The PDZ Domain of Human Erythrocyte p55 Mediates Its Binding to the Cytosplasmic Carboxyl Terminus of Glycophorin C

ANALYSIS OF THE BINDING INTERFACE BY IN VITRO MUTAGENESIS

(Received for publication, December 9, 1996, and in revised form, July 8, 1997)

The PDZ domain, also known as the GLGF repeat/DHR domain, is an ∼90-amino acid motif discovered in recently identified family of proteins termed MAGUKs (membrane-associated guanylate kinase homologues). Sequence comparison analysis has since identified PDZ domains in over 50 proteins. Like SH2 and SH3 domains, the PDZ domains mediate specific protein-protein interactions, whose specificities appear to be dictated by the primary structure of the PDZ domain as well as its binding target. Using recombinant fusion proteins and a blot overlay assay, we show that a single copy of the PDZ domain in human erythrocyte p55 binds to the carboxyl terminus of the cytoplasmic domain of human erythroid glycophorin C. Deletion mutagenesis of 21 amino acids at the amino terminus of the p55 PDZ domain completely abrogates its binding activity for glycophorin C. Using an alanine scan and surface plasmon resonance technique, we identify residues in the cytoplasmic domain of glycophorin C that are critical for its interaction with the PDZ domain. The recognition specificity of the p55 PDZ domain appears to be unique, since the three PDZ domains of hDlg (human lymphocyte homologue of Drosophila discs large tumor suppressor) do not bind the cytoplasmic domain of glycophorin C. Taken together with our previous studies, these results complete the identification of interacting domains in the ternary complex between p55, glycophorin C, and protein 4.1. Implications of these findings are discussed in terms of binding specificity and the regulation of cytoskeleton-membrane interactions.

p55 is a heavily palmitoylated peripheral membrane protein of the red blood cell membrane (1). The primary structure of p55 defines several distinct domains including the PDZ domain, the SH3 domain, the protein 4.1 binding domain, the tyrosine phosphorylation domain, and a carboxyl-terminal guanylate kinase-like domain (2). The domain organization of p55 is similar to a family of proteins termed MAGUKs1 (membrane-associated guanylate kinase homologues), which appear to play important roles in the regulation of signaling pathways and cytoskeleton-membrane interactions (3–6). Among various protein domains of MAGUKs, the PDZ domain has been a focus of intense scrutiny (5–8). Like the SH2 and SH3 domains, the PDZ domains recruit cytoskeletal proteins to specific submembranous sites. For example, the PDZ1 domain of PSD-95 and hDlg interacts with the carboxyl terminus of the NMDA receptors and the Shaker type K+ channels, and this interaction plays an important role in the clustering of ion channels (5, 6). The PDZ domains have also been shown to interact with other PDZ domains. The PDZ domain of neuronal nitric-oxide synthase binds to the PDZ domain of syntrophin at the sarcolemma in skeletal muscle and to the PDZ2 domain of PSD-95 at the synaptic junctions of neuronal cells (9, 10). These observations suggest that the primary structures of both the PDZ domain and its target peptide govern the specificity as well as the affinity of PDZ domain-mediated interactions in vitro and in vivo. The three-dimensional structure of PDZ1 domains in PSD-95 and hDlg (11, 12), together with our understanding of the peptide binding specificities of PDZ domains (13), reveals that the specificity of PDZ domain recognition is indeed dependent upon the primary sequence of the PDZ domain and its cognate ligand.

The biochemical interactions of p55 protein have been well studied in the red cell membrane. We have previously shown that p55 directly binds to protein 4.1 and glycophorin C and may form a ternary complex with these proteins at the cytoplasmic face of the plasma membrane (14). This ternary complex links the spectrin-actin junctions to the lipid bilayer and is presumed to play an important role in maintaining erythrocyte shape and its membrane material properties (15, 16). To delineate the basis of p55 interactions with protein 4.1 and glycophorin C, we have mapped the binding interfaces between protein 4.1 and p55 as well as between protein 4.1 and glycophorin C (17). However, the site where the cytoplasmic domain of glycophorin C binds to p55 had not yet been determined. In this paper, we show that the single copy of the PDZ domain in human erythrocyte p55 binds to the carboxyl-terminal 12 amino acids of glycophorin C. The carboxyl terminus YFI motif of glycophorin C is the critical binding element for PDZ domain recognition.

1 The abbreviations used are: MAGUK, membrane-associated guanylate kinase; GST, glutathione S-transferase; GPC, glycophorin C; FPLC, fast protein liquid chromatography; hDlg, human homologue of the Drosophila discs large tumor suppressor; PSD-95, postsynaptic density-95; SSPC, shape-stabilizing protein complex; MALDI, matrix-assisted laser desorption/ionization; RU, resonance units.

9 The work was supported by National Institutes of Health Grants CA66263, HL51445, and HL37462. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by the Young Investigator Award from the National Neurofibromatosis Foundation.

§ An Established Investigator of the American Heart Association. To whom correspondence should be addressed: St. Elizabeth’s Medical Center, ACH-4 BMR, 736 Cambridge St., Boston, MA 02135. Tel.: 617-789-3118; Fax: 617-789-3111.
main-mediated interaction. The interaction between p55 and glycophorin C appears to be specific, since the PDZ domains of hDlg fail to bind glycophorin C. These findings indicate that the PDZ domains are novel protein modules that target cytoplasmic proteins to the multiprotein complexes at the inner surface of the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Preparation of Recombinant Cytoplasmic Domain of Glycophorin C**—The 47-amino acid cytoplasmic domain of human erythrocyt glycoprotein C was produced as a glutathione S-transferase fusion protein (GST-GPC) truncated at the N terminus (PDZ domain of p55 (amino acids 1–83), the SH3 domain (amino acids 162–180), and the extreme carboxyl-terminal residues of the peptide YFI). These are: 1) peptide YFA, in which Il6-128 was substituted by Ala; 2) peptide AFI, in which Tyr-126 was substituted by Ala; 3) peptide AFA, in which both Tyr-126 and Ile-128 were substituted by Ala. All peptides contained a biotin molecule attached at the N terminus and were purified by high pressure liquid chromatography on an analytical reverse phase column. The purity of the peptides was confirmed by amino acid analysis and mass spectrometry.

**Blot Overlay Assay**—The experimental conditions used for the blot overlay assay have been described previously (14). The binding of glycophorin C to the immobilized proteins was detected using polyclonal antibodies raised against the cytoplasmic domain of human glycophorin C. These antibodies (kindly provided by Dr. Philip Low, Purdue University) were produced in rabbits injected with a synthetic peptide corresponding to the entire cytoplasmic domain of glycophorin C. Alternatively, direct binding of glycophorin C to the immobilized fusion proteins was detected using a biotinylated glycophorin C peptide P24. Bound peptide was then detected using the streptavidin-peroxidase detection system.

**Surface Plasma Resonance Measurements**—Protein interactions were quantified using the BIAcore instrument from Pharmacia. The instrument uses the surface plasmon resonance phenomenon to detect changes in refractive index as a result of biomolecular interactions (5). A surface plasmon resonance signal detection module is configured around a biosensor chip, which contains a dextran-coated gold surface sandwiched between layers of two different refractive indices. Light impinging on the gold surface will cause the outer shell electrons to resonate and hence result in decreased reflected light under conditions of total internal reflection. Binding of soluble analyte molecules to the immobilized ligand on this chip surface changes in mass and, hence, in the refractive index at the chip surface. These changes result in a decreased reflected light detected as the SPR signal and recorded as resonance units (RU) in real time. A change of 1000 RU corresponds to a change in protein surface concentration of about 1.0 ng/mm². A plot of RU versus time (sensorgram) consists of an association phase, an equilibrium phase, and a dissociation phase. To analyze the interaction between p55 and glycophorin C, biotinylated GPC peptides immobilized on a streptavidin surface (sensor chip SA, Research Grade, Pharmacia), and GST-p55 fusion proteins were passed over the immobilized peptides. The GST fusion proteins of p55 were used at a concentration of about 0.41 mg/ml. In kinetic experiments, the concentration of GST fusion proteins of p55 ranged from 1.0 to 22.0 μM. All experiments were carried out at 25 °C at a flow rate of 5.0 μl/min except for the kinetic measurements, which were carried out at a flow rate of 3.0 μl/min. The composition of the running buffer was 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% P20 (BIAcore buffer).

**Quantification of hDlg in Human Red Blood Cell Membranes**—Red blood cells were separated from other blood cells by centrifugation in a microcapillary (hemofuge). Purified red cell membranes and bacterially expressed recombinant hDlg protein of known concentrations were analyzed by 10% SDS-polyacrylamide gel electrophoresis and Western blotting using polyclonal antibodies that were raised against a synthetic peptide of hDlg corresponding to residues 68–80 (DRSKPSEIQPVN) and were affinity-purified on a column of immobilized peptide. A semiquantitative estimate of the amount of hDlg in red cell membranes was made by comparing the intensity of hDlg bands in red cell membranes with the intensities of the hDlg bands of known protein concentration.

**Analysis of the Mutant Red Blood Cell Membranes**—Blood samples were obtained from three members of one family (JC, SS, and TL). All three members are homozygous for the Leach phenotype, and their red blood cells are completely deficient in glycophorin C (23, 24). The control blood samples were selected by taking into account the shipment and age variables. After removal of theuffy coat, red blood cells were passed through the cellulose mixture to remove any residual white blood cells (25). The red cell membranes were analyzed by 10% SDS-polyacrylamide gel electrophoresis (19). Membrane proteins were transferred to nitrocellulose and probed with antibodies against hDlg.
RESULTS

We have previously shown that erythrocyte membrane protein p55 binds to the cytoplasmic domain of glycophorin C (14). To determine the specific binding site on p55, we expressed the glycophorin C cytoplasmic domain and defined segments of human erythrocyte p55 as recombinant GST fusion proteins in bacteria. The GST-GPC82–128 fusion protein was cleaved from GST by thrombin, and the purified GPC82–128 protein at 7.0 mg/ml was used for chromatography. B, Coomassie-stained SDS-polyacrylamide gel electrophoresis (18%). Lane 1 shows the separation of proteins present in the total cell lysate of bacteria expressing the GST-GPC82–128 fusion protein. Note that a significant amount of GST-GPC82–128 protein is broken down to release free GST in the bacterial lysate before purification. Lane 2, protein sample from the 12-kDa peak (panel A) was boiled in the sample buffer containing 2-mercaptoethanol prior to electrophoresis. The asterisk indicates that GPC82–128 migrated as an ~11-kDa species. C, purified 23-pmol GPC82–128 was mixed with 2,5-dihydroxybenzoic acid and analyzed by MALDI. A similar spectrum was obtained when the cytoplasmic domain of glycophorin C (230 pmol) was mixed with sinapinic acid. Inset, analysis of GST by Tricine-containing SDS-polyacrylamide gel electrophoresis (10%). Note that Tricine-containing gels are known to achieve higher resolution of proteins in the lower molecular mass range of 4–40 kDa (27).

FIG. 1. Purification and molecular weight determination of the recombinant cytoplasmic domain of glycophorin C. A, elution profile of the cytoplasmic domain of GPC on a Superdex-75 gel filtration column. The cytoplasmic domain of GPC was expressed as a GST fusion protein in bacteria. The GST-GPC82–128 fusion protein was cleaved from GST by thrombin, and the purified GPC82–128 protein at 7.0 mg/ml was used for chromatography. B, Coomassie-stained SDS-polyacrylamide gel electrophoresis (18%). Lane 1 shows the separation of proteins present in the total cell lysate of bacteria expressing the GST-GPC82–128 fusion protein. Note that a significant amount of GST-GPC82–128 protein is broken down to release free GST in the bacterial lysate before purification. Lane 2, protein sample from the 12-kDa peak (panel A) was boiled in the sample buffer containing 2-mercaptoethanol prior to electrophoresis. The asterisk indicates that GPC82–128 migrated as an ~11-kDa species. C, purified 23-pmol GPC82–128 was mixed with 2,5-dihydroxybenzoic acid and analyzed by MALDI. A similar spectrum was obtained when the cytoplasmic domain of glycophorin C (230 pmol) was mixed with sinapinic acid. Inset, analysis of GST by Tricine-containing SDS-polyacrylamide gel electrophoresis (10%). Note that Tricine-containing gels provide higher resolution of proteins in the lower molecular weight region (27). Again, the FPLC (Mono Q)-purified GPC82–128 migrated as an ~11-kDa species as indicated by an asterisk (lanes 1 and 2). At higher concentrations (lane 2), a relatively weak and diffuse band corresponding to the monomer position of GPC82–128 was also observed (arrowhead).

To resolve this issue, the aggregation state of GPC82–128 protein was analyzed using a MALDI mass spectrometer. As shown in Fig. 1C, purified GPC82–128 protein exists largely as a monomer in solution constituting 70–90% of the total peak area (5575.25 Da). A dimeric peak corresponding to 10–20% of the total protein was also detected (11136.6 Da, Fig. 1C). To test whether purified GPC82–128 protein migrates as a monomer in a modified gel electrophoresis system, we analyzed GPC82–128 by Tricine-containing SDS-polyacrylamide gels. The Tricine-containing gels are known to achieve higher resolution of proteins in the lower molecular mass range of 4–40 kDa (27).

In view of the conflicting results obtained by gel filtration/electrophoresis and MALDI, we reevaluated the self-association state of purified GPC82–128 by sedimentation equilibrium analysis. The data for GPC82–128 were fitted with the rotational form of the solution to the Lamm equation for a single ideal species (22) (see “Experimental Procedures” for details). Using this model, a good fit yielded an apparent whole cell weight average molecular mass of 4340 ± 230 Da, which is in close agreement with the calculated monomeric mass (5297 Da) of GPC82–128 protein (Table I). This result is consistent with the value obtained by MALDI, which shows that GPC82–128 exists as a monomer in solution. Here, the potential to draw erroneous conclusions by using only gel filtration and gel electrophoresis techniques is evident.

The GST-p55 fusion proteins were examined for glycophorin C binding activity using a blot overlay assay. The fusion proteins were first immobilized onto nitrocellulose and then incubated with the purified cytoplasmic domain of glycophorin C. The bound GPC82–128 was detected immunologically using antibodies specific for the cytoplasmic domain of glycophorin C. As shown in Fig. 2, GPC82–128 binding occurred only with GST-p55 fusion proteins containing the PDZ domain, namely 1) full-length p55, 2) N-terminal domain + PDZ domain, and 3) N-terminal domain + PDZ domain + SH3 motif. Neither the N-terminal domain nor the SH3 motif alone bound GPC82–128. Similarly, GPC82–128 did not bind a construct containing only the guanylate kinase domain. A construct containing the guanylate kinase domain, the tyrosine phosphorylation domain (TP), the protein 4.1 binding domain (4.1B), and the SH3 motif of p55 also did not show any glycophorin C binding activity. By process of elimination, the single copy of the PDZ domain in p55 appears to contain the binding site for the cytoplasmic domain of glycophorin C.

From our previous observation that GST-GPC82–128 inhibits up to 65% of the binding between p55 and a synthetic peptide containing the carboxyl-terminal 24 amino acids (Ala-105 to Ile-128; peptide P24) of glycophorin C (14), we inferred that the p55 binding site is located within the carboxyl half of the cytoplasmic domain of glycophorin C. Indeed, peptide P24 bound to the GST-p55 fusion proteins containing the PDZ domain in a blot overlay assay (data not shown). To measure the direct binding of P24 to the p55 PDZ domain, a GST-PDZ fusion protein was expressed in bacteria. The binding was quantified using the surface plasmon resonance technique (BIAcore). The biotinylated P24 was immobilized to the streptavidin surface of the Biosensor SA chip. The GST-p55 fusion proteins containing
Interacting Domains of Erythrocyte p55 and Glycophorin C

The data were fitted with the ASSOC4 model in the XL-A ORIGIN software package to obtain the apparent whole cell weight average mass (m, app). First, the optical base-line offset was set to 0 (assuming that dialysis had equalized the contribution of absorbing, nonsedimenting species at 280 nm). The mass was then floated, and the fits were good (yielding 4010 Da ≤ m, app ≤ 4830 Da), indicating the soundness of using this model. Then the base line was floated together with the mass (yielding 4070 Da ≤ m, app ≤ 4840 Da). Finally, in order to assess the effect of poorly mismatched base lines, the mass was set to 5297 Da (the mass calculated from the amino acid sequence), and the baselines were floated. Again, good fits were obtained. Extrapolation of the concentration dependence of apparent mass to infinite dilution yielded a mass of 4340 ± 230 Da.

| [GPC] (A_{280}) | m, app (base line = 0) | Error | m, app (base line floated) | Error | Base line (if m = 5297 Da) |
|----------------|-----------------------|-------|---------------------------|-------|--------------------------|
| 0.054          | 4465                  | 224   | 4067                      | 202   | 0.005                    |
| 0.104          | 4064                  | 157   | 4326                      | 167   | 0.012                    |
| 0.420          | 4639                  | 58    | 4539                      | 69    | 0.011                    |
| 0.613          | 4010                  | 67    | 4123                      | 69    | 0.042                    |
| 0.563          | 4824                  | 31    | 4641                      | 29    | 0.035                    |
| 0.641          | 4533                  | 56    | 4204                      | 46    | 0.065                    |
| 0.928          | 4563                  | 53    | 4210                      | 43    | 0.072                    |

A summary of the cDNA constructs of human erythrocyte p55. GPC binding activity of the fusion proteins, as determined by the blot overlay assay, is shown on the right. The GST fusion proteins expressing the intact and the truncated PDZ domain of p55 were not included in the blot overlay assay. B, the Ponceau S staining of the fusion proteins. Proteins were analyzed by 10% SDS-polyacrylamide gel electrophoresis. C, autoradiograph of the ECL-developed Western blot. Note that GPC162–466 bound only to the fusion proteins containing the PDZ domain of p55. In panels B and C, the SH3 + GUK construct refers to amino acids 162–466, and the GUK construct refers to amino acids 266–466 as outlined in panel A.

either the N-terminal and PDZ domains or the PDZ domain alone (Fig. 2A) bound to P24, whereas no binding occurred between P24 and the GST-N-terminal domain (Fig. 3A). Moreover, the binding of P24 to GST-PDZ was completely abrogated when 21 amino acids (amino acids 62–82) were deleted from the N terminus of the PDZ domain leaving a truncated PDZ domain (PDZA) (Figs. 2A and 3A). Therefore, the N-terminal 21 residues of the PDZ domain are essential for the interaction of p55 with the cytoplasmic domain of glycophorin C. These observations are consistent with the x-ray crystallographic structure of the PDZ domains of hDlg and PSD-95, which shows that the amino-terminal region of the PDZ domain makes specific contact with the carboxyl-terminal end of the bound peptide and hence is important in stabilizing the PDZ domain–peptide interactions (11, 12).

To identify the minimum segment of glycophorin C required to bind p55, a peptide corresponding to the carboxyl-terminal 12 amino acids of glycophorin C (Asp-117 to Ile-128) was synthesized (see “Experimental Procedures”). This peptide, designated YFI, was conjugated to biotin via its N terminus. Peptide YFI was immobilized on the streptavidin surface of an SA sensor chip, and then was tested for binding to the PDZ domain of p55 using BIACore. Again, both fusion proteins, GST-N-terminal plus PDZ domain and GST-PDZ domain, bound to peptide YFI (Fig. 3B). As expected, no binding was detected with either GST-N-terminal domain or GST-PDZΔ fusion protein (data not shown). These results indicate that the carboxyl-terminal 12 amino acids of glycophorin C are sufficient for its binding to the PDZ domain of p55. The binding capacity of peptide YFI for the PDZ domain is, however, lower than that of peptide P24 (compare the RU values of bound GST-PDZ in Fig. 3A and B). The relatively weaker binding of the 12-amino acid peptide may result from poor flexibility due to immobilization on the chip surface.

We have recently reported that PDZ domains can be classified into two subgroups based on their ability to recognize amino acids located at the carboxy-terminal –2 position of interacting proteins (13). Group I PDZ domains select residues with a hydroxyl side chain (Ser/Thr) at the –2 position, while group II PDZ domains select residues with an aromatic side chain (Tyr/Trp) at the –2 position. Both classes of PDZ domains prefer hydrophobic amino acids at the 0 position of interacting peptides. Using an alanine scan, we examined the role of the glycophorin C carboxy-terminal YFI motif in p55 PDZ domain binding. We produced mutant peptides by selectively replacing the Tyr and Ile residues of the YFI motif (see “Experimental Procedures”): peptide YFA replaced Ile-128; peptide AFI replaced Tyr-126; and peptide AFA replaced both Ile-128 and Tyr-126. All peptides were conjugated to biotin at their amino termini, immobilized on the streptavidin surface of the SA sensor chip, and tested for GST-PDZ domain binding activity. As shown in Fig. 3B, the GST-PDZ domain bound only to peptide YFI and not to any mutant peptides, illustrating that amino acids Tyr-126 and Ile-128 at the carboxy terminus of
glycophorin C are critical for its binding to the PDZ domain of p55.

For the initial attempt to quantify the interaction between the p55 PDZ domain and glycophorin C, we used the 12-amino acid YFI peptide. It should be noted that the determination of kinetic parameters by BIAcore requires regeneration of the sensor chip surface. This process removes bound analyte without affecting the immobilized ligand, so that the interaction of a fixed amount of ligand can be measured with increasing concentrations of the analyte. For unknown reasons, the bound PDZ domain on the YFI peptide-coated sensor chip could not be removed quantitatively. Application of harsher regeneration conditions resulted not only in the removal of bound PDZ domain but also of immobilized YFI peptide. Hence, we selected peptide P24 to quantify the affinity of glycophorin C for the PDZ domain of p55. Peptide P24 (414 RU) was immobilized on the SA chip and introduced to five different concentrations of the GST-PDZ domain (1.2–21.5 μM) (Fig. 3C). Analysis of the resulting sensorgrams gave an apparent association rate constant \(k_\text{a}\) of 231.5 ± 67 M\(^{-1}\) s\(^{-1}\) and an apparent dissociation rate constant \(k_\text{d}\) of 10.2 ± 0.11 × 10\(^{-4}\) s\(^{-1}\). The apparent dissociation constant \(K_\text{D}\) was 4.4 μM. A schematic diagram depicting the interacting domains of p55, glycophorin C, and protein 4.1 is shown in Fig. 4.

We then examined the specificity of glycophorin C binding to the PDZ domain of p55. A GST fusion protein containing all the three PDZ domains of lymphocyte hDlg was produced (5, 28). Using the blot overlay assay, no binding of GPC to p55 was observed with either the three PDZ domains of hDlg or the full-length hDlg protein (data not shown). To supplement these in vitro results, we utilized red cell membranes of individuals with complete glycophorin C deficiency (Leach phenotype) (23, 24). This phenotype was previously shown to exhibit a secondary loss of p55 and protein 4.1 (25% reduction) as compared with normal red cell membranes (29). Using an affinity-purified antibody against lymphoid hDlg, a comparable amount of hDlg protein was detected in the normal and glycophorin C-deficient red cell membranes (Fig. 5). These results confirm that the YFI signature at the carboxyl terminus of glycophorin C determines its specificity for the PDZ domain of p55.

**DISCUSSION**

In this paper, we have established that the single copy of the PDZ domain in human erythrocyte p55 mediates the binding of p55 with the carboxyl terminus of the cytoplasmic domain of glycophorin C; this finding is consistent with recent characterizations of the PDZ domains in other proteins (5–9, 30–32). Recently, using an oriented peptide library technique, we have classified PDZ domains into two subgroups based on the amino acid residues they select at the −2 position from the carboxyl end of the interacting protein (13). Group I PDZ domains recognize a binding sequence having a hydroxy side chain on the −2 amino acid ((S/T/X)V), while group II PDZ domains prefer a residue with an aromatic side chain (Phe/Tyr) at the −2 position. In vitro mutagenesis experiments have shown that replacement of either Thr/Ser at the −2 position or Val at the 0 position completely abolishes binding of the Shaker type K\(^-\) channels to the PDZ domain of PSD-95 (33). In the crystal structure of the PDZ domain of PSD-95, the side chains of Ser/Thr and Val make distinct contacts with specific residues of the PDZ domain, stabilizing binding of the peptide to the PDZ domain (11). The p55 PDZ domain selects Tyr/Phe at the −2 position and Phe at the 0 position (13). The carboxyl terminus of GPC contains Tyr at the −2 position and Ile at the 0 position. Both Ile and Phe are hydrophobic, nonpolar residues with no net charge. The presence of Ile instead of Phe in glycophorin C is consistent with the PDZ domain binding motif in the
inward rectifying K⁺ channel Kir2.3 (34). Similarly, the presence of Tyr instead of Ser/Thr at the −2-position in GPC is also consistent with the PDZ domain binding motif in neuroexins (35). These results underscore the importance of residues at the 0- and −2-positions and support our conclusion that the mutagenesis of these residues abolishes binding of GPC to the p55 PDZ domain (Fig. 3B).

The crystal structures of the PDZ2 domains of PSD-95 and hDlg reveal that the N-terminal 12 amino acids form a β1 strand and a carboxyamide binding loop (11, 12). Although the structure of the p55 PDZ domain is not yet known, we assume that similar structural requirements determine the binding of p55 with GPC. To this effect, a truncated PDZ domain (lacking 21 amino acids) was observed to have completely lost its binding affinity for glycophorin C (Fig. 3A). Our results are also consistent with a previous report showing that the deletion of 9 amino acids from the N terminus of the PDZ2 domain of PSD-95 abolishes its binding to the carboxyl-terminal tail of the Shaker type Kv1.4 channel (33). These results imply that the PDZ domains of p55 homologues such as LIN-2 (36), Dlg2 (37), and Dlg3 (38) may recognize the YXXI motif at the carboxyl termini of their respective ligands.

GPC is believed to exist as a monomer in the red cell membrane (24, 26). This is in contrast to GPA, which is known to be a dimer in the red cell membrane (39). Our results obtained by MALDI mass spectrometry and sedimentation equilibrium analysis show that the bacterially expressed cytoplasmic domain of GPC exists as a monomer in solution (Fig. 1, Table I). For unknown reasons, the purified GPC(82–128) protein migrates as a dimer in the gel filtration and gel electrophoresis assays (Fig. 1). It is noteworthy that the presence of immunoreactive bands corresponding to monomer and dimer forms of GPC has been previously documented (40, 41). It is therefore possible that under certain conditions the intact glycophorin C may self-associate into a dimer in the red cell membrane. In any case, binding data reported in this paper reflect an interaction between monomeric GPC(82–128) and monomeric PDZ domain of p55. The affinity (KeD) of the interaction between recombinant p55 and the synthetic peptide P24 of glycophorin C is 4.4 nM as measured by the surface plasmon resonance technique. Hemming et al. (16) measured a high affinity interaction (KeD = 4.5 nM) between native p55 and alkali-stripped erythrocyte membrane vesicles, indicating that presently unknown factors may modulate the strength of p55-glycophorin C interaction in vivo. The known post-translational modifications of p55, such as its extensive palmitoylation and phosphorylation, are worthy of further examination.

The absence of p55 but not hDlg in the membranes of glycophorin C-deficient red blood cells (Leach phenotype) provides in vivo evidence for the specificity of p55 PDZ domain binding to glycophorin C (Fig. 5). We have previously reported secondary loss of both p55 and hDlg in the red cell membranes of a patient with complete protein 4.1 deficiency (5, 29), demonstrating that protein 4.1 is necessary for the attachment of MAGUKs to the red cell plasma membrane. However, an enigma in the field of elliptocytosis has been the observation that although the red blood cells from both protein 4.1-deficient hereditary elliptocytosis individuals and glycophorin C-deficient Leach phenotype individuals lack the protein 4.1-p55-glycophorin C complex, only protein 4.1-deficient hereditary elliptocytosis red cells show complete elliptocytosis (16); Leach phenotype red cells exhibit a moderate degree of elliptocytosis (15, 42). The presence of nearly 75% of protein 4.1 in Leach phenotype red cell membranes is thought to be responsible for preventing complete loss of discoid shape in these cells (15, 42). The presence
of hDlg in Leach phenotype red cell membranes may offer a new explanation for the membrane association of protein 4.1 in these cells. Based on these findings, we propose that the elliptocytic shape of the Leach phenotype red blood cells is regulated by a membrane-associated, shape-stabilizing protein complex (SSPC). Both p55 and hDlg are components of the SSPC along with protein 4.1 and glycophorin C. In the red cell membranes of patients with protein 4.1-deficient hereditary elliptocytosis, the entire SSPC is lost, resulting in complete elliptocytosis, unlike Leach phenotype membranes, which lack glycophorin C, p55, and 25% of protein 4.1 (29). The binding of hDlg to protein 4.1 may anchor the remains of the SSPC onto the red cell membrane in Leach phenotype. This hypothesis assumes that only a subpopulation of protein 4.1 regulates SSPC at the plasma membrane. The remaining protein 4.1 may independently regulate properties such as membrane deformability, which is restored by the addition of exogenous protein 4.1 in the absence of either p55 or hDlg (43, 44).

The number of copies of GPC per red cell varies from 50,000 to 143,000 (45–47), depending upon the method of estimation. Previously, 80,000 copies of p55 per red cell were estimated by molecular Hydrodynamics, University of Leicester, UK, which is supported by the Biotechnology and Biological Sciences Research Council/Engineering and Physical Sciences Research Council.

REFERENCES

1. Ruff, P., Speicher, D. W., and Chishti, A. H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6595–6599
2. Kim, A. C., Metzenberg, A. B., Sahr, K. E., Marfatia, S. M., and Chishti, A. H. (1996) Genomics 31, 223–229
3. Woolls, D. F., and Bryant, P. J. (1993) Meth. Cell. Biol. 44, 85–89
4. Kim, S. K. (1995) Curr. Opin. Cell Biol. 7, 641–649
5. Marfatia, S. M., Cabrall, J. H. M., Lin, L., Hough, C., Bryant, P. J., Stolz, L., and Chishti, A. H. (1996) J. Cell Biol. 135, 753–766
6. Sheng, M. (1996) Neuron 17, 575–578
7. Fanning, A. S., and Anderson, J. M. (1996) Curr. Biol. 6, 1385–1388
8. Saras, J., and Heldin, C. H. (1996) Trends Biochem. Sci. 21, 455–458
9. Brennan, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Wu, Z., Huang, F., Xia, H., Peters, M. P., Froehlich, S. C., and Bredt, D. S. (1996) Cell 84, 757–767
10. Brennan, J. E., Chao, D. S., Xia, H., Aldape, K., and Bredt, D. S. (1995) Cell 82, 743–752
11. Doyle, D. A., Lee, A., Lewis, J., Kim, E., Sheng, M., and MacKinnon, R. (1995) Cell 85, 1067–1076
12. Cabrall, J. H. M., Petosa, C., Sutcliffe, M. J., Raza, S., Byron, O., Poy, F., Marfatia, S. M., Chishti, A. H., and Liddington, R. C. (1996) Nature 382, 649–652
13. Songyang, Z., Fanning, A. S., Fu, J., Xu, J., Marfatia, S. M., Chishti, A. H., Crompton, A., Chan, A. C., Anderson, J. M., and Cantley, L. C. (1997) Science 275, 73–77
14. Marfatia, S. M., Rue, R. A., Branton, D., and Chishti, A. H. (1994) J. Biol. Chem. 269, 8631–8634
15. Chasis, J. A., and Mohandas, N. (1992) Blood 80, 1869–1879
16. Hemming, N. J., Anstee, D. J., Starchoff, M. A., Tanner, M. J. A., and Mohandas, N. (1995) J. Biol. Chem. 270, 5360–5366
17. Marfatia, S. M., Rue, R. A., Branton, D., and Chishti, A. H. (1995) J. Biol. Chem. 270, 715–719
18. Smith, D. B., and Johnson, K. S. (1988) Gene (Amst.) 67, 31–49
19. Laemmli, U. K. (1970) Nature 227, 680–685
20. Yphantis, D. A. (1964) Biochemistry 3, 297–317
21. Perkins, S. J. (1986) Eur. J. Biochem. 157, 169–180
22. Lamm, O. (1929) Arch. Psychiatr. Nervenkr. 12, 1–4
23. Chishti, A., Palek, J., Fisher, D., Maalouf, G. J., and Liu, S-H. (1995) Blood 87, 3462–3469
24. Reid, M. E., Chasis, J. A., and Mohandas, N. (1987) Blood 69, 1068–1072
25. Beutler, E., West, C., and Blume, K. G. (1976) J. Lab. Clin. Med. 88, 328–333
26. Chong, S., Reid, M. E., Conboy, J., Kan, Y. W., and Mohandas, N. (1993) Blood 77, 644–648
27. Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
28. Lue, R. A., Marfatia, S. M., Branton, D., and Chishti, A. H. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9818–9822
29. Alluisio, N., Venezia, N. D., Rana, A., Andrabhi, K., Texier, P., Gilsanz, F., Cartron, J.-P., Deloumay, J., and Chishti, A. H. (1993) Blood 82, 1233–1237
30. Satô, T., Irie, S., Kitada, S., and Reed, J. C. (1995) Science 268, 411–415
31. Matsunime, A., Ogai, A., Senda, T., Okumure, N., Satô, K., Baeg, G-H., Kawahara, T., Kubayashi, S., Okada, M., Toyoshima, K., and Akiyama, T. (1996) Science 272, 1020–1022
32. Simkse, J. S., Kaech, S. M., Harp, S. A., and Kim, S. K. (1996) Cell 85, 195–204
33. Kim, E., Niethammer, A. R., Rothschild, A., Jan, Y. N., and Sheng, M. (1995) Nature 376, 85–88
34. Cohen, N. A., Brennan, J. E., Snyder, S. H., and Bredt, D. S. (1996) Neuron 17, 759–767
35. Hata, T., Butz, S., and Saudhof, T. C. (1996) J. Neurosci. 16, 2488–2494
36. Hokins, H., Hjalén, A. F., Harp, S. A., and Kim, S. K. (1995) Development 122, 97–111
37. Manzoyer, S., Gayther, S. A., Nagai, M. A., Smith, S. A., Dunning, A., van Rensburg, E. J., Albertson, H., White, R., and Ponder, B. A. J. (1995) Genomics 28, 25–31
38. Smith, S. A., Holik, P., Stevens, J., Manzoyer, S., Melis, R., Williams, B., White, R., and Albertson, H. (1996) Genomics 31, 145–150
39. Anstee, D. J., and Tanner, M. J. A. (1986) Br. J. Haematol. 61, 211–215
40. Reid, M. E., Takakuwa, Y., Conboy, J., Teichnra, G., and Mohandas, N. (1990) Blood 75, 2229–2234
41. King, M. J., Holmes, C. H., Mushena, R. E., Mawby, W., Reid, M. E., and Scott, M. L. (1995) Br. J. Haematol. 89, 440–448
42. Telen, M. J., Le Van Kim, C., Chung, A., Cartron, J.-P., and Colín, Y. (1991) Blood 78, 1603–1606
43. Takakuwa, Y., Chishti, A. H., Rossi, M., Benzadji, M., and Mohandas, N. (1986) J. Clin. Invest. 78, 80–85
44. Discher, D., Parra, M., Conboy, J. G., and Mohandas, N. (1993) J. Biol. Chem. 268, 7186–7195
45. Anstee, D. J., Parsons, S. F., Egidwell, K., Tanner, M. J. A., Merry, A. H., Thomson, E. E., Judson, P. A., Johnson, P., Bates, S., and Fraser, I. D. (1994) Biochem. J. 301, 615–619
46. Lorrat, M. J., Dahr, J. Y., Muller, J. Y., and Blanchard, D. (1994) Transfus. Med. 4, 147–155
47. Smythe, J., Gardner, B., and Anstee, D. J. (1994) Blood 83, 1668–1672