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Isolation and partial characterization of the human erythrocyte band 7 integral membrane protein

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Monoclonal antibodies to the M_r 31000 major integral membrane protein of the human erythrocyte band 7 region were used to identify the corresponding polypeptide chain and epitope-carrying fragments on immunoblots. Analysis of the erythrocyte membrane, membrane fractions, and cytosol revealed that the M_r 31000 band 7 integral membrane protein is unique and not related to any of the other water-soluble or membrane-bound band 7 components. Cross-reacting proteins were identified in the membranes of other mammalian erythrocytes and in cell lines of epithelial and lymphoid origin. Proteolytic digestion of intact human erythrocytes or erythrocyte membranes demonstrated that the band 7 integral membrane protein has an intracellular domain larger than M_r 12000; it does not have an extracellular one. One of the monoclonal antibodies was employed for the isolation of band 7 integral membrane protein by immunoaffinity chromatography; subsequent Edman degradation revealed a blocked N-terminus.

Introduction

Analysis of the human erythrocyte membrane by SDS-PAGE and Coomassie brilliant blue-staining reveals about eight electrophoretic bands, which are numbered by descending M_r values of the corresponding proteins [1,2]. Band 7 protein has a reported M_r of 29000 and represents about 3.4% of the stained erythrocyte membrane proteins [2]. It was found to be the only protein perturbant-resistant component of the cytoplasmic surface of the erythrocyte membrane [2], and to be susceptible to phosphorylation by a cAMP-dependent protein kinase [3]. Differential solubilization of the ^{32}P-phosphorylated erythrocyte membrane proteins and subsequent SDS-PAGE analysis revealed the presence of two band 7 components, a phosphorylated integral membrane protein, and a non-phosphorylated cytoskeleton component [4]. The band 7 region has further been resolved by SDS-PAGE into three components, designated 7.1 (M_r 30000), 7.2 (M_r 28000), and 7.3 (M_r 26000), and by two-dimensional electrophoresis (NEPHGE/SDS-PAGE) into four components (7.1, 7.2a, 7.2b, 7.3), which were also distinguished by differential solubilization [5–7]. Bands 7.1 (also termed 61 × 7) and 7.2a (60 × 8) have been identified as tropomyosin polypeptides [8], bands 7.2b (22 × 8) and 7.3 (8 × 10) were found to be integral membrane proteins. Interestingly, the major component 7.2b was completely absent in the band 7 region of patients with Na^+/K^+ permeability disorders (hereditary stomatocytosis and cryohydrocytosis) [5,6,9], thus raising the possibility that this band 7 polypeptide may have a functional role in $Na^+/K^+/2Cl^-$ or K^+/Cl^- co-transport through the erythrocyte membrane.

The band 7 region is rather complex and little is known about the structural relationships of the membrane-bound and water-soluble polypeptides within this region. In addition to the components described above, five to eight minor components with M_r 21500 to M_r 29000, existing in membrane-bound and cytosolic forms, have been identified as constituents of a hollow cylinder protein (cylindrin) [7,10]. Furthermore, a faintly Coomassie blue-stained M_r 28000 integral membrane protein [11] has been purified and partially characterized. However, the major band 7 integral membrane protein remained as yet incompletely characterized.

Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NEPHGE, non-equilibrium pH gradient electrophoresis; TPCK, 1-chloro-3-tosylamido-4-phenylbutan-2-one.

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In our study we used monoclonal antibodies against the major band 7 integral membrane protein (component 7.2b) of human erythrocytes in order to investigate the relationship of this protein to the various membrane-bound and water-soluble components within the band 7 region. We also studied in situ proteolytic fragmentation of this protein and identified related proteins in other mammalian erythrocytes and cells of epithelial and lymphoid origin. The protein from human erythrocyte membranes was then isolated and some of its characteristics were examined.

Materials and Methods

Materials. Human blood was obtained from the Institute of Blood Group Serology, University of Vienna, and from the Austrian Red Cross. Blood from animals was obtained from Behring Institute (sheep, rabbit), from local slaughter houses (cow, pig, chicken, fish) or was given to us by Dr. O. Scheiner, Univ. Vienna (rat) and Dr. E. Dworkin-Rastl, E. Boehringer Inst. (frog). Human cell lines were K 562 (CCL 243), MOLT-4 (CRL 1582), and HeLa (CCL 2) from ATCC, BALL-1 as described [12]. α-Chymotrypsin (EC 3.4.21.1) and TPCK-trypsin (EC 3.4.21.4) were from Serva, Heidelberg, F.R.G.; *Staphylococcus aureus* V8 proteinase (EC 3.4.21.19), papain (EC 3.4.22.2), proteinase K (EC 3.4.21.14), bovine serum albumin, and molecular weight marker proteins were from Sigma. $Na^{125}I$ (IMS.30), $H_3{}^{32}PO_4$ (PBS.11) and autoradiographic films (Hyperfilm MP) were from Amersham Corp. Cronex Hi-Plus intensifying screens were from Du Pont. Protein G-Sepharose 4 Fast Flow and CNBr-activated Sepharose 4B were obtained from Pharmacia LKB Biotechnology Inc., electrophoresis chemicals and Triton X-100 from Bio-Rad. Nitrocellulose (BA 85) was from Schleicher & Schuell, Dassel, F.R.G., Glassybond glass fiber sheets were from Biometra, Göttingen, F.R.G., and ultrafiltration membranes from Amicon. Tris, cAMP, phenylmethylsulfonyl fluoride, fluorescein isothiocyanate, and *p*-chloromercuribenzene sulfonate were purchased from Sigma. Other analytical grade chemicals were from Merck. Purified glycophorin B was a gift of Dr. H. Furthmayr (Stanford Univ.).

Electrophoresis and immunoblotting. SDS-PAGE on slab gels [13] was generally performed under reducing conditions and the proteins were transferred onto nitrocellulose [14]. The blots were then incubated with mouse monoclonal antibodies (about 2 μg/ml) and ^{125}I-labelled rabbit anti-mouse immunoglobulin G (prepared as described [15], at 200000 cpm/ml), and were subsequently exposed to autoradiographic film at −70°C using an intensifying screen. Quantitative immunoblots were performed as described [11]. Marker proteins were included in each run; for visualisation the blots were stained with Ponceau S.

Preparation of monoclonal antibodies. Three BALB/c mice were immunized four times with electrophoretically purified 30kDa erythrocyte membrane proteins (about 50 μg/injection) in Freund's complete adjuvant. Spleen cells were fused with the mouse myeloma cell line P3-X63-Ag.8.653 as described [16,17] and the hybridoma culture supernatants screened by immunoblotting. Three monoclonal antibodies gave positive reactions with band 7 protein: GARP-50, GARP-61, and GARP-65. The corresponding hybridomas were recloned and grown in cell suspension or as an ascites tumour. The antibodies were purified from the supernatants by Protein G-Sepharose affinity chromatography. GARP-50 was also purified from ascitic fluid by two precipitations at 50% ammonium sulfate saturation. Purified GARP-50 antibody was bound to CNBr-activated Sepharose at 2 mg/ml Sepharose. A monoclonal antibody to glycophorin B (GARP-62) was identified by its immunoblot reaction with purified antigen and was used for controls.

Preparation and solubilization of cell membranes. Mammalian erythrocyte membranes were prepared by hypotonic lysis of the cells in 20 volumes 5 mM Na phosphate buffer (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, for 10 min at 0°C and centrifugation for 30 min at 48000 × *g*. The membranes were washed four times with 20 volumes cold lysis buffer and were solubilized by mixing with an equal volume of 2-fold concentrated SDS-PAGE sample buffer and heating for 3 min at 100°C. Erythrocytes from chicken, frog and fish, as well as cultured cells were incubated for 30 min at 0°C with five volumes 1% (v/v) Triton X-100, 20 mM Na phosphate buffer (pH 7.4), 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride. Solubilized proteins were separated from cellular debris by centrifugation at 10000 × *g* for 10 min at 4°C, the supernatant was mixed with four volumes 1.25-fold concentrated SDS-PAGE sample buffer and heated for 3 min at 100°C.

Washed human erythrocyte membranes were selectively solubilized by the protein perturbants 0.1 M NaOH, *p*-chloromercuribenzene sulfonate [18], or low ionic strength EDTA [5], as described. They were also solubilized by Triton X-100 in 56 mM Na borate buffer (pH 8.0) [19]. Cylindrin (hollow cylinder protein) was prepared from a low ionic strength extract of erythrocyte membranes [7], or from erythrocyte lysate [10], as described.

^{32}P-labelling and immunoprecipitation of band 7 integral membrane protein. Human erythrocytes were obtained from freshly drawn blood of healthy donors and labelled with [^{32}P]phosphate in the presence of cAMP as described [3]. Membranes were prepared and solubilized with five volumes 1% (v/v) Triton X-100, 0.2% (w/v) SDS, 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride for 10 min at 22°C.

The mixture was centrifuged for 5 min at $10000 \times g$ and 1 ml of the supernatant was mixed with 1 ml of a 2.5% (v/v) suspension of GARP-50-Sepharose in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and rotated for 2 h at 22 °C. The suspension was centrifuged for 30 s at $10000 \times g$ and the supernatant retained for further examination. The Sepharose was washed four times with 0.5% (v/v) Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and once with 20 mM Tris-HCl (pH 7.5), and was centrifuged each time for 10 s at $10000 \times g$. The washed pellet was suspended in electrophoresis sample buffer, heated for 3 min to 100°C, centrifuged for 1 min at $10000 \times g$, and the supernatant was applied to a polyacrylamide gel.

Digestion of intact erythrocytes and membranes. Washed human erythrocytes (100 μl packed cells) were resuspended either in 0.9 ml 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, or in 0.9 ml of the same buffer containing 100 μg proteinase. The mixtures were incubated for 1 h at 37°C with shaking, the cells were pelleted for 10 s at $10000 \times g$ and washed three times with 150 mM NaCl. The cells were lysed and the membranes dissolved by mixing with an equal volume of 2-fold concentrated SDS-PAGE sample buffer.

Washed human erythrocyte membranes (100 μl) were taken up in 0.4 ml 20 mM Tris-HCl (pH 7.5), 1 mM NaN_3, with no addition, or in the same volume of buffer containing 10 μg proteinase, and the mixtures were incubated at 37°C. Aliquots (50 μl) were taken at time intervals, mixed with an equal volume 2-fold concentrated SDS-PAGE sample buffer and heated for 3 min. Other aliquots of the membranes were washed with 20 volumes of 20 mM Tris-HCl (pH 7.5), 2 mM phenylmethylsulfonyl fluoride before heating with sample buffer.

Isolation and chemical analysis of band 7 integral membrane protein. One unit of outdated human red blood cells was used for the preparation of membranes. The washed membranes were suspended in two volumes 0.1 M $NaHCO_3$ (pH 9.0), 0.5 M NaCl, and were labelled by addition of 25 mg fluorescein isothiocyanate and rotation of the suspension for 14 h at 4°C. The labelled membranes were sedimented at $27000 \times g$ for 90 min in a Sorvall GSA rotor, washed twice with 500 ml 0.15 M NaCl, and solubilized by mixing with an equal volume of 2-fold concentrated SDS-PAGE sample buffer and heating for 5 min at 100°C. The solution was applied to 30 preparative SDS-PAGE slab gels (12% polyacrylamide, $14 \times 16 \times 0.3$ cm), and electrophoresis was performed for 14 h at 55 V. Fluorescent band 7 was visualized by UV-illumination (254 nm), the corresponding region cut from the gels, and the protein eluted electrophoretically as described [20]. The combined eluates were concentrated by ultrafiltration in an Amicon cell with a YM 30 membrane, and the proteins were washed in the cell with 300 ml 0.2% (v/v) Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM NaN_3 (starting buffer).

The solution was loaded onto an affinity column (1×3 cm) of GARP-50-Sepharose equilibrated with starting buffer at 4°C, the column was washed with 30 ml starting buffer, and eluted with 10 ml 0.1 M glycine-HCl (pH 2.5), 0.1% Triton X-100. The eluted fractions were immediately neutralized with 1 M Tris base, and the column was re-equilibrated with starting buffer. Fractions containing band 7 integral membrane protein were pooled and stored at −20°C.

The relative purity of the preparations was determined by SDS-PAGE, Coomassie blue-staining and densitometry (Beckman DU8); the purity was also examined by silver staining [21]. The recovery was estimated by quantitative immunoblotting [11] using standard solutions of pure band 7 integral membrane protein for calibration. Protein concentration was determined colorimetrically [22].

Purified band 7 integral membrane protein was reduced and carboxymethylated by standard methods [23], subjected to SDS-PAGE (12% polyacrylamide) and blotted onto Glassybond sheets as described [24]. The sheets were stained with Coomassie brilliant blue R-250, bands were cut out and used directly for analyses. Amino acid analysis was performed as described [24]; N-terminal amino acid sequence analysis was performed using an Applied Biosystems AB-470A sequenator.

Results

Production of monoclonal antibodies against band 7 integral membrane protein

When human erythrocyte membranes were analysed by SDS-PAGE and Coomassie blue-staining, band 7 was easily recognized as the major band at M_r 31000. The corresponding band 7 protein was isolated by preparative SDS-PAGE and used for the production of monoclonal antibodies. Three of the antibodies (GARP-50, GARP-61, GARP-65) were identified by their reaction with the isolated protein on immunoblots; they did not react with cytosolic proteins, nor with cylindrin prepared either from erythrocyte membranes or cytosol (not shown). The ability of certain reagents to extract the immunoreactive protein from the membranes was also examined: it was not solubilized by the protein perturbants 0.1 M NaOH, low ionic strength EDTA, or *p*-chloromercuribenzene sulfonate. However, it was partially solubilized by 0.5% Triton X-100. Increasing the Triton X-100 concentration to 5% (v/v) resulted in almost complete solubilization of this protein (not shown).

In order to prove the identity of the immunoreactive protein with band 7 integral membrane protein, we took advantage of the characteristic phosphorylation of

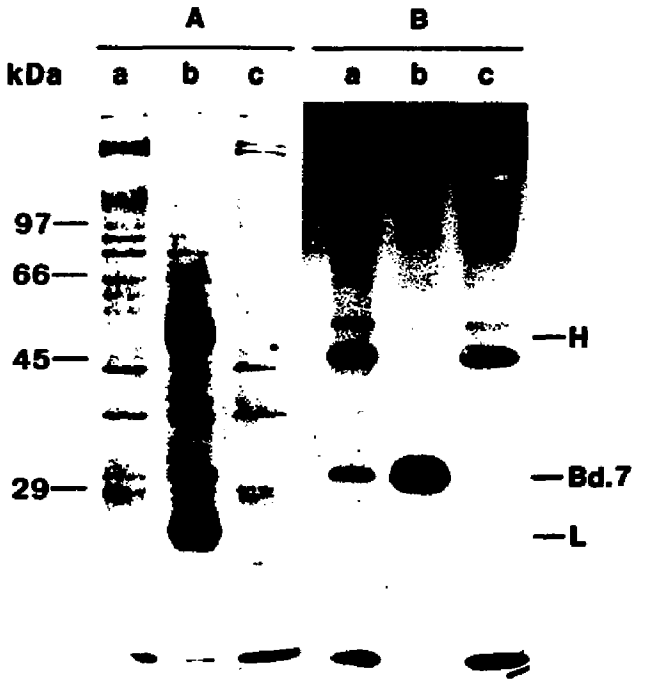

Fig. 1. Immunoprecipitation of ^{32}P-phosphorylated band 7 integral membrane protein. Human erythrocytes were labelled with [^{32}P]phosphate in the presence of cAMP, membranes were prepared and solubilized with 1% Triton X-100, 0.2% SDS. Solubilized band 7 integral membrane protein was immunoprecipitated by monoclonal antibody GARP-50. Whole membranes (lane a), the immunoprecipitate (lane b) and supernatant (lane c) were electrophoresed into a 12% SDS-PAGE gel, which was stained with Coomassie blue (panel A), and exposed to autoradiographic film (panel B). The positions of band 7 (Bd. 7), antibody heavy (H) and light (L) chains are indicated.

band 7 protein by a cAMP-dependent protein kinase. Human erythrocytes were labelled with [^{32}P]phosphate in the presence of cAMP, the membranes were isolated and solubilized, and the antigen was precipitated by GARP-50-Sepharose. Analysis by SDS-PAGE, Coomassie blue-staining, and autoradiography of the pellet and supernatant showed that band 7 integral membrane protein was detected by both staining and autoradiography, and that it was completely adsorbed by the immune affinity material (Fig. 1). No ^{32}P-labelled protein was found in the erythrocyte lysate.

Identification of related proteins

Immunoblot analysis of erythrocytes from various species showed the presence of cross-reacting proteins in all the mammalian red cells tested (Fig. 2). These proteins have a M_r very similar to human band 7 integral membrane protein and are thus related. Cross-reacting proteins were also found in chicken and frog erythrocytes, with M_r values of 14000–18000. These low M_r values were reproducibly found in different samples; however, we cannot completely exclude the possibility of degradation during sample handling. In fish (trout) erythrocytes, no related protein was found.

Several human cell lines were also screened for the presence of cross-reacting proteins. Large amounts of a band 7-related protein were found in HeLa cells, with small amounts also being found in the cells of the erythroid cell line K 562 and the cell lines BALL-1 (B-lymphoid) and MOLT-4 (T-lymphoid).

Fig. 2. Identification of band 7 integral membrane protein-related proteins by immunoblot analysis. Cell membranes of various vertebrate erythrocytes and cultured human cells were solubilized, electrophoresed into 12% SDS-PAGE gels, and immunoblotted with monoclonal antibody GARP-50. Erythrocyte samples were taken from man (lane a), cow (lane b), pig (lane c), sheep (lane d), rabbit (lane e), rat (lane f), chicken (lane g) and frog (lane h); cultured cells were from the cell lines HeLa (lane i), K 562 (lane j), BALL-1 (lane k), and MOLT-4 (lane l).

Proteolytic degradation of band 7 integral membrane protein

The location of the M_r 31000 band 7 integral membrane protein at the erythrocyte membrane was examined by proteolytic digestion of intact red cells or their membranes and subsequent immunoblot analysis. When washed intact erythrocytes were incubated with proteinase K and then analysed, band 7 integral membrane protein was not degraded (Fig. 3). In contrast, proteinase K did remove an extracellular portion of glycophorin B. On the other hand, when erythrocyte membranes were digested with proteinase K and then analysed, the specific epitope was lost. These results indicate that band 7 integral membrane protein is located solely at the cytoplasmic surface of the erythrocyte membrane.

In order to study the degradation of band 7 integral membrane protein in situ, the membranes were digested with various proteinases in a time-dependent manner; the membranes were then dissolved in sample buffer and immunoblotted with the monoclonal antibody GARP-50. Within 5 min, a degradation product of M_r 29000 formed, irrespective of the proteinase used. A fragment of M_r 29000 was also recognized when the erythrocyte membranes were stored for several days at 4°C in the absence of EDTA or when the membranes had been prepared from stored blood containing citrate as the only additive (Fig. 4). Trypsin degraded the band 7 integral membrane protein to fragments of M_r 29000, M_r 22000, and to a stable end product of M_r 19000. Chymotrypsin generated fragments of M_r 29000, 26000, 24500, 23500, 23000, and a stable M_r 19000 fragment. *Staphylococcus aureus* V8 proteinase digested the protein to fragments of M_r 29000, 27500, 25500, 24500 and 23000. After 5 h incubation the loss of the GARP-50 epitope signal was noticed, indicating that the epitope is accessible to the V8 proteinase. This result also shows that the GARP-50 epitope is located within the cytoplasmic region of band 7 integral membrane protein. The tryptic or chymotryptic M_r 19000 fragments further indicate that at least one third of the protein is accessible to the enzymes. Digestion of erythrocyte membranes with papain or proteinase K led to the quick fading of the M_r 31000 signal without the appearance of degradation products. Water-soluble fragments of band 7 integral membrane protein were not identified in any of the experiments, probably because they were degraded much faster than the membrane-bound fragments.

Identical immunoblotting results were obtained when the band 7-specific monoclonal antibodies GARP-61 and GARP-65 were used instead of GARP-50 (not shown). This indicates that the epitopes of the three antibodies are located close to one another.

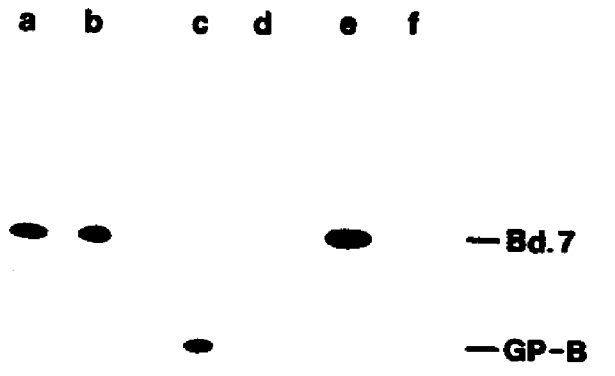

Fig. 3. Band 7 integral membrane protein is located at the cytoplasmic surface of the human erythrocyte membrane. 100 μl human erythrocytes (lanes a-d) were resuspended in 0.9 ml 0.15 M NaCl, 20 mM Tris-HCl (pH 7.5), and incubated for 1 h at 37°C with no addition or in the presence of 100 μg proteinase K. 100 μl erythrocyte membranes (lanes e, f) were resuspended in 0.4 ml 20 mM Tris-HCl (pH 7.5), and incubated for 1 h at 37°C with no addition or in the presence of 10 μg proteinase K. Membranes were isolated, washed, and solubilized in sample buffer. Aliquots (50 μl) of the untreated (lanes a, c, e) or proteinase-treated (lanes b, d, f) samples were electrophoresed into 15% SDS-PAGE gels and immunoblotted with monoclonal antibody GARP-50 (lanes a, b, e, f) or anti-glycophorin B monoclonal antibody GARP-62 (lanes c, d). The positions of band 7 (Bd. 7) and glycophorin B (GP-B) are indicated.

Isolation and chemical analysis of band 7 integral membrane protein

Band 7 integral membrane protein was isolated from fluorescein isothiocyanate-labelled human erythrocyte membranes by preparative SDS-PAGE. Fluorescent band 7 was excised from the gels and the protein eluted. In order to obtain pure band 7 integral membrane protein, the SDS-containing solution was diluted with 0.2% Triton X-100 and then applied to a GARP-50-Sepharose affinity column. The protein eluted from the column was indeed the electrophoretically pure M_r 31000 protein of the band 7 region, as judged from SDS-PAGE analysis and Coomassie blue-staining (Fig. 5). The relative purity of the preparation was consistently higher than 95% by densitometry. Band 7 integral membrane protein was also pure by silver staining. For an unknown reason it is not stained well with the silver reagent; however, contaminating proteins are stained correctly.

Employing the purified protein for standard solutions, the amount of band 7 integral membrane protein estimated by quantitative immunoblot analysis was 32 mg in one unit of erythrocytes. This value corresponds to 410000 copies per erythrocyte, in good agreement with the published value [2]. From one unit of erythrocytes 0.8 mg of purified protein were obtained, corresponding to 2.5% recovery. The amino acid composition (Table I) containing 49% hydrophobic amino acids was in accordance with compositions of other mem-

brane proteins. Edman degradation of band 7 integral membrane protein was not possible, as the N-terminus appeared to be blocked. A blocked N-terminus was also found when other methods of isolation were applied, which did not bear the risk of creating protein modifications (first step immunoaffinity chromatography, second step agarose gel electrophoresis). However, tryptic digestion [23] of the isolated protein produced suitable internal peptides for sequencing. The internal sequences revealed no homology to a known protein when compared to the sequences in the SWISSPROT™ library (release 16.0, October 1990) with the FASTA computer program [25] (Maurer-Fogy, I., unpublished data).

Discussion

We have developed monoclonal antibodies to the major band 7 integral membrane protein of the human erythrocyte and have used them as specific reagents for the identification of this protein, for its isolation, and partial characterization.

Immunoblot analyses of membrane-bound and water-soluble band 7 components showed that the integral membrane protein is unique and is not related to any of the water-soluble peripheral or cytosolic erythrocyte proteins. Thus, the band 7 polypeptide chains appear to be different from the cylindrin components, which are known to exist in both, a membrane-bound

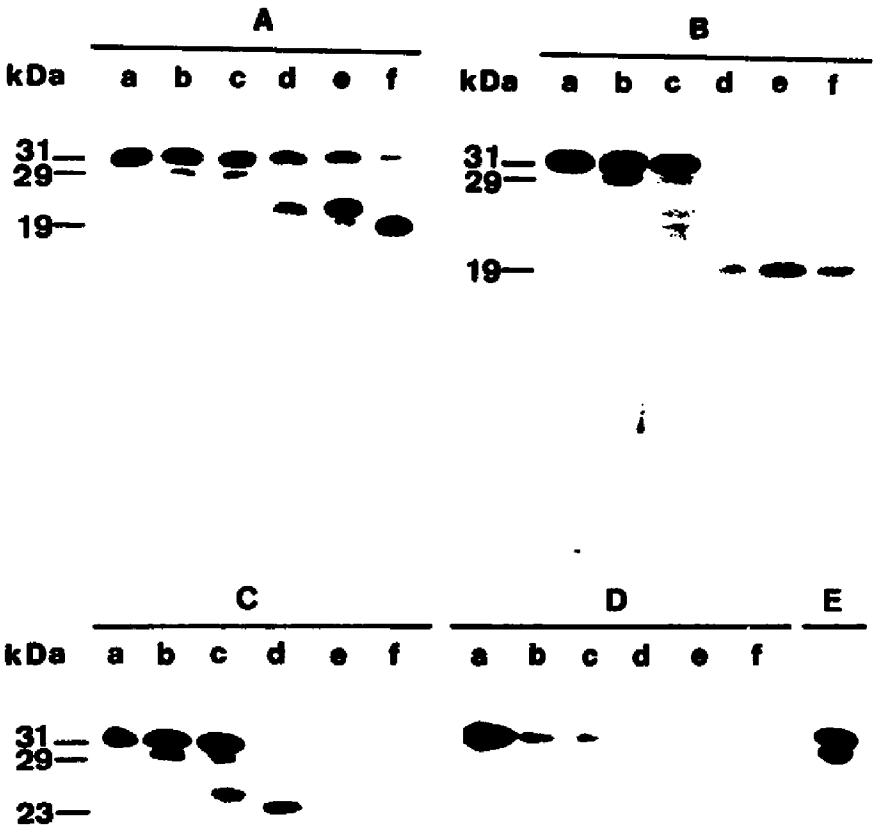

Fig. 4. Proteolytic degradation of band 7 integral membrane protein at the cytoplasmic surface of the human erythrocyte membrane. Fresh human erythrocyte membranes (100 μl) were resuspended in 0.4 ml 20 mM Tris-HCl (pH 7.5), 1 mM NaN_3, and incubated in the presence of 10 μg TPCK-trypsin (panel A), α-chymotrypsin (panel B), *Staphylococcus aureus* V8 proteinase (panel C), or papain (panel D) at 37°C. Aliquots (50 μl) were taken at the intervals: 0 min (lanes a), 5 min (lanes b), 30 min (lanes c), 60 min (lanes d), 120 min (lanes e), or 300 min (lanes f), mixed with sample buffer and electrophoresed into a 17% SDS-PAGE gel (panel A) or into 15% SDS-PAGE gels (panels B-D). A sample of membranes (10 μl) prepared from stored erythrocytes (5 days at 4°C) was included (lane E). The gels were immunoblotted with monoclonal antibody GARP-50.

TABLE I

Amino acid composition of purified erythrocyte band 7 integral membrane protein

Results are the averages of two determinations.

Amino acid	mol%
Asx	9.6
Thr	5.8
Ser	10.8
Glx	11.4
Pro	2.9
Gly	11.2
Ala	10.3
Cys	n.d. [a]
Val	5.0
Met	0.5
Ile	6.0
Leu	8.3
Tyr	1.7
Phe	3.3
His	1.6
Lys	5.5
Arg	6.1
Trp	n.d.

[a] n.d., not determined.

and a cytosolic form [7,10]. Band 7 protein was shown to be located at the cytoplasmic surface of the erythrocyte membrane [2] and to be only partially solubilized by Triton X-100 [19], giving rise to the speculation that band 7 protein may be linked to the cytoskeleton. By immunoblot analysis we found that the solubility of band 7 membrane protein increased with increasing Triton X-100 concentrations, therefore interactions with the cytoskeleton are not very likely. Also, by cross-linking experiments according to Wang and Richards [26] with subsequent immunoblot analysis, we have shown, that band 7 integral membrane protein forms mainly dimers and a small amount of higher oligomers, but it is not cross-linked to the cytoskeletal proteins (Glotzmann, C., unpublished data), in accordance with the earlier results [26]. We therefore conclude that this membrane protein is not part of the cytoskeleton.

Immunoprecipitation of ^{32}P-labelled band 7 integral membrane protein proved that it is the only ^{32}P-phosphorylated component of the band 7 region, in accordance with the previous finding that ^{32}P-labelled band 7 protein is not solubilized by NaOH [4].

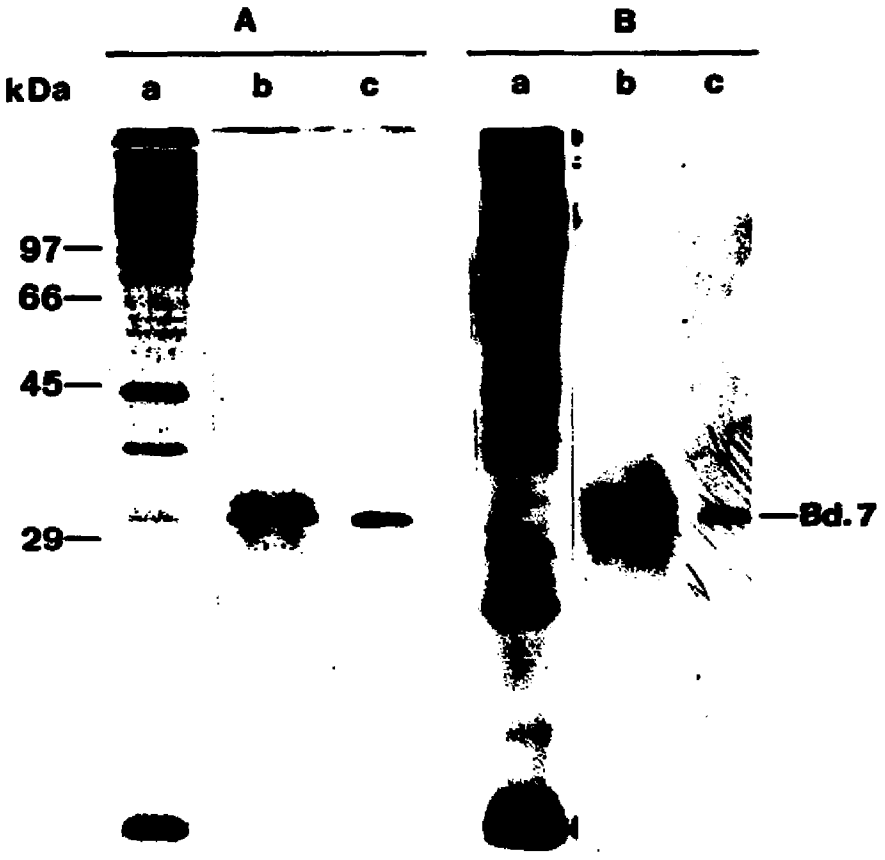

Fig. 5. Isolation of band 7 integral membrane protein from human erythrocyte membranes. Samples from various stages of band 7 integral membrane protein purification were electrophoresed into a 12% SDS-PAGE gel and stained with Coomassie blue (panel A) or silver reagent (panel B): solubilized human erythrocyte membranes (lanes a), eluate of the preparative SDS-PAGE gels (lanes b), affinity purified band 7 integral membrane protein (lanes c). The position of band 7 (Bd. 7) is indicated.

Degradation of band 7 integral membrane protein at the cytoplasmic surface of the human erythrocyte membrane gave some insight into its spatial arrangement. A M_r 2000 terminal peptide seems to be exposed to the aqueous medium, because of the rapid appearance of a M_r 29000 fragment upon digestion with proteinases. About one third (12 kDa) of the protein is accessible to trypsin or chymotrypsin, as judged by the formation of a membrane-bound M_r 19000 end product. The cytoplasmic domain of the band 7 integral membrane protein may well be larger than 12 kDa, as indicated by the fading of the GARP-50 epitope signal on prolonged digestion with V8 proteinase. Obviously the epitope is located within the cytoplasmic domain and is cleaved off by V8 proteinase digestion.

It is not known, whether band 7 integral membrane protein spans the lipid bilayer, or if the molecule is anchored to the membrane via a covalently bound lipid. Digestion experiments of intact red cells have shown that the protein's M_r was not reduced. Furthermore, ^{125}I-iodination of the protein in situ was only successful when erythrocyte membranes were used (Prohaska, R., unpublished data), but not when intact red cells were used [27]. These results indicate that either there is no extracellular part, or the part is too small to be detectable by the methods applied. Amino acid sequence information will be necessary to identify the domains interacting with the lipid bilayer.

The N-terminal amino acid sequence analysis of purified band 7 integral membrane protein showed that the N-terminus is blocked. The blocking is not due to the fluorescein isothiocyanate-labelling or the method of isolation, because only a small fraction of the erythrocyte membrane proteins was labelled, and the N-terminal blocking was also noticed when the protein was isolated without labelling and the use of preparative SDS-PAGE.

The function of band 7 integral membrane protein is still unclear. In the light of the lack of this band 7 component in erythrocytes of patients with Na^+/K^+ permeability disorders, it is conceivable that it is a component of the $Na^+/K^+/2Cl^-$ and/or K^+/Cl^- co-transport systems, responsible for cell volume control. Our observation that band 7 integral membrane protein is expressed in all the analysed mammalian erythrocytes suggests that it is a functionally important erythrocyte component. Furthermore, its presence in other human cells suggests that its role is not limited to red cell-specific functions. Immunohistochemical analyses of the tissue distribution and the exact localization of band 7 integral membrane protein in various cells may help to shed light on the function of this interesting protein.

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References

1 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617.
2 Steck, T.L. (1974) J. Cell Biol. 62, 1–19.
3 Plut, D.A., Hosey, M.M. and Tao, M. (1978) Eur. J. Biochem. 82, 333–337.
4 Johnson, R.M., McGowan, M.W., Morse, P.D. and Dzandu, J.K. (1982) Biochemistry 21, 3599–3604.
5 Lande, W.M., Thiemann, P.V.W. and Mentzer, W.C. (1982) J. Clin. Invest. 70, 1273–1280.
6 Eber, S.W., Lande, W.M., Iarocci, T.A., Mentzer, W.C., Höhn, P., Wiley, J.S. and Schröter, W. (1989) Br. J. Haematol. 72, 452–455.
7 Lande, W.M., Thiemann, P.V.W., Fisher, K.A. and Mentzer, W.C. (1984) Biochim. Biophys. Acta 778, 105–111.
8 Fowler, V.M. and Bennett, V. (1984) J. Biol. Chem. 259, 5978–5989.
9 Morlé, L., Pothier, B., Alloisio, N., Féo, C., Garay, R., Bost, M. and Delaunay, J. (1989) Br. J. Haematol. 71, 141–146.
10 Malech, H.L. and Marchesi, V.T. (1981) Biochim. Biophys. Acta 670, 385–392.
11 Denker, B.M., Smith, B.L., Kuhajda, F.P. and Agre, P. (1988) J. Biol. Chem. 263, 15634–15642.
12 Miyoshi, I., Hiraki, S., Tsubota, T., Kubonishi, I., Matsuda, Y., Nakayama, T., Kishimoto, H., Kimura, I. and Masuji, H. (1977) Nature 267, 843–844.
13 Laemmli, U.K. (1970) Nature 227, 680–685.
14 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
15 Prohaska, R. and Adolf, G.R. (1987) Immunobiology 174, 93–106.
16 Köhler, G. and Milstein, C. (1975) Nature 256, 495–497.
17 Kearney, J.F., Radbruch, A., Liesegang, B. and Rajewski, K. (1979) J. Immunol. 123, 1548–1550.
18 Steck, T.L. and Yu, J. (1973) J. Supramol. Struct. 1, 220–232.
19 Yu, J., Fischman, D.A. and Steck, T.L. (1973) J. Supramol. Struct. 1, 233–248.
20 Mendel-Hartvig, I. (1982) Anal. Biochem. 121, 215–217.
21 Merril, C.R., Goldmann, D., Sedman, S.A. and Ebert, M.H. (1981) Science 211, 1437–1438.
22 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
23 Allen, G. (1989) Sequencing of Proteins and Peptides, 2nd Edn. (Burdon, R.H. and Van Knippenberg, P.H., eds.), p. 58, Elsevier, Amsterdam.
24 Eckerskorn, C., Mewes, W., Goretzki, H. and Lottspeich, F. (1988) Eur. J. Biochem. 176, 509–519.
25 Pearson, W.R. and Lipman, D.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2444–2448.
26 Wang, K. and Richards, F.M. (1974) J. Biol. Chem. 249, 8005–8018.
27 Krahmer, M. and Prohaska, R. (1987) FEBS Lett. 226, 105–108.