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Identification, Purification, and Partial Characterization of a Novel M_r 28,000 Integral Membrane Protein from Erythrocytes and Renal Tubules*

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A novel M_r 28,000 integral membrane protein ("28kDa") was identified in human erythrocytes and found entirely associated with the Triton X-100 insoluble membrane skeletons. Antibodies to 28kDa reacted strongly on immunoblots with 28kDa and a diffuse region of M_r 35,000–60,000 ("HMW-28kDa"). Selective proteolytic digestions of membranes demonstrated that HMW-28kDa has an extracellular domain, and both 28kDa and HMW-28kDa have intracellular domains. 28kDa and HMW-28kDa were purified to homogeneity. Quantitative immunoblots indicate that each erythrocyte contains 120,000–160,000 copies of 28kDa. Two-dimensional iodopeptide maps of 28kDa and HMW-28kDa were nearly identical; peptide-*N*-glycosidase digestion of purified HMW-28kDa demonstrated that it is the *N*-glycosylated form of 28kDa. When concentrated, 28kDa formed a series of larger oligomers which were stable in sodium dodecyl sulfate. Of several nonerythroid tissues studied with anti-28kDa immunoblots, only kidney displayed immunoreactive 28kDa. Purified rat kidney 28kDa was nearly identical to rat erythrocyte 28kDa when compared by two-dimensional iodopeptide mapping. Immunohistochemical staining of human kidney with anti-28kDa demonstrated prominent staining over the apical brush borders of proximal convoluted tubules.

A novel integral membrane protein has been purified from erythrocyte and kidney membranes. This new protein may play a role in linkage of the membrane skeleton to the lipid bilayer.

The human erythrocyte membrane is an accessible, pure membrane source which has provided information upon which general understandings of membrane architecture are based (see reviews: Cohen, 1983; Bennett, 1985; Marchesi, 1985; Lazarides, 1987). The membrane is composed of integral membrane proteins, which penetrate the lipid bilayer and play roles in transport and structure, and the membrane skeleton, which is located just below the cytoplasmic face of the lipid bilayer and provides the shape and reversible deformability characteristic of the erythrocyte.

The nature of the linkage of the membrane skeleton to the lipid bilayer has been a topic of intense investigation. Ankyrin is well understood to provide a major linkage between spectrin in the membrane skeleton and a site on the cytoplasmic domain of the anion transporter (Bennett and Stenbuck, 1979b, 1980; Hargreaves *et al.*, 1980; Tyler *et al.*, 1980). Other skeleton to bilayer linkages have been identified but are less well understood. Glycophorin C is associated with the membrane skeleton, but the specific linkage remains to be elucidated (Mueller and Morrison, 1981). Glycophorin A has been shown to relay stimuli from the extracellular surface of the membrane to the membrane skeleton (Anderson and Lovrien, 1981; Chasis *et al.*, 1985). Protein 4.1 has been shown to associate with cytoplasmic domains of glycophorin A (Anderson and Lovrien, 1984; Anderson and Marchesi, 1985), the anion tranporter (Pasternack *et al.*, 1985), and phosphatidylserine in the lipid bilayer (Cohen *et al.*, 1988; Rybicki *et al.*, 1988). The Rh polypeptide is an integral membrane protein which was found to be associated with the membrane skeleton (Gahmberg and Karhi, 1984; Ridgwell *et al.*, 1984). The Rh polypeptide can be extracted from membrane skeletons with high detergent concentrations (Bloy *et al.*, 1987) and lacks an identifiable cytoplasmic domain (Agre *et al.*, 1987). This suggests that the Rh polypeptide linkage with the membrane skeleton results from a side-to-side association with another skeleton-linked integral membrane protein.

The role of the membrane skeleton in the attachment and maintenance of the normal lipid bilayer is still not well understood. Integral membrane proteins linked to the membrane skeleton and lipid-translocating enzymes must all be identified. Associations between the integral proteins within the bilayer, and associations between integral membrane proteins and components in the membrane skeleton must be characterized. This paper describes the isolation and partial characterization of an abundant erythrocyte integral membrane protein of M_r 28,000 ("28kDa") which is linked entirely to the membrane skeleton. While the physiologic importance of 28kDa remains uncertain, a role in linkage of the membrane skeleton to bilayer is a possibility.

EXPERIMENTAL PROCEDURES

Materials—Human blood was obtained from the Johns Hopkins Hospital Blood Bank. α-Chymotrypsin and TPCK[1]-trypsin were from Cooper Biomedical. Carrier free $Na^{125}I$ was from Amersham Corp. Iodogen was from Pierce Chemical Co. Ultro-Gel AcA 34 was from LKB. High resolution hydroxylapatite and protein A-bearing *Staphylococci* were from Behring Diagnostics. The Mono Q column, Q-Sepharose, protein A, and protein A-Sepharose were from Pharmacia

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[1] The abbreviations used are: TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid.

LKB Biotechnology Inc. Phenylmethylsulfonyl fluoride, leupeptin, dithiothreitol, EDTA, EGTA, Folin reagent, low molecular weight protein standards, neuraminidase, papain, *N*-lauroylsarcosine, and Triton X-100 were from Sigma. Polyethylene glycol (Carbowax 8000) was from Fisher. Pentex fraction V pure albumin was from Miles. Ultra pure urea and Tris were from Schwarz/Mann. Affi-Gel 15, silver stain kits, and electrophoresis reagents including pure SDS and acrylamide were from Bio-Rad. X-Omat AR autoradiographic film, chromagram microgranular cellulose thin layer sheets, and developing reagents were from Kodak. Cronex intensifier screens were from Du Pont. Millipore Millex-HV 0.45 μm and Alltech nylon-66 0.2 μm filters were used.

Methods—SDS-PAGE slabs were prepared using the buffer system of Laemmli (1970); samples were reduced with 40 mM dithiothreitol. 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 Bennett and Davis (1981). Most blots were incubated with 0.1 μg/ml of affinity-purified anti-28kDa IgG.

Quantitative immunoblots were performed to estimate the amount of 28kDa in erythrocyte membranes. Serial dilutions of erythrocyte membranes (32, 16, 8, and 4 μg of protein) and highly purified 28kDa eluted from preparative gels (0.64, 0.32, 0.16, and 0.08 μg) were electrophoresed into the same gel and immunoblotted with anti-28kDa. Segments containing 28kDa (not higher molecular weight component, M_r 35,000–60,000,"HMW-28kDa") were excised from the blots and counted for ^{125}I. Slopes were compared from plots of protein *versus* cpm.

Preparation of Antibodies—Approximately 200 μg of protein (28kDa with HMW-28kDa) in 1.5 ml of 0.2% (w/v) SDS was mixed with 1.5 ml 10% (w/v) $KAlSO_4$. NaOH was slowly added until a precipitate formed (approximately 140 μl of 10 M NaOH). The precipitate was pelleted by centrifugation at 700 × *g* for 10 min and resuspended to 3 ml in 0.15 M NaCl, 10 mM $NaPO_4$, pH 7.4. Young New Zealand White rabbits were immunized on three alternate days by slow intravenous injection of 0.1 ml of the suspension. A second series of injections was begun 2 weeks later, and the animals were serially bled at intervals thereafter. Four of the rabbits developed strong responses as determined by immunoblots. Serum containing 0.6 mM phenylmethylsulfonyl fluoride was incubated for 30 min at 60 °C and was subsequently diluted 1:1 with 1% (v/v) Triton X-100, 0.15 M NaCl, 10 mM $NaPO_4$, pH 7.4, 1 mM NaN_3, and frozen in aliquots.

An affinity column was made from approximately 500 μg of 28kDa in 4 M urea, 0.2% (w/v) SDS, 10 mM $NaPO_4$, pH 7.4, 1 mM NaN_3 which was added to 3 ml of Affi-Gel 15 which had been washed with chilled isopropyl alcohol and water. The slurry was gently shaken for 4 h at 4 °C. The gel was poured into a column, washed extensively with the same buffer, and subsequently with 4 M urea, 1% (v/v) Triton X-100, 0.1 M glycine. The column was then washed, loaded with 50 ml of treated antiserum, and eluted as described (Bennett and Stenbuck, 1979a). The peak fractions were combined, dialyzed extensively against 0.15 M NaCl, 50 mM $NaPO_4$, pH 7.4, 1 mM NaEDTA, 1 mM NaN_3 and snap frozen at approximately 0.2 mg of protein/ml. Preimmune globulin was similarly isolated from protein A-Sepharose columns.

Antibodies to human ankyrin and the M_r 43,000 cytoplasmic domain of the anion transporter (band 3) were provided by Vann Bennett, Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC. Antibodies to spectrin and protein 4.1 were prepared in our laboratory as described (Bennett and Stenbuck, 1979a).

Two-dimensional Iodopeptide Maps—Approximately 10 μg of purified 28kDa (Fig. 5, *lane 4*) was ^{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).

Preparation of Erythrocyte Membranes—Human erythrocytes were obtained from blood-bank units stored up to 5 weeks at 4 °C. Rat erythrocytes were obtained by cardiac puncture. All membrane preparations employed the methods described by Bennett (1983) which included hypotonic lysis, spectrin-actin elution, and preparation of stripped membrane vesicles with 1 M KI.

Purification of 28kDa—KI-stripped erythrocyte membrane vesicles from 1 unit of erythrocytes were suspended to 400 ml in 1% (w/v) sodium *N*-lauroylsarcosine, 5 mM $NaPO_4$, pH 7.4, 1 mM dithiothreitol, 1 mM NaN_3, 1 mM NaEDTA (final concentrations) and incubated for 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 $NaPO_4$, pH 7.4, and another centrifugation. The pellet was partially solubilized by shaking for 7 h at 22°C in 800 ml of 1% (v/v) Triton X-100, 20 mM Tris-HCl, 1 mM NaN_3, 1 mM dithiothreitol, pH 7.8. A significant pellet remained after centrifugation at 30,000 × *g* for >6 h. The supernatant was removed and filtered through a 0.2-μm filter and loaded onto a 1.6 × 35-cm column packed with Q-Sepharose which had been equilibrated with the same buffer at 4 °C. 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 eluted near the end of the gradient.

28kDa was purified in larger amounts in denatured form by solubilizing the *N*-lauroylsarcosine insoluble pellet (above) in 200 ml of 1% (w/v) SDS, 10 mM $NaPO_4$, pH 7.4, 1 mM NaN_3, 1 mM dithiothreitol for 60 min at 22 °C. The soluble proteins were removed after centrifugation at 30,000 × *g* for >6 h, filtered, and loaded onto a 1.6 × 30-cm column packed with hydroxylapatite. The column was eluted at 25 ml/h at 22°C with a 300-ml linear gradient of 0.3–0.8 M $NaPO_4$ in the same buffer; the peak containing both 28kDa and HMW-28kDa eluted midway through the gradient. The peak fractions were concentrated, dialyzed against 0.2% (w/v) SDS, 10 mM $NaPO_4$, pH 7.4, 1 mM NaN_3 and electrophoresed into two 0.3 × 14 × 16-cm preparative 12% SDS-PAGE slabs. The slabs were cut into 6-mm horizontal strips which were eluted with 5 ml of the same buffer by shaking overnight at 22 °C.

Samples for amino acid analysis (approximately 3 μg of protein) were precipitated with acetone at −20 °C and were analyzed in the Peptide Laboratory in the Johns Hopkins University Department of Biological Chemistry as described (Bidlingmeyer *et al.*, 1984).

Rat Tissue Preparations—Tail veins were injected with 100 units of sodium heparin, and the animals were killed by ether inhalation. The carcasses were perfused with 200 ml of 0.15 M NaCl by cardiac puncture after thoracotomy. Nonmuscle tissues were placed in 5 volumes of 0.3 M sucrose, 2 mM NaEGTA at 0 °C in a Potter Elvehjem

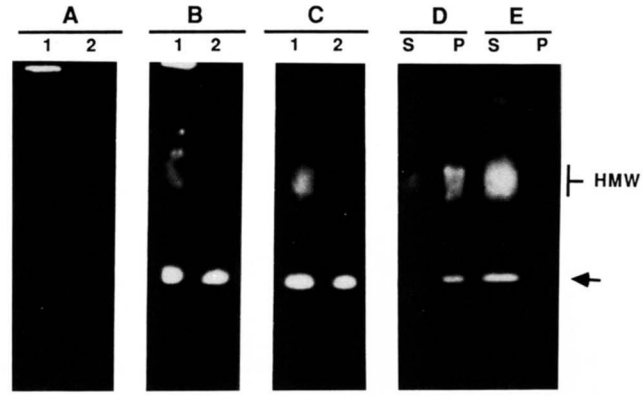

FIG. 1. **Immunoblot analysis of antibodies to 28kDa.** Development of antibodies (*panels A–C*): erythrocyte membranes, 10 μg of protein (*lanes 1*) and isolated 28kDa, 0.1 μg (*lanes 2*) were electrophoresed into 12% SDS-PAGE slabs and blot transferred onto nitrocellulose strips which were incubated with 1:200 preimmune rabbit sera (*panel A*), 1:200 immune sera (*panel B*), or 0.1 μg/ml affinity-purified anti-28kDa immunoglobulin (*panel C*). The blots were washed, incubated with ^{125}I-labeled protein A, and visualized by autoradiography (see "Experimental Procedures"). Immunoprecipitation of 28kDa with affinity purified anti-28kDa antibody (*panels D–E*): KI-stripped membrane vesicles, 40 μg of protein, were entirely solubilized in 0.1 ml 1% (v/v) Triton X-100, 150 mM NaCl, 20 mM $NaPO_4$, pH 7.4, 1 mM NaN_3. Four μg of affinity-purified anti-28kDa immunoglobulin (*panel D*) or preimmune IgG (*panel E*) were added and incubated for 36 h at 4 °C. Twenty μl of 10% (v/v) washed protein A bearing *Staphylococci* were added for 2 h at 4 °C, and immune complexes were precipitated as described (O'Keefe and Bennett, 1980). The immunoprecipitates (*P*) and unprecipitated supernatants (*S*) were analyzed by immunoblot as described for *panel C* (above). The *arrow* identifies 28kDa and *HMW brackets* HMW-28kDa. Note that anti-28kDa quantitatively immunoprecipitated 28kDa, and preimmune globulin failed to precipitate any 28kDa.

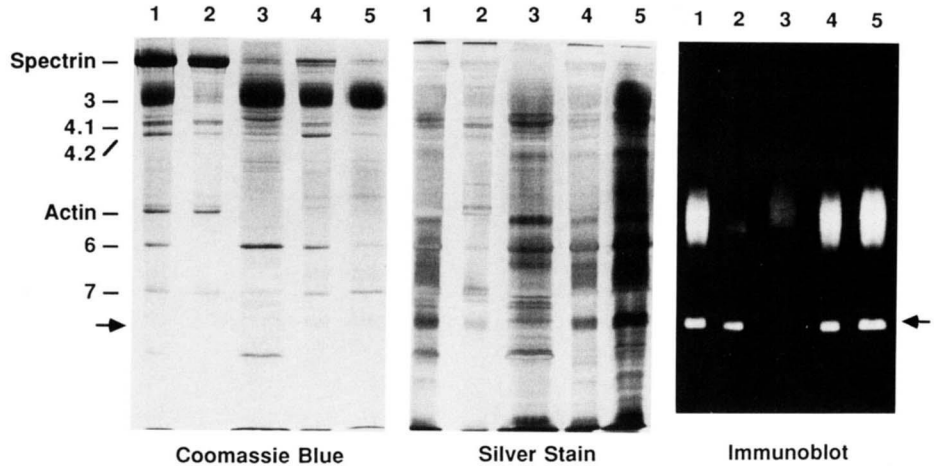

FIG. 2. **Membrane distribution of 28kDa.** Whole erythrocyte membranes (*lanes 1*), Triton X-100 insoluble membrane skeletons (*lanes 2*), Triton-soluble extract (*lanes 3*), spectrin-actin-depleted membrane vesicles (*lanes 4*), and KI-stripped membrane vesicles (*lanes 5*) were electrophoresed into 12% SDS-PAGE slabs which were then stained with Coomassie, or silver reagent, or immunoblotted with anti-28kDa as indicated. Each lane is equivalent to membranes prepared from 15–30 μl of erythrocytes.

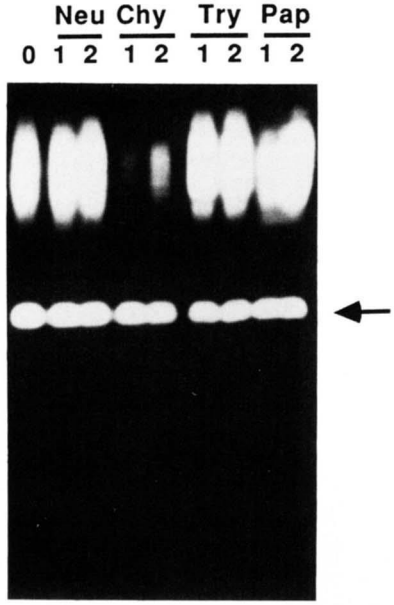

FIG. 3. **The lipid bilayer protects 28kDa but not HMW-28kDa from digestion at the extracellular surface of the membrane.** Intact washed erythrocytes were suspended to a hematocrit of 10% in 0.25 ml of 0.15 M NaCl, 20 mM $NaPO_4$, pH 7.4, 1 mM NaEDTA and incubated at 37 °C for 60 min with no addition (*lane 0*), 625 μg (*lanes 1*), or 125 μg (*lanes 2*) of neuraminidase (*Neu*), α-chymotrypsin (*Chy*), TPCK-trypsin (*Try*), or papain (*Pap*). The erythrocytes were washed free of enzyme. Membranes were prepared by lysis and electrophoresed into a 14% SDS-PAGE slab and immunoblotted with anti-28kDa.

apparatus for five pulses at 3,000 rpm in the presence of 200 μg/ml phenylmethylsulfonyl fluoride and 4 μg/ml leupeptin. Tissue homogenates were centrifuged 10 min at 1,000 × *g*; 3 ml of supernatant was layered over a 15-ml barrier of 0.8 M sucrose, 2 mM NaEGTA and spun 40 min at 32,000 × *g*.

Cardiac muscle membranes were prepared by placing the tissue in 10 volumes of 0.1 M KCl, 5 mM $NaPO_4$, pH 7.5, 0.75 mM NaEGTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 200 μg/ml phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin. Muscle was homogenized in a polytron for 3–4 min and then treated as described above.

Purification of 28kDa from Rat Kidney—The presence of the kidney 28kDa analog was monitored during isolation with anti-28kDa immunoblots. Kidneys from eight rats were thoroughly perfused. Membranes were prepared (above) and extracted in 10 ml of 0.1% (w/v) *N*-lauroylsarcosine for 30 min at 37 °C, and centrifuged for 45 min at 44,000 × *g*. The pellet was solubilized in 10 ml of 9 M urea, 30 mM pyrophosphate, 1% (v/v) Triton X-100, 1 mM dithiothreitol, and 1 mM NaN_3, pH 10, by mixing for 15 min at 22 °C. The sample was diluted 1:2 with 20 mM $NaPO_4$, pH 7.4, 1% (v/v) Triton X-100, 1 mM dithiothreitol, 1 mM NaN_3, filtered through a 0.45-μm Millipore membrane, and loaded onto a 5 × 100-cm column packed with Ultro-Gel AcA 34 eluting at 100 ml/h with the same buffer. The peak 28kDa fractions were pooled and loaded onto a 1 × 15-cm column packed with hydroxylapatite equilibrated with the same buffer. The column was eluted at 30 ml/h with a 60-ml linear gradient of 0.1–0.3 M $NaPO_4$ in the same buffer. The peak 28kDa fractions were pooled, dialyzed against 20 mM Tris-HCl, pH 7.8, 1% (v/v) Triton X-100, 1 mM dithiothreitol, 1 mM NaN_3 for 24 h at 0 °C, and loaded onto a Mono Q anion exchange column (HR 5/5) equilibrated with the same buffer. The protein was eluted at 0.5 ml/min with a linear gradient of 0–0.2 M NaCl in the same buffer.

Histochemical Staining—Human kidney was processed by the surgical pathology service at The Johns Hopkins Hospital employing standard methods. Six μm sections of fixed tissue were mounted on slides, incubated 15 min with 3% (v/v) H_2O_2 in H_2O, and then preincubated with nonimmune goat serum. The rinsed slides were incubated overnight at 4 °C with 2 μg/ml rabbit anti-28kDa or preimmune globulin. The slides were then rinsed and incubated 30 min at 25 °C with biotinylated goat-anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at a concentration of 1:100 in 0.5 M Tris, pH 7.5. The slides were rinsed and incubated for 30 min with avidin-horseradish peroxidase (Vector) at a concentration of 1:150 in 0.5 M Tris, pH 7.5, stained in aminoethyl-carbazole (Biomeda Laboratories, Foster City, CA) for 10 min, and counterstained with Mayer's hematoxylin.

RESULTS

A Newly Recognized Erythrocyte Membrane Protein—A M_r 28,000 polypeptide copurified with the Rh polypeptide during several steps of its isolation and was initially considered to be a degradation product of the M_r 32,000 Rh polypeptide (Agre *et al.*, 1987). Approximately 20% of the initial Rh purifications consisted of this M_r 28,000 polypeptide, referred to in this text as 28kDa. Rabbits immunized with this material developed an immune response to 28kDa but not to the M_r 32,000 Rh polypeptide. Additional animals immunized with isolated 28kDa developed antibodies which reacted strongly with 28kDa on immunoblots. Affinity-purified anti-28kDa antibodies quantitatively immunoprecipitated 28kDa from Triton X-100-solubilized membrane vesicles, whereas preimmune globulin failed to immunoprecipitate 28kDa (Fig. 1). In addition to the notable reaction with the M_r 28,000 component,

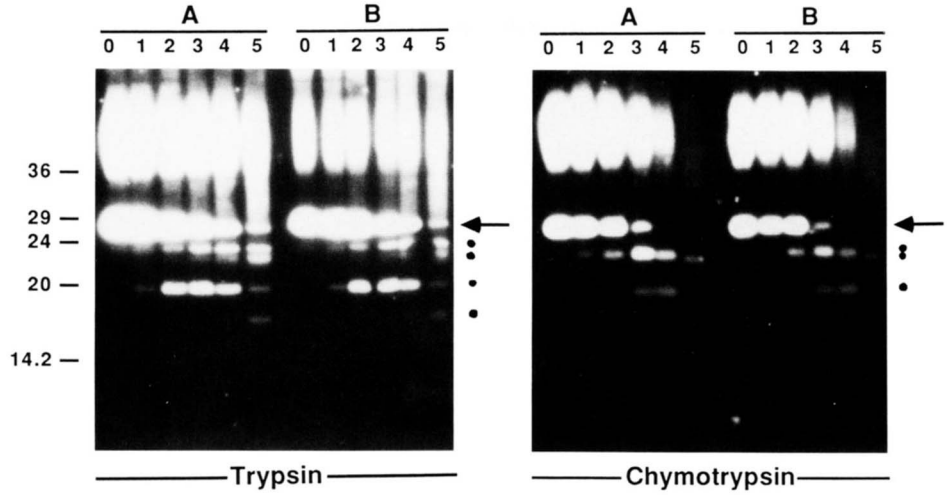

FIG. 4. **28kDa is vulnerable to proteolytic digestion at the intracellular surface of the lipid bilayer.** KI-Stripped inside-out membrane vesicles, 0.5 mg, were suspended to 0.5 ml in 10 mM $NaPO_4$, pH 7.4, 1 mM dithiothreitol, 1 mM NaEDTA, 1 mM NaN_3 and incubated 60 min at 37 °C with no addition (*lanes 0*) or in the presence of 0.01 μg (*lanes 1*), 0.05 μg (*lanes 2*), 0.2 μg (*lanes 3*), 1 μg (*lanes 4*), or 5 μg (*lanes 5*) of TPCK-trypsin or α-chymotrypsin as indicated. The entire digest containing vesicles and water-soluble peptide fragments was solubilized in 5% (w/v) SDS containing 0.2 mM phenylmethylsulfonyl fluoride at 60 °C prior to electrophoresis (*panels A*). The membrane vesicles were washed free of water-soluble peptide fragments in 10 mM $NaPO_4$, pH 7.4, 0.2 mM phenylmethylsulfonyl fluoride at 0 °C prior to solubilization in 5% (w/v) SDS and electrophoresis (*panels B*). The samples were electrophoresed into 16% SDS-PAGE gels and immunoblotted with anti-28kDa. Proteolytic fragments of 28kDa are identified by *dots* at the right margin of each panel.

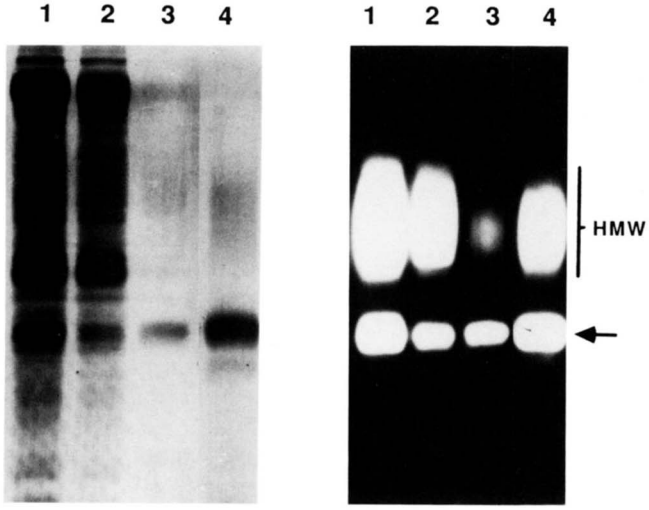

FIG. 5. **Purification of 28kDa from erythrocyte membranes.** Samples from various stages of isolation (see "Experimental Procedures") were electrophoresed into a 14% SDS-PAGE slab and stained with silver reagent (*left*) or immunoblotted with anti-28kDa (*right*): KI-stripped membrane vesicles (*lanes 1*); *N*-lauroylsarcosine-solubilized proteins (*lanes 2*); *N*-lauroylsarcosine-insoluble proteins subsequently solubilized in Triton X-100 (*lanes 3*); peak fractions of 28kDa from the Q-Sepharose elution (*lanes 4*).

TABLE I

Amino acid composition of purified erythrocyte 28kDa polypeptide

Amino acid	mol %
ASX	9.0
GLX	5.8
SER	8.3
GLY	14.0
HIS	1.8
ARG	4.3
THR	5.5
ALA	10.3
PRO	3.6
TYR	2.0
VAL	6.9
MET	1.2
CYS	0.3
ILE	8.1
LEU	12.2
PHE	4.2
LYS	2.5

TABLE II

Purification of 28kDa from human erythrocytes

Step	Protein	28kDa recovery[a]	Fold purified	Relative purity[b]
	mg	%		%
1. Erythrocyte membranes	794	100	1.0	
2. KI-stripped vesicles	434	100	1.8	<5
3. *N*-lauroylsarcosine-insoluble pellet	16	40	20	50
4. Triton X-100-soluble, Q-Sepharose peak[c]	0.1			~95
5. SDS-soluble, hydroxylapatite peak[c]	0.93			>99
6. SDS-PAGE elution[d]	0.31	2.9	77	>99

[a] Estimated from quantitative immunoblots (see "Experimental Procedures").
[b] Estimated from silver-stained SDS-PAGE slabs (see Fig. 5).
[c] Contains both 28kDa and HMW-28kDa.
[d] Contains only 28kDa.

the antibody consistently reacted with a diffuse, higher molecular weight component (M_r 35,000–60,000) suspected to be related to the 28kDa polypeptide and is referred to here as HMW-28kDa. These antibodies never reacted with the M_r 32,000 Rh polypeptide even in highly concentrated pure form indicating that 28kDa is not derived from the Rh polypeptide.

28kDa does not correspond to any of the previously described erythrocyte membrane proteins seen on Coomassie Blue-stained SDS-PAGE slabs (Steck, 1974). When identical SDS-PAGE slabs were stained with Coomassie or silver reagent, a striking number of polypeptides became visible on the silver-stained slabs including a prominent band or nar-

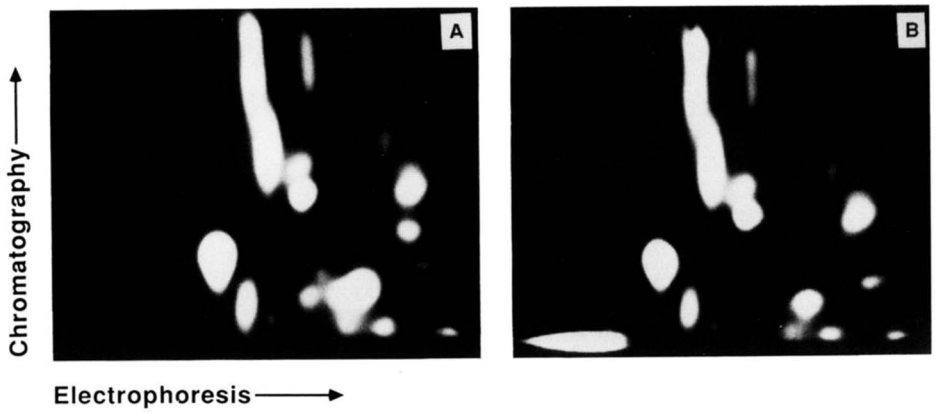

FIG. 6. **Comparison of two-dimensional iodopeptide maps of 28kDa and HMW-28kDa.** Purified 28kDa and HMW-28kDa were denatured in SDS, ^{125}I-labeled, and electrophoresed into a 12% SDS-PAGE slab. Gel slices containing 28kDa (*panel A*) and HMW-28kDa (*panel B*) were separately excised, digested with α-chymotrypsin, and analyzed in two dimensions (see "Experimental Procedures").

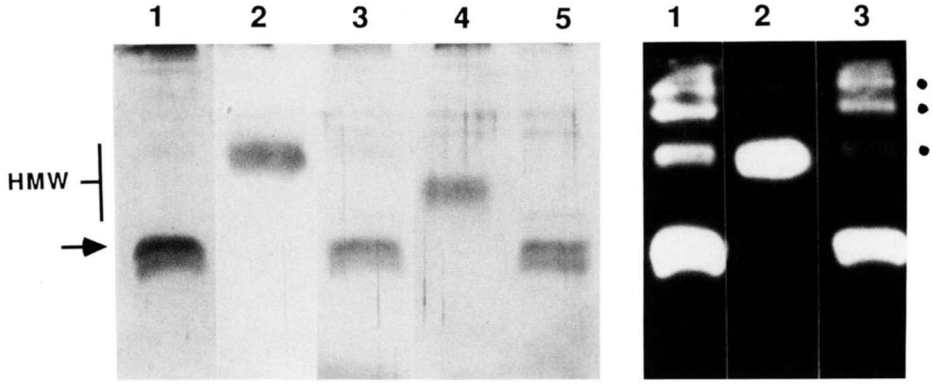

FIG. 7. **HMW-28kDa is *N*-glycosylated and 28kDa exists as a series of oligomers.** Pure 28kDa and HMW-28kDa of M_r 50,000–60,000 and M_r 35,000–50,000 were eluted from preparative SDS-PAGE slabs, denatured in SDS, and digested with peptide-*N*-glycosidase (PNGase) or incubated identically without the enzyme as described (Holt and Hart, 1986). The samples were then electrophoresed into 14% SDS-PAGE slabs and stained with silver reagent (*left*) or immunoblotted with anti-28kDa (*right*). Purified 28kDa was incubated without PNGase (*lanes 1*); purified M_r 50,000–60,000 HMW-28kDa was incubated without (*lanes 2*) or with PNGase (*lanes 3*); purified M_r 35,000–50,000 HMW-28kDa was incubated without (*lane 4*) or with PNGase (*lane 5*). The location of 28kDa oligomers are identified by the *dots* at the right margin. Note the appearance of a M_r 27,500 polypeptide just beneath 28kDa which probably represents a 28kDa degradation product.

rowly spaced doublet at M_r 28,000. This polypeptide corresponded to a very faint band seen midway between bands 7 and 8 of the Coomassie-stained slab and comigrated exactly with 28kDa on immunoblots (Fig. 2). 28kDa did not correspond with any of the known sialoglycoproteins visualized with the periodic acid-Schiff procedure and failed to react with anti-glycophorin C on immunoblots (not shown).

The membrane organization of 28kDa was studied with anti-28kDa immunoblots (Fig. 2). 28kDa was found entirely in the Triton X-100 insoluble membrane skeletons. None was found in the Triton X-100 soluble extract, indicating that 28kDa is linked to the membrane skeleton. The distribution of HMW-28kDa was similar to that of 28kDa, except that some of the former appeared in the Triton X-100 extract. It is presently uncertain whether the partial elution of HMW-28kDa in Triton X-100 results from increased solubility in the detergent or whether 28kDa and HMW-28kDa have different membrane skeleton linkages. 28kDa is an integral membrane protein (penetrates the bilayer), since it was located in inside-out membrane vesicles after all peripheral proteins were stripped with 1 M KI. Band 7 is composed of several components of which some are integral membrane proteins and some are linked to the membrane skeleton. 28kDa is not band 7, however, since the electrophoretic mobilities are distinctly different.

Extra- and Intracellular Domains—The existence of extracellular or intracellular domains of integral membrane proteins may be studied by selective proteolytic degradations at the extracellular and intracellular faces of the lipid bilayer. Intact erythrocytes were digested with two concentrations of neuraminidase, chymotrypsin, trypsin, or papain (Fig. 3). None of these enzymes qualitatively or quantitatively altered the immunoblot reactivity of 28kDa. HMW-28kDa was selectively lost from the erythrocytes digested with both concentrations of chymotrypsin and was partially lost from cells digested with the larger concentration of papain. Smaller polypeptides derived from the HMW-28kDa were not identified, but the existence of smaller components comigrating exactly with the 28kDa cannot be excluded.

Digestion at the intracellular face of the bilayer was accomplished by incubating KI-stripped inside-out membrane vesicles with increasing concentrations of trypsin or chymotrypsin (Fig. 4). Both enzymes reduced the amount of HMW-28kDa and 28kDa while a series of smaller polypeptides appeared which still reacted with anti-28kDa on immunoblots. Trypsin generated fragments of M_r 24,000, 23,000, 20,000 and 17,000, and chymotrypsin generated fragments of M_r 24,000 23,000 and 19,000. Trypsin also led to the increased appearance of a M_r 27,500 band (not seen well here). This M_r 27,500

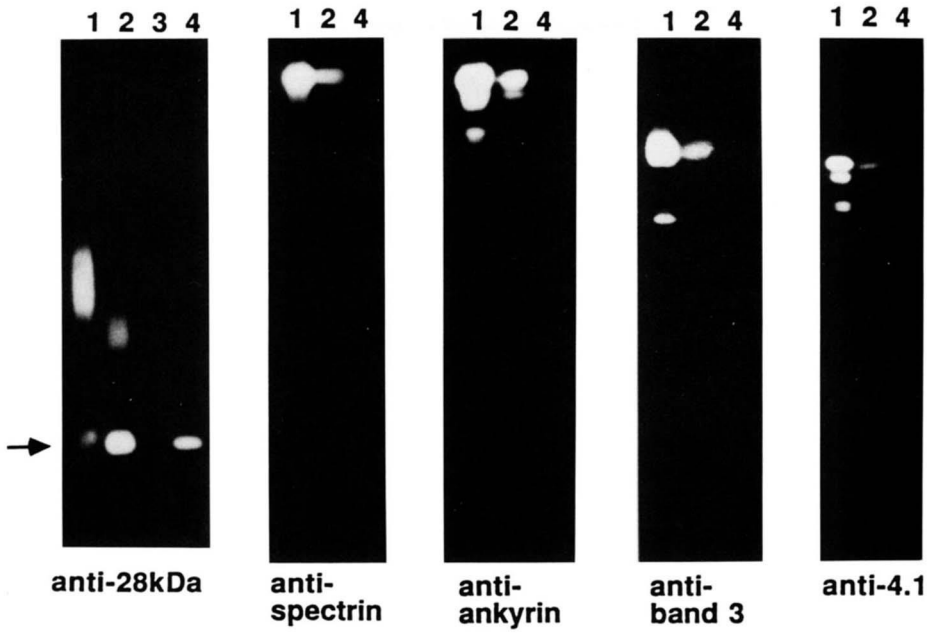

FIG. 8. **Immunoblot analysis of membranes from selected rat tissues employing antibodies specific for human 28kDa and other human erythrocyte proteins.** Membranes were prepared after thorough perfusion (see "Experimental Procedures"); 10–20 μg of protein were solubilized in SDS and electrophoresed into 12% SDS-PAGE slabs and immunoblotted with anti-28kDa (*left panel*): human erythrocytes (*lane 1*), rat erythrocytes (*lane 2*), rat lung (*lane 3*), and rat kidney (*lane 4*). Membranes from human erythrocytes (*lanes 1*), rat erythrocytes (*lanes 2*), and rat kidney (*lanes 4*) were electrophoresed similarly but immunoblotted with antibodies to human erythrocyte proteins as indicated (*right panels*): anti-spectrin, anti-ankyrin, anti-43kDa cytoplasmic domain of anion transporter (*band 3*), and anti-protein 4.1.

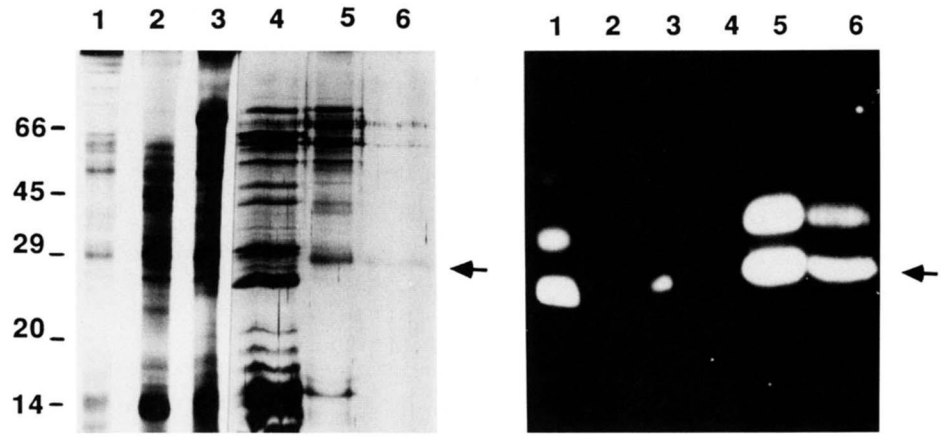

FIG. 9. **Purification of 28kDa from rat kidney.** The analog to 28kDa was isolated from thoroughly perfused rat kidneys (see "Experimental Procedures"). Samples from various stages of purification were solubilized in SDS and electrophoresed into a 14% SDS-PAGE slab and stained with silver reagent (*left panel*) or analyzed by anti-28kDa immunoblot (*right panel*): kidney membranes (*lanes 1*); *N*-lauroylsarcosine-soluble proteins (*lanes 2*); *N*-lauroylsarcosine-insoluble pellet subsequently solubilized in 9 M urea, 1% Triton X-100 (*lanes 3*); peak from gel filtration (*lanes 4*); peak from hydroxylapatite chromatography (*lanes 5*); peak from mono-Q anion exchange chromatography (*lanes 6*).

polypeptide may represent a naturally occurring fragment derived from 28kDa, since it comigrated exactly with a band frequently seen just beneath 28kDa on membranes prepared from erythrocytes after prolonged storage. The digested vesicles and supernatants were directly analyzed together on anti-28kDa immunoblots and compared to digested vesicles which had been washed free of water-soluble peptides. The washed vesicles had an identical pattern, indicating that the smaller immunoreactive fragments all remain associated with the bilayer.

The digested portion of 28kDa may consist of one or more cytoplasmic domains, and the total mass of the digested region(s) could be as large as 11,000 daltons. No water-soluble peptide was identified, but the protease concentrations employed were large compared to those needed for release of the M_r 43,000 cytoplasmic fragment of the anion transporter (Steck *et al.*, 1976). It is probable that released 28kDa fragment(s) were further degraded. It is possible that much of the 28kDa polypeptide is located between the leaflets of the lipid bilayer where it is protected from proteolytic digestions, or 28kDa may be associated with the membrane in some other conformation which is resistant to proteolytic degradation.

Isolation of 28kDa and HMW-28kDa—The purification of 28kDa and HMW-28kDa were performed beginning with KI-stripped membrane vesicles (Fig. 5). Unlike the Rh polypeptide which was not soluble in conventional purification deter-

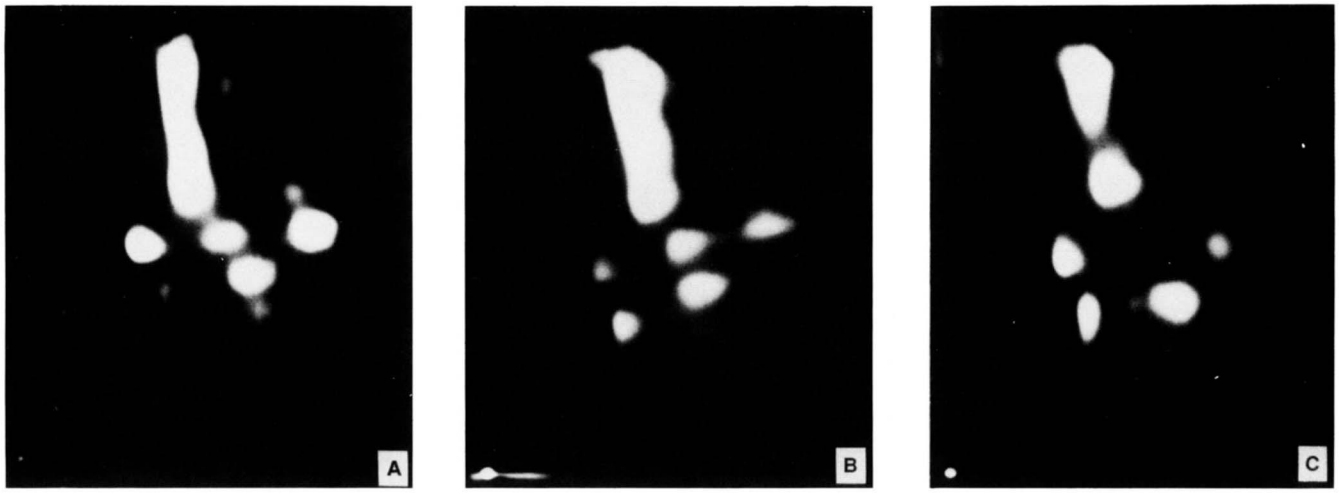

FIG. 10. **Comparison of two-dimensional iodopeptide maps of 28kDa from kidney and erythrocytes.** 28kDa proteins purified from rat kidneys (*A*), rat erythrocytes (*B*), and human erythrocytes (*C*) were denatured in SDS and ^{125}I-labeled and digested with α-chymotrypsin and analyzed in two dimensions (see "Experimental Methods").

gents (Agre *et al.*, 1987), 28kDa was relatively soluble in Nonidet P-40 and Triton X-100 but was relatively insoluble in Tween 20. *N*-Lauroylsarcosine effectively solubilized nearly all erythrocyte membrane vesicle proteins except 28kDa. The *N*-lauroylsarcosine-insoluble pellet was composed primarily of 28kDa which was thereafter partially soluble in Triton X-100 and was purified to near homogeneity by anion exchange chromatography (Fig. 5). The principal contaminant was a smaller polypeptide which is presumably a breakdown of 28kDa. 28kDa and HMW-28kDa copurified during ion exchange and hydroxylapatite chromatography. Consistent with other integral membrane proteins, the amino acid content of purified 28kDa was found to be hydrophobic consisting of 49% aromatic and nonpolar residues (Table I).

The majority of the *N*-lauroylsarcosine-insoluble material was thereafter soluble in SDS and was purified by hydroxylapatite chromatography (Table II). 28kDa and HMW-28kDa were individually eluted from preparative SDS-PAGE slabs in highly purified form. Interestingly, the apparently heterogeneous electrophoretic behavior of the HMW-28kDa was preserved after isolation. HMW-28kDa was eluted from the upper (M_r 50,000–60,000) and lower (M_r 35,000–50,000) regions of preparative SDS-PAGE slabs, and the relative electrophoretic mobilities on analytical SDS-PAGE gels were identical to the electrophoretic mobilities on the preparative gels from which they were eluted (not shown). When erythrocyte membranes and highly purified 28kDa were compared by quantitative immunoblotting, it was determined that 28kDa constitutes approximately 1.3% of the original membrane protein, 120,000–160,000 copies/erythrocyte. The number of copies of HMW-28kDa was estimated to be 1/4 to 1/10 of this, although variable reactivity of HMW-28kDa on immunoblots made precise determination impossible.

28kDa and HMW-28kDa Are Related Polypeptides—The electrophoretic behavior of 28kDa and HMW-28kDa were distinct, but the immunological and chromatographic behavior were nearly identical. To identify a potential relationship between the two polypeptides, the isolated 28kDa and HMW-28kDa components were compared by two-dimensional mapping of chymotryptic iodopeptide fragments. The 28kDa and HMW-28kDa maps were found to be nearly identical (Fig. 6). All but three of the major iodopeptide fragments found in 28kDa were also found in HMW-28kDa, but the latter also demonstrated a notable amount of poorly digested material which remained at the origin (lower left of *panel B*).

HMW-28kDa Is Glycosylated and 28kDa Forms Larger Oligomers—Electrophoretically purified 28kDa and HMW-28kDa were digested extensively with peptide-*N*-glycosidase and analyzed by SDS-PAGE and anti-28kDa immunoblot (Fig. 7). After incubation, the HMW-28kDa of M_r 50,000–60,000 and M_r 35,000–50,000 comigrated exactly with 28kDa, indicating that *N*-glycosylation is the major difference between HMW-28kDa and 28kDa. When immunoblots were developed, it was noted that the concentrated forms of 28kDa and deglycosylated HMW-28kDa both consisted of a ladder of bands including the M_r 28,000 monomer and oligomers corresponding to dimers, trimers, tetramers, and larger oligomers which were stable in SDS. The amount of the dimer form was diminished relative to the larger forms indicating that the latter were more stable. Oligomerization of 28kDa was enhanced by concentration prior to electrophoresis. A small amount of 28kDa oligomer was detected in other experiments but was obscured on immunoblots by HMW-28kDa.

Kidney 28kDa—Polypeptides related to the major erythrocyte membrane proteins have subsequently been identified in nonerythroid tissues where they play important structural roles. Existence of proteins related to 28kDa were sought by immunoblot analyses of membranes prepared from several other tissues which had each been thoroughly perfused to eliminate persistent erythrocytes. Membranes prepared from rat erythrocytes and kidney reacted strongly with anti-28kDa, but there was no detectable 28kDa immunoreactivity with membranes prepared from rat lung (Fig. 8), nor was immunoreactivity detected with the following rat tissues: liver, brain, myocardium, spleen, lens, testicle, small intestine, and colon (not shown). The 28kDa immunoreactivity in kidney was not the result of contaminating erythrocytes, since the kidneys had been thoroughly perfused, and kidney membrane failed to react comparably with antibodies to erythrocyte spectrin, ankyrin, cytoplasmic domain of anion transporter, or protein 4.1.

Rat kidney 28kDa was isolated employing anti-28kDa immunoblots to follow the protein during isolation. Kidney membranes were extracted in *N*-lauroylsarcosine, and the insoluble pellet was subsequently solubilized in buffer containing 1% (v/v) Triton, 30 mM sodium pyrophosphate, 9 M

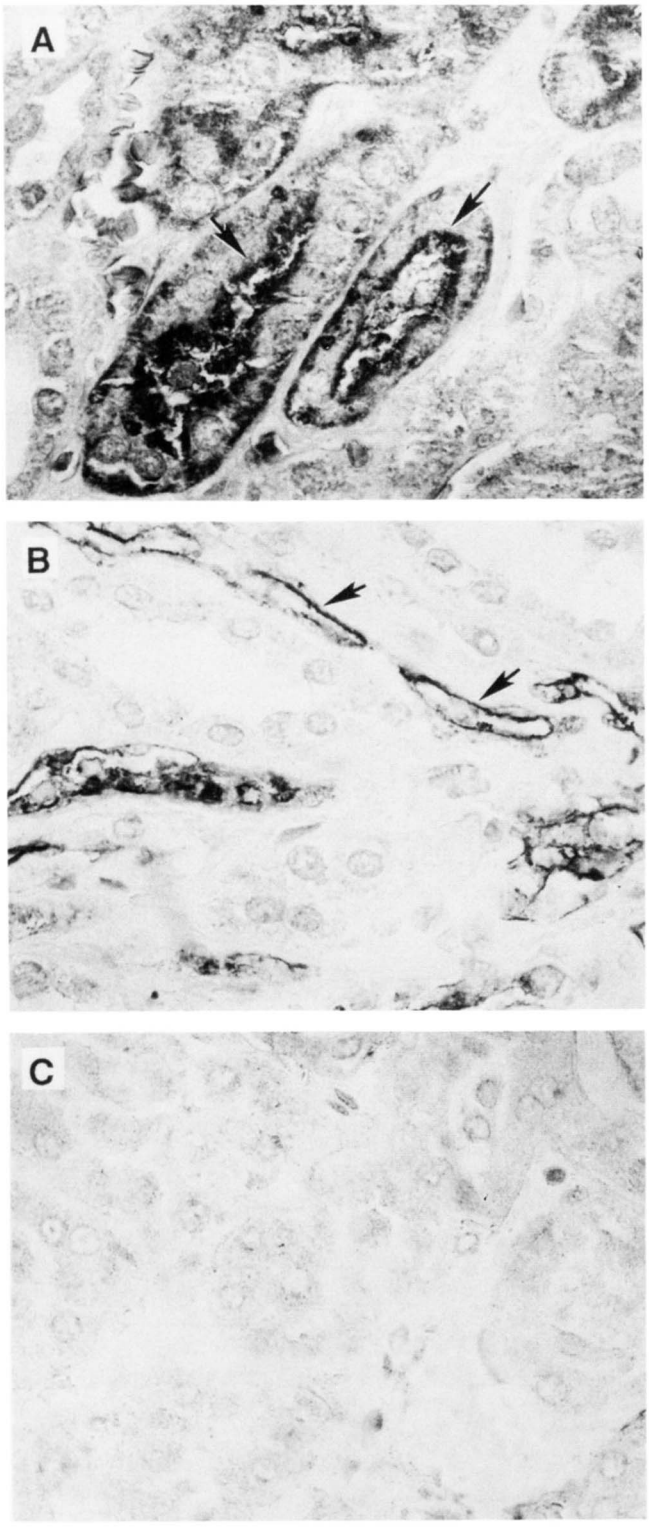

FIG. 11. **Immunohistochemical localization of 28kDa in human kidney.** Human kidney sections were incubated with anti-28kDa immunoglobulin (*panels A* and *B*) or preimmune globulin (*panel C*); immunoreactivity was identified with biotinylated goat-anti-rabbit IgG and avidin-horseradish peroxidase, magnification × 800 (see "Experimental Procedures"). Specific reactivity was noted with anti-28kDa over the apical brush border of proximal-convoluted tubules (*panel A, arrows*) and over the descending thin limb of Henle's loop (*panel B, arrows*) but not in any areas of tissue incubated with preimmune globulin (*panel C*).

urea and fractionated by gel filtration, hydroxylapatite, and Mono Q anion exchange chromatography (Fig. 9). The final peak consisted primarily of a single band of M_r 28,000 which reacted intensely on anti-28kDa immunoblots. Subsequent studies characterizing the relative concentrations of 28kDa in different kidney membrane populations with quantitative immunoblots demonstrated approximately a 20-fold enrichment of 28kDa in apical brush border preparations. Such preparations would therefore be a preferred point to begin future purifications of kidney 28kDa.

28kDa isolated from rat kidney, rat erythrocytes, and human erythrocytes were compared by two-dimensional iodopeptide maps (Fig. 10). The pattern of iodopeptides derived from rat kidney and erythrocyte 28kDa were strikingly similar with the major difference being a reciprocal change in the relative intensities of two neighboring iodopeptides at the left-center of the autoradiographs. This suggests that 28kDa from erythrocytes and kidney have closely related structures. Maps of iodopeptides derived from 28kDa from rat and human erythrocytes bear overall similarities but are clearly not identical.

Immunohistochemical staining of fixed human kidney was undertaken with anti-28kDa (Fig. 11). The staining was localized to proximal convoluted tubules where the immunoreactivity was most intense over the apical brush borders. A smaller degree of immunoreactivity was noted over the basolateral surface to the same tubules, and reactivity was also frequently noted over the descending thin limb of Henle's loop. The intensity of staining of proximal convoluted tubules was somewhat variable. Some tubules exhibited strikingly intense staining while others stained weakly. The variability in staining most likely results from differences between populations of tubules, although staining was most intense over the most proximal segments of individual tubules. Staining over glomeruli and collecting ducts was uniformly negative with anti-28kDa as was staining in all areas with preimmune IgG.

DISCUSSION

This report describes the identification, purification, and partial characterization of a novel integral membrane protein (28kDa) from erythrocytes and kidney. 28kDa is surprisingly abundant (approximately 140,000 copies/erythrocyte) and has apparently escaped previous attention due to its poor Coomassie staining. 28kDa bears a significant cytoplasmic domain, since a truncated remnant of M_r 17,000 remains associated with vesicles after rigorous digestion of inside-out membranes with trypsin. HMW-28kDa bears an extracellular domain which is *N*-glycosylated. The nonglycosylated 28kDa molecules form a series of larger oligomers which remain stable in SDS. Preliminary cross-linking studies with membrane vesicles indicate that the majority of 28kDa molecules exist as dimers or larger oligomers while in the native lipid bilayer (not shown).

HMW-28kDa is not well focused on SDS-PAGE slabs and migrates as a smear between M_r 35,000–60,000. The level of electrophoretic migration within that range is constant presumably due to variations within the carbohydrate moiety. This behavior is reminiscent of the polylactosaminoglycan proteins, long repetitive carbohydrate side chains attached to the extracellular domains of the erythrocyte anion transporters and protein 4.5 (Jaernefelt *et al.*, 1978; Fukuda *et al.*, 1979a). HMW-28kDa bears a strong resemblance to protein 4.5, a group of membrane constituents most of which are poorly understood. Polylactosaminoglycans contain the determinants for certain blood group antigens and are known to

be important in development (Fukuda *et al.*, 1979b). The existence of a glycosylated and non-glycosylated form of the same protein in a fully differentiated cell is quite surprising, and at this time it is impossible to state which is precursor and which is product. The lack of glycosylation on the majority of 28kDa molecules may be consistent with a selective loss of the polylactosaminoglycan carbohydrate during erythrocyte differentiation or during aging.

The apparent linkage of 28kDa to the membrane skeleton is more likely the result of a direct interaction of the cytoplasmic domain of the polypeptide with sites on the membrane skeleton rather than a phenomenon related to solubility, since 28kDa can be solubilized from vesicles in Triton X-100. The membrane skeleton site for 28kDa attachment may be the junctional complex which consists of a short actin filament, tropomyosin, protein 4.1, and the ends of 5–7 spectrin oligomers. The known number of junctional complexes (30,000) and 28kDa monomers (120,000–160,000) is consistent with direct attachment of one member of a 28kDa oligomer to the membrane skeleton, and side-to-side associations of other members of the 28kDa oligomer within the bilayer. Alternatively, the number of copies of ankyrin (100,000) could nearly accommodate all 28kDa monomers.

The physiologic role of the 28kDa polypeptide is uncertain. The existence of a polypeptide nearly identical to 28kDa in the apical brush borders of renal proximal-convoluted tubules is provocative. There is yet no evidence that 28kDa is itself a membrane transporter, but such a role is consistent with its physical behavior. The 28kDa could conceivably be a noncatalytic subunit of one or more transporters by interacting side-to-side between the leaflets of the lipid bilayer with catalytic subunits which would be restricted to a favorable orientation within the cell membrane. Renal proximal-convoluted tubule epithelia are highly polarized cells, and the apical brush borders are rich in a variety of transporters which are not found in the basolateral side of the tubule (see review by Murer and Gmaj, 1986). Cytoskeletal elements related to those of the erythrocyte have recently been colocalized to the basolateral surfaces of polarized kidney epithelia apparently in association with specific transport polypeptides. Analogs to ankyrin and spectrin were colocalized with the anion transporter in basolateral membranes of rat kidney distal tubules (Drenckhahn *et al.*, 1985). Fodrin colocalized with Na,K-ATPase in basolateral membranes of Madin-Darby canine kidney cells (Nelson and Veshnock, 1986). The localization of 28kDa primarily in the apical brush borders implies that 28kDa does not interact directly with the kidney analogs to spectrin and ankyrin which are primarily located on basolateral membranes. The abundance of 28kDa in apical brush borders suggests that it may be a structural component of transport. Moreover, a skeleton-linked integral membrane protein like 28kDa may provide the mechanism whereby the state of cell volume is sensed from the degree of membrane skeleton distension and transduced back to the appropriate membrane transporters or channels which respond by restricting the entry of additional water or electrolyte. The relative ease with which 28kDa can be isolated and the existence of potent antibodies to 28kDa may make the evaluation of such potential roles feasible.

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