sucrose, pH 7.4). Protein was measured using the bincinchoninic acid procedure.

Ankyrin was purified from human erythrocytes as previously described (Bennett '83). Band 4.1 was purified from different fractions of the anion-exchange purification. Both proteins were greater than 95% pure as determined by silver staining of pooled and concentrated fractions. Both proteins were iodinated using Bolton-Hunter reagent. Ankyrin was iodinated to a specific activity of 35,000–45,000 cpm/mg and band 4.1 to 27,000–38,000 cpm/mg protein.

**Binding studies**

Binding of iodinated band 4.1 or ankyrin to the KI-IOV was essentially as described previously (Thevinin and Low, '90; Musch and Goldstein, '96). KI-IOV were used at a final concentration of 100 µg/ml from preparations of 1,250–2,350 µg/ml. 125I-band 4.1 was used at concentrations of 0–200 µg/ml and ankyrin from 1.25–250 µg/ml (from stocks
at approximately 1,250 μg/ml). Final volumes were made to 350 μl with addition of binding buffer. The binding was terminated after 60 min by layering over a 500 μl cushion of binding buffer with additional 15% w/v sucrose (to a final of 20% w/v sucrose). Pellets were prepared (35,000 g for 30 min at 20°C). A sample of the top phase was collected to determine specific activity of free iodinated band 4.1 or ankyrin probe, and the bottom was cut off to measure bound ligands.

RESULTS

Characterization of band 4.1 binding to skate erythrocyte KI-IOV

Band 4.1 is known to interact with a number of erythrocyte proteins including glycophorins C and D, p55, as well as band 3. To determine band 4.1 binding to band 3 in the skate erythrocyte, two conditions were tested. First, the purified cytoplasmic domain of human band 3 was included in the binding reaction (at 400 μg/ml). Second, the peptide IRRRY (4 mM) was included in the binding reaction. The peptide IRRRY has been demonstrated to specifically interfere with the interaction of band 4.1 with band 3 but not to disturb the interaction of band 4.1 with the glycophorins (Jons and Drenckhahn, 92; An et al., '96). Varying concentrations of iodinated band 4.1 were incubated with KI-IOV made from skate erythrocytes (Fig. 1). Both band 3 and the peptide IRRRY competed for a population of the binding sites. As in other species, it appears that the major portion of the binding is to proteins other than band 3. When analyzed by Scatchard analysis the maximal binding was 175 ± 19 μg/mg KI-IOV protein. Of this total, 41.7 ± 3.6 μg/mg KI-IOV protein was competed for by either the cytoplasmic domain of band 3 or the peptide IRRRY. The effects of these two agents were not additive, suggesting that they affected the same interactions. The affinity of the interactions for both the IRRRY-insensitive portion (including glycophorins) was calculated to be 0.185 ± 0.043 nM and for the IRRRY-sensitive portion 0.391 ± 0.072 nM.

Effect of volume expansion on band 4.1 interaction with band 3

To determine if band 4.1 interaction with band 3 was altered by cell swelling, KI-IOV were isolated from cells after varying times of exposure...
to medium of one-half osmolarity. The skate erythrocyte transiently swells to nearly 150% of its volume within 15 min, and its volume returns to near baseline levels within 60–120 min (Dickman and Goldstein, 90). The binding of band 4.1 to band 3 decreased significantly within 2 min of exposure to hypotonic medium, and this effect was maintained at a significant level for at least 30 min (Fig. 2). The altered band 4.1:band 3 interaction was reversible since by 60 min this interaction had returned to near baseline levels.

Skate erythrocytes may be swollen either by exposure to hypotonic medium, or these cells may be swollen by inclusion of the permeant solutes ethylene glycol or ammonium chloride in the medium. As shown in Fig. 3, when analyzed at 10 min, all three conditions decreased the amount of band 4.1 that bound to band 3 in KI-IOV isolated from cells swollen by the three protocols.

**Effect of band 4.1 inclusion on high-affinity ankyrin binding in swollen cells**

We have previously demonstrated that under swollen conditions, ankyrin interaction with the cytoplasmic domain of band 3 becomes higher affinity (Musch and Goldstein, ’96). Band 4.1 and ankyrin may interact with similar sites on certain membrane proteins, including band 3. Therefore, ankyrin binding was measured under isotonic and hypotonic conditions (after 10 min), and the effect of including (400 μg/ml band 4.1 in these ankyrin binding reactions was determined. Varying concentrations of labeled ankyrin were incubated with KI-IOV from cells under isotonic or hypotonic conditions. As demonstrated previously, a shift of some binding to a higher-affinity state was confirmed (Fig. 4). Inclusion of band 4.1 had minimal effect on binding under isotonic conditions; however, band 4.1 decreased the amount of the high-affinity binding observed under hypotonic conditions.

**Effect of band 3 oligomerization under isotonic conditions**

Two treatments that alter the interaction of band 3 with ankyrin were also tested for their effects on band 4.1:band 3 interaction. Pyridoxal-5-phosphate and DIDS cause the shift to a higher-affinity ankyrin binding to band 3 as well as promoting the formation of tetramers under isotonic conditions. KI-IOV isolated from cells exposed to hypotonic medium, or PLP or DIDS under isotonic conditions is shown in Fig. 5. All three conditions which induce oligomerization cause a decrease in the band 4.1 binding to band 3. The IRRRY-insensitive binding did not change with PLP or DIDS (data not shown). Although the decrease with PLP or DIDS is less than that of hypotonic medium, it should be noted that the
VOLUME EXPANSION DECREASES BAND 4.1:BAND 3 INTERACTION

Discussion

Band 4.1 is known to interact with a number of erythrocyte membrane proteins including glycoporphins C and D, p55, and band 3 in a number of species (see introduction). Specific amino acid domains have been identified that may regulate some of these interactions (Hemming et al., ’94; An et al., ’96). However, little is known about the functional consequences of altered interaction of band 4.1 with its membrane protein anchors. In the skate erythrocyte, approximately one-quarter of the interaction of band 4.1 with the membrane protein anchors occurs through interaction with band 3. The remaining interaction may be with the glycophorins as has been demonstrated with other species. The present results are important because the data suggest that transport activity of the anion exchanger band 3 may be modulated by its interaction with band 4.1.

The ability of band 3 to act as an osmolyte chan-

Formaldehyde fixation or detergent extraction of the membrane was used to study the interaction. In peptides that disrupt interaction of band 4.1 with band 3, a 30% decrease in band 4.1 binding to band 3 was observed. This decrease was not observed in control peptides that do not disrupt interaction. The decrease in band 4.1 binding to band 3 was observed in the presence of high concentrations of 125I-ankyrin was measured in the absence and presence of unlabeled band 4.1 (300 µg/ml). Values are means ± SE for four different experiments.

**Fig. 4.** Effect of band 4.1 on ankyrin binding. KI-IOV were isolated from cells under isotonic conditions (ISO) or hypotonic (HYPO) for 10 min. Iodinated ankyrin binding at varying concentrations of 125I-ankyrin was measured in the absence and presence of unlabeled band 4.1 (300 µg/ml). Values are means ± SE for four different experiments.

**Fig. 5.** Effect of band 3 oligomerizing agent on band 4.1 binding to band 3. KI-IOV were isolated from cells incubated in isotonic medium with or without PLP or DIDS for 10 min or hypotonic medium for 10 min. Binding was measured in the presence of 40 µg/ml 125I-band 4.1 and with or without IRRRY peptide. Values shown are means ± SE for three different experiments. *0.1 < P < 0.05, +P < 0.05, ++P < 0.01 compared with isotonic by analysis of variance. PLP = pyridoxal 5-phosphate; DIDS = 4,4’-diisothiocyanatostilbene-2,2’ disulfonic acid.
nel was first hypothesized based on studies done on the effects of pharmacological inhibitors on hypotonic-stimulated fluxes of osmolytes such as taurine in skate and trout red blood cells. A number of agents (stilbenes, niflumic acid, pyridoxal phosphate) known to inhibit band 3 all reduced the hypotonic-stimulated efflux of taurine and other osmolytes from these cells (Garcia-Romeu et al., '91; Goldstein and Brill, '91). Furthermore, RBC from fish (lamprey, hagfish) lacking band 3 do not demonstrate a volume-stimulated taurine efflux as observed in RBC from fish possessing band 3 (Brill et al., '92).

Studies in skate RBC (Haynes and Goldstein, '93) demonstrated that the volume-activated osmolyte transporter had the properties of a channel: high flux rates with no evidence of substrate saturation, lack of substrate stereospecificity, and size limitation of permeating substrates. That band 3 could function as this channel or as a channel activator was shown in oocyte expression studies (Fievet et al., '95, '98). Expression of trout band 3 in Xenopus oocytes induces a taurine channel in the cell membrane of the oocytes that was inhibited by band 3 blocking agents. As in the trout RBC, the channel was permeable to a variety of different osmolytes (Fievet et al., '98). It is not clear, however, whether band 3 itself acts as the channel or whether it activates an endogenous native channel in the oocyte.

It is not certain how volume expansion leads to activation of the band 3-associated channel. It is likely that changes in the cytoskeleton produced by volume expansion are transmitted to transport proteins in the cell membrane. We presently demonstrate that under conditions that volume-expand cells, the interaction between band 4.1 and band 3 largely decreases. At the same time, the affinity of ankyrin, another cytoskeletal protein, for band 3 increases (Musch and Goldstein, '96). This reciprocal relationship could play a role in the ability of band 3 to form tetramers (a condition known to be associated with an increase in taurine efflux) in the skate red blood cell under volume expanded conditions (Musch et al., '94). It is noteworthy that two agents that cause the oligomerization of band 3 under non-volume-expanded conditions, PLP and DIDS, also cause the interaction of band 4.1 and band 3 to decrease and also promote the higher-affinity interaction of ankyrin with band 3.

The interaction of band 4.1 and band 3 may be manifest not only at the level of band 3 function but also in the mechanical changes that may occur in the membrane, despite the fact that the band 4.1:band 3 interaction comprises only a modest proportion of the band 4.1 membrane interaction. The disruption of the band 4.1 interaction by the peptide IRRRY causes decreased membrane deformability and increased mechanical stability (Jons and Drenckhahn, '92; An et al., '96). In seeming contrast, strains of mouse and bovine erythrocytes which have a complete deficiency of band 3 demonstrate instability of the membrane (Peters et al., '95; Inaba et al., '96). Therefore, a complex interaction between many membrane components may regulate the mechanical changes that would occur under volume expansion of the cells. In the skate erythrocyte, we might anticipate that decrease in band 4.1 binding might be essential for binding of ankyrin to its high-affinity sites on band 3. Whether this would create greater or lesser mechanical instability is unknown. These mechanical changes could potentially be translated into alterations in the functions of membrane proteins, including the transport activities of band 3.

**LITERATURE CITED**


