Biochemical Analysis of Potential Sites for Protein 4.1-mediated Anchoring of the Spectrin-Actin Skeleton to the Erythrocyte Membrane∗

(Received for publication, September 5, 1997, and in revised form, November 24, 1997)

Ryan F. Workman and Philip S. Low‡
From the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907-1393

Erythrocyte protein 4.1 has been hypothesized to link the spectrin-actin junctional complex directly to the cytoplasmic domain of glycophorin C, but this bridging function has never been directly demonstrated. Because an alternative protein-mediated bridge between the junctional complex and the cytoplasmic domain of band 3 is also plausible, we have undertaken to characterize the membrane sites to which protein 4.1 can anchor the spectrin and actin skeleton. We demonstrate that proteolytic removal of the cytoplasmic domain of band 3 has minimal effect on the ability of protein 4.1 to promote 125I-labeled spectrin and actin binding to KI-stripped erythrocyte membrane vesicles. We also show that quantitative blockade of all band 3 sites with either monoclonal or polyclonal antibodies to band 3 is equally ineffective in preventing protein 4.1-mediated association of spectrin and actin with the membrane. In contrast, obstruction of protein 4.1 binding to its docking site on the cytoplasmic pole of glycophorin C is demonstrated to reduce the same protein 4.1 bridging function by ~85%. We conclude from these data that (i) glycophorin C contributes the primary anchoring site of the protein 4.1-mediated bridge to the spectrin-actin skeleton; (ii) band 3 is incapable of serving the same function; and (iii) additional minor protein 4.1 bridging sites may exist on the human erythrocyte membrane.

Spectrin, actin, and protein 4.1 form the bulk of the protein network that underlies and stabilizes the human erythrocyte membrane (1–7). Polymerization of spectrin with actin into a two-dimensional network is strongly dependent on protein 4.1, an ~78-kDa polypeptide that binds avidly to the β subunit of spectrin (8–13) and thereby forms a calmodulin-dependent binding site for actin (14). The approximate stoichiometry of this ternary complex, as estimated from the composition of the dense gel that rapidly forms when protein 4.1 is added to a solution of spectrin and actin, is 1.2:1 of spectrin:actin:protein 4.1 (15, 16). Not surprisingly, defects in the structure or level of expression of protein 4.1 in erythrocytes result in fragile, abnormally shaped cells (17–19). More importantly, when membrane mechanical instability arises from the absence of protein 4.1, the membrane fragility can be corrected by resealing either intact protein 4.1 or its spectrin-actin binding domain into the defective erythrocytes (20).

In addition to its association with spectrin and actin, protein 4.1 also interacts with at least two prominent integral proteins of the red cell membrane. The more avid of these membrane ligands is glycophorin C, which binds protein 4.1 with a Kd ~50 nM and provides up to ~1/5 of its total anchoring sites on KI-IOVs1 (21–23). P55, a protein comprised of several classical signal transduction domains (24, 25), is thought to significantly stabilize this association (26–28). Of lower affinity than glycophorin C is the interaction of protein 4.1 with band 3, the anion transport protein that also links ankyrin to the red cell membrane. Band 3 associates with protein 4.1 approximately 30-fold less avidly than glycophorin C; however, the anion transporter may also provide up to twice the number of membrane binding sites as glycophorin C (23, 29, 30). In addition to glycophorin C and band 3, protein 4.1 is also known to interact with anionic lipids, especially phosphatidylinerzine and phosphatidylglycerol, 4,5-bisphosphate (31–35).

With both the lipid bilayer and membrane skeletal attachment sites for protein 4.1 established, the question naturally arises as to which protein 4.1 sites can be simultaneously occupied, i.e. from which membrane sites might protein 4.1 form a bridge to the spectrin-actin skeleton. Evidence in support of a glycophorin C linkage to the membrane skeleton includes the following: (i) retention of glycophorin C in detergent-extracted membrane skeletons correlates with the content of protein 4.1 in the same skeletons under a variety of conditions (36, 37); (ii) addition of protein 4.1 to protein 4.1-deficient erythrocytes converts glycophorin C from a detergent-soluble membrane protein to a skeletally linked membrane protein (37); and (iii) migration of glycophorin C in membrane distentions of protein 4.1-deficient cells follows the behavior of a freely diffusing membrane protein, whereas migration in similar tethers of normal membranes conforms to the distribution pattern of the spectrin-actin skeleton (38). Taken together, these data argue that some type of protein 4.1-mediated bridge between glycophorin C and the spectrin-actin skeleton must exist. Nevertheless, the hypothesized physical linkage has never been directly demonstrated in any defined biochemical system.

Data exploring the possible role of band 3 in anchoring a protein 4.1 bridge to the membrane skeleton are essentially nonexistent. Analogous studies on the migration and extractability of band 3 in protein 4.1-deficient membranes are obviously meaningless, because band 3 is independently linked via ankyrin to the spectrin-actin skeleton (1). Furthermore, no direct binding studies have ever been conducted to examine whether band 3-linked protein 4.1 can simultaneously bind spectrin and actin. Consequently, we have undertaken to characterize the direct protein 4.1-mediated bridging of spectrin-actin complexes to band 3 and glycophorin C in KI-stripped erythrocyte membrane vesicles (IOVs); IOVs, inside-out vesicles.

† This work was supported by National Institutes of Health Grant GM24417. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed. Tel.: 765-494-5273; Fax: 765-494-0238; E-mail: lowps@omni.cc.purdue.edu.

1 The abbreviations used are: KI-IOVs, KI-stripped inside-out vesicles; IOVs, inside-out vesicles.
inside-out erythrocyte membrane vesicles. We report here that glycophorin C, as expected, constitutes the primary attachment site of the protein 4.1-tethered spectrin-actin skeleton. We also demonstrate that band 3 is unable to serve an analogous bridging function.

**EXPERIMENTAL PROCEDURES**

**Protein Purifications**—Protein 4.1 was purified by a novel purification protocol (39) based on the method of Tyler et al. (40). Spectrin and actin were extracted from red cell membranes using low ionic strength buffer, as described by Bennett (41), except the membranes were prepared in the presence of 2 mM MgCl₂. Spectrin and actin were subsequently concentrated by dehydration through a dialysis membrane against polyethylene glycol, and the concentrated proteins were labeled with ¹²⁵I Bolton-Hunter reagent (see below). Spectrin and actin were then transferred to binding buffer (10 mM HEPES, 130 mM KCl, 20 mM NaCl, 2 mM MgCl₂, pH 7.4) and stored at 4 °C until used.

**Membrane Preparations**—IOVs were prepared essentially as described elsewhere (41), except during removal of spectrin and actin the IOVs were incubated at 37 °C for 30 min in a minimum of 100 volumes of extraction buffer (0.5 mM EDTA, 1 mM dithiothreitol, pH 8.0). KI-stripped IOVs were prepared, when desired, by incubating the IOVs at 37 °C for 30 min in 50 volumes of neutralization buffer (2 M KI, 25 mM Na₂HPO₄, 1 mM MgCl₂) prior to dilution with an equal volume of double distilled water and centrifugation at 23,400 × g for 1 h. The resulting membranes were washed twice with lysis buffer (5 mM Na₂HPO₄, 1 mM EDTA, pH 8.0) before resuspension in binding buffer. Membranes showed no aggregation upon resuspension in binding buffer.

**¹²⁵I Protein Labeling**—All ¹²⁵I-labeled proteins were prepared by the method of Bennett (41) with minor modifications. Briefly, spectrin and actin were labeled in labeling buffer (20 mM Na₂HPO₄, 100 mM NaCl, 1 mM EDTA, pH 7.6) at a concentration of 8.2 μg/ml. Following labeling, the proteins were extensively dialyzed at 4 °C against binding buffer to remove unreacted label. Protein stocks of the appropriate concentration were then prepared by dilution with binding buffer just before use.

**Antibodies**—Polyclonal antibodies were raised in rabbits against a synthetic glycoporphin C peptide comprising residues 85–96, according to published procedures (42). The antibody was purified using the synthetic peptide as an affinity ligand. A monclonal antibody (m00-10) directed against the N-terminal 10 residues of the cytoplasmic domain of band 3, and polyclonal antibodies against the entire cytoplasmic domain of band 3 were also prepared, as described previously (43). Nonspecific IgG was partially purified by ammonium sulfate precipitation of rabbit preimmune serum followed by DEAE chromatography.

**Binding Assays**—For determination of protein 4.1 polymerization with spectrin and actin, 30 μg/ml protein 4.1 was added to increasing concentrations of ¹²⁵I-labeled spectrin and actin in binding buffer, and the solution was allowed to incubate for 3 h at 4 °C. After layering onto 40% sucrose cushion, the protein 4.1-facilitated binding. As shown in Fig. 2, spectrin and actin associated much more extensively with membranes preincubated with protein 4.1 (solid diamonds) than those lacking protein 4.1 (open diamonds). These observations document biochemically that protein 4.1 can indeed function to bridge the spectrin-actin skeleton to the membrane.

**Evaluation of the Role of Band 3 in Anchoring a Protein 4.1 Bridge to the Membrane Skeleton**—To identify the integral membrane protein(s) that participate in the protein 4.1-mediated tether to the spectrin-actin skeleton, several additional studies were conducted. First, the cytoplasmic domain of band 3 was proteolytically removed with trypsin, and the above described protein 4.1 binding and bridging functions were again evaluated. As shown in Fig. 3, ¹²⁵I-protein 4.1 association with the trypsin-cleaved KI-IOVs was reduced to 45% of normal, consistent with earlier observations that band 3 might contribute up to 60% of the sites on KI-stripped erythrocyte membranes (21, 23, 29–30, 44). Importantly, protein 4.1-mediated bridging of the spectrin-actin complex to the same digested membranes was only slightly altered, displaying somewhat reduced binding at high spectrin-actin concentrations but normal binding at lower concentrations (Fig. 2, solid squares). Since >95% of the band 3 was digested in these membrane preparations, we conclude that band 3 is not a major participant in the protein 4.1-mediated skeletal anchor.

To resolve more thoroughly the question of whether band 3 plays even a minor role in anchoring a protein 4.1 bridge to the spectrin-actin network, we directly blocked the protein 4.1 binding sites on band 3 with a monoclonal antibody to the N terminus of band 3, and then we examined the effect of this modification on the interaction of ¹²⁵I-labeled spectrin and actin with the opsonized KI-IOVs. The monoclonal antibody employed in this study (m00-01) has been shown previously to quantitatively prevent protein 4.1 binding to the cytoplasmic domain of band 3 (44). Furthermore, as observed previously for the proteolytically digested KI-IOVs (Fig. 3), the monoclonal Fab reduces ¹²⁵I-labeled protein 4.1 binding to KI-IOVs to <50% of control values (Fig. 4). Despite this loss of roughly half of the protein 4.1 binding sites on the membrane, no diminu-
Protein 1.0-dependent binding of 125I-labeled spectrin and actin to KI-IOVs and trypsin-digested KI-IOVs. 45 μg/ml KI-IOVs (●, □) or trypsin-digested KI-IOVs (●, □) were incubated for 4 h at 4 °C in the presence (●, □) or absence (○, △) of 50 μg/ml protein 4.1. After thorough washing to remove unbound protein 4.1, increasing concentrations of 125I-labeled spectrin and actin were allowed to bind. Unbound 125I-spectrin and actin were then separated from membrane-bound material by centrifugation, and the membrane fraction was counted in a gamma counter, as described under “Experimental Procedures.” All data points represent the mean ± S.D., where n = 3.

FIG. 2. Protein 1.0-dependent binding of 125I-labeled spectrin and actin to KI-IOVs and trypsin-digested KI-IOVs in the presence and absence of antibodies to the protein 4.1 binding site on glycophorin C. 50 μg/ml 125I-protein 4.1 was incubated for 4 h at 4 °C with control KI-IOVs (A), KI-IOVs plus 8.6 mg/ml nonspecific IgG (B), KI-IOVs plus 1.5 mg/ml affinity purified anti-glycophorin C IgG (C), trypsinized KI-IOVs (D), trypsinized KI-IOVs plus 8.6 mg/ml nonspecific IgG (E), or trypsinized KI-IOVs plus 1.5 mg/ml affinity purified anti-glycophorin C IgG (F). Bound and free 125I-protein 4.1 were then separated by pelleting the membranes through a 20% sucrose cushion, after which the bound fraction was counted in a gamma counter. Data shown represent the mean ± S.D. of three separate assays.
Data points presented represent the mean clonal antibody (m00-01) to residues 1–10 of band 3; digestion in protein 4.1-mediated attachment of 125I-labeled spectrin and actin to the membrane was observed (Fig. 5A). Rather, the protein 4.1-facilitated spectrin-actin binding to opsonized KI-IOVs matched the binding isotherm of control KI-IOVs. Similar results were also obtained with a polyclonal antibody to the whole cytoplasmic domain of band 3 (Fig. 5B). Thus, loss of all band 3 sites can be concluded to have no impact on protein 4.1-mediated attachment of the membrane skeleton to the erythrocyte membrane.

**Evaluation of the Role of Glycophorin C in Anchoring a Protein 4.1 Bridge to the Membrane Skeleton**—To determine whether glycophorin C might provide the membrane anchor for the protein 4.1 bridge to the spectrin-actin skeleton, a similar series of studies to those described above was performed with an antibody to glycophorin C. In this case, the antibody was raised against the amino acid sequence identified by two other groups (27, 28) as the protein 4.1 binding site on glycophorin C (Fig. 6A). Not surprisingly, the antibody competitively displaced ~27% of protein 4.1 binding to KI-IOVs and ~66% of the residual protein 4.1 binding to trypsin-digested KI-IOVs (Fig. 3). It can, therefore, be concluded that the antibody effectively prevents protein 4.1 binding to glycophorin C sites on the red cell membrane.

In stark contrast to the effect of anti-band 3 antibodies, the anti-glycophorin C antibody also blocked the majority of protein 4.1-mediated 125I-spectrin and actin binding to the red cell membrane (Fig. 6B). Indeed, ~85% of all bridging sites on the KI-IOVs were eliminated by anti-glycophorin C opsonization. The conclusion, therefore, follows that glycophorin C serves as the primary anchoring site of protein 4.1-mediated tethers to the spectrin and actin skeleton. However, because ~15% of protein 4.1-assisted connections to the membrane skeleton consistently survived competition with anti-glycophorin C, we also propose that an unidentified anchor for protein 4.1 may still remain on the red cell membrane.

**DISCUSSION**

Two lines of evidence were presented to demonstrate that band 3 does not participate in a protein 4.1-mediated bridge to the spectrin-actin skeleton. First, monoclonal and polyclonal antibodies to band 3 reduced protein 4.1 binding to KI-IOVs by >50% but had no effect on protein 4.1-mediated association of spectrin and actin with the membrane. Second, trypsin removal of the cytoplasmic domain of band 3, which reportedly does not cleave glycophorin C (22) but may well digest other less prominent protein 4.1 binding sites, reduced protein 4.1-promoted spectrin and actin binding to KI-IOVs only minimally. In fact, in three independent replicates of this experiment, trypsin digestion decreased spectrin and actin binding only at elevated 125I-labeled spectrin and actin concentrations, suggesting an unidentified class of lower affinity sites might have been eliminated by the trypsin proteolysis. In this respect, it is interesting to note that protein 4.1-related polypeptides connect CD44 to the cytoskeleton in nonerythroid cells (45, 46) and that CD44 has been recently shown to bind protein 4.1 in mature erythrocytes.2

There are major discrepancies in the literature over the distribution of protein 4.1 binding sites between band 3 and glycophorin C. Hemming et al. (28) report that ~85% of all sites on stripped IIOVs reside on glycophorin C. Cohen and co-workers (21) and Low and co-workers (30) measure only ~1/3 of the total sites on glycophorin C, the remainder locating primarily on band 3. Although differences in binding assays could ac-
petes with ankyrin for a site on band 3 (44, 49). Because the band 3-ankyrin-spectrin linkage constitutes the major attachment site of the spectrin-actin skeleton to the bilayer, any protein 4.1-mediated displacement of ankyrin might be expected to destabilize the cell. This has indeed been observed (49), suggesting that the mechanical properties of the erythrocyte membrane might be regulated in part by the distribution of protein 4.1 between glycoporphin C and band 3. In this scenario, stimuli that displace protein 4.1 from the junctional complex (e.g. cAMP, Refs. 50 and 51), allowing the protein 4.1 to compete with ankyrin for band 3, might be expected to weaken the membrane’s structure, whereas stimuli that promote the opposite translocation would be expected to strengthen it (21, 52).

Finally, it should be noted that ~40% of glycoporphin C is free to diffuse laterally in erythrocyte membranes, suggesting that this population of glycoporphin C is not skeletally attached (40). Since there are maximally 150,000 copies of glycoporphin C per red cell membrane (53), it can be calculated that at most 84,000 of the 200,000 total copies of red cell protein 4.1 will be tethered to glycoporphin C. The remainder could be complexed with spectrin-actin but unattached to the lipid bilayer or could be bound to band 3 in place of the usual ankyrin bridge. It would seem, therefore, that protein 4.1 has not evolved to maximize its bridging capabilities to glycoporphin C but instead to serve as a broker of membrane association, where enhanced association with glycoporphin C might be induced to increase membrane-skeletal tethers, whereas decreased association with glycoporphin C coupled with a rise in interaction with band 3 might be exploited to weaken skeletal interactions. With the many kinases that regulate the association of protein 4.1 with glycoporphin C (51, 54), with the spectrin-actin complex (50–51, 55), and with band 3 (21), one can anticipate that protein 4.1 may eventually prove critical to pathways that modulate erythrocyte behavior.

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