Selective Modulation of Band 4.1 Binding to Erythrocyte Membranes by Protein Kinase C*

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We have studied the effects of band 4.1 phosphorylation on its association with red cell inside-out vesicles stripped of all peripheral proteins. Band 4.1 bound to these vesicles in a saturable manner, and binding was characterized by a linear Scatchard plot with an apparent $K_d$ of $1-2 \times 10^{-7}$ M. Phosphorylation of band 4.1 by purified protein kinase C reduced its ability to bind to membranes, resulting in a reduction in the apparent binding capacity of the membrane by 60-70% but little or no change in the apparent $K_d$ of binding. By contrast, phosphorylation of band 4.1 by cAMP-dependent kinase had no effect on membrane binding. Digestion of the stripped inside-out vesicles with trypsin cleaved 100% of the cytoplasmic domain of band 3 but had little or no effect on glycophorin. Binding of band 4.1 to these digested vesicles was reduced by 70%. Phosphorylation of band 4.1 by protein kinase C had no effect on its binding to the digested vesicles, suggesting that the cytoplasmic domain of band 4.1 contained the phosphorylation-sensitive binding sites. This was confirmed by direct measurement of band 4.1 binding to the purified cytoplasmic domain of band 3. Phosphorylation of band 4.1 by protein kinase C reduced its binding to the purified 43-kDa domain by as much as 90%, while phosphorylation by cAMP-dependent kinase was without effect. These results show a selective effect of protein kinase C phosphorylation on the binding of band 4.1 to one of its membrane receptors, band 3, and suggest a mechanism whereby one of the key red cell-skeletal membrane associations may be modulated.

Underlying the human erythrocyte membrane is a self-assembled network of proteins termed the membrane skeleton (reviewed in Refs. 1 and 2). This self-assembled network interacts with integral membrane proteins, confers mechanical strength on the otherwise fragile lipid bilayer, and plays a major role in conferring a biconcave shape on the normal erythrocyte. Three principal components of the membrane skeleton are: 1) spectrin molecules, largely in the form of 200-nm-long flexible $\alpha\beta_2$ tetramers; 2) short oligomers of actin containing perhaps 12-15 actin monomers each; and 3) band 4.1. While these three components together can form two- or three-dimensional arrays which have some features reminiscent of the membrane skeleton, other proteins such as protein 4.9, adducin, tropomyosin, and myosin are likely necessary for the assembly of a complete skeletal network.

Recently, evidence from a variety of sources has led to the suggestion that some of the key skeletal protein associations may be subject to regulation or modification via one or several pathways. For example, the ability of adducin to promote spectrin-actin binding is markedly inhibited by Ca$^{2+}$ in the presence of calmodulin (3, 4). Calmodulin also interacts weakly with spectrin (5-9) and in the presence of Ca$^{2+}$ may affect spectrin binding to band 4.1 and actin (9), and possibly spectrin phosphorylation (10, 11).

Beyond the effects of Ca$^{2+}$ and calmodulin, phosphorylation is likely the principal pathway by which red cell skeletal protein associations may be modulated. With the exception of actin, all of the major skeletal components are phosphorylated by one or several kinases in the intact red blood cell, and evidence shows that phosphorylation can have major effects on skeletal protein associations (reviewed in Ref. 12). In vitro phosphorylation of band 4.1 by a CAMP-independent membrane-associated kinase (13), protein kinase C, and CAMP-dependent kinase (14) reduces band 4.1 binding to spectrin, and the latter two kinases also reduce the ability of band 4.1 to promote spectrin binding to actin (14). Phosphorylation of ankyrin by casein kinase or CAMP-independent membrane-associated kinase reduces its affinity for spectrin tetramers or oligomers (15, 16). Also, phosphorylation of ankyrin by cAMP-independent membrane-associated kinase reduced its binding to the purified cytoplasmic domain of band 3 (17). These and other observations suggest that many of the principal associations in the red cell membrane skeleton are subject to modulation by one or several endogenous red cell kinases.

Recently, we have focused our attention on the association of band 4.1 with components on the cytoplasmic surface of the red cell membrane. In addition to promoting the tight association of spectrin with actin (reviewed in Refs. 1 and 2) band 4.1 also serves to anchor the skeleton to the membrane via its association with glycophorin (18), band 3 (19), and lipids (20, 21). Since other associations of band 4.1 are not dramatically affected by phosphorylation, we investigated the role of phosphorylation in controlling one or several of these membrane associations. Our results show that the association of band 4.1 with the red cell membrane can be modulated in vitro by changes in the level of band 4.1 phosphorylation. This modulation is interesting not only because there is selectivity in the kinases which affect the association but also phosphorylation apparently affects band 4.1 binding to band 3 and not glycophorin. This type of selectivity in the regulation of skeletal protein associations may have important implication...
consequences for the maintenance and possible alteration of red cell mechanical properties. Moreover, because of the wide spread presence of band 4.1 analogues in nonerythroid cells (22-29) our results suggest a possible mechanism by which protein kinase C may affect cytoskeletal organization in many cell types.

**EXPERIMENTAL PROCEDURES**

**Materials**
- cAMP, ATP, bovine serum albumin, DTT, α-chymotrypsin, trypsin, phosphatidylserine, 12-O-tetradecanoylphorbol 13-acetate, diisopropylfluorophosphate, Triton X-100 were obtained from Sigma. 125I-labeled Bolton-Hunter reagent was obtained from Du Pont-New England Nuclear. Reagents for gel electrophoresis were obtained from Bio-Rad. \( \gamma ^{32} P \)ATP was synthesized using PO, from Du Pont-New England Nuclear and a \( \gamma ^{32} P \)ATP synthesis kit from Promega.

**Methods**

**Preparation of Band 4.1** — Band 4.1 was prepared by a modification (14) of the method of Ohanian and Gratzer (30). Band 4.1 prepared in this way is devoid of contaminating polyproteins (see Fig. 1B), contains no endogenous or contaminating protein kinase activity, and contains \( \leq 0.1 \) mol of PO/mol of band 4.1 (14). Band 4.1 was iodinated with \( ^{125} \)I-labeled Bolton Hunter reagent as described (14).

**Preparation of 43-kDa Band 3 Cytoplasmic Domain** — Band 3 cytoplasmic domain was prepared from pH 11 stripped inside-out vesicles by α-chymotrypsin digestion as described in Korsgren and Cohen (31). This preparation consisted of a mixture of about 40- and 43-kDa polypeptides (see Fig. 1D) derived from the cytoplasmic portion of band 3 (see Ref. 32 for review). The protein was iodinated with \( ^{125} \)I-labeled Bolton Hunter reagent as described (33).

**Preparation of pH 11-stripped Inside-out Vesicles** — Red cell inside-out vesicles devoid of all peripheral proteins (see Fig. 1C) were prepared by treating inside-out vesicles at alkaline pH (which is known to elute tightly bound peripheral membrane proteins (34)) as follows. White ghosts (prepared as described (35)) were depleted of band 6 (glyceraldehyde-3-phosphate dehydrogenase) by incubation in 10 volumes of 150 mM NaCl, 5 mM sodium phosphate, pH 8.0, 0.5 mM EGTA on ice for 20 min. The membranes were washed once in the above buffer and once in 5 mM sodium phosphate, pH 8.0, 0.5 mM EGTA. Spectrin and actin were removed by incubation in 20 volumes of 0.1 M EGTA, pH 8.5, at 37 °C for 30 min. The resulting inside-out vesicles were washed once in 5 mM sodium phosphate, pH 8.0, 0.5 mM EGTA. The remaining peripheral proteins (ankyrin, band 4.1, and band 4.2) were removed by incubation of the vesicles in 10 ghost volumes of 0.1 mM EGTA titrated to pH 11 with 0.1 M NaOH, followed by incubation at 25 °C for 20 min. The stripped membranes were washed once in 5 mM sodium phosphate, pH 8.0, 0.5 mM EGTA. Unsealed vesicles were removed by centrifugation on a shelf of Dextran T-150 (1.03 g/ml in 5 mM sodium phosphate, 0.5 mM EGTA, pH 8.0) as described (36) with minor modifications. These vesicles are referred to in the text as stripped inside-out vesicles.

In some cases the stripped vesicles were trypsinized by incubating vesicles at a protein concentration of 0.1-0.2 mg/ml with 0.5 \( \mu \)g/ml trypsin in 5 mM sodium phosphate, pH 7.6, 0.5 mM Na-EGTA on ice for 15 min. Digestion was terminated by the addition of 4 \( \mu \)M (final concentration) diisopropylfluorophosphate. After 20 min the vesicles were labeled with \( \gamma ^{32} P \)ATP, at a concentration of 200 \( \mu \)M, for 20 min at 0 °C. The reaction was terminated by the addition of 50 mM EDTA. The samples were centrifuged at 20,000 rpm for 20 min in a Beckman 42.2 Ti rotor and, after careful aspiration of the supernatant, \( ^{125} \)I in the pellet was measured in a \( \gamma \) counter. Control samples containing all of the components except vesicles were treated identically except that ATP was omitted. Phosphorylation reactions were terminated, and ATP and CAMP were removed by filtration as described for protein kinase C above. Molar \( ^{32} P \) incorporation into parallel samples was measured as described above.

**Measurement of Band 4.1 Binding to Stripped Vesicles** — Band 4.1 (at the concentrations indicated in the figure) was incubated with 18 \( \mu \)g/ml stripped inside-out vesicles in 100 mM KCl, 2 mM MgCl2, 1 mM EGTA, 1 mM DTT, 10 \( \mu \)M CAMP, and 50 \( \mu \)M ATP at 24 °C for 2 h. Nonphosphorylated band 4.1 used for controls was treated identically except that ATP was omitted. Phosphorylation reactions were terminated, and ATP and CAMP were removed by filtration as described for protein kinase C above. Molar \( ^{32} P \) incorporation into parallel samples was measured as described above.

**Measurement of Band 4.1 Binding to Cytosolic Domain of Band 3** — Band 4.1 (at the concentrations indicated in the figure) was incubated with 18 \( \mu \)g/ml stripped inside-out vesicles in 100 mM KCl, 2 mM MgCl2, 1 mM EGTA, 1 mM DTT, 10 \( \mu \)M CAMP, and 50 \( \mu \)M ATP at 24 °C for 2 h. Nonphosphorylated band 4.1 used for controls was treated identically except that ATP was omitted. Phosphorylation reactions were terminated, and ATP and CAMP were removed by filtration as described for protein kinase C above. Molar \( ^{32} P \) incorporation into parallel samples was measured as described above.
Band 3 fragment complex (see Fig. 5) a procedure was used to correct for these background counts. Alternating gel lanes were loaded with increasing amounts of 125I-43-kDa band 3 fragment alone. After electrophoresis, the gel was cut to separate the region containing the complex from the region containing free 43-kDa fragment, and the upper portion of these lanes which contained only the 43-kDa band 3 fragment were counted for 125I. These counts were used to construct a background calibration plot of 125I counts in the complex region versus free 125I-43-kDa fragment. After determining the amount of free 125I-43-kDa fragment in the lanes in which band 4.1 was present, the appropriate background counts were obtained from the calibration curve and subtracted from total counts in the region of the complex to give the values shown in the figures. These corrections were routinely 10% or less of the total counts.

Data Analysis—All binding data were fit using the Enzfitter computer program (Elsevier-Biosoft) using a single site binding isotherm.

Other Procedures—Gels were run according to Laemmli (40) in 10% acrylamide and stained with Coomassie Blue. Protein was determined by the method of Bradford (41) with bovine serum albumin as the standard. Vesicle protein was determined in the presence of 0.1% SDS.

Glycoprotein Quantitation—To quantify glycophorin a (dimer), glycoproteins of intact red cells were prelabeled with NaB3H3 as described (42). Stripped vesicles were prepared from these cells, and some were trypsinized as described above. The 3H-labeled vesicles were electrophoresed on a SDS slab gel, and glycoproteins were localized by fluorography. 3H-Containing bands were excised and counted in a scintillation counter, and the percentage of counts in the region corresponding to glycophorin a (dimer) was determined in each case. Glycophorin a dimer was chosen as an index protein to monitor glycoprotein digestion since our results indicated that the other glycoproteins were either equally or less susceptible to trypsinization.

RESULTS

Band 4.1 binding to erythrocyte integral membrane proteins was studied using inside-out vesicles which were stripped of all peripheral proteins by a brief dilute alkali treatment (Fig. 1C). As judged by the Coomassie Blue-stained gels these vesicles contained only band 3; labeling the glycoproteins in the cells from which the vesicles were derived with 3H (42) revealed they also contained the full complement of glycoproteins (not shown).

In order to test the effects of phosphorylation on band 4.1 binding to the stripped vesicles, we incubated band 4.1 with protein kinase C or cAMP-dependent kinase either in the presence or absence of ATP. The rationale behind this approach was that the only difference between phosphorylated and nonphosphorylated band 4.1 would be whether ATP was present or absent from the phosphorylation reaction mixture (see Ref. 14 also). Samples were, in addition, treated either by gel filtration or ultrafiltration after the phosphorylation reaction incubation to remove ATP, Ca2+, etc. (see "Methods").

Fig. 2A shows that nonphosphorylated 125I-band 4.1 bound saturably to the stripped vesicles, and the inset shows that the binding was characterized by an approximately linear Scatchard plot giving an apparent \( K_d = 0.8 \times 10^{-7} \) M and a binding capacity of 292 ± 4 μg of band 4.1 bound/mg of vesicle protein. Control experiments using noniodinated band 4.1, in which binding was measured by scanning Coomassie Blue-stained SDS gels of vesicles, gave comparable results (not shown) indicating that iodination of band 4.1 had little or no effect on binding. Fig. 2A also shows that in the same experiment band 4.1 phosphorylated by protein kinase C to 2.5 mol of PO4/mol of band 4.1 had a considerably reduced binding to the vesicles. Scatchard analysis of the binding data (inset) shows that phosphorylation resulted in a reduction in the apparent binding capacity of the vesicles by 67% (binding capacity, 105 ± 4 μg of band 4.1/mg of vesicle protein) while the apparent \( K_d \) remained unchanged (\( K_d = 0.9 \pm 0.1 \times 10^{-7} \) M). Similar effects of protein kinase C phosphorylation of band 4.1 were found in eight other separate experiments in which phosphorylation ranged from 1.5 to 3.0 mol of PO4/mol of band 4.1. In these other experiments, apparent vesicle binding capacity was reduced by 50-80%, and \( K_d \) values were either unchanged (four experiments) or reduced by 40-60% (four experiments).

By contrast to the effect of protein kinase C, phosphorylation of band 4.1 with cAMP-dependent kinase had little or no effect on binding. Fig. 2B shows that band 4.1 incubated with purified red cell membrane cAMP-dependent kinase in the presence of ATP (0.8 mol of PO4 incorporated/mol of band 4.1) associated with vesicles to the same extent as nonphosphorylated band 4.1 (no ATP in phosphorylation incubation). Similar results were found in four other experiments in which phosphorylation by cAMP-dependent kinase ranged from 0.7 to 1.4 mol of PO4/mol of band 4.1. It is interesting to note that we have shown in previous work (14) that this degree of band 4.1 phosphorylation by the cAMP-dependent kinase did not change the binding of band 4.1, while similar kinase activity had no effect on band 4.1 binding. Inhibition of band 4.1 phosphorylation by 125I-band 4.1 binding to pH 11-stripped inside-out vesicles. A, 125I-band 4.1 was incubated with purified protein kinase C in the presence (○) or absence (●) of ATP. The indicated concentrations of phosphorylated (+ATP, 2.5 mol of PO4/mol of band 4.1) and nonphosphorylated (−ATP) band 4.1 were incubated with 18 μg/ml stripped vesicles, and binding was measured as described under "Methods." Inset, Scatchard plot of binding of phosphorylated (○, \( K_d = 0.8 \pm 0.08 \times 10^{-7} \) M, capacity 292 ± 4 μg/mg) and nonphosphorylated (●, \( K_d = 0.9 \pm 0.1 \times 10^{-7} \) M, capacity = 105 ± 4 μg/mg) band 4.1 to vesicles. B, 125I-band 4.1 was incubated with purified cAMP-dependent kinase in the presence (●) or in the absence (○) of ATP as described under "Methods." The indicated concentrations of phosphorylated (+ATP, 0.8 mol of PO4/mol of band 4.1 incorporated) and nonphosphorylated (−ATP) band 4.1 were incubated with 18 μg/ml stripped vesicles, and binding was measured as described under "Methods."
dependent kinase reduced band 4.1 binding to spectrin and actin by 50–70%.

The results of Fig. 2A suggested that phosphorylation of band 4.1 by protein kinase C reduced or abolished the binding of band 4.1 to a subset of vesicle binding sites (although it is perhaps unexpected that the $K_d$ of binding to the unaffected sites is identical to the affected sites (see "Discussion")). Band 4.1 is known to bind to sites on inside-out vesicles (43, 44) including the cytoplasmic domain of band 3 (19), glycophorin (18), and possibly to lipids (20, 21). In order to test whether band 4.1 phosphorylation affected its ability to bind to the cytoplasmic domain of band 3 we performed selective proteolysis of vesicles. Steck (45) has previously shown that mild trypsinization of inside-out vesicles cleaves the 43 kDa cytoplasmic domain of band 3 from the membrane while having little or no effect on the glycophorins. Table I summarizes the results of trypsinization of our stripped inside-out vesicles. While band 3 was completely proteolyzed, the glycophorin α (dimer) band was reduced by only 15%.

Fig. 3 shows that trypsinization of vesicles significantly reduced binding of control (nonphosphorylated) band 4.1. Scatchard analysis (Fig. 3, inset) indicates that the binding capacity was reduced by 64%, and the $K_d$ was reduced by nearly 50%. Similar results were found in four other experiments. (Note: to limit interexperimental variability this experiment was performed simultaneously with, and using the same vesicles as, the experiment shown in Fig. 2, which is why the same binding curves are shown for nonphosphorylated band 4.1 binding to stripped vesicles in both cases.)

The 36% of the band 4.1 binding sites remaining on the trypsinized vesicles presumably represent contributions of glycophorins, lipids, and possibly other components as well. However, Fig. 3 shows that phosphorylation of band 4.1 by protein kinase C (the same phosphorylated band 4.1 was used in the experiment shown in Fig. 2A containing 2.5 mol of PO$_4$/mol of band 4.1) had no effect on band 4.1 binding to these remaining sites.

The above results show that phosphorylation of band 4.1 by protein kinase C and trypsinization of vesicles both result in a similar reduction in the apparent binding capacity of stripped inside-out vesicles for band 4.1 (see Table I). Since trypsinization eliminated the effect of phosphorylation on band 4.1 binding to vesicles it can be concluded that the phosphorylation-sensitive sites were removed from the membrane. Two observations suggest that these sites are associated with band 3 and not glycophorin. 1) Trypsinization removes 100% of the phosphorylation-sensitive sites, and band 3 is 100% proteolyzed by trypsinization. The glycophorins were affected by 15% at best. 2) It has been estimated by others (19) that approximately 65% of band 4.1 binding to inside-out vesicles is due to band 3 and 35% to glycophorin. It is therefore unlikely that a 70% reduction in band 4.1 binding could be due to an altered association of band 4.1 with glycophorin. These arguments are, however, indirect, and additional experiments were done to address the question more directly.

We purified the 43-kDa band 3 cytoplasmic domain and measured directly the effect of phosphorylation on band 4.1 binding to it. Fig. 4 shows that binding of $^{125}$I-43-kDa band 3 fragment to band 4.1 can be measured using a nondenaturing gel system similar to that used by Fasternack et al. (19). In order to accurately quantitate binding, the region of the gel containing the $^{125}$I-43-kDa band 3 fragment-band 4.1 complex was cut out and counted in a γ counter. Correction for a small percentage of $^{125}$I-43-kDa band 3 fragment counts in this region of the gel not associated with the complex was made as described under "Methods." Fig. 5A shows the results of a typical experiment in which the effect of protein kinase C phosphorylation of band 4.1 was measured. As with the vesicle binding experiments, phosphorylated (in this case 1.86 mol of PO$_4$/mol of band 4.1) and nonphosphorylated band 4.1 differed only in the presence and absence, respectively, of ATP from the phosphorylation reaction mixture. Fig. 5A shows that phosphorylation by protein kinase C reduced binding of band 4.1 to the 43-kDa band 3 fragment significantly (51% reduction in maximum binding capacity). Fig. 5B shows the cumulative results of four separate binding experiments done under conditions similar or identical to that in Fig. 5A. In each case the percent reduction in band 4.1 binding capacity for the 43-kDa band 3 fragment was computed and plotted versus molar phosphate incorporation into band 4.1 by protein kinase C. The data suggest that increased phosphorylation correlates with a decrease in band 4.1 binding to band 3 fragment.

By contrast, Fig. 5C shows that in a separate study phosphorylation of band 4.1 by cAMP-dependent kinase (1.25 mol of PO$_4$/mol of band 4.1) had no effect on binding to $^{125}$I-43-kDa band 3 fragment. Three other separate studies confirmed that phosphorylation of band 4.1 by cAMP-dependent kinase either had no effect on binding to the 43-kDa band 3 fragment or slightly increased the binding. In conjunction with the studies in Figs. 2 and 3, this experiment shows that protein kinase C but not cAMP-dependent kinase phosphorylation of band 4.1 reduces band 4.1 binding to the cytoplasmic domain of band 3.

### DISCUSSION

Our data show that phosphorylation of band 4.1 by protein kinase C results in a reduction in band 4.1 binding to the inner surface of red cell membrane. Three lines of evidence support this conclusion

| Type of vesicles          | Amount of intact band 3 on membrane | Amount of glycophorin α on membrane | $K_d$ of band 4.1 binding | Band 4.1 binding capacity | Reduction$^*$ of band 4.1 binding capacity by trypsinization | Reduction$^*$ of band 4.1 binding by protein kinase C |
|---------------------------|-----------------------------------|-----------------------------------|--------------------------|---------------------------|-----------------------------------------------------------|--------------------------------------------------------|
| Stripped vesicle          | 100                                | 100                               | 0.8 ± 0.08               | 292 ± 4                   | 64                                                        | 70                                                     |
| Trypsinized stripped vesicle | 0                                 | 85                                | 0.44 ± 0.1              | 85 ± 5                    | 0                                                         | 0                                                      |

$^*$ Percent reduction of band 4.1 binding capacity was computed from the change in the intercepts of the Scatchard plots in each case.
FIG. 3. Effect of trypsinization on $^{125}$I-band 4.1 binding to pH 11-stripped inside-out vesicles. A portion of the stripped vesicles used in the experiment shown in Fig. 2A was digested with trypsin as described under "Methods" to cleave the 43-kDa cytoplasmic domain from band 3. An aliquot of the same nonphosphorylated $^{125}$I-band 4.1 used in Fig. 2A was tested for binding to control vesicles (same as in Fig. 2A) (●) and to trypsinized vesicles (▲). To facilitate comparison of binding to digested and undigested vesicles, binding to digested vesicles was normalized to an equivalent volume of stripped vesicles, treated identically but without proteolysis. Binding of band 4.1 phosphorylated by protein kinase C (+ATP, 2.5 mol of $P_{i}$/mol of band 4.1) and nonphosphorylated band 4.1 (−ATP) were incubated at a concentration of 63.3 µg/ml with increasing concentrations of $^{125}$I-43-kDa band 3 fragment. Binding measurements and background corrections were done as described under "Methods." B, data from four separate binding experiments similar to that shown in A. In each case, the binding of phosphorylated and nonphosphorylated band 4.1 to the 43-kDa band 3 fragment was measured, as in A, and the percent maximum binding capacity of the phosphorylated band 4.1 relative to nonphosphorylated band 4.1 was computed from the predicted maximum binding capacity in each case. The error bars show the standard deviation of each measurement. C, band 4.1 was incubated with cAMP-dependent kinase in the presence (●) or absence (○) of ATP as described under "Methods." After the incubation, phosphorylated band 4.1 (+ATP, 1.25 mol of $P_{i}$/mol of band 4.1) and nonphosphorylated band 4.1 (−ATP) were incubated at a concentration of 75 µg/ml with increasing concentrations of $^{125}$I-43-kDa fragment. Binding and background corrections were done as described under "Methods."

with band 4.1 depleted inside-out vesicles (18, 43, 44). Band 3 and one or more of the glycophorins have been suggested as possible high affinity protein binding sites (18-21). Although Shiffer and Goodman (44) found that partial proteolysis of band 3 with trypsin or chymotrypsin had little effect on band 4.1 binding to vesicles, Pasternack et al. (19) showed that purified 43-kDa band 3 fragment could competitively reduce band 4.1 binding to vesicles by approximately 65%. This figure is nearly identical to the reduction in band 4.1 binding to stripped vesicles which we found when band 3 cytoplasmic domain was completely removed from vesicles by proteolysis. Along with our phosphorylation data, these results suggest that under our binding conditions about 65% of the total band 4.1 binding capacity of inside-out vesicles is accounted for by band 3. The apparent discrepancy with the results of Shiffer and Goodman could be accounted for by the fact that band 3 in their studies was incompletely proteolyzed as well as by the presence on their vesicles of other proteins, such as band 4.2, which may have weak interactions with band 4.1 (31).

We have no direct evidence for the identity of the 35% of the non-band 3 (phosphorylation-insensitive) binding sites on the stripped vesicles. However, it seems likely that these sites are associated with the glycophorins and possibly lipids as well. The above discussion suggests that the binding curve in Fig. 2A for nonphosphorylated band 4.1 binding to stripped vesicles is composed of at least two components, one representative of binding to band 3, and at least one other, representing the contribution of non-band 3 sites. The contribution of the non-band 3 sites can be inferred from the curve in Fig. 3 representing band 4.1 binding to trypsinized stripped vesicles. The Scatchard plot of this data (Fig. 3 inset) suggests that the non-band 3 binding sites have an apparent $K_{d}$ of 0.44 × 10⁻⁷ M and a capacity of 85 µg of band 4.1/mg of vesicle protein. If the binding curve in Fig. 2A (nonphosphorylated band 4.1 binding to stripped vesicles containing band 3 and

suggest that the phosphorylation selectively inhibits the ability of band 4.1 to bind to the cytoplasmic domain of band 3. 1) Selective proteolysis of inside-out vesicles to remove the cytoplasmic domain of band 3 reduces binding of band 4.1 by about the same extent as protein kinase C phosphorylation; 2) phosphorylation of band 4.1 by protein kinase C has no effect on band 4.1 binding to vesicles from which the cytoplasmic domain of band 3 has been cleaved; and 3) phosphorylation of band 4.1 by protein kinase C reduces the binding of band 4.1 to the purified 43-kDa band 3 cytoplasmic domain in solution.

Numerous studies have shown that band 4.1 will reassocia
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non-band 3 binding sites) is fit with a two-component binding isotherm using a $K_d = 0.44 \times 10^{-7}$ M and capacity $-85 \mu g$/mg for the non-band 3 sites, it is found that the other (band 3) binding sites must have a $K_d = 0.86 \times 10^{-7}$ M and capacity $= 207 \mu g$/mg. Thus, the $K_d$ for the non-band 3 sites is half that of the band 3 sites. In principle, if phosphorylation of band 4.1 completely eliminated its binding to band 3 then the $K_d$ of phosphorylated band 4.1 binding to stripped vesicles should be $0.44 \times 10^{-7}$ M, while Fig. 2B shows it to be $0.9 \times 10^{-7}$ M. This discrepancy cannot be due to a possible effect of band 4.1 phosphorylation on its association with the non-band 3 sites, as Fig. 3 shows that binding to these sites is unaffected by band 4.1 phosphorylation. The most likely explanation is that not all of the band 3 binding was abolished by phosphorylation, and the lower affinity sites have affected our estimation of the $K_d$ for non-band 3 sites in Fig. 2B.

While the above results suggest that the $K_d$ of band 4.1 binding to the cytoplasmic domain of band 3 is in the range of $0.8 \times 10^{-7}$ M direct measurement of the $K_d$ in solution using the purified 43-kDa band 3 fragment gave higher values. In seven separate experiments the $K_d$ ranged from $10 \times 10^{-7}$ to $1.9 \times 10^{-7}$ M with a mean of $5.8 \times 10^{-7}$ M. The range of values likely reflects differences in the protein preparations themselves. One variable which was difficult to control was the proportion of 43-kDa versus 41-kDa fragments in the preparations. It is possible that each of these fragments has a slightly different affinity for band 4.1 (see Ref. 32 for discussion). The most likely explanations for the higher $K_d$ of the purified 43-kDa fragment compared with the membrane-associated form are either that the band 4.1 binding site partially overlaps the trequinal cleavage site in band 3 and/or that the conformation of the site is altered in the isolated fragment.

Anderson and Lovrien (18) have estimated that the $K_d$ for band 4.1 binding to glycoporin is on the order of $9.0 \times 10^{-7}$ M which is very close to the $K_d$ for band 4.1 binding to non-band 3 binding sites deduced above. By contrast, Pasternack et al. (19), on the basis of a visual analysis of a binding isotherm, have suggested a $K_d$ of $2 \times 10^{-8}$ M for one set of binding sites (likely glycoporin) and $5 \times 10^{-8}$ M for another (likely band 3). While the latter value is consistent with our in-solution binding data, neither value agrees with our or Anderson and Lovrien's (18) membrane-binding studies. A wide variety of factors is expected to play a role in such measurements including the level of membrane-associated polyphosphoinositides (47), the methods used to strip band 4.1 from vesicles, as well as the endogenous PO4 content of the band 4.1 used in the studies. Until the effects of each of these variables is clearly defined and controlled, it will be difficult to compare quantitatively different types of binding studies.

By contrast to the effects of protein kinase C, cAMP-dependent kinase phosphorylation of band 4.1 had little or no effect on its binding to vesicles or to purified 43-kDa band 3 fragment (although in some cases a slight increase in binding was observed). This lack of effect was observed even when band 4.1 phosphorylation by cAMP-dependent kinase was comparable with that induced by protein kinase C. This observation is interesting in light of our previous work which showed that phosphorylation of band 4.1 by cAMP-dependent kinase, as well as protein kinase C, resulted in a dramatic reduction in spectrin binding and spectrin-actin-band 4.1 complex formation (14). Studies by Leto et al. (48, 49) and Horne et al. (50) have shown that protein kinase C phosphorylates a site within a 16-kDa domain of band 4.1, while cAMP-dependent kinase phosphorylates principally an adjacent 10-kDa domain with some 16-kDa phosphorylation observed. In conjunction with our results, this suggests that the 16-kDa domain contains regulatory sites both for spectrin binding and band 3 binding. Moreover, our results suggest that if cAMP-dependent kinase does in fact phosphorylate sites on the 16-kDa domain they may be distinct from those affected by protein kinase C. Since the spectrin-binding domain of band 4.1 is within the 10-kDa domain (61) which is not a substrate for protein kinase C, the effect of protein kinase C phosphorylation on spectrin binding may be indirect or conformational in nature. The locus of the band 3 binding region with the domain structure of band 4.1 is unknown, but it would not necessarily lie within the 16-kDa domain affected by protein kinase C.

To what degree do the results of these in vitro measurements reflect the associations of band 4.1 in the red cell? Our data suggest a maximum binding capacity of stripped vesicles for band 4.1 of about 300 $\mu g$/mg vesicle protein, while normal ghosts contain approximately 50 $\mu g$ of band 4.1/mg of membrane protein (52). If we normalize band 4.1 content to band 3 instead of total membrane protein (since the vesicles are stripped of many proteins which ghosts contain) we find a maximum capacity of 375 $\mu g$ of band 4.1/mg of band 3 in stripped vesicles (assuming that band 3 is 80% of the protein of these vesicles) and a content of 200 $\mu g$ of band 4.1/mg of band 3 in ghosts (assuming that band 3 is 25% of the protein of ghosts). That the stripped vesicles have nearly twice the binding capacity of ghosts is perhaps not surprising since removal of other peripheral proteins may expose new binding sites and since inside-out vesicles containing almost all of the normal band 4.1 content can bind additional added band 4.1 (43).

It is difficult to evaluate to what extent the proportion of band 4.1 bound to band 3 and glycoporin suggested by our data reflects in vivo values. Pasternack et al. (19) have suggested that the purified 43-kDa fragment of band 3 may compete with spectrin for band 4.1 binding, suggesting that in vivo band 4.1 may be in equilibrium between several potential binding sites. The relevance of the glycoprotein binding sites is difficult to evaluate. Even though band 4.1 can bind to glycoporin $\alpha$ in vitro (47), red cells which lack completely glycoporin $\alpha$ have normal shape and mechanical properties and normal band 4.1 content (53). Evidence suggests that glycoporin $\beta$ (or $\gamma$ which are structurally related (54)) are involved with the in vivo anchorage of band 4.1 to the membrane (46, 55, 56). While the membranes of individuals lacking these glycoproteins have decreased mechanical stability and deformability (54) they nevertheless contain normal quantities of skeletal proteins, specifically band 4.1. In these individuals it may be that all of the band 4.1 is associated with the cytoplasmic domain of band 3.

A physiological role for a modulation of band 3-band 4.1 association by protein kinase C remains speculative, but several possibilities can be suggested. 1) Regulation of skeletal-membrane contacts may be important during erythroid biogenesis when developing erythroid precursor cells must undergo dramatic alterations in shape and content while retaining the nascent skeletal network. 2) Red cells may respond to mechanical stress by activation of kinases leading to transient changes in skeletal properties. While there is no direct evidence for such a response, there is evidence suggesting that cells with increased levels of membrane protein phosphorylation are more deformable and more resistant to osmotic stress than controls (57); and 3) steady state phosphorylation of band 4.1 via the ongoing action of phosphatases and kinases may be required in order to maintain the appropriate degree of association with band 3, spectrin, and other
proteins. This steady state phosphorylation may be required in order for the cell to achieve the mechanical properties appropriate for its high degree of flexibility and mechanical strength. Further work will be required to explore each of these possibilities.

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