Erythrocyte M, 28,000 Transmembrane Protein Exists as a Multisubunit Oligomer Similar to Channel Proteins*

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A novel M, 28,000 erythrocyte transmembrane protein was recently purified and found to exist in two forms, “28kDa” and “gly28kDa,” the latter containing N-linked carbohydrate (Denker, B. M., Smith, B. L., Kuhajda, F. P., and Agre, P. (1988) J. Biol. Chem. 263, 15644–15648). Although 28kDa protein resembles the Rh polypeptides biochemically, structural homologies were not identified by immunoblot or two-dimensional iodopeptide maps. The NH2-terminal amino acid sequence for the first 35 residues of purified 28kDa protein is 37% identical to the 26-kDa major intrinsic protein of lens (Gorin, M. B., Yancey, S. B., Cline, J., Revel, J.-P., and Horwitz, J. Cell 39, 49–59). Antibera to a synthetic peptide corresponding to the NH2-terminus of 28kDa protein gave a single reaction of molecular mass 28kDa on immunoblot of erythrocytemembranes. Selective digestions of intact erythrocytes and inside-out membrane vesicles with carboxypeptidase Y indicated the existence of a 5-kDa COOH-terminal cytoplasmic domain. Multiple studies indicated that 28kDa and gly28kDa proteins exist together as a multisubunit oligomer: 1) similar partial solubilizations in Triton X-100; 2) co-purification during ion exchange and lectin affinity chromatography; 3) cross-linking in low concentrations of glutaraldehyde; and 4) physical analyses of purified proteins and solubilized membranes in 1% (v/v) Triton X-100 showed 28kDa and gly28kDa proteins behave as a large single unit with Stokes radius of 61 Å and sedimentation coefficient of 5.7 S. These studies indicate that the 28kDa and gly28kDa proteins are distinct from the Rh polypeptides and exist as a multisubunit oligomer. The 28kDa protein has NH2-terminal amino acid sequence homology and membrane organization similar to major intrinsic protein and other members of a newly recognized family of transmembrane channel proteins.

Because of accessibility, abundance, and relative ease of preparation, erythrocyte membranes have proven to be the model from which most understandings of membrane structure have been derived (for reviews, see Bennett, 1989; Steck, 1989). A complex of cytoskeletal proteins known as the “membrane skeleton” is associated with certain transmembrane proteins which penetrate the lipid bilayer which covers the cell. Together these proteins and lipids define the erythrocyte membrane and determine the shape, provide reversible deformability, and regulate the intracellular fluid and electrolyte composition of the erythrocyte. Individual transmembrane proteins are known to contribute to the structural integrity of the membrane and/or the transport of small molecules across the lipid bilayer. Well characterized erythrocyte transmembrane proteins include the glycophorins A and B, glyco- porins C and D, the band 3 anion exchanger, Na,K-ATPase, Ca,Mg-ATPase, and the glucose transporter.

Despite extensive previous investigation, new erythrocyte transmembrane proteins have recently been identified including certain clinically important blood group antigens (for reviews, see Rosse and Telen, 1989; Mollison et al., 1987). While certain antigens, such as ABH, Lewis, and Ii, are specific carbohydrate structures attached to various membrane sites, other blood groups including the MN, Ss, Rh, LW, Kell, Duffy, and Lutheran antigens are specific membrane proteins and glycoproteins. The structures of these erythrocyte protein blood group antigens are sometimes complex, and associated proteins have been found which may be antigenically or genetically related. Despite initial characterizations of these blood group antigen-transmembrane proteins, identification of their physiological roles remains.

During isolation of the molecular mass 32-kDa Rh polypeptide, a slightly smaller protein (referred to as “28kDa”[1]) was found to co-purify and was distinct from band 7 (Agre et al., 1987; Saboori et al., 1988). Although initially thought to represent a breakdown product of Rh, 28kDa protein was subsequently shown to be a separate protein which is abundant in the membranes of erythrocytes and renal proximal convoluted tubules (Denker et al., 1988). The Rh polypeptides comprise a group of related polypeptides with several shared and few variable domains (Blanchard et al., 1988). It was considered that the 28kDa protein and Rh polypeptides may also be related, thereby explaining several of their similar characteristics including approximate size, amino acid composition, staining characteristics, solubility, unusual chromatographic behavior on hydroxylapatite, and existence of glycosylated isoforms (Table I). Unlike the common Rh negative phenotypes which only lack the Rh D antigen, rare Rh deficiency phenotypes lack all Rh C, c, D, E, and e antigens

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[1] The abbreviations used are: 28kDa protein, M, 28,000 integral membrane protein; gly28kDa protein, glycosylated 28kDa protein which has a M, ranging from 35,000 to 60,000; anti-28kDa, affinity-purified polyclonal rabbit IgG against denatured 28kDa protein; anti-N-peptide, polyclonal rabbit antiserum raised against synthetic peptide corresponding to the 10 NH2-terminal residues of 28kDa protein conjugated to albumin; MIP, major intrinsic protein of lens (Gorin et al., 1984); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; DTT, dithiothreitol; TPC, 1,1-tosylamido-2-phenylethyl chloromethyl ketone.

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and are also deficient in the glycosylated Rh isofrom (Moore and Green, 1987) as well as a newly recognized glycoprotein.²

Whether Rh and 28kDa are evolutionarily or functionally related proteins has remained uncertain. Moreover, the physiologic significance of 28kDa protein as well as the membrane organization and amino acid sequence have not yet been reported. The findings in this study indicate that the 28kDa protein and Rh polypeptides are not closely related, but 28kDa protein appears to be related to MIP, the 28kDa major intrinsic protein of lens (Gorin et al., 1984) which is the prototype of a recently recognized family of transmembrane channel proteins including Drosophila Big Brain, soy bean nodulin 26, and Escherichia coli glyceral facilitator proteins (Baker and Saier, 1990) (see "Discussion"). It is postulated that 28kDa protein is a member of this newly recognized family of transmembrane channel proteins, although 28kDa protein bears channel-like activities remains to be established.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human blood was obtained from the Johns Hopkins Hospital Blood Bank and from normal healthy volunteers. α-Chymotrypsin (50 units/mg) and TPK-trypsin (250 units/mg) were from Cooper Biomedical. Carrier-free Na¹²⁵I was from Amersham Corp. High resolution hydroxyapatite and protein A-bearing Staphylococcus were from Behring Diagnostics. The Mono family of transmembrane channel proteins, although whether organization and amino acid sequence have not yet been established.

**TABLE I**

Comparison of known properties of 28kDa protein and Rh polypeptides

| Similarities/property | 28kDa | Rh |
|-----------------------|-------|----|
| Retained in membrane vesicles | Yes* | Yes* |
| Retained in membrane skeletons | Yes* | Yes* |
| (insoluble in 1% Triton X-100) | | |
| Molecular weight, SDS-PAGE | 28,000 | 32,000 |
| Cytoplasmic domain | Yes* | Yes* |
| Glycosylated subpopulation | g28kDa | Isoform |
| SDS-PAGE staining | | |
| Coomassie | Very weak | Very weak |
| Silver | Strong | Strong |
| Amino acid content | Similar | Similar |
| Na,HPO₄ elution from hydroxyapatite | 0.5 mM | 0.5 mM |
| Present in nonhuman erythrocytes | Yes* | Yes* |
| Differences | | |
| Surface ¹²⁵I-labeled | No | Yes |
| Acylated with [¹⁴C]palmitate | No | Yes |
| Immunoprecipitated with Rh antibodies | No* | Yes* |

* Denker et al., 1988.
² Agre et al., 1987.
³ Gahmberg and Karbi, 1984; Ridgwell et al., 1984.
* Moore et al., 1982; Gahmberg, 1983.
* Suyama and Goldstein, 1990.
³ Moore and Green, 1987; Avent et al., 1988.
* Saiboori et al., 1988.
³ Saiboori et al., 1989.
² de Vetten and Agre, 1988.

**Preparation of Antisera**—Preparation of polyclonal rabbit antiserum to denatured 28kDa protein ("anti-28kDa") and purification over a 28kDa protein affinity column were described previously (Denker et al., 1988). Antiserum was raised to the synthetic peptide corresponding to the 10 NH₂-terminal amino acids of 28kDa protein conjugated to bovine serum albumin ("28kDa peptide"). Bovine serum albumin (10 mg/ml), synthetic peptide (1 mg/ml), and glutaraldehyde (0.02%, v/v) in 0.1 M NaCl, 50 mM Na₂HPO₄ (pH 7.2) were mixed and shaken at room temperature for 1 h. Completion of cross-linking was assessed by gel filtration over Superose 12 with monitoring at 214 nm. Glycine was added to a final concentration of 0.2 M and the incubation was continued 90 min more before dialysis against several liters of 0.15 M NaCl, 20 mM Na₂HPO₄ (pH 7.2). New Zealand White rabbits were injected at numerous subcutaneous, intradermal, and intramuscular sites with the equivalent of 0.5 ml of the conjugate (1.5 mg of albumin, 0.5 mg of peptide) mixed into complete Freund's adjuvant. Antiserum was boosted 3-week intervals with the equivalent of 0.1-0.2 ml of the conjugate (0.3-0.6 mg of albumin, 0.1-0.2 mg of peptide) into incomplete Freund's adjuvant. Sera from two of four rabbits gave positive responses after the sixth boost when analyzed for reaction with purified 28kDa protein and erythrocyte membrane glycoproteins by immunoblotting; preimmune sera gave no reaction.

**Two-dimensional Iodopeptide Maps**—Approximately 10 μg of purified 28kDa protein or Rh polypeptides were ¹²⁵I-labeled, digested with 100 μg/ml of α-chymotrypsin, and analyzed in two dimensions on thin layer plates as adapted by Saboori et al. (1988) from the method of Elder et al. (1977). Recoveries of ¹²⁵I-labeled peptides eluted from the plates were >50% of the total.

**Preparation of Erythrocyte Membranes**—Human erythrocytes were obtained from blood bank units stored up to 5 weeks at 4 °C or from whole blood drawn from healthy normal volunteers into acid citrate dextrose anticoagulant and stored up to 1 day at 0 °C. All membrane preparations employed the methods described by Bennett (1983), hypotonic lysis, spectrin-actin elution, and preparation of 1 M KI-stripped inside-out membrane vesicles. In Fig. 8, the extracellular domain of g28kDa protein was degraded by digestion of intact erythrocytes with α-chymotrypsin (600 μg/ml) for 3 h at pH 5 prior to preparation of membranes, otherwise as described (Denker et al., 1988).

**Purification of 28kDa and gly28kDa Proteins, and Rh Polypeptide**—Purifications of 28kDa and gly28kDa proteins were performed basically as reported (Denker et al., 1988). Note that under nondenaturing conditions, 28kDa and gly28kDa protein purified; and the proteins were monitored during purification by immunoblot with anti-28kDa. In large scale purifications, KI-stripped inside-out membrane vesicles prepared from 1 unit of erythrocytes were suspended to 400 μl in 1% (w/v) Na N-lauroylsarcosine, 1 mM Na₂HCO₃, and shaken hypotonic lysis, spectrin-actin elution, and preparation of 1 M KI-stripped inside-out membrane vesicles. In Fig. 8, the extracellular domain of g28kDa protein was degraded by digestion of intact erythrocytes with α-chymotrypsin (600 μg/ml) for 3 h at pH 5 prior to preparation of membranes, otherwise as described (Denker et al., 1988).
Sepharose which had been equilibrated at 4 °C with 1% (v/v) Triton X-100 in the same buffer. The column was eluted at 50 ml/h with a 250-ml linear gradient of 0-0.4 M NaCl in the same buffer; pure 28kDa and gly28kDa proteins eluted together near the end of the gradient. Smaller scale purifications were performed similarly using one of the 15 cm steps and employing a Mono Q column.

28kDa and gly28kDa proteins were purified separately in denatured form by solubilizing the N-lauroylsarcosine-insoluble pellet (above) in 100 ml of 5% (w/v) SDS, 20 mM Na2HPO4, (pH 7.4) for 30 min at 60 °C. The soluble proteins were removed after centrifugation at 44,000 x g for 4 h, filtered, and loaded onto a 1.6 x 30-cm column packed in hydroxyapatite equilibrated with 0.5% (w/v) Na2HPO4, (pH 7.2), 1 mM NaNO3, 1 mM DTT. The column was eluted at 25 ml/h at 30 °C with a 300-ml linear gradient of 0-0.3 M Na2HPO4, in the same buffer; the 28kDa and gly28kDa proteins eluted near the end of the gradient. The peak fractions were combined, dialyzed against 0.5% (w/v) SDS, 10 mM Na2HPO4, (pH 7.4), 1 mM NaNO3, concentrated against polyethylene glycol, again dialyzed, and electophoresed into multiple 0.3 x 14 x 16 cm preparative 12% SDS-PAGE slabs. The slabs were cut into 3-mm horizontal strips, and proteins were eluted with 25 ml of 0.1% (w/v) SDS, 10 mM Na2HPO4, (pH 7.2), 1 mM NaNO3 by shaking 48 h at 22 °C.

Rh polypeptides were purified from solubilized membranes by hydroxyapatite chromatography (Saboori et al., 1988).

**Determination of NH2-terminal Amino Acid Sequence**—NH2-terminal amino acid sequence was determined from 28kDa and gly28kDa proteins purified by six different variations of the procedures described above, and quantitative protein determinations were performed using the method of Lowry et al. (1951) using bovine albumin as a standard. All purifications began with KI-stripped membrane proteins after extraction with N-lauroylsarcosine, but on one occasion, the erythrocytes had been previously digested extensively with chymotrypsin (to degrade extracellular domains of gly28kDa protein as well as possible contaminants). Five of the purifications were accomplished in Triton X-100 employing anion exchange chromatography, which was followed by high resolution gel filtration over a Superose 12 column on one occasion. One purification employed solubilization of membrane vesicles in SDS and hydroxyapatite chromatography followed by preparative SDS-PAGE. The products of all purifications were >90% pure, as assessed by silver staining of SDS-PAGE slabs. Pure 28kDa proteins (20-60 µg of protein) was precipitated in chilled ethanol. NH2-terminal amino acid sequence analysis was carried out in the University Protein/Peptide Laboratory using an Applied Biosystems model 470A protein sequenator.

**Determination of Stokes Radius**—A 50-µg aliquot of 28kDa and gly28kDa proteins purified under nondenaturing conditions was solubilized in 200 µl of 1% Triton X-100 (v/v), 0.1 M NaCl, 20 mM Tris-HCl (pH 7.8), 1 mM Na2EDTA, 1 mM DTT and loaded onto a 1 x 30-cm Superose 12 column previously equilibrated with the same buffer and run at a flow rate of 0.5 ml/min. Fractions of 30 µl each were collected. The peak fractions containing 28kDa and gly28kDa proteins were identified by immunoblot with anti-28kDa. In separate experiments, identical elution behavior was determined when erythrocyte membrane vesicles (500 µg of protein) were solubilized in the same volume of the same buffer, injected onto the column, and analyzed similarly. This column was previously calibrated with each of six water-soluble protein standards of known Stokes radius (listed in legend to Fig. 9). A 200-µg aliquot of each protein standard was solubilized in the same buffer and injected separately. The elution volumes were determined by Coomassie staining of SDS-PAGE slabs.

**Determination of Sedimentation Coefficient**—A 25-µg aliquot of 28kDa and gly28kDa proteins purified under nondenaturing conditions was solubilized in 150 µl of 1% Triton X-100 (v/v), 20 mM Tris (pH 7.5), 1 mM Na2EDTA, 1 mM DTT, 5% (w/v) sucrose in H2O, loaded onto 4-ml linear gradients of 5-20% sucrose (w/v) in the same buffer, followed by ultracentrifugation at 58,000 rpm for 5 h in a TST 60 Ti rotor (Beckman). Identical mobility of the 28kDa-gly28kDa aliquots was found when membrane vesicles (approximately 100 µg of protein) were solubilized in the same volume of the same buffer and analyzed similarly. Fifty to 100 µg of each of seven protein standards of known sedimentation coefficient (listed in legend to Fig. 9) was analyzed under identical conditions. The overall recoveries were always low, varying from 5 to 15% of the anticipated yield, indicating partial NH2-terminal block or incomplete solubilization during sequencing. To protect against the possibility that the sequence obtained might represent the NH2-terminal amino acid sequence of a contaminating protein, different purification schemes were employed for each preparation (see “Experimental Procedures”). On five of the determinations, the first and second cycles of the amino acid sequence contained multiple other peaks besides alanine and serine, but the remaining cycles always corresponded to the sequence in Fig. 3 with the number of unambiguous cycles for the six samples ending after 9, 20, 21, 23, and 35 residues, respectively.

When the amino acid sequence of 28kDa protein was compared to that of Rh polypeptides, seven of the 35 amino acids were identical (Fig. 3). When the 28kDa protein sequence was

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**RESULTS**

**Comparison of 28kDa Protein and the Rh Polypeptides**—The possibility that 28kDa protein and Rh polypeptides may have some shared domains was explored with anti-28kDa affinity purified polyclonal rabbit antibodies to denatured 28kDa protein. Purified Rh polypeptides demonstrated no cross-reaction with anti-28kDa on immunoblots, whereas purified 28kDa and gly28kDa proteins reacted strongly (Fig. 1). Relative degrees of homology between proteins can also be estimated by 125I labeling, proteolytic digestion, and two-dimensional analysis of the iodopeptides, electrophoresis followed by thin layer chromatography (Elder et al., 1977). When such analyses were conducted with pure Rh polypeptides, pure 28kDa protein, and the mixture of the two preparations, only a single iodopeptide appeared to be contained in all preparations (Fig. 2, arrow). Interestingly, this iodopeptide appeared to correspond to one shared among human Rh c, D, and E polypeptides (Blanchard et al., 1988) as well as all nonhuman homologs of the Rh polypeptides (Saboori et al., 1989). However, elution of these apparently identical iodopeptides from the thin layer plates and analysis by anion exchange chromatography demonstrated that the iodopeptides from Rh and 28kDa were not identical (Fig. 2, insets).

**NH2-terminal Amino Acid Sequence of 28kDa Protein**—NH2-terminal amino acid sequence determination was carried out on six different samples of purified 28kDa protein, and a consistent sequence was obtained from all preparations (Fig. 3). The overall recoveries were always low, varying from 5 to 15% of the anticipated yield, indicating partial NH2-terminal block or incomplete solubilization during sequencing. To protect against the possibility that the sequence obtained might represent the NH2-terminal amino acid sequence of a contaminating protein, different purification schemes were employed for each preparation (see “Experimental Procedures”). On five of the determinations, the first and second cycles of the amino acid sequence contained multiple other peaks besides alanine and serine, but the remaining cycles always corresponded to the sequence in Fig. 3 with the number of unambiguous cycles for the six samples ending after 9, 20, 21, 23, and 35 residues, respectively.

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FIG. 2. Comparison of two-dimensional iodopeptide maps of Rh polypeptides and 28kDa protein. Purified Rh polypeptides or purified 28kDa protein were denatured in SDS, 125I-labeled, electrophoresed into SDS-PAGE slabs, digested with α-chymotrypsin, spotted on thin layer plates, and separated in two dimensions: first, electrophoresis and second, thin layer chromatography (see "Experimental Procedures"). Analyses were performed on pure Rh polypeptides, pure 28kDa protein, or a mixture of both. A single iodopeptide appeared to be shared by all preparations (arrows) and was eluted into 0.2 ml of H2O and injected onto a Mono Q column equilibrated with 20 mM Tris (pH 7.5) and eluted with a 0-1 M linear gradient of NaCl (insets).

FIG. 3. Alignment of NH2-terminal amino acid sequences of 28kDa and selected proteins. Amino acid sequences for an Rh polypeptide (Cherif-Zahar et al., 1990), 28kDa protein (see "Experimental Procedures"), bovine lens major intrinsic protein (MIP) (Gorin et al., 1984), and Drosophila Big Brain (BIB) (Rao et al., 1990) are aligned to maximize homology. Closed boxes include identical residues. The number of the residue at the left of each sequence is identified. Analyzed by computer for homology with known amino acid sequences, a single significant homology was identified, MIP, the 26-kDa major intrinsic protein from bovine lens (Gorin et al., 1984). When the actual NH2-termini of 28kDa protein and MIP were aligned, 13 of the 35 amino acids were identical. Nine of these 13 residues plus four additional residues were shared with Big Brain, a putative homolog of MIP which is essential to neural development in Drosophila (Rao et al., 1990). Moreover several other residues were found to be conservative substitutions for amino acids in MIP or Big Brain. Smaller degrees of homology were identified between 28kDa protein and soybean nodulin 26 (Fortin et al., 1984) and E. coli glycerol facilitator (Muranatsu and Mizuno, 1989), other MIP homologs which, respectively, had 7 and 9 residues identical to 28kDa protein (not shown).

Membrane Organization of 28kDa Protein—A synthetic peptide corresponding to the first 10 NH2-terminal amino acids of the 28kDa protein was conjugated to albumin and used to raise a polyclonal antiserum in rabbits (anti-N-peptide). Anti-N-peptide gave a strong reaction with purified 28kDa protein on immunoblots, whereas none of the preimmunization sera reacted (not shown). In addition, anti-N-peptide gave a single clear reaction with a molecular mass 28kDa band on blots containing SDS-solubilized KI-stripped inside-out membrane vesicles (Fig. 4, right panels). However, anti-N-peptide gave only a very faint reaction with gly28kDa protein on blots. While this is consistent with a polymorphism at the NH2-terminus of the gly28kDa protein, it more likely results from the relatively low affinity of the antibody, the diffuse migration of gly28kDa protein during SDS-PAGE and existence of large amounts of contaminating immunoglobulin in the antiserum creating a high background which obscures immunoreactivity over the gly28kDa protein.

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When KI-stripped inside-out membrane vesicles were digested with carboxypeptidase Y, 28kDa protein was entirely degraded to a 23-kDa fragment which stained weakly with anti-28kDa but strongly with anti-N-peptide (Fig. 4, top panels), consistent with the presence of a 5-kDa cytosolic
COOH-terminal domain which contains most of the epitopes recognized by anti-28kDa. Removal of the cytosolic 5-kDa fragment also apparently reduced the solubility of the remaining protein, and oligomeric forms of 28kDa protein were found after digestion (Fig. 4, arrow). When intact erythrocytes were digested with carboxypeptidase Y or aminopeptidase M, there was no degradation of 28kDa protein (not shown), indicating that neither carboxyl nor amino termini extend significantly outside of the cell.

When KI-stripped inside-out membranes were digested with aminopeptidase M, 28kDa protein was largely resistant to degradation, although a small amount of multiple breakdown products was found (Fig. 4, bottom panels). A fragment of approximately 24 kDa reacted weakly with anti-28kDa but strongly with anti-N-peptide. A second fragment of approximately 19-kDa reacted with the anti-28kDa but gave no reaction with anti-N-peptide (Fig. 4, bottom). These fragments probably represent the products of small amounts of contaminating proteases, and chymotrypsin or trypsin digestion of KI-stripped inside-out membrane vesicles generated similar fragments (Fig. 5). The studies with chymotrypsin and trypsin also indicate that much of the 28kDa protein is protected from degradation to small fragments. Such proteolysis could occur if most of the protein should reside between the leaflets of the lipid bilayer, however, specific protein folding and assembly of multiple protein subunits could also confer relative inaccessibility of the proteases to potential digestion sites within cytosolic domains of the gly28kDa protein. Interestingly, the cytoplasmic domain of the gly28kDa protein was relatively less vulnerable to carboxypeptidase Y and trypsin digestions than was the 28kDa protein (Figs. 4 and 5), and the reason for this disparity is uncertain. This is also consistent with potential polymorphic differences between the two forms of the protein, although differences in overall gly28kDa protein conformation could conceivably result from attachment of the large glycan to an extracellular domain.

**Solubilization of 28kDa and gly28kDa Proteins in Nonde-naturing Detergent**—Previous efforts to solubilize 28kDa protein from erythrocyte membranes with Triton X-100 demonstrated minimal solubility at 0 °C, and it was concluded that the protein was linked to the underlying membrane skeleton (Denker et al., 1988). This was re-evaluated employing Triton X-100 concentrations varying from 0.5 to 4% (v/v) with physical shaking during the extraction at 22 °C. The Triton-soluble and insoluble fractions were separated by centrifugation and analyzed by SDS-PAGE. The membrane skeleton was judged to be intact at all concentrations of Triton X-100, since spectrin remained in the insoluble fraction while the non-ankyrin linked fraction of band 3 was extracted (Fig. 6, top panel). Extraction of 28kDa and gly28kDa proteins was evaluated by anti-28kDa immunoblot (Fig. 6, bottom panel). Neither 28kDa nor gly28kDa proteins were significantly solubilized in 0.5% Triton, whereas both were nearly totally solubilized at 4% Triton. At intermediate concentrations, both were partially solubilized, suggesting that they are associated in a complex. These studies also suggest that 28kDa protein is not tightly linked to the membrane skeleton but has limited solubility in nonionic detergents. In addition, when immunoblots of 28kDa protein immunoprecipitated from inside-out membrane vesicles solubilized in 1% (v/v) Triton X-100 were stained with anti-ankyrin, anti-protein 4.1, or anti-43kDa fragment of band 3, co-precipitation of these other proteins with 28kDa protein was not detected.3

28kDa and gly28kDa Proteins Behave as an Oligomer—The possibility that 28kDa and gly28kDa proteins associate with each other was explored by affinity chromatography with immobilized wheat germ agglutinin (WGA). Both forms of the protein co-purified under denaturing conditions, but 28kDa and gly28kDa protein were separated from each other by preparative SDS-PAGE, and analyzed for the ability to adsorb to the WGA columns (Fig. 7, panels a and b). By itself, pure 28kDa protein failed to adsorb to the WGA column,

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3 B. M. Denker and P. Agre, unpublished.
Fig. 7. Wheat germ agglutinin affinity chromatography of pure 28kDa and pure gly28kDa proteins and the 28kDa-gly28kDa oligomer. The experiments were conducted with SDS-purified 28kDa protein (containing no gly28kDa protein, panel a), SDS-purified gly28kDa protein (containing no 28kDa protein, panel b), or with 28kDa and gly28kDa proteins purified together under nondenaturing conditions (panels c and d). Approximately 20–50 µg of the proteins were solubilized in 1 ml of 2% Triton X-100 (v/v), 20 mM Tris (pH 7.2), 1 mM DTT, 1 mM NaCl, and loaded onto a 0.1-ml wheat germ agglutinin-Sepharose column in the absence (panels a, b, and c) or presence (panel d) of 1 M N-acetylglucosamine (GlcNAc), and the flow-through was collected. After wash, the columns were eluted with 1 M GlcNAc. Fractions corresponding to equivalent concentrations of Triton X-100-solubilized membranes (lanes c), flow-through (lanes f), or GlcNAc eluate (lanes e) were analyzed by SDS-PAGE immunoblot with anti-28kDa. Note that pure 28kDa protein is not adsorbed onto the affinity columns (panel a) unless complexed with gly28kDa protein (panel c).

Fig. 8. Cross-linking of 28kDa protein in membrane vesicles with glutaraldehyde. KI-stripped inside-out membrane vesicles were prepared from undigested erythrocytes (left panel) or from erythrocytes that were previously digested with α-chymotrypsin to degrade gly28kDa protein at extracellular sites (right panel, see “Experimental Procedures”). The vesicles (1 mg of protein/ml) were then incubated at 22 °C in 0.1 M NaCl, 13 mM Na2HPO4 (pH 7.2), 1 mM DTT, 0.5 mM NaEDTA, 0.5 mM NaN3, containing 0.005% (v/v) glutaraldehyde for 0 min (lanes 1), 7.5 min (lanes 2), 15 min (lanes 3), 30 min (lanes 4), 1 h (lanes 5), or 2 h (lanes 6) prior to solubilization and SDS-PAGE and immunoblot with anti-28kDa (see “Experimental Procedures”). Cross-linked complexes of approximately 60, 87, and 115 kDa, were noted at the right.

whereas the pure gly28kDa protein adsorbed to the WGA column and was eluted with N-acetylglucosamine. 28kDa and gly28kDa proteins purified under nondenaturing conditions behaved similar to gly28kDa protein and specifically adsorbed to the WGA columns (Fig. 7, panels c-d).

Chemical Cross-linking of 28kDa and gly28kDa Proteins in Membranes—Close physical association between two molecules within the membrane may permit selective covalent cross-linking with low concentrations of agents such as glutaraldehyde. KI-stripped inside-out membrane vesicles were incubated in 0.08% (v/v) glutaraldehyde for varying intervals prior to SDS-PAGE and anti-28kDa immunoblot (Fig. 8, left panel). 28kDa protein disappeared with increased time of incubation and a smear of a larger molecular weight complex appeared. (The interruption at 90 kDa probably results from impaired transfer of the cross-linked protein complex by the glycophorin A dimer.)

It has previously been shown that digestion of intact erythrocytes with chymotrypsin results in proteolytic degradation of the extracellular domain(s) of gly28kDa protein without degradation of 28kDa protein when assessed by anti-28kDa immunoblot (Denker et al., 1988). KI-stripped inside-out membrane vesicles prepared from chymotrypsin-digested erythrocytes were incubated in glutaraldehyde, and a discrete pattern of higher molecular weight complexes appeared including discernible bands at approximately 60 kDa, and more prominent bands at 87 and 115 kDa, as well as a smear extending to the top of the gel (Fig. 8, right panel). These bands may correspond to dimers, trimers, tetramers, and higher order oligomers of 28kDa protein, although the existence of other proteins in the cross-linked complex cannot be refuted. Likewise, cross-linking of 28kDa protein to fragments of digested gly28kDa protein could also be present.

Physical Characterization of 28kDa and gly28kDa Protein—Purified 28kDa and gly28kDa proteins were electrophoresed into SDS-PAGE slabs containing 20, 16, 12, and 10% gels, and the mobilities relative to protein standards were assessed after staining with silver reagent. Electrophoretic mobility of the pure 28kDa protein increased slightly but consistently as the concentration of acrylamide was reduced, corresponding to apparent molecular masses ranging from 30 to 27.5 kDa (Fig. 9A). Increased electrophoretic mobility at lower acrylamide concentrations is characteristic of extremely hydrophobic proteins, since they bind relatively more SDS (Helenius and Simons, 1975), and the electrophoretic mobility of the Rh polypeptide has been previously shown to increase even more dramatically at lower acrylamide concentrations (Gahmberg, 1983; Agre et al., 1987). The electrophoretic mobility of gly28kDa protein was more difficult to establish precisely, but the apparent molecular mass was approximately 36–55 kDa on all determinations.

The mobility of purified 28kDa and gly28kDa proteins was assessed by gel filtration under nondenaturing conditions, and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit. The apparent Stokes radius under denaturing conditions (SDS) and the proteins consistently migrated as a discrete unit.

Estimation of the sedimentation coefficient was performed by ultracentrifugation into gradients of 5–20% (w/v) sucrose containing 1% (v/v) Triton X-100. 28kDa protein purified under nondenaturing conditions (SDS) migrated with a sedimentation coefficient of approximately 2 S, most likely corresponding to a 28kDa protein monomer (not shown). However, 28kDa and gly28kDa proteins purified under nondenaturing conditions migrated as a discrete unit of approximately 5.7 S when the gradients were poured from buffers made in H2O (Fig. 9C, upper profile). Interestingly, identical mobility was found when Triton X-100-solubilized membrane vesicles were analyzed. In order to correct for detergent binding to the proteins, sedimentation was conducted in buffers made in D2O (Reynolds and Tanford, 1976). This resulted in a significant shift (Fig. 9C, lower profile) which permitted calculation of partial specific volume (0.795 ml/g) and amount of detergent bound to the oligomer (0.41 mg of Triton X-100/mg of protein). The calculated molecular mass of the oligomer was thereby corrected from 190 kDa (the 28kDa-gly28kDa oligomer-detergent complex) to 135 kDa (the 28kDa-gly28kDa oligomer, see Table II).

DISCUSSION

This study further characterizes an unusual erythrocyte transmembrane protein which exists in two forms, 28kDa and gly28kDa, the latter containing asparagine-linked carbohydrate (Denker et al., 1988). Although several biochemical similarities to the Rh polypeptides suggested that these proteins may share common domains, none were identified. Sev-
A, through 4-ml linear gradients of 5-20% sucrose gradients containing proteins eluted together, as indicated by the proteins eluted together, as indicated by the under nondenaturing conditions were analyzed by sedimentation.

Gly28kDa proteins relative to the molecular weight markers were solubilized in SDS and electrophoresed into soluble standard proteins with known Stokes radius and sedimentation coefficient were used for this and for sedimentation analyses (panel C). The slabs were stained with silver reagent and the mobilities were measured (listed in Fig. 1).

The size of 28kDa and gly28kDa proteins purified under nondenaturing conditions were analyzed in 1% (v/v) Triton X-100 (see "Experimental Procedures"). The following water-soluble standard proteins with known Stokes radius and sedimentation coefficient were used for this and for sedimentation analyses (panel C): (a) cytochrome c, 17 Å, 1.8 S; (b) carbonic anhydrase, 24 Å, 2.9 S; (c) bovine serum albumin, 37 Å, 4.3 S; (d) alcohol dehydrogenase, 42 Å, 7.6 S; (e) β-amylase 8.9 S; (f) ferritin, 61 Å, 17.6 S; and (g) thyroglobulin, 85 Å, 21.4 S. O, 28kDa and gly28kDa proteins eluted together, as indicated by the arrow.

Panel C, determination of sedimentation coefficient. 28kDa and gly28kDa proteins purified under nondenaturing conditions were analyzed by sedimentation through 4-ml linear gradients of 5-20% sucrose gradients containing 1% (v/v) Triton X-100 (see "Experimental Procedures"). To correct for detergent binding to proteins, sedimentations were conducted employing gradients of sucrose in D2O, 0.28kDa and gly28kDa proteins eluted together, as indicated by the arrows.

**Fig. 9.** Determination of the physical size of 28kDa and gly28kDa proteins and the 28kDa-gly28kDa oligomer. Panel A, analysis by SDS-PAGE. A 2-μg aliquot of purified 28kDa and gly28kDa proteins was solubilized in SDS and electrophoresed into 14 × 16-cm slabs of 20, 16, 12, and 10% gels (see "Experimental Procedures"). The size of 28kDa and gly28kDa proteins relative to the molecular weight markers were calculated by nonlinear regessions employing the equation y = ae^x. Panel B, determination of Stokes radius. 28kDa and gly28kDa proteins purified under nondenaturing conditions were analyzed in 1% (v/v) Triton X-100 by filtration through a Superose 12 column as described (see "Experimental Procedures"). The following water-soluble standard proteins with known Stokes radius and sedimentation coefficient were used for this and for sedimentation analyses (panel C).

### TABLE II

| Property                                      | Value     |
|-----------------------------------------------|-----------|
| Stokes radius, \( R_s \)                       | 61 Å      |
| Sedimentation coefficient, \( \phi^{580} \)    | 5.7 S     |
| Partial specific volume, \( \phi \)            | 0.795 ml/g|
| Triton X-100 bound \( M_r \)                   | 0.41 mg/mg protein |
| Molecular weight of protein                   | 190,000   |
| Molecular weight, SDS-PAGE \( M_r \)           | 36,000-60,000 (gly28kDa) |
| Frictional ratio, \( f/f_0 \)                  | 1.63      |

* Estimated by gel filtration (see Fig. 9).
* Estimated by sedimentation on 5-20% sucrose gradients (see Fig. 9).
* Calculated from sucrose gradients in H2O and D2O by the method of Sadler et al. (1979).
* Calculated using \( \psi_{protein} = 0.735 \) and \( \psi_{TritonX-100} = 0.94 \) (Steele et al., 1978).
* Calculated from the equations:

\[
M_r = \frac{4\pi N M_r}{3 M_r (1 - \psi_{protein})}
\]

where \( N = 6.02 \times 10^{23} \) and \( M_r \) was assumed to be 0.2 g of solvent/g of protein (Tanford, 1961).

**TABLE II**

| Property                                      | Value     |
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When the NH2 termini are aligned, the amino acid sequence of the 28kDa protein was found to be related to that of MIP, the 25-kDa major intrinsic protein of lens (Gorin et al., 1984). Furthermore, several physical similarities between the two proteins are apparent including: similar size, a cytoplasmic COOH-terminal domain, peculiar detergent solubilities, and the ability to form multisubunit transmembrane oligomers. Significant homologies are known to exist between other membrane proteins of erythrocytes and lens fiber cells (Allen et al., 1987), and the existence of such homologies is most likely related to specialized features common to both of these cell types. Erythrocytes and differentiated lens fiber cells lack nuclei and intracellular organelles, both being essentially membrane sacks which contain cytoplasm composed nearly entirely of uniform species of proteins (hemoglobin and crystallins, respectively). 28kDa and MIP are not likely to be species variants of the same protein, since they are only 37% identical, while the amino acid sequences of MIP from bovine and rat lens are >90% identical overall. Also, immunoblots of rat lens membranes failed to react with anti-28kDa despite
strong reactions of rat erythrocyte and kidney membranes (Denker et al., 1988). The extent of the homology between 28kDa protein and MIP will not be known until the full primary sequence of the 28kDa protein is determined.

Unfortunately, recognition of a potential homology between 28kDa protein and MIP does not clarify what the physiologic role of 28kDa protein might be. The physiologic role of MIP is still uncertain, and investigators do not agree whether or not MIP is a component of gap junctions. Lens fiber membranes contain an enormous amount of MIP (Benedetti et al., 1976; Broekhuysen et al., 1976). Lens fiber cells behave as if metabolically linked by gap junctions (Goodenough, 1979), and MIP was therefore thought to be a specialized gap junction protein forming intercellular channels. The amino acid sequence deduced from the isolated cDNA predicted MIP to have six membrane-spanning domains of which one is amphiphilic, consistent with the potential role of MIP as a junctional protein (Gorin et al., 1984). To our knowledge high conductance has not been reported in native membranes containing MIP. When reconstituted into synthetic membranes, however, MIP can form high conductance channels (Kistler J., Christie, D., and Bullivant, S.).

Recent identification of amino acid sequence homologies between MIP and the products of other genes from diverse species suggests that they belong to a family of transmembrane proteins which form channels permeable to small molecules. Big Brain (BiB) is a neural development gene from Drosophila needed for differentiation of ectodermal cells into epidermoblasts instead of neuroblasts; the product of the BiB gene was felt to be a major component of lens fiber junctions by some investigators (Saé et al., 1985; Bok et al., 1982), but not others (Paul and Goodenough, 1983). In addition, the amino acid sequence of MIP bears no homology with the sequence of other gap junction proteins (Nicholson et al., 1983; Paul, 1986; Kumar and Gilula, 1986; Beyer et al., 1987). A second lens fiber cell protein, MP70, has been localized to lens junctions (Gruitjers et al., 1987), and MP70 has amino acid sequence homologous to the recognized liver and heart gap junction proteins (Kistler et al., 1988). It was recently shown that MIP complexes are not aligned between the membranes of the two cells at lens fiber junctions but exist in a checkerboard distribution and may form volume-regulating channels by which the cells individually communicate with the extracellular space (Zampi et al., 1989).

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REFERENCES

Agre, P., Saboori, A. M., Asimos, A., and Smith, B. L. (1987) J. Biol. Chem. 262, 17497-17502
Allen D. P., Low, P. S., Dola, A., and Maisel, H. (1987) Biochem. Biophys. Res. Commun. 149, 266-275
Avent, N. D., Ridgwell, K., Mawby, W. J., Tanner, M. J. A., Ansee, D. J., and Kumpel, B. (1988) Biochem. J. 256, 1043-1046
Baker, M. E., and Saier, M. H. (1990) Cell 60, 185-186
Benedetti, E. C., Dunia, L., Bentzel, C. J., Vermorken, A. J. M., Kibbilaar, M., and Bloemendal, H. (1976) Biochim. Biophys. Acta 457, 353-384
Bennett, V. (1983) Methods Enzymol. 96, 313-324
Bennett, V. (1989) Biochim. Biophys. Acts 988, 107-121
Ber, E. C., Paul, D. L., and Goodenough, D. A. (1987) J. Cell Biol. 105, 2621-2629
Blanchard, D., Bloy, C., Hermand, P., Carton, J.-P., Saboori, A., Smith, B. L., and Agre, P. (1988) Blood 72, 1424-1427
Bok, D., Dockstader, J., and Horwitz, H. (1982) J. Cell Biol. 92, 213-220
Broekhuysen, R. M., Kuhlman, E. D., and Stols, A. L. H. (1976) Exp. Eye Res. 28, 365-371
Cherif-Zahar, B., Bloy, C., Le Van Kim, C., Blanchard, D., Bailly, P., Hermand, P., Salmon, C., Carton, J.-P., and Colon, Y. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6243-6246
Davis, J., and Bennett V. (1990) J. Biol. Chem. 257, 5816-5820
Denker, B. M., Smith, R. B., Kuhajda F. P., and Agre, P. (1988) J. Biol. Chem. 263, 15654-15662
de Vetten, M. P., and Agre, P. (1988) J. Biol. Chem. 263, 18193-18196
Elder, J. H., Pickett, R. A., I., Hampton, H., and Lerner, R. A. (1977) J. Biol. Chem. 252, 6510-6515
Fortin, M. G., Morrison, N. A., and Verma, D. P. S. (1987) Nucleic Acids Res. 15, 813
Gahmberg, C. G. (1983) EMBO J. 2, 223-227
Gahmberg, C. G., and Karhi, K. K. (1984) J. Immunol. 133, 334-337
Goodenough, D. M., Rintoul, D. A., Takemoto, L. J., Hunkapiller, M. W., and Goodenough, D. A. (1987) J. Cell Biol. 104, 556-572
Helenius, A., and Simons, K. (1972) J. Biol. Chem. 247, 3656-3661
Helenius, A., and Simons, K. (1975) Biochim. Biophys. Acta 415, 29-79
Hunter, W., and Greenwood, F. (1962) Nature 194, 495-496
Kistler J., Christie, D., and Bullivant, S. (1988) Nature 331, 721-723
Kumar, N. M., and Gilula, N. B. (1986) J. Cell Biol. 103, 767-776
Laemmli, U. K. (1970) Nature 227, 680-685
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
Mollison, P. L., Engelfriet, C. P., and Contreras, M. (1987) Blood Transfusion in Clinical Medicine, 8th Ed, Blackwell Scientific Publications, Oxford
Moore, S., Woodrow, C. F., and McClelland, D. B. L. (1982) Nature 298, 528-530
Moore, S., and Green, C. (1987) Biochem. J. 244, 735-742
Muramatsu, S., and Mizuno, T. (1989) Nucleic Acids Res. 17, 4378
Nicholson, B. J., Takemoto, L. J., Hunkapiller, M. W., Hood, L. E., and Revel, J. P. (1983) Cell 32, 967-978
Paul, D. (1986) J. Cell Biol. 103, 123-134
Paul, D. L., and Goodenough, D. A. (1985) J. Cell Biol. 96, 625-632
Peracchia, C., and Girsch, S. J. (1985) Curr. Eye Res. 4, 431-439
Rao, Y., Jan, L. Y., and Jan, Y. (1985) Nature 318, 163-167
Reynolds, J. A., and Tanford, C. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 4467-4470
Ridgwell, K., Tanner, M. J. A., and Antee, D. J. (1984) FEBS Lett. 174, 7-10
Rosse, W. F., and Telen, J. J. (1989) in Red Blood Cell Membranes (Agre, P., and Parker, J. C., eds) pp. 299-324, Marcel Dekker Inc., New York
Erythrocyte M, 28,000 Transmembrane Protein

Saboori, A. M., Smith, B. L., and Agre, P. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 4042–4045
Saboori, A. M., Denker, B. M., and Agre, P. (1989) *J. Clin. Invest.* **83**, 187–191
Sadler, J. E., Rearick, J. I., Paulson, J. C., and Hill, R. L. (1979) *J. Biol. Chem.* **254**, 4434–4442
Sas, D. F., Sas, J., Johnson, K., Menko, A. A., Johnson, R. G. (1985) *J. Cell Biol.* **100**, 216–225
Steck T. L. (1989) in *Cell Shape: Determinants, Regulation and Regulatory Role* (Stein, W., and Bronner, F., eds) pp. 205–246, Academic Press, Orlando, FL

Steele, J. C. H., Tanford, C., and Reynolds, J. A. (1978) *Methods Enzymol.* **48**, 11–23
Suyama, K., and Goldstein, J. (1990) *Blood* **75**, 255–260
Tanford, C. (1961) *Physical Chemistry of Macromolecules*, pp. 364–396, John Wiley & Sons, New York
Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350–4354
Zampighi, G. A., Hall, H. E., and Kreman, M. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 8468–8472
Zampighi, G. A., Hall, J. E., Ehring, G. R., and Simon, S. A. (1989) *J. Cell Biol.* **108**, 2255–2275