Localization of the protein 4.1-binding site on human erythrocyte glycoporphins C and D

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The flexibility of the human erythrocyte membrane is mediated by an underlying network of skeletal proteins which interact with the membrane through ankyrin and protein 4.1. The nature of the membrane attachment site(s) for protein 4.1 has yet to be fully elucidated. In this paper we show that purified protein 4.1 binds much less strongly to alkali-stripped membranes from erythrocytes of individuals with total glycoporphin C and D deficiency (Leach phenotype) than to alkali-stripped normal membranes. We further show that a synthetic peptide corresponding to amino acid residues 82–98 of the cytoplasmic

domain of glycoporphin C specifically binds to purified protein 4.1 and inhibits protein 4.1 binding to alkali-stripped normal membranes. The same synthetic peptide binds directly to membranes from individuals with glycoporphin C and D deficiency but not to normal membranes. These results indicate that glycoporphins C and D provide major membrane attachment sites for protein 4.1 in normal erythrocytes and that this interaction is mediated by protein 4.1 binding to amino acid residues 82–98 of glycoporphin C and 61–77 of glycoporphin D.

INTRODUCTION

The human erythrocyte relies on a network of proteins underlying the membrane to maintain its structural integrity and deformability. This network, known as the skeleton, consists predominantly of spectrin, actin and protein 4.1, as well as the minor components protein 4.2, protein 4.9, tropomyosin and myosin (reviewed in Bennett, 1985). The skeleton is anchored to the membrane through at least two protein interactions involving ankyrin and protein 4.1 respectively. While the ankyrin–band-3 link has been well studied (Bennett, 1985), the nature of the protein 4.1 membrane-attachment site is less clear. Possible candidates include band 3 (Pasternack et al., 1985; Jons and Drenckhahn, 1992; Lombardo et al., 1992), glycoporphin C/D (GPC/D) (Mueller and Morrison, 1981; Alloisio et al., 1985; Reid et al., 1990a), glycoporphin A (Lovrien and Anderson, 1980) and the lipid bilayer (Sato and Ohnishi, 1983; Cohen et al., 1988).

A number of workers have provided evidence for an interaction between protein 4.1 and GPC/D. Erythrocytes lacking GPC and GPD (Leach phenotype) are elliptocytic (Anstee et al., 1984; Daniels et al., 1986), suggesting that these proteins play a role in regulating the stability and mechanical properties of normal erythrocytes. An attachment to the skeleton is likely, as GPC and GPD remain firmly associated with the skeletal proteins after extraction of erythrocyte membranes with non-ionic detergent (Mueller and Morrison, 1981). Evidence suggesting that the site of attachment is protein 4.1 arose from the findings that GPC levels are dramatically reduced in patients with homozygous protein 4.1 deficiency (Mueller and Morrison, 1981; Alloisio et al., 1985) and, more recently, that protein 4.1 regulates the GPC content of human erythrocytes (Reid et al., 1990a). In this paper, we provide direct evidence of an interaction between protein 4.1 and GPC/D and show that the protein 4.1-binding region of GPC/GPD is contained within residues 82–98 of GPC (61–77 of GPD).

MATERIALS AND METHODS

Materials

Fresh normal erythrocytes were available from Blood Services South West, Southmead Road, Bristol BS10 5ND, U.K. Fresh Leach-phenotype erythrocytes were obtained from donor PL (Anstee et al., 1984). Leach-phenotype erythrocytes from donors KW (Anstee et al., 1984), SS, JJ and TL (Daniels et al., 1986) were available as frozen cells. Erythrocytes from individuals heterozygous and homozygous for the Memphis variant of band 3 were obtained from Dr. Y. Okubo, Japanese Red Cross Transfusion Centre, Osaka, Japan. Rabbit anti-(protein 4.1) antibody was a gift from Professor N. Mohandas, Lawrence Berkeley Laboratories, Berkeley, San Francisco, CA, U.S.A.

Chemicals for peptide synthesis were purchased from Millipore (Watford, Herts., U.K.). Peptide cross-linkers were from Pierce and Warriner (Chester, Cheshire, U.K.).

Peptide synthesis and coupling to carriers

GPC peptides corresponding to amino acid residues 112–128 (Asp-Pro-Ala-Leu-Gln-Asp-Ala-Gly-Asp-Ser-Ser-Arg-Lys-Glu-Tyr-Phe-Ile) (GPC peptide 1), 99–111 (Thr-Glu-Phe-Ala-Glu-Ser-Ala-Asp-Ala-Ala-Leu-Gln-Gly) (GPC peptide 2) and 82–98 (Arg-Tyr-Met-Tyr-Arg-His-Lys-Gly-Thr-Tyr-His-Thr-Asn-Glu-Ala-Lys-Gly) (GPC peptide 3) were synthesized using fluoren-9-ylmethoxycarbonyl (Fmoc)-protected amino acids (Atherton et al., 1981), as preactivated pentafluorophenyl esters (Kisfaludy and Schon, 1983), with a continuous-flow solid-phase polyamide resin (Dryland and Sheppard, 1986), on a Milligen 9050 PepSynthesizer. The synthesized peptides were cleaved from the resin and deprotected with trifluoroacetic acid/ethanedithiol/water/phenol (93:2.5:2.5:2, by vol.) for 2 h at 25 °C. The mixture was extracted three times with a large excess of petroleum ether (60–80 b.p.) and precipitated with diethyl ether. The peptides were redissolved in water and their purity

was assessed by h.p.l.c., amino acid sequencing and analysis (Gardner et al., 1991).

The peptides were coupled to keyhole-limpet haemocyanin (KLH) or BSA using a modification of the method of Green et al. (1982). KLH (10 mg) was reacted with 2 mg of the cross-linker sulphosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate for 30 min at room temperature. Excess cross-linker was removed on a Sephadex G-25 column equilibrated with argon-purged 50 mM sodium phosphate buffer (pH 6.0). In parallel, approx. 8 mg of peptide was reacted with 1 mM dithiothreitol (DTT) for 1 h at room temperature, and excess DTT removed on a Sephadex G-10 column equilibrated in argon-purged sodium phosphate buffer (pH 5.0). Column eluates were monitored at 230 nm. The reduced peptide was added slowly to the activated KLH, the pH was adjusted to 7.0 and the reaction was allowed to proceed for 3 h at room temperature. Coupling to BSA was performed as for KLH except that *N*-maleimidobenzoyl-*N*-hydroxysulphosuccinimide was used as a cross-linker, and the peptide was reduced, desalted and stored on ice under argon before the reaction of BSA with cross-linker.

Concentrations of uncoupled peptides used for inhibition studies were assayed by amino acid analysis.

Production of antisera

Rabbits were given one subcutaneous injection of either KLH-GPC peptide 2 or KLH-GPC peptide 3 (100 µg) in an equal volume of Freund's complete adjuvant. Subsequent injections (100 µg of KLH peptide in an equal volume of Freund's incomplete adjuvant) were given at fortnightly intervals. Animals were bled from the marginal ear vein 10 days after each booster. A pre-immune bleed was taken before the first immunization. Serum was recovered from blood after clot retraction and heated to 56 °C for 10 min. Antisera [anti-(GPC peptide-2) and anti-(GPC peptide-3)] were exhaustively absorbed with membranes from Leach-phenotype erythrocytes before use.

Immunoblotting

Erythrocyte membranes were electrophoretically separated under reducing conditions on a 10% (w/v) acrylamide homogeneous Laemmli gel. Immunoblotting was as described by Mallinson et al. (1986), except that 5% (w/v) bovine milk powder, resuspended in PBS at pH 7.3, was used as the blocking agent.

Purification of protein 4.1

Protein 4.1 was isolated using a modified method of Tyler et al. (1979). Washed 'ghosts' were mixed with 30 vol. of PBS (pH 7.4) and incubated on ice for 30 min to remove band 6. After centrifugation (17000 *g*; 10 min), the pellet was washed with 0.3 mM sodium phosphate/0.1 mM EDTA/0.1 mM phenylmethanesulphonyl fluoride (PMSF), pH 9.0. The pellet was resuspended in this buffer and incubated at 37 °C for 30 min. Spectrin-depleted vesicles were obtained after centrifugation (17000 *g*; 3 h). The vesicles were resuspended in 210 ml of 5 mM sodium phosphate/1 mM EDTA/20 mM KCl/0.5 mM DTT, pH 7.6, brought to 1 M KCl and incubated at 37 °C for 30 min before centrifugation (55000 *g*, 95 min). The resulting supernatant, containing protein 4.1 and ankyrin, was dialysed overnight against 5 mM sodium phosphate/1 mM EDTA/20 mM KCl/0.5 mM DTT, pH 7.6, at 4 °C. It was then mixed with DEAE-cellulose (DEAE-Sephacyl, Sigma) for 2 h at 4 °C, centrifuged (2500 *g*, 5 min) and then the DEAE-cellulose was used to

prepare a column. The proteins were eluted sequentially by means of a 50–200 mM KCl gradient.

Alkali-stripping of human erythrocyte membranes

Leaky membranes were prepared by the method of Dodge et al. (1963). Membranes were incubated with 2 mM PMSF at 0 °C for 5 min, before stripping of peripheral proteins by washing with ice-cold 0.1 M NaOH. They were then washed three times with 5 mM sodium phosphate buffer, pH 8.0, at 4 °C.

Low-ionic-strength extraction of protein 4.1 from Leach-phenotype membranes

Leaky membranes were prepared by the method of Dodge et al. (1963). After washing in 0.3 mM sodium phosphate/0.1 mM EDTA/0.1 mM PMSF/0.1 mM 2-mercaptoethanol, pH 8.0, the membranes were incubated in the same buffer for 30 min at 37 °C, then centrifuged at 93000 *g* for 25 min. The resulting supernatant contained the extracted proteins.

Quantification of protein 4.1 bound to alkali-stripped erythrocyte membranes

The wells of a round-bottomed microtitre plate (Sterilin) were coated with 75 µl of lectin (from *Agaricus bisporus*; Sigma) (7 µg/ml in 20 mM sodium phosphate buffer, pH 7.0) and incubated for 1 h at room temperature (Scott, 1992). All other procedures were carried out on ice and all washes were in PBS, pH 7.4/0.055% Tween. The lectin-coated plate was washed and 75 µl of a 5% suspension of alkali-stripped membranes was added and the plate centrifuged at 930 *g* for 2 min. The lectin, from *Agaricus bisporus*, binds O-glycosidically linked oligosaccharides having the structure galactose β (1→3)*N*-acetyl-galactosamine-serine (or threonine) (Presant and Kornfeld, 1972). These oligosaccharides are located on several glycoproteins present on the extracellular surface of erythrocytes. Untreated and stripped membranes are permeable, thus allowing protein 4.1 and antibodies access to the intracellular surface of the membranes. Protein 4.1 (75 µl per well) was added and the plate incubated overnight at 4 °C, washed and incubated with rabbit anti-(protein 4.1) (75 µl/well for 2 h). After washing, the secondary antibody [pig anti-(rabbit peroxidase) conjugate, from DAKO] was added and incubated for 2 h on ice. After further washing, 75 µl of substrate was added (1 mg of *o*-phenylenediamine and 0.4 µl of H₂O₂ in 1 ml of 0.05 M citrate-phosphate buffer, pH 5.0). The colour reaction was terminated by the addition of 75 µl of 1 M HCl after 3 min. The plate was read on a Titertek Multiskan e.l.i.s.a. reader (Flow Laboratories) at 492 nm. Dilutions of protein 4.1 were used to produce a standard curve in order to quantify the amount of bound material in each assay.

Quantification of binding of GPC peptides to purified protein 4.1

Purified protein 4.1 was diluted to 150 µg/ml in 50 mM sodium carbonate buffer, pH 9.6, and incubated directly with the microplates (75 µg/well) for 15 min at 37 °C. All other procedures were carried out on ice and all washes were in PBS, pH 7.4/0.055% Tween 20. After washing, GPC peptide (75 µg/well) was added to the plate, incubated overnight at 4 °C, washed and incubated with the relevant anti-(GPC peptide) (75 µg/well, 2 h). After washing, the secondary antibody [pig anti-(rabbit peroxidase) conjugate; DAKO] was added and incubated for 2 h. After further washing, the plate was developed as described above.

Quantification of binding of GPC peptides to membranes from erythrocytes of the Leach phenotype

The plates were coated with lectin, as described for quantification of protein 4.1 bound to alkali-stripped erythrocyte membranes. All procedures were carried out on ice and all washes were in PBS, pH 7.4/0.055% Tween 20. The lectin-coated plate was washed and 75 μ l of a 1% suspension of leaky membranes was added and the plate centrifuged at 930 *g* for 2 min. GPC peptides (75 μ l/well) were added and the plate incubated overnight, washed and incubated with the relevant anti-(GPC peptide) (75 μ l/well, 2 h). After washing, the secondary antibody [pig anti-(rabbit peroxidase) conjugate; DAKO] was added and incubated for 2 h. After further washing, the plate was developed as described above.

Inhibition of protein 4.1 binding to alkali-stripped erythrocyte membranes

Serial dilutions of synthetic peptides were made in 3% (w/v) BSA and incubated with an equal volume of protein 4.1 (final concentration 150 μ l/ml) overnight at 4 °C before addition to the microtitre plate.

RESULTS

Low-ionic-strength extraction of protein 4.1 from Leach-phenotype erythrocyte membranes

Under low-ionic-strength conditions, spectrin and actin were readily released from normal membranes together with small quantities of protein 4.1. When Leach-phenotype erythrocyte membranes were used, spectrin and actin were extracted in similar amounts to normal, but there was a 3-fold increase in protein 4.1 released (Figure 1). These results confirm those of Pinder (1991) and clearly indicate that Leach-phenotype membranes lack a binding site for protein 4.1 which is present in normal membranes.

Binding of protein 4.1 to alkali-stripped membranes from normal and Leach-phenotype erythrocyte membranes

The ability of purified protein 4.1 (Figure 2b) from normal membranes to re-associate with alkali-stripped normal and Leach-phenotype membranes was studied using an e.l.i.s.a. assay (see the Materials and methods section). Figure 3 shows that protein 4.1 bound saturably to stripped normal membranes, with a binding capacity of 225 ± 3 μ g of protein 4.1 bound/mg of vesicle protein (Figure 3a) and an apparent K_d of 6.25×10^{-8} M (Figure 3b). In contrast, protein 4.1 binding to stripped Leach-phenotype membranes was reduced to 42.5 ± 5 μ g of protein 4.1 bound/mg of membrane protein (Figure 3a) with an apparent K_d of 1.25×10^7 M (Figure 3b). These results clearly demonstrate that substantially more protein 4.1 bound to stripped normal membranes than bound to stripped Leach-phenotype membranes, and that stripped normal membranes possess a high-affinity binding site that is lacking in stripped Leach-phenotype membranes.

Characterization of rabbit antisera to synthetic peptides comprising regions of the cytoplasmic domain of GPC/D

In order to examine more precisely the region of GPC/D involved in protein 4.1 binding, three peptides were synthesized. Peptide 1 corresponded to GPC amino acid residues 112–128, peptide 2 corresponded to residues 99–111 and peptide 3 to residues 82–98. The three peptides together comprise the entire linear sequence

of the cytoplasmic domain common to GPC and GPD. In the present study rabbit antisera for peptides 2 and 3 were produced. A rabbit antiserum and mouse monoclonal antibody (BGRL 100) to peptide 1 have been described previously (Reid et al., 1990b). Each rabbit antiserum was shown to react specifically with the appropriate peptide by dot-blot analysis of peptide coupled to BSA and with the free peptide by e.l.i.s.a. The pre-immune sera did not react with peptide coupled to BSA or free peptide. The antisera did not cross-react with the other synthetic peptides. The antisera were exhaustively absorbed with membranes from Leach-phenotype erythrocytes, and thus did not

Figure 1 Densitometry analysis of low-ionic-strength extracts from normal (●) and Leach-phenotype (PL) (△) membranes

Samples were run on a 4–16% gradient gel, stained with Coomassie Blue and scanned at 530 nm using a Joyce Loebl Chromoscan 3 densitometer.

Figure 2 SDS/PAGE analysis of membranes and purified protein 4.1 and immunoblotting of membranes with anti-GPC1 antibody

Samples in lanes a and b were run on a 4–16% polyacrylamide gradient Laemmli gel and stained with Coomassie Blue. Samples in lanes c and d were run on a 10% homogeneous Laemmli gel, transferred to nitrocellulose paper and probed with anti-GPC1 (as described in the Materials and methods section). Lane a, membranes; lane b, purified protein 4.1; lane c, normal membranes; lane d, Leach-phenotype membranes

Figure 3 Protein 4.1 binding to alkali-stripped normal and Leach-phenotype membranes

(a) Binding of protein 4.1 was measured as described in the Materials and methods section. ●, Normal; △, Leach. Results are plotted with error bars corresponding to mean $\pm$ S.D. ($n = 5$). The values for Leach-phenotype membranes represent the results of five experiments using erythrocytes from five different individuals of Leach phenotype (PL, KW, SS, JJ, TL). (b) Scatchard plot of binding of protein 4.1 to normal (●) and Leach-phenotype (△) membranes.

react with Leach-phenotype erythrocyte membranes or alkali-stripped Leach-phenotype membranes. However, the antisera did react with normal erythrocyte membranes and alkali-stripped normal membranes by e.i.s.a. While anti-GPC1 was reactive with GPC/D by immunoblotting (Figures 2c and 2d), anti-GPC2 and anti-GPC3 were non-reactive. These results demonstrate that the rabbit antisera were specific for GPC/D in normal erythrocyte membranes. The binding of rabbit antisera to normal membranes and alkali-stripped normal membranes was abolished when the antisera were preincubated with the synthetic peptide to which they were raised, but was unaffected by preincubation with the other synthetic peptides (results not shown). These results demonstrate that the antibodies produced are specific for the peptide to which they were raised and that they recognize the appropriate peptide in its native conformation.

Figure 4 Inhibition of protein 4.1 binding to alkali-stripped normal membranes

Protein 4.1 (150 μg/ml) was incubated with GPC peptide for 5 h at 4 °C before incubation with stripped normal membranes. Binding was measured as described in the Materials and methods section. Results are plotted with error bars corresponding to mean $\pm$ S.D. ($n = 5$). ■, GPC peptide 1; △, GPC peptide 2; ●, GPC peptide 3.

Figure 5 Binding of synthetic peptides to protein 4.1

GPC peptides were incubated with protein 4.1 (150 μg/ml) for 1 h at room temperature. Binding was measured as described in the Materials and methods section. Results are plotted with error bars corresponding to mean $\pm$ S.D. ($n = 6$). ■, GPC peptide 1; △, GPC peptide 2; ●, GPC peptide 3.

Localization of the protein 4.1-binding site on GPC/D

The localization of the protein 4.1-binding site on GPC/D was examined in three experiments. The first experiment assessed the ability of peptides 1, 2 and 3 to inhibit the binding of purified protein 4.1 to alkali-stripped normal membranes. The results clearly demonstrate a substantial inhibition of binding by peptide 3 (Figure 4). Some inhibition (maximum 20%) was observed with peptide 1 and at higher concentrations of peptide 2.

Figure 6 Binding of synthetic peptides to Leach-phenotype membranes

GPC peptides were incubated with Leach-phenotype membranes (30 μg/ml) overnight at 4 °C. Binding was as described in the Materials and methods section. Results are plotted with error bars corresponding to mean ± S.D. ($n = 4$). ■, GPC peptide 1; △, GPC peptide 2; ●, GPC peptide 3.

In the second experiment purified protein 4.1 was used to directly coat microplate wells and the ability of synthetic peptides 1, 2 and 3 to bind to protein 4.1 was examined. The results (Figure 5) clearly showed a concentration-dependent binding of peptide 3 to bound protein 4.1. The binding was saturable at 12 μg of peptide 3/mg of protein 4.1. This compares with a theoretical value of 15 μg of peptide 3/mg of protein 4.1, assuming one peptide 3-binding site per molecule of protein 4.1. Some binding of peptide 2 was apparent, but this was consistently much lower than that of peptide 3. No significant binding of peptide 1 was observed at peptide concentrations as high as 20 μg/ml.

The third experiment examined the binding of peptides 1, 2 and 3 to membranes of the Leach phenotype. The results showed that peptide 3 bound strongly and specifically to Leach-phenotype membranes (Figure 6). Maximal binding was observed at 0.6 μg of GPC peptide 3/mg of Leach-phenotype membranes. This binding was not observed after alkali-stripping of Leach-phenotype membranes (results not shown). Control experiments showed that none of the peptides bound directly to normal membranes (results not shown).

These results clearly indicate that the protein 4.1-binding site on GPC/D is located in the region of peptide 3 and that peptide 3 can bind to protein 4.1 in Leach-phenotype membranes.

Residual protein 4.1 binding to Leach-phenotype membranes is unrelated to band 3 Memphis

The results presented in Figure 3 indicate that under the assay conditions employed some protein 4.1 can bind to alkali-stripped Leach-phenotype membranes, albeit with lower affinity than binding to GPC/D. Several alternative binding sites for protein 4.1 have been proposed [band 3 (Pasternack et al., 1985; Lombardo et al., 1992; Jons and Drenkhahn, 1992), glyophorin A (Lovrien and Anderson, 1980) and phosphatidylserine (Cohen et al., 1988)]. The best-studied of these is that involving band 3. During the course of the present work it became apparent that

the original Leach-phenotype propositus (PL) is heterozygous for the band 3 Memphis variant. As the Memphis variant is known to result from a Lys-56 → Glu change in the N-terminal cytoplasmic domain of band 3 (Yannoukakos et al., 1991), and this domain of band 3 has been implicated as containing the protein 4.1-binding site (Lombardo et al., 1992; Jons and Drenkhahn, 1992), it was necessary to explore the significance of the Memphis variant in our reassociation assays. Protein 4.1-binding assays using alkali-stripped membranes from four other individuals having the Leach phenotype but lacking the Memphis variant of band 3 gave comparable results to those obtained with the Leach-phenotype cells of donor PL (Figure 3). These results clearly show that the band 3 Memphis variant does not affect protein 4.1 binding in the assays employed. Similarly, no alteration of protein 4.1 binding was observed when alkali-stripped normal membranes from a donor homozygous for the Memphis variant was examined (results not shown).

DISCUSSION

There seems little doubt that the protein 4.1-GPC/D interaction has physiological significance *in vivo*. There are numerous reports of shape abnormalities in erythrocytes deficient in either of the components (Anstee et al., 1984; Alloisio et al., 1985; Conboy et al., 1986; Reid et al., 1987; McGuire et al., 1988). Nevertheless, direct evidence for protein 4.1-GPC/D interaction is lacking. The study of erythrocytes from individuals of the rare Leach phenotype, which have a total deficiency of GPC/D, offers an attractive experimental approach to this problem. In this paper we have confirmed the observation of Pinder (1991) that protein 4.1 is more readily extracted from Leach-phenotype membranes than normal membranes. We have also compared the binding of purified protein 4.1 to alkali-stripped normal membranes with that to alkali-stripped membranes of the Leach phenotype. The results (Figure 3) show that alkali-stripped normal membranes bind 225 ± 3 μg of protein 4.1/mg of membrane protein at saturation, while only 42.5 μg of protein 4.1 is bound per mg of stripped membrane of Leach phenotype. Furthermore, the binding affinity of protein 4.1 to Leach-phenotype membranes is approx. 50 times lower than to normal membranes (1.25×10^{-7} M compared with 6.25×10^{-8} M for normal membranes). It is clear that under the conditions of this experiment GPC/D in normal membranes provides a major high-affinity binding site for protein 4.1 as the absence of GPC/D is the only known difference between Leach-phenotype membranes and normal membranes.

In an attempt to localize the region of GPC/D involved in protein 4.1 binding, three synthetic peptides corresponding to different regions of the cytoplasmic domain of GPC/D were prepared. The results show that peptide 3 inhibited the binding of protein 4.1 to alkali-stripped normal membranes (Figure 4) and that the same peptide binds directly to purified protein 4.1 (Figure 5). Furthermore, peptide 3 binds directly to membranes of the Leach phenotype, indicating that at least a proportion of protein 4.1-binding sites are unoccupied in these membranes. This is consistent with the finding that protein 4.1 is more readily extracted from Leach-phenotype membranes than normal membranes. GPC peptide 3 corresponds to residues 82–98 of GPC and 61–77 of GPD. This region of GPC/D contains the sequence YRHKG which has recently been suggested as a possible binding site for protein 4.1 by analogy with experimental evidence, indicating that the binding site for protein 4.1 on band 3 contains a triplet of similarly charged residues flanked by residues with hydrophobic side chains (LRRRY/IRRRY) (Jons and Drenkhahn, 1992).

These results provide clear evidence that GPC/D is a major

protein 4.1-binding site in normal erythrocyte membranes. However, the amount of protein 4.1 bound to GPC/D *in vivo* is more difficult to determine. Under the conditions of our protein 4.1 reassociation assay $225 \pm 3 \mu\text{g}$ of protein 4.1 binds/mg of alkali-stripped membrane protein, whereas we detect $59 \pm 4 \mu\text{g}$ of endogenous protein 4.1/mg of membrane protein in normal membranes. These figures suggest that the protein 4.1-binding capacity of alkali-stripped membranes is much higher than normal membranes, and are consistent with those of Steck (1974) and Cohen and Foley (1982).

One approach to determining the amount of protein 4.1 bound to GPC/D in normal erythrocytes is suggested by our observation that peptide 3 binds directly to Leach-phenotype membranes. If it is assumed that all of the peptide 3 bound to Leach-phenotype membranes is binding to sites on protein 4.1, that 1 mg of protein 4.1 binds $12 \mu\text{g}$ of peptide 3 at saturation (Figure 5) and that $0.6 \mu\text{g}$ of peptide 3 binds/mg of Leach-phenotype membrane protein at saturation (Figure 6), then $50 \mu\text{g}$ of protein 4.1/mg of protein would be bound in Leach-phenotype membranes. We have measured the protein 4.1 content of Leach-phenotype membranes as $572 \mu\text{g}/\text{mg}$ of membrane protein. These results suggest that most, if not all, of the protein 4.1 in Leach-phenotype membranes is available to bind GPC peptide 3. Until recently it was believed that there was insufficient GPC/D in normal membranes to bind all of the available protein 4.1 (200000 copies/cell). However, studies using Fab fragments from several GPC monoclonal antibodies suggest that there are around 150000 copies of GPC and 50000 copies of GPD (Smythe et al., 1992) and so this is not a limiting factor.

These results are in marked contrast with several studies which have considered the N-terminal cytoplasmic domain of band 3 to be the major protein 4.1-binding site in normal erythrocytes (Pasternack et al., 1985; Danilov et al., 1990; Lombardo et al., 1992). Clearly, further studies are required to elucidate the respective roles of band 3 and GPC/D in binding protein 4.1 *in vivo*.

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