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

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A novel M_r 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, 15634–15642). Although 28kDa protein resembles the Rh polypeptides biochemically, structural homologies were not identified by immunoblot or two-dimensional iodopeptide maps. The NH_2-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). Antisera to a synthetic peptide corresponding to the NH_2-terminus of 28kDa protein gave a single reaction of molecular mass 28kDa on immunoblots of erythrocyte membranes. 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 NH_2-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, glycophorins 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_r 28,000 integral membrane protein; gly28kDa protein, glycosylated 28kDa protein which has a M_r ranging from 35,000 to 60,000; anti-28kDa, affinity-purified polyclonal rabbit IgG against denatured 28kDa protein; anti-N-peptide, polyclonal rabbit antisera raised against synthetic peptide corresponding to the 10 NH_2-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; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

TABLE I
Comparison of known properties of 28kDa protein and Rh polypeptides

	28kDa	Rh
Similarities/property		
Retained in membrane vesicles	Yes[a]	Yes[b]
Retained in membrane skeletons (insoluble in 1% Triton X-100)	Yes[a]	Yes[c]
Molecular weight, SDS-PAGE	28,000[a]	32,000[d]
Cytoplasmic domain	Yes[a]	Yes[e]
Glycosylated subpopulation	gly28kDa[a]	Isoform[f]
SDS-PAGE staining		
Coomassie	Very weak[a]	Very weak[b]
Silver	Strong[a]	Strong[b]
Amino acid content	Similar[a]	Similar[b]
Na_2HPO_4 elution from hydroxylapatite	0.5 M[g]	0.5 M[g]
Present in nonhuman erythrocytes	Yes[a]	Yes[h]
Differences		
Surface ^{125}I-labeled	No[b]	Yes[d]
Acylated with [^3H]palmitate	No[i]	Yes[i]
Immunoprecipitated with Rh antibodies	No[a]	Yes[d]

[a] Denker *et al.*, 1988.
[b] Agre *et al.*, 1987.
[c] Gahmberg and Karhi, 1984; Ridgwell *et al.*, 1984.
[d] Moore *et al.*, 1982; Gahmberg, 1983.
[e] Suyama and Goldstein, 1990.
[f] Moore and Green, 1987; Avent *et al.*, 1988.
[g] Saboori *et al.*, 1988.
[h] Saboori *et al.*, 1989.
[i] de Vetten and Agre, 1988.

and are also deficient in the glycosylated Rh isoform (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 structures, but 28kDa protein appears to be related to MIP, the 26kDa 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* glycerol 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 whether 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 TPCK-trypsin (250 units/mg) were from Cooper Biomedical. Carrier-free $Na^{125}I$ was from Amersham Corp. High resolution hydroxylapatite and protein A-bearing Staphylococci were from Behring Diagnostics. The Mono Q column, Q-Sepharose, and protein A were from Pharmacia Inc. Phenylmethylsulfonyl fluoride, dithiothreitol, glutaraldehyde, EDTA, carboxypeptidase Y (100 units/mg), aminopeptidase M (30 units/mg), protein standards (used for determination of molecular weight, sedimentation coefficient, and Stokes radius), wheat germ agglutinin-Sepharose (5 mg/ml), *N*-lauroylsarcosine, and Triton X-100 were all from Sigma. Polyethylene glycol (Carbowax 8000) was from Fisher. Pentex fraction V albumin was from Miles. Ultra-pure sucrose and Tris were from Schwarz/Mann. Silver stain kits and electrophoresis reagents including nitrocellulose sheets, pure SDS, and acrylamide were from Bio-Rad. Chloramine-T, X-Omat AR autoradiographic film, and developing reagents were from Kodak. TLC cellulose/plastic sheets (No. 5577) were from E. Merck. Cronex intensifier screens were from Du Pont. Millipore Millex-HV 0.45-μm filters were used. A synthetic peptide (A-S-E-F-K-K-K-L-F-W-C) corresponding to the 10 NH_2-terminal amino acids of 28kDa with COOH-terminal cysteine was synthesized by Peninsula Laboratories, Belmont, CA.

Methods—Except where indicated, 0.1 × 9 × 6-cm miniature SDS-PAGE slabs were prepared using 12% gels with the buffer system of Laemmli (1970); most samples were incubated in 40 mM dithiothreitol and 1% (w/v) SDS for 10 min at 60 °C prior to electrophoresis. (Incubations at 95 °C lead to irreversible aggregation of 28kDa protein.) Samples containing Triton X-100 were incubated in higher SDS concentrations in order to produce at least a 5-fold excess of SDS. Protein determinations were performed by the method of Lowry *et al.* (1951) employing the appropriate buffer controls and bovine serum albumin as standard.

Immunoblots—Proteins were electrophoretically transferred onto nitrocellulose sheets as described by Towbin *et al.* (1979) employing the buffers, wash conditions, and ^{125}I-labeled protein A described by Davis and Bennett (1982). Protein A was ^{125}I-labeled employing chloramine-T as an oxidant (Hunter and Greenwood, 1962) to a specific activity of approximately 20 μCi/μg. Most blots were incubated with 0.1 μg/ml of affinity-purified anti-28kDa IgG. The anti-N-peptide antiserum was used at a dilution of 1:200 on immunoblots.

Preparation of Antibodies—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). Antisera was raised to the synthetic peptide corresponding to the 10 NH_2-terminal amino acids of 28kDa protein conjugated to albumin ("anti-N-peptide"). Bovine serum albumin (3 mg/ml), synthetic peptide (1 mg/ml), and glutaraldehyde (0.02%, v/v) in 0.1 M NaCl, 50 mM Na_2HPO_4 (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 Na_2HPO_4 (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. The animals were boosted at 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) mixed 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 membranes on immunoblots; preimmune sera gave no reaction.

Two-dimensional Iodopeptide Maps—Approximately 10 μg of purified 28kDa protein or Rh polypeptides were ^{125}I-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 ^{125}I-labeled peptides eluted from the gel 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 gly28kDa protein was degraded by digestion of intact erythrocytes with α-chymotrypsin (600 μg/ml) for 3 h at pH 8 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 proteins co-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 ml in 1% (w/v) Na *N*-lauroylsarcosine, 1 mM Na_4HCO_3 and shaken 60 min at 22 °C. The solubilized proteins were separated from the insoluble pellet by centrifugation for >6 h at 30,000 × *g*. The pellet was washed by suspending to 1.5 liters in 10 mM Na_2HPO_4 (pH 7.4) followed by another centrifugation. The pellet was partially solubilized by shaking for 3–6 h at 22 °C in 800 ml of 2% (v/v) Triton X-100, 20 mM Tris-HCl, 1 mM NaN_3, 1 mM dithiothreitol (pH 7.8), and unsolubilized material was removed by centrifugation at 30,000 × *g* for >6 h. The supernatant was removed and filtered through a 0.45-μm filter and loaded onto a 1.6 × 35-cm column packed with Q-

[2] B. L. Smith and P. Agre, manuscript in preparation.

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-tenth volumes at each step 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 Na_2HPO_4 (pH 7.4) for 30 min at 60 °C. The soluble proteins were removed after centrifugation at 44,000 × *g* for >4 h, filtered, and loaded onto a 1.6 × 30-cm column packed with hydroxylapatite equilibrated with 0.2% SDS, 10 mM Na_2HPO_4 (pH 7.2), 1 mM NaN_3, 1 mM DTT. The column was eluted at 25 ml/h at 30 °C with a 300-ml linear gradient of 0.3–0.8 M Na_2HPO_4 in the same buffer; the 28kDa and gly28kDa proteins eluted near the end of the gradient. The peak fractions were combined, dialyzed against 0.2% (w/v) SDS, 10 mM Na_2HPO_4 (pH 7.4), 1 mM NaN_3, concentrated against polyethylene glycol, again dialyzed, and electrophoresed into multiple 0.3 × 14 × 16-cm preparative 12% SDS-PAGE slabs. The slabs were cut into 3-mm horizontal strips, and proteins were eluted with 2.5 ml of 0.1% (w/v) SDS, 10 mM Na_2HPO_4 (pH 7.2), 1 mM NaN_3 by shaking 48 h at 22 °C.

Rh polypeptides were purified from SDS-solubilized membranes by hydroxylapatite chromatography (Saboori *et al.*, 1988).

Determination of NH_2-terminal Amino Acid Sequence—NH_2-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 by the method of Lowry *et al.* (1951) using bovine serum 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 hydroxylapatite chromatography followed by preparative SDS-PAGE. The products of all purifications were >99% pure, as assessed by silver staining of SDS-PAGE slabs. Pure 28kDa proteins (20–60 μg of protein) was precipitated in chilled ethyl alcohol. NH_2-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 NaN_3, 1 mM DTT and loaded onto a 1 × 30-cm Superose 12 column previously equilibrated with the same buffer and running at 30 ml/h while 0.5-ml fractions were collected. 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 NaEDTA, 1 mM DTT, 5% (w/v) sucrose in H_2O, 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 60.4 rotor (Sorvall). Identical mobility of the 28kDa-gly28kDa oligomer 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 identically in separate gradients. Forty fractions of 100 μl were removed for analysis of protein mobilities. Fractions containing 28kDa and gly28kDa proteins were identified by immunoblot with anti-28kDa. Fractions containing protein standards were identified by Coomassie staining of SDS-PAGE slabs. To correct for detergent binding to proteins, sedimentations were conducted employing gradients of sucrose in D_2O with calculations performed as described by Sadler *et al.* (1979).

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 ^{125}I 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*).

NH_2-terminal Amino Acid Sequence of 28kDa Protein—NH_2-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 NH_2-terminal block or incomplete solubilization during sequencing. To protect against the possibility that the sequence obtained might represent the NH_2-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, 26, and 35 residues, respectively.

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

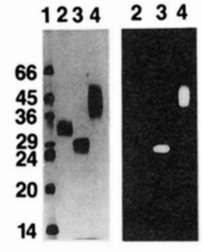

FIG. 1. **SDS-PAGE and anti-28kDa immunoblot analysis of Rh polypeptides, 28kDa protein, and gly28kDa protein.** Standard protein molecular mass markers were employed (*lane 1*): bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa). Approximately 2 μg of the following purified proteins were analyzed: Rh polypeptides (*lanes 2*), 28kDa protein (*lanes 3*), and gly28kDa protein (*lanes 4*). SDS-PAGE slabs were stained with silver reagent (*left*) or blot transferred onto nitrocellulose, reacted with anti-28kDa, decorated with ^{125}I-protein A, and visualized by autoradiography (see "Experimental Procedures").

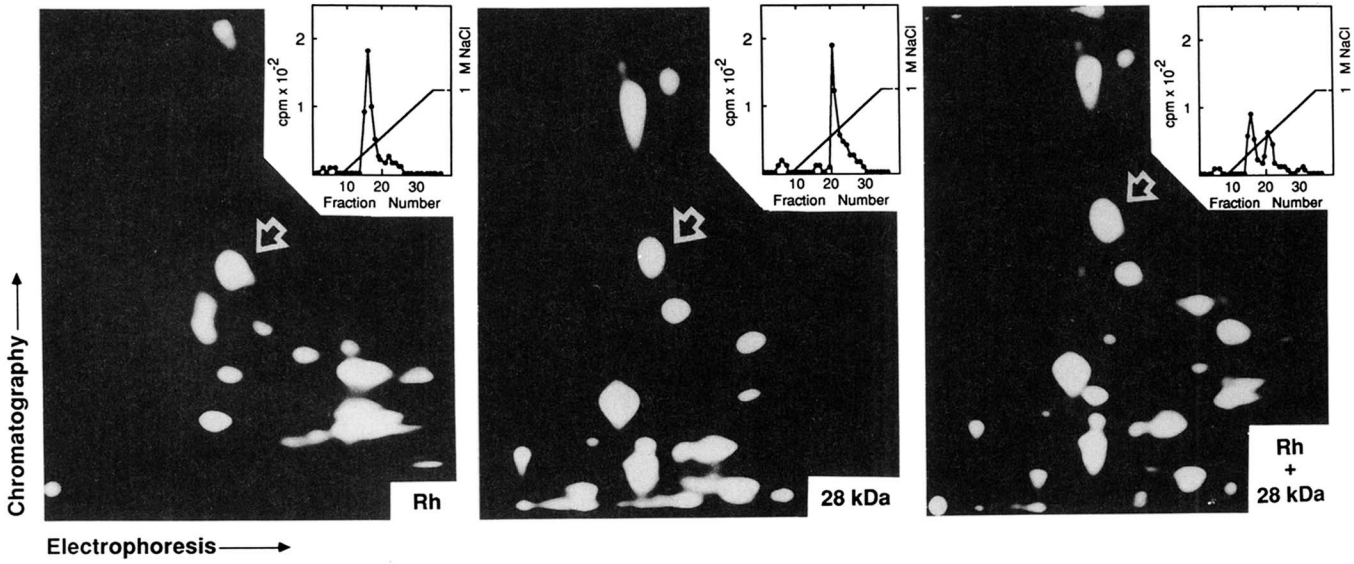

FIG. 2. **Comparison of two-dimensional iodopeptide maps of Rh polypeptides and 28kDa protein.** Purified Rh polypeptides or purified 28kDa protein were denatured in SDS, ^{125}I-labeled, electrophoresed into SDS-PAGE slabs, digested with α-chymotrypsin, spotted on thin layer plates, and separated in 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 H_2O 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*).

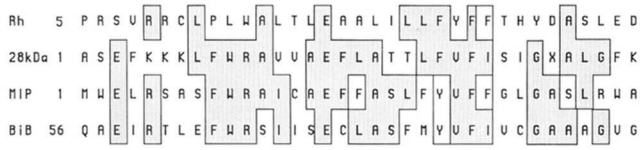

FIG. 3. **Alignment of NH_2-terminal amino acid sequences of 28kDa and selected proteins.** Amino acid sequences for an Rh polypeptide (Cherif-Zahar *et al.*, 1990), 28kDa protein (see "Experimental Procedures"), bovine lens major intrinsic protein (MIP) (Gorin *et al.*, 1984), and *Drosophila* Big Brain (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.

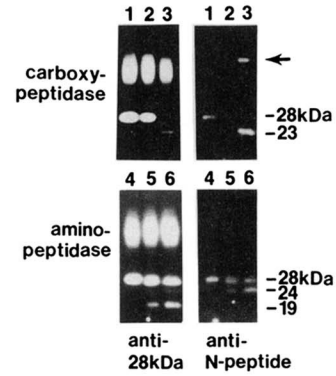

FIG. 4. **Immunoblot of inside-out membranes after digestion with carboxypeptidase or aminopeptidase.** KI-stripped inside-out membrane vesicles (250 μg of protein/ml) were incubated 17 h at 22 °C in 25 mM Na acetate (pH 6), 0.5 mM DTT (carboxypeptidase Y digestions, *upper panels*); or in 200 mM Na_2HPO_4 (pH 7), 0.5 mM DTT (aminopeptidase M digestions, *lower panels*) without peptidase (*lanes 1* and *4*), with 40 units peptidase/ml (*lanes 2* and *5*), or 200 units/ml (*lanes 3* and *6*). The membranes were pelleted, and approximately 10 μg of protein was electrophoresed into SDS-PAGE slabs and analyzed on immunoblots with anti-28kDa (*left panels*) or anti-N-peptide (*right panels*) as described (see "Experimental Procedures"). The approximate size of the reactive fragments are identified at the right.

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 NH_2 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.*, 1987) and *E. coli* glycerol facilitator (Muramatsu 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 NH_2-terminal amino acids of the 28kDa protein was conjugated to albumin and used to raise a polyclonal antisera 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 NH_2 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.

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 protection 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 Nondenaturing 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

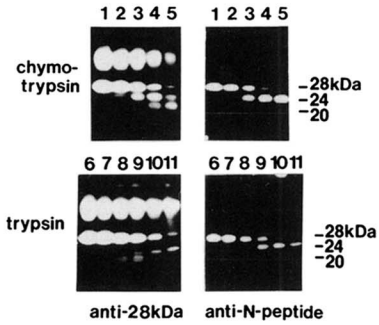

FIG. 5. **Immunoblot of inside-out membranes after digestion with chymotrypsin or trypsin.** KI-stripped inside-out membrane vesicles (800 μg of protein/ml) were incubated 1 h at 37 °C in 10 mM Na_2HPO_4 (pH 7.2), 1 mM DTT, 1 mM NaN_3 with no protease (*lanes 1* and *6*), 0.02, 0.1, 0.4, or 2 μg/ml with α-chymotrypsin (*lanes 2–5*), or 0.02, 0.1, 0.4, 2, or 10 μg/ml with TPCK-trypsin (*lanes 7–11*). Approximately 10 μg of protein was electrophoresed into SDS-PAGE slabs and analyzed on immunoblots with anti-28kDa (*left panels*) or anti-N-peptide (*right panels*) as described (see "Experimental Procedures"). The approximate size of the reactive fragments are identified at the right.

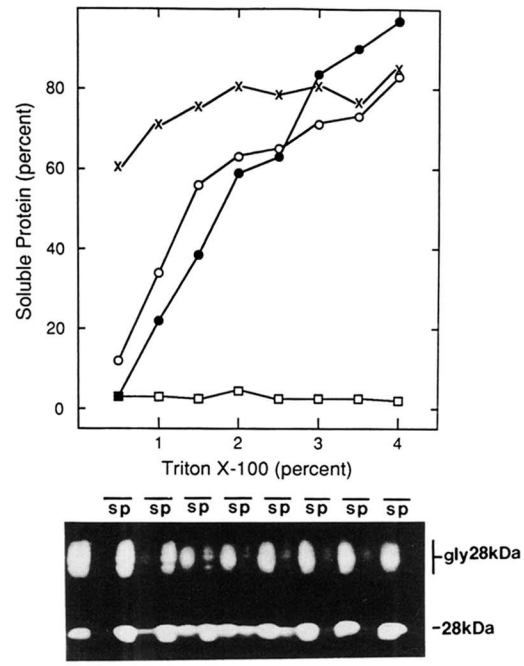

FIG. 6. **Solubilization of 28kDa and gly28kDa proteins from erythrocyte membranes with Triton X-100.** Erythrocyte membranes (2 mg of protein/ml) were incubated 60 min in 10 mM Na_2HPO_4 (pH 7.2) containing from 0 to 4% (v/v) Triton X-100 while shaking at 22 °C (see "Experimental Procedures"). Soluble fractions (*lanes s*) and insoluble pellets (*lanes p*) were separated by centrifugation at 44,000 × *g* for 30 min, resuspended to comparable volumes, and analyzed by Coomassie-stained SDS-PAGE or by immunoblot with anti-28kDa (*lower panel*). Spectrin (□) and band 3 (×) were quantitated in stained gels and 28kDa protein (●) and gly28kDa protein (○) were quantitated in autoradiographs by laser densitometry (*upper panel*).

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,

[3] B. M. Denker and P. Agre, unpublished.

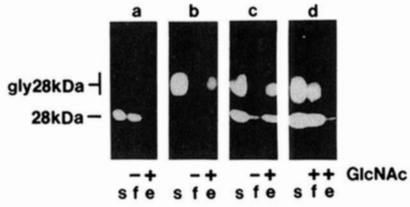

FIG. 7. **Wheat germ agglutinin affinity chromatography of pure 28kDa and pure gly28kDa proteins and the 28kDa-gly28kDa oligomer.** The experiments were conducted with SDS-purified 28kDa protein (containing no gly28kDa protein, *panel a*), SDS-purified gly28kDa protein (containing no 28kDa protein, *panel 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 7.8), 1 mM DTT, 1 mM NaN_3 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 s*), 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*).

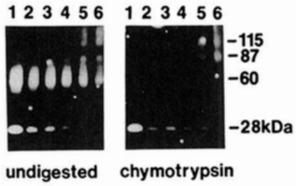

FIG. 8. **Cross-linking of 28kDa protein in membrane vesicles with glutaraldehyde.** KI-stripped inside-out membrane vesicles were prepared from undigested erythrocytes (*left panel*) or from erythrocytes previously digested with α-chymotrypsin to degrade gly28kDa protein at extracellular sites (*right panel*, see "Experimental Procedures"). The vesicles (1 mg of protein/ml) were then incubated at 22 °C in 0.1 M NaCl, 10 mM Na_2HPO_4 (pH 7.2), 1 mM DTT, 0.5 mM NaEDTA, 0.5 M NaN_3 containing 0.008% (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, are 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. 9*A*). 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 in 1% (v/v) Triton X-100 determined by comparison to six water-soluble protein standards was found to be 61 Å, corresponding to the radius of the 28kDa-gly28kDa oligomer-detergent complex. Of note, an identical Stokes radius was determined when Triton X-100 solubilized membrane vesicles were analyzed.

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 denaturing 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 H_2O (Fig. 9*C*, *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 D_2O (Reynolds and Tanford, 1976). This resulted in a significant shift (Fig. 9*C*, *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-

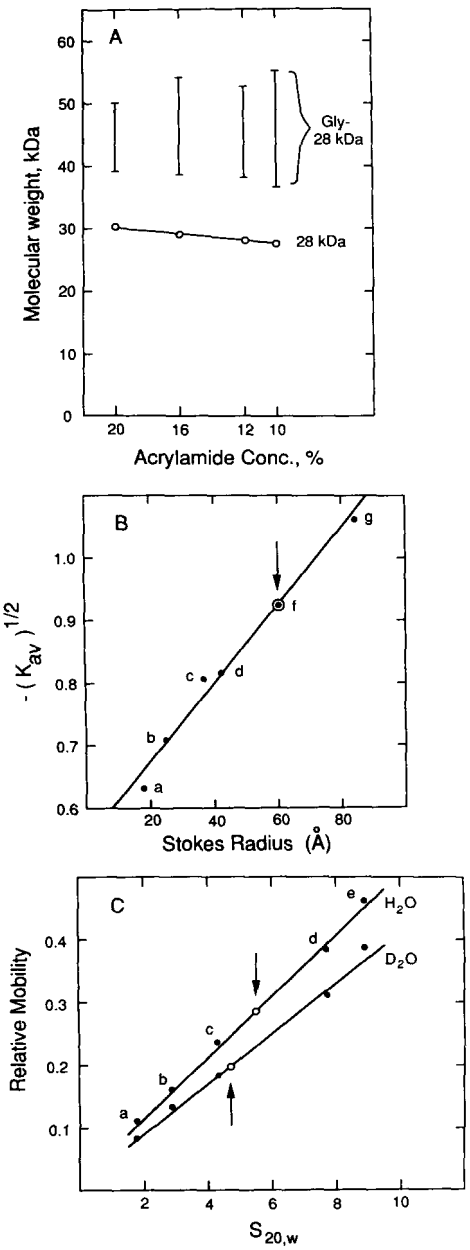

FIG. 9. **Determination of the physical size of 28kDa and gly28kDa proteins and the 28kDa-gly28kDa oligomer.** *Panel A*, analysis by SDS-PAGE. A 2-μg aliquot of purified 28kDa and gly28kDa proteins was solubilized in SDS and electrophoresed into 14 × 16-cm slabs of 20, 16, 12, and 10% gels (see "Experimental Procedures"). The slabs were stained with silver reagent and the mobilities were measured (listed in Fig. 1). The size of 28kDa and gly28kDa proteins relative to the molecular weight markers were calculated by nonlinear regressions employing the equation $y = ae^{bx}$. *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 coeficient 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, 19.4 S. O, 28kDa and gly28kDa proteins eluted together, as indicted 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 D_2O. O, 28kDa and gly28kDa proteins eluted together, as indicated by the *arrows*.

TABLE II

Physical properties of the 28kDa-gly28kDa oligomer in 1% Triton X-100

Property	Value
Stokes radius, R_S[a]	61 Å
Sedimentation coefficient, $s_{20,w}$[b]	5.7 S
Partial specific volume, $\bar{v}$[c]	0.795 ml/g
Triton X-100 bound[d]	0.41 mg/mg protein
M_r[e]	190,000
Molecular weight of protein	135,000
Molecular weight, SDS-PAGE[f]	28,500 (28kDa) 36,000–60,000 (gly28kDa)
Frictional ratio, f/f_0[e]	1.63

[a] Estimated by gel filtration (see Fig. 9).

[b] Estimated by sedimentation on 5–20% sucrose gradients (see Fig. 9).

[c] Calculated from sucrose gradients in H_2O and D_2O by the method of Sadler *et al.* (1979).

[d] Calculated using $\bar{v}_{protein} = 0.735$ and $\bar{v}_{Triton\ X\text{-}100} = 0.94$ (Steele *et al.*, 1978).

[e] Calculated from the equations:

$$M_r = \frac{6\pi N R_S s_{20,w}}{1 - \bar{v}\rho_{20,w}} \quad \text{and} \quad f/f_0 = R_S \left(\frac{4\pi N}{3M_r(\bar{v} + \delta\rho)}\right)^{1/3}$$

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

[f] SDS-PAGE using buffers of Laemmli (1970) (see Figs. 1 and 9).

eral studies reported here all indicated that the 28kDa and gly28kDa proteins exist as a multisubunit transmembrane oligomer. The hydrodynamic studies are most consistent with the 28kDa-gly28kDa oligomer being a tetramer containing both 28kDa protein and gly28kDa protein subunits. The overall contribution of detergent binding to the Stokes radius cannot be directly assessed and the reported value is likely to be too large. By velocity sedimentation in H_2O and D_2O, the mass of the detergent was estimated to be 0.4 mg of Triton X-100/mg of protein which is typical of hydrophobic membrane proteins (Helenius and Simons, 1972; Sadler *et al.*, 1979). The frictional ratio of 1.63 indicates that the 28kDa-gly28kDa oligomer is very asymmetric, and such physical behavior most likely results from the attached carbohydrate which is a single large glycan.[2] In addition, physical studies performed upon purified 28kDa and gly28kDa proteins under nondenaturing conditions were identical to those performed upon solubilized membranes, indicating that the native 28kDa-gly28kDa oligomer does not contain significant amounts of other components.

When the NH_2 termini are aligned, the amino acid sequence of the 28kDa protein was found to be related to that of MIP, the 26-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 physiological 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; Broekhuyse *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 (Zampighi *et al.*, 1985; Peracchia and Girsch, 1985) which can be inhibited with antibodies to MIP (Gooden *et al.*, 1985). Employing immunolocalization techniques, MIP was felt to be a major component of lens fiber junctions by some investigators (Sas *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 (Gruijters *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 (Zampighi *et al.*, 1989).

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 shows partial sequence homology with MIP (Rao *et al.*, 1990) as well as 28kDa protein (Fig. 3). Two other transmembrane channel-forming proteins of approximate molecular mass 28kDa have been identified which share sequence homologies with MIP. The glycerol facilitator from *E. coli* (GlpF) forms a channel in the inner membrane which is permeable to small uncharged molecules including glycerol (Muramatsu and Mizuno, 1989). A transmembrane protein (nod 26) is formed in the nitrogen fixing root nodules of soybeans after the symbiotic infection with *Bradyrhizobium japanicum*, and nod 26 is thought to form multisubunit channels permeable to nutrients (Fortin *et al.*, 1987). Analysis of the cDNA sequences from these proteins suggests that they arose from the gene of an ancestral prokaryotic channel protein (Baker and Saier, 1990). Although less notable than the homology with MIP, GlpF and nod 26 have amino acid sequence homologies with 28kDa protein (see "Results"). While it is interesting to speculate that the 28kDa protein will have similar permeability for small molecules, the existence of such putative channel-forming capacities remain to be established.

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