Regulation of Linkages between the Erythrocyte Membrane and Its Skeleton by 2,3-Diphosphoglycerate*

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Ryuichi Moriyama, Christian R. Lombardo, Ryan F. Workman, and Philip S. Low§

From the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907-1393

In addition to reducing hemoglobin-O₂ affinity, 2,3-diphosphoglycerate (DPG) is known to modulate the mechanical properties of the erythrocyte membrane. By fluorescence spectroscopy and differential scanning calorimetry, we demonstrate that DPG binds the cytoplasmic domain of erythrocyte membrane band 3 in two stages characterized by apparent Kᵦ values of ~-2 and 12 mM. DPG was also shown to perturb the stability of ankyrin, protein 4.1, and protein 4.2 in situ and to directly bind to protein 4.1. In studies of membrane-skeleton interactions, DPG was observed to inhibit the fast and slow phases of ankyrin binding to band 3 and to reduce both the number of ankyrin sites and affinity of ankyrin for each class of site. The inhibition was biphasic, similar to the band 3-DPG binding isotherm; however, at physiological DPG concentrations a reduction in only 15% of the ankyrin sites was observed. In contrast, inhibition of protein 4.1 binding to the membrane reached 65% at physiological DPG concentrations (~5.9 mM); at more elevated concentrations, blockade was nearly quantitative, affecting glycoporphin and band 3 sites alike. Taken together with previous observations, these data suggest that DPG's effect on O₂ delivery may extend beyond its well recognized impact on hemoglobin-O₂ affinity.

Despite years of considerable progress, regulation of the shape, deformability, and stability of the human erythrocyte membrane is still not well understood. Early studies suggested that interactions within the spectrin-based membrane skeleton might account for most of the morphological and mechanical properties of the cell (Lux et al., 1979; Mohandas et al., 1983; Wolfe et al., 1982; Chasis et al., 1988a). Subsequent research, however, has revealed significant contributions from the lipid bilayer to maintenance of cell morphology (Daleke and Huestis, 1989; Lange et al., 1982). More recently, a critical role for the major linkages between the bilayer and the underlying skeleton in control of membrane stability has been demonstrated (Reid et al., 1987; Chasis et al., 1988b; Low et al., 1991; Mohandas et al., 1992; Liu et al., 1990). Thus, disruption or deletion of the interaction between ankyrin or protein 4.1 and their integral membrane protein anchors has been found to cause altered morphology, enhanced cell fragility, and sometimes hemolysis (Reid et al., 1989; Low et al., 1991; Agre et al., 1981). While the relative importance of the above membrane structural regions is still a matter of debate, it seems likely that the rheology and durability of the circulating erythrocyte will be affected by a significant perturbation of any of the three.

One factor that has been suspected of participating in the physiological regulation of membrane mechanical properties is the intracellular metabolite, 2,3-diphosphoglycerate (DPG). In the oxygenated erythrocyte, free DPG may rise to 5.9 mM, while in the deoxygenated cell, due to its association with deoxyhemoglobin, free diphosphoglycerate concentration may diminish to 0.5 mM (Bunn et al., 1971). When added to leaky membranes, DPG enhances the lateral diffusion of integral membrane proteins (Schindler et al., 1980) and enhances the fragility while reducing the deformability of the membrane (Chasis and Mohandas, 1986) without affecting its shear modulus or viscosity (Waugh, 1986). Furthermore, in isolated systems, DPG inhibits formation of the spectrin-actin-protein 4.1 ternary complex, i.e., the fundamental unit of the erythrocyte membrane skeleton (Cohen and Foley, 1984; Sheetz and Casaly, 1980). Perhaps for these reasons, DPG has been suspected of exacerbating the mechanical instability of poikilocytic and elliptocytes, especially in infants where free DPG is elevated due to the preponderance of fetal hemoglobin (Mentzer et al., 1987).

Because membrane mechanical properties were initially thought to derive almost exclusively from the membrane skeleton, once the effect of DPG on skeletal interactions was discovered, no further investigation into its mechanism of action was undertaken. However, since recent studies have documented that membrane-skeletal linkages may also be important, we have undertaken to examine whether these tethers might also be subject to DPG modulation. We report here that the two major attachment sites of the membrane skeleton to the bilayer are specifically dissociated by physiological concentrations of DPG. Taken together with previous data on spectrin-actin-protein 4.1 interactions, these results suggest that DPG may influence erythrocyte function far beyond its role in regulating hemoglobin-oxygen affinity.

EXPERIMENTAL PROCEDURES

Materials—Human blood was purchased from the Central Indiana Regional Blood Center and used within 1 week of its drawing date. Diasopropylfluorophosphate, DEAE-Sepharose, and bovine serum albumin were purchased from Sigma. Electrophoresis reagents were obtained from Bio-Rad. Acceill QMA anion exchange resin was from Millipore, Inc., and Sephacryl S-500 HR, Sephacryl S-500 HR, and Dextran T-500 were obtained from Pharmacia LKB Biotecnology Inc. PMSF and DTT were purchased from Research Organics, Inc., and sucrose was from Sigma/Mann. Chymotrypsin was obtained

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† Recipient of a Herman and Margaret Sokol Graduate Fellowship.

§ To whom correspondence and reprint requests should be addressed.

3 The abbreviations used are: DPG, 2,3-diphosphoglycerate; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; PBS, phosphate-buffered saline; TPCB, tosylphenylalanoyl chloromethyl ketone; BSA, bovine serum albumin; IOD, inside-out vesicle; cd3, cytoplasmic domain of band 3.
from Worthington, Bolton-Hunter reagent from ICN, and Triton X-100 from Boehringer Mannheim.

**Protein Purification**—Unless otherwise noted, all procedures were performed at 0–4 °C. Human blood (1 unit) was washed three times with PBS (138 mM NaCl, 1 mM EDTA, 5 mM NaHPO₄, 50 μg/ml PMSF, pH 7.5) and then sedimented at 1 × through 0.75% Dextran T-70 in PBS (0.14 M) on ice to remove white cells. Membranes were obtained by repeated washing with lysis buffer (5 mM NaHPO₄, 1 mM EDTA, 1 mM NaCl, 20 μg/ml PMSF, pH 8.0) in a Pellicon tangential flow filtration apparatus until the membranes were white. Ankyrin was purified from a 1.0 M KCl extract of Triton X-100 shells prepared from membranes using modifications of published procedures (Bennett, 1983; Pinder et al., 1989). Briefly, Triton X-100-insoluble membrane skeletons were extracted with 1.0 M KCl by Lombardo et al., (1992). The cytoplasmic domain of band 3 (cdb3) was isolated from freshly prepared IOVs that had been stripped with 0.17 M acetic acid (Bennett, 1983). Chymotryptic digestion of acetic acid-stripped IOVs was performed in 10 mM NaHPO₄, 0.2 mM DTT, and 10% methanol, pH 7.4, at −5 °C with 1.25 μg/ml chymotrypsin and 20 μg/ml 1-chloro-3-tosylamido-7-amino-2-heptanone for 25 min.

Digestions were terminated with 0.5 mM diisopropylfluorophosphate, and then the digestion with 1 mM diisopropylfluorophosphate, digested with 0.5 μg/ml TPCK-trypsin (Worthington) for 15 min on ice to remove the cytoplasmic domain of band 3. After terminating the digestion with 1 mM diisopropylfluorophosphate, digested stripped IOVs were collected and washed by centrifugation in protein 4.1 binding buffer (see below). Polyacrylamide gels and immunoblots of the digested IOVs revealed that >95% of the band 3 had been digested while less than 5% of the glycophorin C had been removed.

**Binding Assays**—Assays for the binding of ankyrin to K1-IOVs (Thevenin and Low, 1990) and protein 4.1 binding to pH 11 stripped IOVs (Lombardo et al., 1992) were performed as described. The composition of the ankyrin binding buffer was 50 mM NaHPO₄, 50 mM H₂BO₃, 30 mM NaCl, 1 mM EDTA, 0.2 mM DTT, 0.25 mg/ml BSA, 5% sucrose, pH 7.5 or pH 6.5. The concentrations of [131I]ankyrin and the K1-stripped IOVs used for these studies were 7 and 35 μg protein/ml, respectively. The composition of the protein 4.1 binding buffer was 130 mM KCl, 20 mM NaCl, 10 mM HEPES, 1 mM EDTA, 0.5 mM DTT, 1 mM NaCl, pH 7.5, and the concentrations of [131I]labeled protein 4.1 and pH 11 stripped IOVs required for these experiments were both 20 μg/ml. Before each binding assay, all tubes were coated with 10 mg/ml BSA in the appropriate binding buffer for 20 min, then rinsed with binding buffer lacking BSA, and finally allowed to air dry (Joy and Purich, 1990). This procedure significantly reduced variability in the data. Bound and free ligand were quantitated by centrifuging the IOVs through a sucrose cushion in binding buffer containing 2 mg/ml BSA. Tubes were frozen in liquid N₂, the tips containing the pellets were cut off with a heated razor, and then the tips were counted in a gamma counter (Thevenin and Low, 1990).

**Miscellaneous Methods**—Differential scanning calorimetry was performed as previously described (Davio and Low, 1982) in a Microcal-1 calorimeter (Amherst, MA) equipped with 1 ml of matched protein plus buffer containing 120 mM NaCl, 5 mM NaH₂PO₄, pH 7.4 (for membranes) or 10 mM imidazole acetate, 3 mM β-mercaptoethanol, 0.1 mM EDTA, pH 7.2 (for the isolated cytoplasmic domain of band 3). The heating rate was 1 °C/min. Measurement of intrinsic cdb3 fluorescence was monitored at 24 °C in ankyrin binding buffer in a Perkin-Elmer MFP-44 fluorospectrophotometer (Low et al., 1984). The excitation and emission wavelengths were 290 nm and 335 nm, respectively, and the slit widths were 6 nm. Samples of the cytoplasmic domain were preincubated with DPG for 30 min prior to fluorescence measurements.

Measurement of intrinsic protein 4.1 fluorescence was monitored while heating the protein from 20 °C to 80 °C at a rate of 2 °C/min in 4.1 binding buffer in a Perkin-Elmer LS50 fluorometer. The excitation and emission wavelengths were set at 272 and 348 nm, respectively, and the slit width was 5 nm. Samples containing 44 μg/ml protein 4.1 with or without DPG were incubated at 25 °C for 20 min prior to measurements.

**RESULTS**

**Effect of DPG on Erythrocyte Membrane Components**—To identify membrane components that might be regulated by 2,3-diphosphoglycerate, differential scanning calorimetry was performed on erythrocyte ghosts in the presence of 10 mM DPG. The usual five major membrane thermal transitions were observed (Fig. 1), each of which has been at least partially characterized (Brandts et al., 1977, 1978; Lysko et al., 1981; Davio and Low, 1982; Appell and Low, 1982). Importantly, all of the transitions, except D, were affected by DPG, but the magnitude of the perturbations differed among the various endotherms (Table I). The A transition, deriving from the denaturation of spectrin (Brandts et al., 1977; Lysko et al., 1981), was shifted down 1 °C in the presence of DPG. Although this perturbation is small, the destabilization is still real and may relate to the fact that DPG inhibits self-associations of the integral membrane proteins.
ation of spectrin dimers (Morrow and Marchesi, 1981). The $B_1$ transition, a poorly characterized endotherm arising from denaturation of ankyrin, protein 4.2, and protein 4.1, or more likely a complex of the three components (Lysko et al., 1981), is destabilized by DPG. Some decrease in the enthalpy of $B_1$ is also seen upon DPG treatment. Unfortunately, insufficient information on the nature of the $B_1$ endotherm precludes any physical interpretation of the perturbation other than to conclude that destabilization of some complex involving one or more of these proteins must result from DPG administration. The $B_1$ transition, unambiguously assigned to the cytoplasmic domain of band 3 (Appell and Low, 1982), was also shifted significantly ($\Delta T_m = -2.6{\degree}C$) by DPG, suggesting the metabolite may bind directly to cdb3. Again, the decrease in denaturation temperature implies that the metabolite destabilizes the cytoplasmic domain. The $C$ transition, in contrast, rises 1.8°$C$ in the presence of DPG, suggesting it is stabilized by the ligand. Since “C” is due to unfolding of the anion transport domain of band 3 (Davio and Low, 1982), the increase in $T_m$ may simply imply that band 3 is stabilized by substrate anions. Because the most prominent effects of DPG were those involving cdb3, ankyrin, band 4.1, and band 4.2, we felt an examination of the metabolite’s effects on these proteins and their interactions with the membrane deserved further scrutiny.

To determine whether DPG’s effect on the $B_1$ endotherm was due to a direct association with cdb3 or a perturbation of some tightly coupled protein, the impact of DPG on the structure of the isolated cytoplasmic domain of band 3 was examined. As shown in Fig. 2, 10 mM DPG also destabilizes purified cdb3 ($\Delta T_m = -5.5{\degree}C$), indicating the ligand indeed associates directly with the polypeptide. To obtain a more accurate determination of this interaction, the cytoplasmic domain of band 3 was titrated with increasing concentrations of DPG at room temperature, and its intrinsic fluorescence was measured. A shift in cdb3 tryptophan fluorescence has been previously shown to be a sensitive indicator of a change in cdb3 conformation (Low et al., 1984). As seen in Fig. 3, DPG causes a substantial decrease in cdb3 fluorescence and a concomitant shift in emission wavelength from 335 to 329 (not shown). The dramatic modulation of tryptophan fluorescence was reversible, independent of the pH of the medium near neutrality, and biphasic, with transitions at 2 and 12 mM comprising a 16% and 23% decrease, respectively, in the fluorescence of the protein. Taken together, the data of Figs. 1–3 demonstrate that DPG binds directly to cdb3 at physiological concentrations.

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**Fig. 2.** Differential scanning calorimetry of the isolated cytoplasmic domain of band 3 in the presence of DPG. Heat capacity is plotted as a function of temperature. Scans were performed in 10 mM imidazoleacetate, 5 mM $\beta$-mercaptoethanol, 0.1 mM EDTA, pH 7.2, at a protein concentration of 1.67 mg/ml.

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**Fig. 3.** The effect of DPG on the intrinsic fluorescence of the cytoplasmic domain of band 3. The isolated cdb3 was suspended at 50 µg/ml in ankyrin binding buffer, pH 7.5, containing various concentrations of DPG. After 30 min equilibration at 24°$C$, the intrinsic fluorescence ($\lambda_{ex}$ = 290 nm (excitation); $\lambda_{em}$ = 335 nm (emission)) of cdb3 was measured. Similar results were also obtained in separate studies using 35 and 100 µg/ml cdb3.

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**Effect of DPG on the Band 3: Ankyrin Interaction—**Since cdb3 links the membrane skeleton through interactions with ankyrin and possibly protein 4.1 (Bennett, 1985; Lombardo et al., 1992; Pasternack et al., 1985), we examined the ability of DPG to regulate the binding of these peripheral proteins to the membrane. Ankyrin binding to band 3 has been shown to occur in two kinetics phases (Thevenin and Low, 1990). The rapid phase, which reaches completion within 1.5 h of mixing, leads to formation of a low affinity complex whose stability is governed by the reversible, pH-dependent conformational equilibrium in band 3. The slow phase, requiring several hours to reach completion, generates a higher affinity complex whose stability appears to be independent of pH and the above cdb3 conformation. Because DPG could conceivably affect either of these two binding phases, we compared both the kinetics and equilibrium of ankyrin association with KI-IOVs in the presence and absence of DPG.

As shown in Fig. 4, DPG reduces ankyrin binding to KI-IOVs over the same concentration range where it associates with cdb3. Further, the concentration dependence of the inhibition displays a biphasic response reminiscent of the effect of DPG on cdb3 intrinsic fluorescence (compare Fig. 3). Most importantly, regardless of pH, the inhibitory effect is very mild, reducing the amount of ankyrin bound by only ~15% at the highest physiologically free DPG concentration of 5.9 mM (Bunn et al., 1971).

Comparison of the kinetics of ankyrin association with KI-IOVs in the presence and absence of 10 mM DPG reveals that both fast and slow phases of ankyrin binding are affected (Fig. 5). By ~160 min, a major reduction in ankyrin association has been realized, and as the reaction proceeds, the inhibition appears to continue, albeit in a somewhat mitigated manner. Assuming DPG concentrations rise and fall with each pass of the erythrocyte through the circulatory system, only the impact of DPG on the fast kinetic phase may have physiological relevance.

A more precise comparison of the influence of DPG on the low and high affinity complexes of ankyrin with band 3 is obtained by measuring the inhibition as a function of ankyrin concentration. As seen in the data of Fig. 6 and Table II, both high and low affinity sites are reduced in number. Furthermore, the affinity of ankyrin for each set of sites appears to
DPG Regulation of Ankyrin and Protein 4.1 Binding

FIG. 4. DPG inhibition of ankyrin binding to stripped IOVs. KI-IOVs (35 μg protein/ml) were incubated in a total volume of 120 μl with increasing concentrations of DPG for 30 min in ankyrin binding buffer, pH 7.5 (A), or pH 6.5 (B) prior to the addition of [125I]-labeled ankyrin (7 μg/ml). After addition of [125I]-labeled ankyrin, the incubations were allowed to proceed for 40 min, and then bound and free [125I]-labeled ankyrin were separated and quantitated as described under "Experimental Procedures."

FIG. 5. Effect of DPG on the kinetics of [125I]-labeled ankyrin binding to stripped IOVs. KI-IOVs and [125I]-labeled ankyrin were preincubated in the absence (closed circles) or presence (open circles) of 10 mM DPG in ankyrin binding buffer, pH 6.45. Samples were assayed for bound and free [125I]-labeled ankyrin at the times indicated in the figure and binding was quantitated as described under "Experimental Procedures." Other conditions were as indicated in the legend to Fig. 4.

be mildly reduced. Thus, the interaction of DPG, band 3, and ankyrin appears to be complex and not interpretable in terms of a single structural form or kinetic phase of the complex.

Effect of DPG on the Association of Band 4.1 with the Membrane—Protein 4.1 binds to multiple sites on the erythrocyte membrane. The most abundant site appears to be the cytoplasmic domain of band 3 (Pasternack et al., 1985; Danilov et al., 1991; Lombardo et al., 1992), while glycoporphin C (Reid et al., 1990) and possibly phosphatidyserine (Cohen et al., 1988; Sato and Oshini, 1983) also contribute to membrane association. To evaluate whether any of these interactions might be modulated by DPG, band 4.1 was incubated with stripped IOVs in the presence of increasing concentrations of DPG. As seen in Fig. 7, DPG inhibits protein 4.1 binding to the membrane with an apparent Ki of 4 mM and a maximum inhibition near 100%. At the highest free physiological DPG concentration of 5.9 mM, ~65% of band 4.1 binding was eliminated.

Because band 3 represents the predominant protein 4.1 site on stripped IOVs (compare top and middle curves of Fig. 8),
it can be concluded that the band 3-band 4.1 interaction is modulated by DPG. To confirm that the less prominent glycophorin C association is also inhibited by DPG, mildly digested stripped IOVs that lacked >95% of their band 3 sites but still retained their entire complement of glycophorin C were tested for DPG sensitivity. Addition of 10 mM DPG was also found to reduce protein 4.1 binding to the digested IOVs, decreasing the number of sites at high protein 4.1 concentration from 86 to 28 \( \mu \text{g/mg membrane protein} \). This ~66% inhibition of protein 4.1 binding to band 3-depleted membranes is similar to the ~77% inhibition obtained at the same DPG in band 3-containing IOVs (Fig. 7). It would thus appear that DPG modulates protein 4.1 binding to membrane sites unrelated to band 3 with roughly equal potency. Because the majority of these sites are thought to reside on glycophorin A or C, we suggest that a glycophorin interaction is also downregulated by DPG.

Finally, the fact that the interactions of protein 4.1 with one or more glycophorins and cdh3 are similarly inhibited by DPG suggests that DPG may bind protein 4.1 directly. Because sufficient quantities of protein 4.1 for calorimetry were difficult to obtain, this hypothesis was tested by examining the effect of DPG on the intrinsic fluorescence of protein 4.1. Although DPG had no effect on the fluorescence of protein 4.1 at room temperature (data not shown), DPG was found to modify the thermal quenching of protein 4.1 as the protein was heated. As shown in Fig. 9, in the absence of DPG protein 4.1 displays a transient increase in intrinsic fluorescence near 47 °C, probably due to a structural transition preceding or accompanying thermal denaturation. However, in the presence of 4 mM DPG this transition is measurably broadened and shifted to near 51 °C. By 10 mM DPG, the structural transition is no longer detected. For DPG to modify protein 4.1 unfolding in this manner, the two must obviously interact. Thus, DPG modulation of protein 4.1 association with IOVs may derive from a direct interaction of DPG with protein 4.1. It should also be noted that the apparent affinity of the interaction may permit the ligation state of protein 4.1 to change during fluctuations in DPG concentration that occur during normal oxygenation-deoxygenation cycles in the cell.

**DISCUSSION**

On a macroscopic scale, DPG increases red cell fragility and reduces erythrocyte deformability (Chasis and Mohandas, 1986). At the molecular level, DPG releases spectrin from membrane skeletons (Sheetz and Casaly, 1980), interferes with spectrin-actin-protein 4.1 association (Cohen and Foley, 1984), increases the lateral mobility of integral membrane proteins (Schindler et al., 1980), and decreases the number of connecting links between the membrane and its skeleton. Based on this information, DPG would seem to weaken virtually every major interaction stabilizing the membrane. Since...
most of the above effects occur at physiological DPG concentrations, albeit predominantly at the higher end, it is difficult to imagine that the metabolite's destabilizing effects did not evolve to improve the fitness of the erythrocyte. What remains puzzling is why it might be desirable to have a more fragile, less deformable cell when the blood is oxygenated (high free DPG) than when it is deoxygenated (i.e. when most of the DPG is bound by hemoglobin).

A second unresolved question is whether the 15% reduction in ankyrin binding seen at physiological DPG concentrations is sufficient to affect red cell function. Using pH to alter the number of ankyrin linkages in membranes and whole cells, we have shown that gradual elimination of ankyrin sites leads to a decrease in membrane mechanical stability (Low et al., 1991). Agre et al. (1981) have further demonstrated that the defect in two unrelated families with hemolytic poikilocytic anemia derives from a <50% diminution in the number of high affinity ankyrin sites on the membrane, suggesting a decrease in linkages of this magnitude significantly exceeds adaptive limits and pushes the cell toward hemolysis. The fact that involvement of less than 2% of the glycoporphin A population in new junctions with the membrane skeleton (presumably protein 4.1) leads to a 10-fold increase in membrane rigidity (Chasis et al., 1988b) also emphasizes the potential impact of minor changes in membrane-skeleton interactions on global cell behavior. Taken together, it seems reasonable that physiological modulations of the ankyrin and protein 4.1 linkages by DPG could affect red cell deformability. It should also be mentioned that maintenance of membrane mechanical stability may not represent the sole function of protein 4.1 and ankyrin. Thus, it is also conceivable that changes in ankyrin and protein 4.1 tethers may impact cell shape recovery processes (Reinhart et al., 1988; Jinbu et al., 1982) or band 3 aggregation reactions regulating cell removal (Kannan et al., 1991) and thereby affect other unrelated properties of the erythrocyte.

Although the function of ankyrin to tether spectrin to band 3 is quite well established (Bennett, 1985), the role of band 4.1 in red cell membrane architecture is not so unambiguously defined. While the association of protein 4.1 with glycoporphin C appears to be important for membrane stability (Chasis et al., 1988a; Reid et al., 1990), glycoporphin C is present in quantities sufficient to link only 25% of the protein 4.1 to the membrane. Since at least 80% of the protein 4.1 remains membrane-associated following removal of spectrin and actin (Bennett, 1989), it is currently believed that the remaining 4.1 is anchored primarily to band 3 (Pasternack et al., 1986; Danilov et al., 1990; Lombardo et al., 1992) and glycoporphin A (Anderson, 1989). What is peculiar about these latter interactions is that, among the potential membrane-skeletal linkages, they appear to be the most heavily regulated. Whereas the ankyrin-band 3 and the protein 4.1-glycoporphin C associations may be nearly constitutive, the interaction of protein 4.1 with these other sites has been shown to be up-regulated by phosphorylated phosphatidylinositol and down-regulated by protein kinase C (Anderson, 1989; Danilov et al., 1990). With addition of our current data, DPG can also be included as a modulator of these interactions.
Although the present data provide the first direct evidence for an interaction between DPG and both band 3 and protein 4.1, there has been one previous report implying at least an indirect association of DPG with protein 4.1. Thus, Chao and Tao (1991) have shown that DPG enhances the phosphorylation of protein 4.1 by protein kinase C even though it inhibits the activity of protein kinase C toward other substrates. Not only was the rate of protein 4.1 phosphorylation increased by DPG, but the number of phosphates per protein 4.1 was also doubled. It is conceivable that this phosphorylation might further modulate protein 4.1 interactions in vivo, but in our purified systems lacking protein kinase C, only a direct binding effect can account for part of the cytoplasmic domain’s sequence divergence.

REFERENCES
Agre, P., Orringer, E. P., Chen, D. H. K., and Bennett, V. (1986) *J. Clin. Invest.* 88, 1566-1576
Anderson, R. A. (1989) in *Red Blood Cell Membranes: Structure, Function, Clinical Implications* (Agre, P., and Parker, J. C., eds) pp. 187-236, Marcel Dekker, Inc., New York
Appell, K. C., and Low, P. S. (1982) *Biochemistry* 21, 2151-2157
Bennett, V. (1983) *Methods Enzymol.* 96, 313-324
Bennett, V. (1985) *Annu. Rev. Biochem.* 54, 273-304
Bennett, V. (1988) *Biochim. Biophys. Acta* 988, 107-121
Bolton, A. E., and Hunter, W. M. (1973) *Biochem. J.* 133, 529-536
Brandts, J. F., Lysko, K. A., Schwartz, A. T., and Taverna, R. D. (1977) *Biochemistry* 16, 3450-3454
Brandts, J. F., Taverna, R. D., Sadasivan, E., and Lysko, K. A. (1978) *Biochim. Biophys. Acta* 512, 560-578
Burn, H. P., Ransil, B. J., and Chao, A. (1971) *J. Biol. Chem.* 246, 5273-5279
Chao, T.-S., and Tao, M. (1991) *Arch. Biochem. Biophys.* 285, 221-226
Chasis, J. A., and Mohandas, N. (1986) *J. Cell Biol.* 103, 345-350
Chasis, J. A., Agre, P., and Mohandas, N. (1988) *J. Clin. Invest.* 82, 617-623
Chasis, J. A., Reid, M. E., Jensen, R. H., and Mohandas, N. (1988b) *J. Cell Biol.* 107, 1351-1357
Cohen, A. M., Liu, S. C., Lawler, J., Derick, L., and Palek, J. (1988) *Biochemistry* 27, 614-619
Cohen, C. M., and Foley, S. F. (1984) *Biochemistry* 23, 6091-6098
Daleke, D. L., and Huestis, W. H. (1989) *J. Cell Biol.* 108, 1375-1385
Danilov, Y. N., Fennell, R., Ling, E., and Cohen, C. M. (1990) *J. Biol. Chem.* 265, 2556-2562
Davio, S. R., and Low, P. S. (1982) *Biochemistry* 21, 3585-3593
Jinbu, Y., Sato, S., Nakao, T., and Nakao, M. (1992) *Biochem. Biophys. Res. Commun.* 104, 1087-92
Joly, J. C., and Purich, D. L. (1990) *Biochemistry* 29, 8916-8920
Kannan, R., Yuan, J., and Low, P. S. (1991) *Biochem. J.* 278, 57-62
Kopito, R. R. (1990) *Int. Rev. Cytol.* 123, 177-199
Lange, Y., Hadesman, R. A., and Steck, T. L. (1983) *J. Cell Biol.* 92, 714-721
Liu, S. C., Zhai, S., Palek, J., Golan, D. E., Amato, D., Hassan, K., Nurse, G. T., Babone, D., Coetzer, T., Jarolim, P., Zaik, M., and Borwein, S. (1990) *N. Engl. J. Med.* 322, 1530-1538
Lombardo, C. R., Willardson, B. M., and Low, P. S. (1992) *J. Biol. Chem.* 267, 9540-9546
Low, P. S., Westfall, M. A., Allen, D. P., and Appell, K. C. (1984) *J. Biol. Chem.* 259, 13070-13076
Low, P. S., Willardson, B. M., Mohandas, N., Rossi, M., and Shohet, S. (1991) *Blood* 77, 1581-1586
Luo, S. E., Pease, B., Tomasselli, M. H., John, K. M., and Bernstein, S. E. (1979) *Normal and Abnormal Red Cell Membranes* pp. 463-469, Alan R. Liss, Inc., New York
Lysko, K. A., Carlson, R., Taverna, R. D., Snow, J., and Brandts, J. F. (1981) *Biochemistry* 20, 5570-5576
Mentzer, Jr., W. C., Iarocci, T. A., Mohandas, N., Lane, P. A., Smith, B., Larsson, J., and Huy, T. (1987) *J. Clin. Invest.* 79, 942-949
Mohandas, N., Chasis, J. A., and Shohet, S. H. (1983) *Semin. Hematol.* 20, 225-242
Mohandas, N., Winardi, R., Knowles, D., Leung, A., Parra, M., George, E., Cowboy, J., and Chasis, J. A. (1992) *J. Clin. Invest.* 89, 686-692
Morrow, J. S., and Marchesi, V. T. (1986) *J. Cell Biol.* 108, 463-468
Patterson, C. G., Anderson, R. A., Leto, T. L., and Marchesi, V. T. (1985) *J. Biol. Chem.* 260, 3676-3683
Pinder, J. C., Smith, K. S., Pfekrun, A., and Grutzer, W. B. (1989) *Biochem. J.* 253, 429
Reid, M., Chasis, J., and Mohandas, N. (1987) *Blood* 69, 1068-1072
Reid, M. E., Takakawa, Y., Chermni, G., Jensen, R. H., Chasis, J. A., and Mohandas, N. (1989) *The Red Cell Seventh Ann Arbor Conference*, pp. 553-575, Alan R. Liss, Inc., New York
Reid, M. E., Takakawa, Y., Cowboy, J., Chermni, G., and Mohandas, N. (1990) *Blood* 75, 2239-2234
Reinhart, W. H., Sung, L.-P. A., Sung, K.-L., Bernstein, S. E., and Chein, S. (1984) *Am. J. Hematol.* 19, 185-200
Sato, S. B., and Oshimi, S. (1983) *Eur. J. Biochem.* 130, 19-25
Schnider, M., Koppel, D. E., and Schoetz, M. P. (1980) *Proc. Natl. Acad. Sci. U. S. A.* 77, 1457-1461
Sheetz, M. P., and Casaly, J. (1980) *J. Biol. Chem.* 255, 9985-9980
Tveeven, B. J. M., and Low, P. S. (1980) *J. Biol. Chem.* 255, 16168-16172
Tyler, J. M., Hargreaves, W. R., and Brantzen, D. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 5192-5196
Wright, R. E. (1980) *Blood* 68, 231-238
Wolfe, L. C., John, K. M., Falcone, J. C., Byrne, A. M., and Lux, S. E. (1982) *N. Engl. J. Med.* 307, 1367-1374