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TWO-DIMENSIONAL ELECTROPHORETIC ANALYSIS OF HUMAN ERYTHROCYTE CYLINDRIN

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Cylindrin, a macromolecule isolated from the human erythrocyte, and the band 7 proteins of the erythrocyte membrane were analyzed by one- and two-dimensional electrophoresis. Cylindrin was recovered from both the cytosol and cell membranes of hypotonically lysed erythrocytes, and its identity was confirmed by electrophoresis and transmission electron microscopy. Cylindrin from either source produced eight bands on one-dimensional SDS gels, and seventeen spots on two-dimensional gels, revealing a more complex composition than previously reported. It is unlikely that this complexity was due to proteolysis, since preparations of cylindrin with various protease inhibitors gave the same electrophoretic patterns. Mixing experiments showed that the polypeptide subunits of the cylindrin complex are distinct from the band 7 proteins of the erythrocyte membrane. This finding failed to support a role for the cylindrin macromolecule in the permeability disorders of the erythrocyte membrane associated with a missing band 7 protein.

Introduction

Cylindrin is a macromolecule of approx. 747 kDa which has a 'hollow cylinder' appearance when negatively stained and examined by electron microscopy [1–4]. It can be isolated from both the cytosol and cell membranes of human erythrocytes, but its function is not yet known. We were interested in cylindrin because the molecular weights of its polypeptide subunits are close to those of the band 7 proteins of the erythrocyte membrane. In a previous study [5], we showed that band 7 is composed of four proteins, one of which is completely absent in certain congenital hemolytic anemias with increased Na^+/K^+ membrane permeability. In the work described here, we analyze cylindrin by one and two-dimensional electrophoresis and demonstrate a greater number of subunits than previously reported. We then compare the polypeptides of the cylindrin complex to those of membrane band 7 and show that they are completely different.

Methods

Preparation of cylindrin. Cylindrin was prepared from either outdated (30–40 days old) or underweight (1–5 days old) units of type A + and O + human blood anticoagulated with citrate-phosphate-dextrose solution. The blood was washed three times with 0.154 M NaCl, and leukocytes removed by aspiration. Erythrocytes were then lysed in 20 volumes of 5 mM sodium phosphate, pH 8.0 (5P8), and hemolysate centrifuged at $12000 \times g$ for 10 min in a Sorvall SS-34 rotor. The supernatant was saved for the isolation of cytosolic cylindrin and the membrane pellet either saved or washed twice in 20 volumes of 5P8. Unwashed membrane pellets appeared pink, whereas washed pellets were white. Membranes

were extracted with 5 volumes of 0.5 mM EDTA/0.5 mM β-mercaptoethanol at pH 8.5 for 16 h [6] and then pelleted by centrifugation at 37000 × *g* for 40 min (SS-34 rotor). Thereafter, isolation of cylindrin from the supernatant and the membrane extract proceeded in parallel and followed the procedure of Malech and Marchesi [6]. Briefly, cylindrin was precipitated from the supernatant or the membrane extract by adjusting the pH to 5.2 with 0.5 M citric acid. The precipitate was solubilized in 100 mM sodium phosphate, pH 8.0, and purified by three successive centrifugations on 5–17% (w/v) linear sucrose density gradients.

To prevent proteolysis, all steps were performed at 0–4°C, and the following protease inhibitors added to all of the solutions except the 0.154 M NaCl: 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM EDTA, 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), and 5 μg/ml pepstatin A. For the five separate preparations of cytosolic cylindrin, PMSF alone was used for two, PMSF and EDTA were employed for one, and all four inhibitors were used for two. For the three preparations of cylindrin from unwashed membranes, PMSF alone was used for two, and all four inhibitors were used for one. The single preparation from washed membranes employed all four inhibitors. Samples for transmission electron microscopy (TEM) were stored at 0–4°C; samples for electrophoresis were stored in 500 μl aliquots at −80°C.

Electrophoresis. Non-denaturing, detergent-free electrophoresis was performed with 4% acrylamide gels in 6 mm internal diameter tubes as described by Malech and Marchesi [6].

SDS gel electrophoresis (the discontinuous Laemmli system) was performed as described by O'Farrell [7] with a 4.5% stacking gel and either a 10% or a 5–15% linear acrylamide gradient separating gel. Cylindrin was mixed with an equal volume of SDS sample buffer (3 g/dl SDS, 0.38 M dithiothreitol, 8 g/dl glycerol, and 0.19 M Tris-HCl, pH 6.8); washed erythrocyte membranes were mixed with two volumes of SDS sample buffer. After incubating in a boiling water bath for 3 min, either 3 μg of cylindrin, as measured by the Bio-Rad protein assay (Bio-Rad, Richmond, CA), or 50 μg of erythrocyte membrane protein, as measured by the Lowry assay [8], was loaded in each sample well. Gels were run at 15 mA constant current for approx. 3 h until the tracking dye reached approx. 9 cm, fixed, washed and stained with either Coomassie brilliant blue or silver [9]. The gels were calibrated using the known molecular weights of the major erythrocyte membrane proteins [10] and the following Bio-Rad molecular weight standards: phosphorylase B (92500), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000), and soybean trypsin inhibitor (21500).

Two-dimensional electrophoresis (nonequilibrium pH gradient electrophoresis combined with SDS gel electrophoresis (NEPHGE-SDS)) was performed as described by O'Farrell and colleagues [11]. For the first dimension separation, 3 μg of cylindrin was loaded on pH 3.5–10 gels and electrophoresed at 400 V for 4 h. For the second dimension separation, a 5–15% linear acrylamide gradient was used, and gels stained with Coomassie brilliant blue. In the figures, the basic end of the first dimension separation is on the left and the acidic end is on the right.

One- and two-dimensional electrophoretic patterns were correlated by a concomitant 1-D and 2-D electrophoretic technique [5]. NEPHGE tube gels were sealed on top of SDS slab gels with hot agarose containing protein (either 70 μg cylindrin or 2 mg membrane proteins) solubilized in SDS sample buffer. The resulting gels displayed both bands (the one-dimensional pattern of the protein in agarose) and spots (the two-dimensional pattern from the NEPHGE tube gel). Bands and spots with the same SDS migration intersected on the gel.

For co-electrophoresis experiments, cylindrin (3 μg) and washed erythrocyte membranes (26 μg protein) were solubilized, sequentially loaded on each NEPHGE tube gel, and electrophoresed in two dimensions as described above. To identify spot positions, a coordinate system was established with the origin at the membrane-derived glucose-3-phosphate dehydrogenase spot. The coordinates of a spot are the horizontal (left to right) × vertical (top of bottom) distances in millimeters from the origin. The coordinates of the second-most basic (leftward) cylindrin spot (7 × 11.5) were used to place axes on gels of cylindrin alone.

Transmission electron microscopy. Samples (2–4 μl) were diluted with distilled water and applied to glow-discharged carbon films on 400 mesh grids. After 30 s, liquid was removed and replaced with about 5 μl of 1% uranyl acetate, pH 4.2. The first drop was immediately removed and replaced with a second drop. After 15 s, excess liquid was removed, samples were air-dried, and examined within 15 min using a Siemens 101 electron microscope at 75 kV. Standard magnifications of 50 800 × were calibrated with a cross-ruled carbon replica grating.

Results

Preparation of cylindrin from hypotonically lysed human erythrocytes

Cylindrin was recovered from both cytosol and unwashed membranes. Washed membrane also contained cylindrin, but it was not possible to complete isolation of the macromolecule from this source. Cylindrin polypeptides were seen on silver stained SDS gels of fractions from the first sucrose gradient of the washed membrane preparation. However, the amount of protein in subsequent gradients was below our level of detectability (gels not shown).

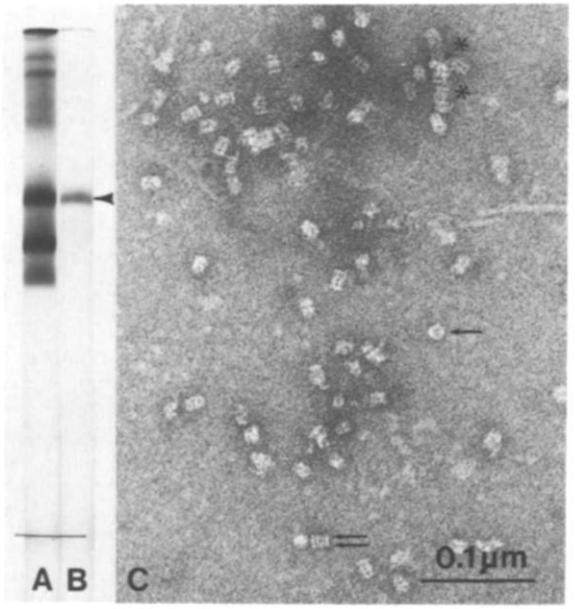

Fig. 1. Nondenaturing electrophoresis and electron microscopy of cylindrin prepared from erythrocyte cytosol. Pooled fractions from the first (A) and third (B) sucrose gradients. Purified cylindrin (arrow), top of gel, and tracking dye front (pin) are shown. (C) Electron micrograph of purified protein from the third gradient, stained with 1% uranyl acetate. Note both cylindrical (double arrows) and disc (arrow) shapes and rouleau formations (asterisks). (×150000)

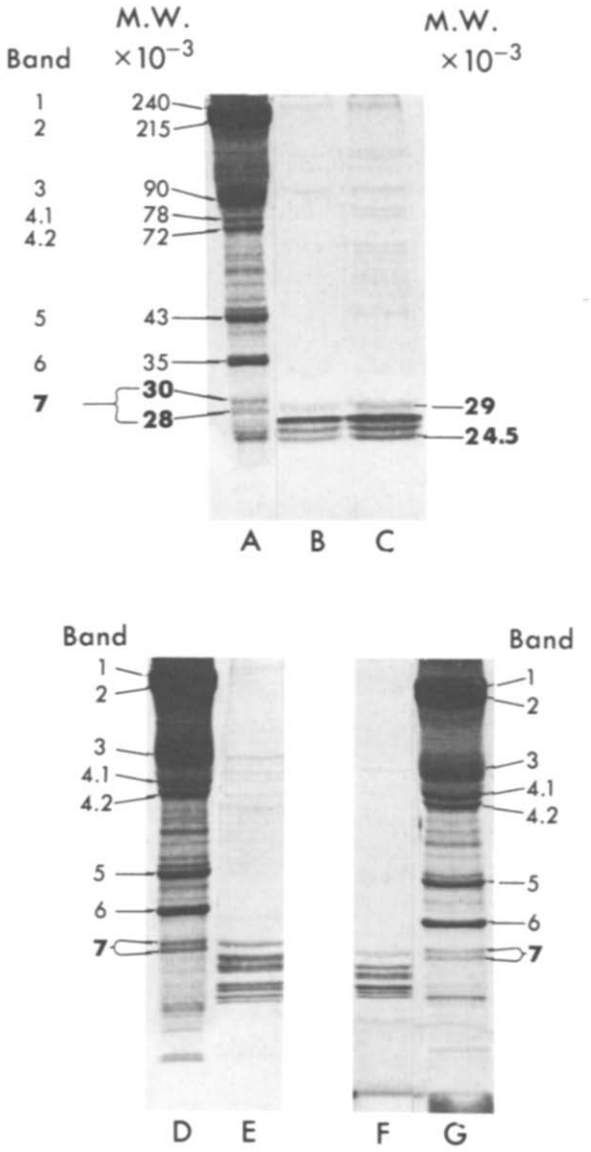

Fig. 2. One-dimensional SDS gel electrophoresis of cylindrin. Upper panel, 10% acrylamide gel; lower panel, 5–15% gradient gels. Erythrocyte membrane polypeptides (lanes A, D and G) for comparison to cylindrin isolated from the cytosol (lanes B and E) and from unwashed membranes (lanes C and F).

The purification of cytosolic and unwashed membrane cylindrin was monitored by non-denaturing, detergent-free gel electrophoresis. After the first sucrose gradient centrifugation, the pooled material from the cylindrin-rich fractions migrated as several bands on non-denaturing gels (Fig. 1A). However, following the third centrifugation, only a single band was observed (Fig. 1B). The final product from the third sucrose gradient was identified as cylindrin by its characteristic electron microscopic appearance (Fig. 1C). Two types of profiles were observed: a ring with external diameter of approx. 11 nm, and a four-part rectangular aggregate, 11 nm × 18 nm. Aggregates were occasionally assembled in rouleau formation as initially seen by Harris in ox erythrocyte ghost preparations (Fig. 4 of Ref. 1). Occasional filaments were also observed.

The electrophoretic findings described below were unaffected by either the age of the unit of

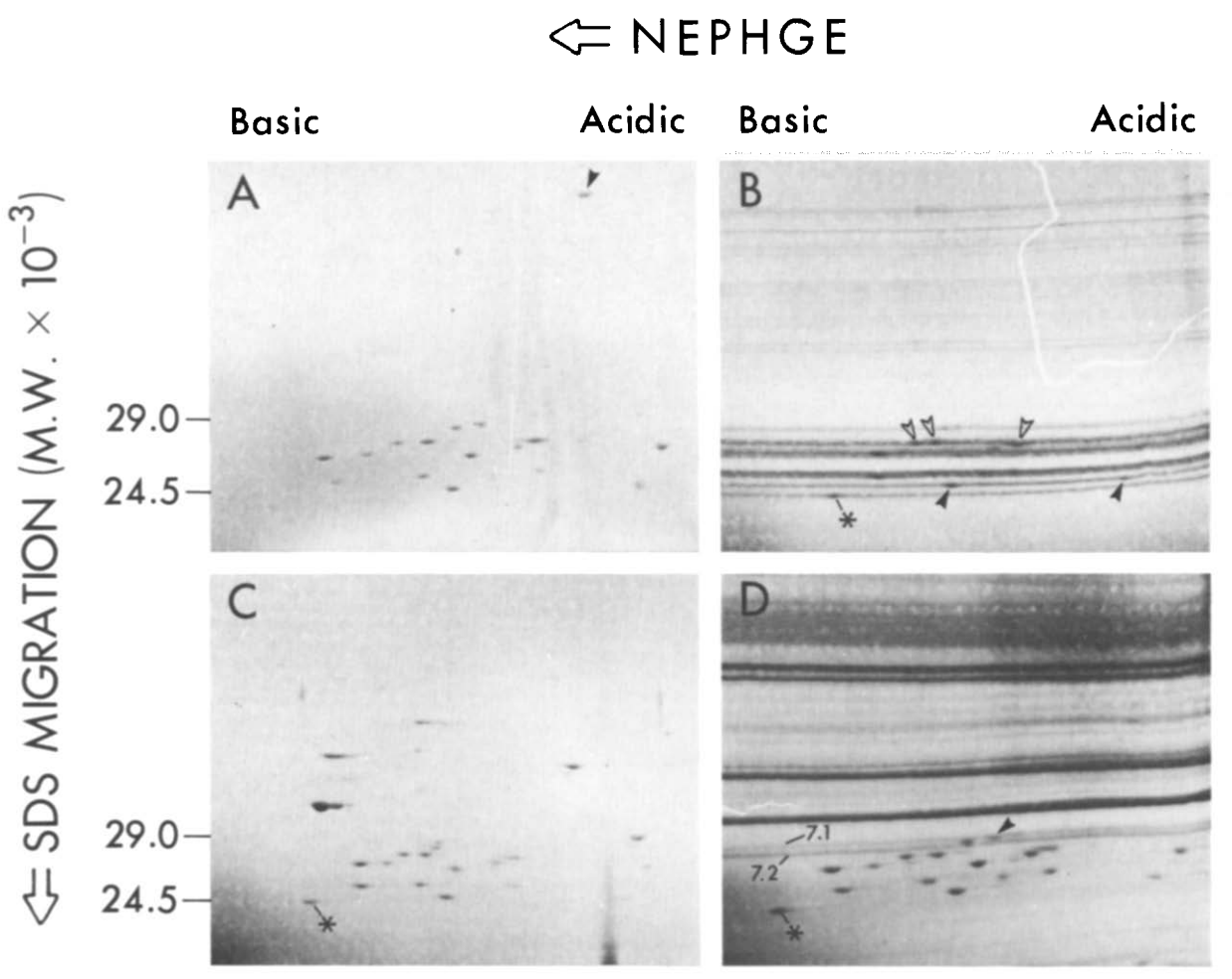

Fig. 3. Two-dimensional analysis of cylindrin. (A) NEPHGE-SDS gel pattern of cylindrin isolated from the cytosol. The spot indicated by the arrow is a contaminant. (B) Concomitant one- and two-dimensional electrophoresis of cylindrin from unwashed membranes. Individual cylindrin bands correspond to either one (asterisk), two (closed arrows) or three (open arrows) cylindrin spots. (C) Co-electrophoresis of cylindrin (unwashed membrane preparation) with erythrocyte membrane proteins. All the cylindrin and band 7 polypeptides can be separately identified, as is shown schematically in Fig. 4C. (D) Concomitant one- and two-dimensional electrophoresis of erythrocyte membrane proteins (bands) and cytosolic cylindrin (spots). Band 7 is resolved into two components labeled 7.1 and 7.2. The uppermost cylindrin spot (arrow) is very close to band 7.1. Although not seen in gel A, the most basic (leftward) cylindrin spot is well-visualized in gels B, C and D (asterisks).

blood, or the number of protease inhibitors used during the preparation of cylindrin. The only effect of storage at −80 °C that we noted was a decrease in the uppermost (29 kDa) band on SDS gels, which occurred after approximately three months of storage and several cycles of freezing and thawing.

One-dimensional SDS gel electrophoresis of cylindrin

Ten percent acrylamide gels resolved 4–6 bands (Fig. 2B and C), whereas 5–15% acrylamide gels revealed 7–8 bands (Fig. 2E and F) consistent with the greater resolution of gradient gels. The molecular weights of the cylindrin bands ranged from 24 500 to 29 000, and cylindrin prepared from the cytosol was identical to that from unwashed membranes (compare Fig. 2B to 2C and Fig. 2E to 2F). The gradient gels (E and F) were run at different times, accounting for the difference between the two gels in the heights of the bands. To evaluate the possibility of proteolysis, we repeated the preparation of cylindrin from the cytosol five times, and from unwashed membranes three times, using one or more protease inhibitors as described under Methods. All preparations gave the same results.

Two-dimensional electrophoresis of cylindrin

The number of polypeptide subunits in the cylindrin macromolecule was examined by two-dimensional electrophoresis (NEPHGE-SDS). As shown in Fig. 3, cylindrin was resolved into 15–17 polypeptide spots. The most basic (leftward) cylindrin spot is not seen on gel A, but is well visualized on gels B, C and D (asterisks). The polypeptide beneath the arrow on gel A was identified as a contaminant, since it was present in fractions of the third sucrose gradient which did not contain any of the other cylindrin spots. Whether cylindrin was isolated from the cytosol or from unwashed membranes, we observed the same 2-D pattern.

Concomitant one- and two-dimensional electrophoresis

To correlate one- and two-dimensional gel patterns, NEPHGE tube gels were sealed on top of SDS slab gels with hot agarose containing solubilized protein. Intersection of a band and a spot on the final gels (co-migration of one- and two-dimensional polypeptides) was interpreted as evidence for peptides having the same apparent molecular weight.

Electrophoresis of cylindrin revealed that each band is composed of one to three polypeptides. As shown in Fig. 3B, the lowermost cylindrin band intersects a single cylindrin spot (asterisk), whereas other bands intersect two (closed arrows) or three (open arrows) spots. The one- and two-dimensional cylindrin patterns are compared schematically in Figs. 4A and 4B.

The electrophoretic migration of the cylindrin polypeptides was also compared to that of the erythrocyte membrane proteins by loading cylindrin and membrane proteins for the spot and band patterns, respectively. As shown in Fig. 3D, the membrane band 7 proteins are resolved into two components with approximate molecular weights of 30 000 and 28 000 (designated 7.1 and 7.2, respectively). The two uppermost cylindrin spots migrate between bands 7.1 and 7.2, although one of the spots (arrow) is very close to band 7.1. None of the cylindrin spots intersect band 7.2.

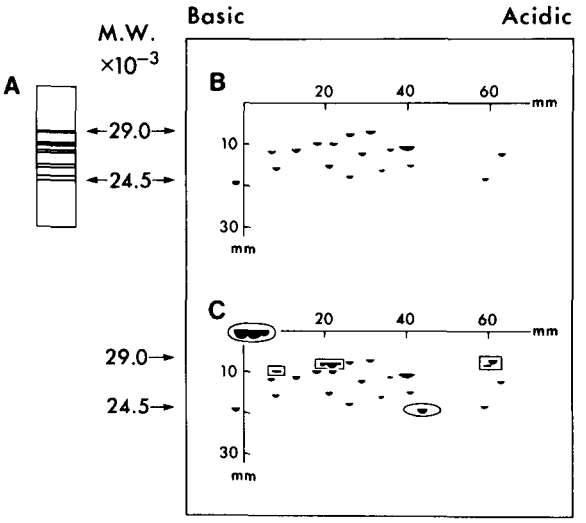

Fig. 4. Schematic synopsis of the one- and two-dimensional electrophoretic analyses of cylindrin. (A, B) Note the clear relationship between the one- and two-dimensional patterns. (C) Summary of the co-electrophoresis experiment shown in Fig. 3C. Erythrocyte membrane polypeptides are outlined: band 7 proteins by boxes, and glucose-3-phosphate dehydrogenase and band 8 by ellipses. Note that all membrane polypeptide spots are distinct from cylindrin spots.

Co-electrophoresis of cylindrin and erythrocyte membrane proteins

To test further whether cylindrin and the band 7 proteins of the erythrocyte membrane have any polypeptides in common, cylindrin and erythrocyte membranes were solubilized, mixed, and electrophoresed in two dimensions. As shown in Fig. 3C, all the cylindrin and band 7 spots are well-resolved and can be separately accounted for. The mixing experiment is drawn schematically in Fig. 4C.

Discussion

Cylindrin is composed of a number of polypeptides which can be partially resolved by one-dimensional SDS gel electrophoresis. Investigators have reported two to five subunits depending on the 1-D gel system used [2–4, 6]. In the study by Malech and Marchesi [6], each of the five subunits produced a unique chymotryptic peptide map, diminishing the possibility that those polypeptides resulted from either proteolysis or precursor-product relationship. In the work reported here, we demonstrate by two-dimensional electrophoresis that cylindrin is composed of many more polypeptides than were resolved by one-dimensional electrophoresis. Furthermore, by concomitant one- and two-dimensional electrophoresis, we show that many of the single bands seen on one-dimensional gels are composed of two or more polypeptides with different isoelectric points. We excluded proteolytic artifact by repeating the analysis with several combinations of protease inhibitors, but we did not investigate possible relationships between the polypeptides seen on two-dimensional gels. Since the two-dimensional analysis we employed is sensitive to single charge differences [7], the spots seen on two-dimensional gels may represent either unique polypeptides or similar but charge-modified polypeptides.

The distribution of cylindrin between the membrane and the cytosol has been studied by several authors. Harris [1] originally isolated cylindrin from a low ionic strength extract of erythrocyte membranes. Subsequently, White and Ralston [4] found that the recovery of cylindrin from pink membranes was greater than that from extensively washed membranes. More recently, Malech and Marchesi [6] isolated significant amounts of cylindrin from the cytosol of erythrocytes disrupted by either hypotonic lysis, homogenization, or freeze-thaw, and found cylindrin in the extract of washed membranes as well. Thus, there is convincing evidence that cylindrin is present in both the membrane and cytosolic fractions of the erythrocyte, but the quantitative relationship between the two forms is unknown.

Although the polypeptide composition and cellular distribution of cylindrin have been studied, its function has remained elusive. Our interest in cylindrin was sparked by the possibility that the complex might play a role in erythrocyte membrane permeability, since the molecular weights of the subunits are close to those of the band 7 proteins of the erythrocyte membrane. We previously reported that band 7 can be resolved into four polypeptides on two-dimensional gels, and that one of these polypeptides (with coordinates 22×8 in Fig. 4C) is absent in certain congenital hemolytic anemias with increased Na^+/K^+ membrane permeability [5]. In the present study, we compared the polypeptide subunits of the cylindrin complex to band 7 proteins by two-dimensional electrophoresis. We found that the cylindrin polypeptides are distinct from the band 7 polypeptide implicated in permeability disorders. The structural or functional significance of membrane associated cylindrin thus remains unknown.

Acknowledgements

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