A Spectrin-like Protein in Retinal Rod Outer Segments†

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ABSTRACT: Biochemical and immunochemical studies indicate that rod outer segments (ROS) of bovine photoreceptor cells contain a M_r 240 000 polypeptide related to the α-subunit of red blood cell (RBC) spectrin. With the use of sodium dodecyl sulfate gel electrophoresis in conjunction with the immunoblotting technique, monoclonal antibody 4B2 was found to bind to a M_r 240 000 polypeptide in ROS that is distinct from the prominent M_r 220 000 concanavalin A binding glycoprotein. The M_r 240 000 polypeptide is highly susceptible to degradation by endogenous proteases. It does not appear to be an integral membrane protein but is tightly membrane associated since it can be partially extracted from ROS membranes with urea in the absence of detergent. The 4B2 antibody cross-reacted with RBC ghosts and bovine brain microsomal membranes. Radioimmune assays and immunoblotting analysis of purified bovine RBC spectrin further revealed that the 4B2 antibody predominantly labeled the α-chain of RBC spectrin having an apparent molecular weight of 240 000. Polyclonal anti-spectrin antibody that bound to both the α- and β-chain of RBC spectrin predominantly labeled a M_r 240 000 polypeptide of ROS membranes. Two faintly labeled bands in the molecular weight range of 210 000–220 000 were also observed. These components may represent variants of the β-chain of spectrin that are weakly cross-reacting or present in smaller quantities than the α-chain. Immunocytochemical labeling studies using the 4B2 antibody and immunogold–dextran markers indicated that the ROS spectrin-like protein is preferentially localized in the region where the disks come in close contact to the plasma membrane of ROS. These studies indicate that ROS contain a protein related to RBC spectrin, which may constitute a major component of a filamentous network lining the inner surface of the ROS plasma membrane as previously seen by electron microscopy. This membrane skeletal system may serve to stabilize the ordered ROS structure and maintain a constant distance between the rim region of the disks and the plasma membrane.

The outer segment of the retinal rod photoreceptor cell is a specialized organelle that serves as the site of phototransduction in vision. It is a highly ordered structure consisting of a stack of hundreds of closed disks equally spaced apart (Cohen, 1972). This assembly is enclosed by a plasma membrane that is osmotically (Korenbrot et al., 1973) and electrically (Hagins & Ruppel, 1971) separated from the disks over most of the length of the ROS.[1] The disks are composed of two apparently distinct membrane domains, the flat lamellar domain and the highly curved rim region (Falk & Fatt, 1969).

Studies in several laboratories have been directed toward identifying and characterizing proteins of ROS and defining their role in visual excitation and maintenance of the ROS structure. The most extensively studied protein is the photoreceptor protein rhodopsin (Hargrave, 1982). It is a transmembrane glycoprotein of M_r 39 000 that constitutes about 90% of the disk membrane protein. Several membrane-associated proteins have been identified (Godchaux & Zimmerman, 1979; Kühn, 1982) and shown to be involved in the regulation of cGMP levels in ROS during photobleaching of rhodopsin (Fung et al., 1981; Liebman & Pugh, 1982). These include transducin or G-protein exhibiting GTPase activity (Kühn, 1980), cGMP-dependent phosphodiesterase (Pannbacker et al., 1972; Miki et al., 1973), a 48 K protein (Kühn, 1982), and rhodopsin kinase (Kühn, 1978), which catalyzes the light-dependent phosphorylation of rhodopsin along its carboxyl-terminal segment. Several high molecular weight proteins have been found in frog and bovine ROS preparations. Papermaster et al. (1978) have used immunocytochemical techniques to localize a M_r 290 000 disk membrane protein, referred to as the rim protein, along the margins and incisures of frog ROS disks. Two proteins of M_r 220 000 and 240 000 have recently been reported by Szuts (1985) to undergo a light-mediated phosphorylation reaction in frog ROS. The M_r 220 000 protein appears to be equivalent to the rim protein. In bovine ROS disk membrane preparations, a M_r 220 000 transmembrane glycoprotein designated as ROS 1.2 has been identified (Molday & Molday, 1979), but its localization to the rim regions of bovine ROS disks has not been reported. Usukura and Yamada (1981) and Roof and Heuser (1982) have observed thin filaments extending from the rims of ROS disks by electron microscopy. In the latter study, one type of filamentous structure was observed to link adjacent disks within a stack of disks and another filamentous structure appeared to link the disks to the plasma membrane. The molecular composition of these filaments has not been determined, but it has been suggested that the filaments that link disks together may be composed of the high molecular weight rim protein as found in frog ROS.

Recently, we have generated several monoclonal antibodies against ROS disk membrane proteins and have used these reagents as probes to study the organization of rhodopsin and minor proteins in disk and plasma membranes (MacKenzie & Molday, 1982). One monoclonal antibody designated as 4B2 was found to bind to a high molecular weight polypeptide

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[1] Abbreviations: ROS, rod outer segments; $NaDodSO_4$, sodium dodecyl sulfate; ConA, concanavalin A; FCS, fetal calf serum; BSA, bovine serum albumin; Ig, immunoglobulin; RIA, radioimmune assay; RBC, red blood cell; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; PBS, phosphate-buffered saline.

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having a similar mobility to ROS 1.2 on $NaDodSO_4$–polyacrylamide gels. This protein was shown to be associated with ROS disk membranes, but a detailed study of its properties was not carried out. In this paper we have carried out a more in-depth study of the 4B2-binding protein in bovine ROS. Our results indicate that monoclonal antibody 4B2 does not bind to the M_r 220000 glycoprotein ROS 1.2 but instead binds to a polypeptide of M_r 240000. This polypeptide appears to be related to the α-subunit of spectrin from red blood cells.

Experimental Procedures

Preparation of ROS and Disk Membranes. Purified ROS membranes were prepared either from frozen retinas (Hormel) as previously described (MacKenzie & Molday, 1982) or from freshly dissected retinas as follows. Fifty bovine retinas in 15 mL of homogenizing solution containing 20% sucrose, 0.25 mM $MgCl_2$, 10 mM taurine, 10 mM glucose, and 20 mM Tris–acetate, pH 7.4, were swirled gently for 1 min and filtered through a single layer of cheesecloth. Retinas were then washed with an additional 5 mL of homogenizing solution, swirled, and filtered as above. Filtrates were combined, refiltered twice through two layers of cheesecloth, and placed on ice for 5 min. Filtrate (5 mL/gradient) was applied to four 22-mL 25–60% (w/w) linear sucrose gradients containing 10 mM taurine, 10 mM glucose, and 20 mM Tris–acetate, pH 7.4. The gradients were centrifuged at 25000 rpm for 50 min in a SW27 rotor. Purified ROS membranes were collected as a single band at the upper region of the gradient and washed in 20 mM Tris buffer, pH 7.4. Disk membranes were subsequently prepared by hypotonic lysis of ROS membranes followed by floatation on 5% Ficoll according to the method of Smith et al. (1975).

Preparation of RBC Ghosts, Spectrin, and Brain Homogenate. RBC ghosts were prepared from bovine blood by using the method of Dodge et al. (1963). Bovine brain microsomal membranes were prepared from bovine brain tissue by using the method of Burridge et al. (1982). Spectrin was purified from RBC ghosts by using the method of Marchesi and Steers (1968). Protein concentration was measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Monoclonal Antibodies. Monoclonal antibodies 4B2 and rho 1D4 were obtained from hybridoma cell lines generated by fusion of NS-1 mouse myeloma cells with lymphocytes from mice immunized with ROS membranes (MacKenzie & Molday, 1982; Molday & McKenzie, 1983). Culture fluid obtained from hybridoma cells cloned 3 times was centrifuged at 4000 rpm to remove any cellular debris and used in indirect immunochemical and immunocytochemical studies as described below.

Radioiodination of Proteins. Goat anti-mouse Ig, RBC spectrin, ConA (Sigma Chemical Co., St. Louis, MO) and protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) were labeled with ^{125}I (sp act. (1–2) $\times 10^6$ dpm/μg) by using the chloramine T method (Hunter & Greenwood, 1962). The iodinated proteins were separated from free [^{125}I]iodide and other reactants as previously described (Molday & MacKenzie, 1985).

Solid-Phase Radioimmune and Competitive Inhibition Assays. The binding of monoclonal antibodies to ROS membranes and RBC spectrin was measured by using indirect solid-phase RIA as previously described (MacKenzie et al., 1984). Briefly, bleached ROS membranes or RBC spectrin were solubilized with 1% Triton X-100, and 25 μL of a 0.25 mg/mL solution was dried onto Flex vinyl microtiter wells at 60 °C. The wells were rinsed with H_2O and incubated in RIA buffer (phosphate-buffered saline containing 1% BSA, 1% FCS, and 0.1% NaN_3) for 60 min. The wells were then rinsed in phosphate-buffered saline and incubated with 25 μL of serially diluted 4B2 (MacKenzie & Molday, 1982) or rho 1D4 (Molday & MacKenzie, 1983) hybridoma culture fluid for 60 min at 23 °C. Finally, the wells were rinsed in phosphate-buffered saline and incubated with 25 μL of ^{125}I-labeled goat anti-mouse Ig [10–30 μg/mL; (1–2) $\times 10^6$ dpm/μg] in RIA buffer for 30–60 min at 23 °C. The plates were then rinsed in phosphate-buffered saline and individual wells were counted in a Beckman 8000 γ counter.

The binding of rabbit anti-human RBC spectrin antibodies was measured by a similar procedure. Variable amounts of Triton X-100 treated ROS membranes, bovine RBC spectrin, or BSA were dried onto microtiter wells. In the first step the wells were treated with 25 μL of rabbit anti-human RBC spectrin antibody, a generous gift of Dr. Rinehart Reithmeier. After 30 min, the wells were rinsed and incubated with 25 μL of ^{125}I-labeled protein A (30 μg/mL). The wells were rinsed in phosphate-buffered saline, and the radioactivity was measured as described above.

The effect of ROS membranes, RBC ghosts, and brain microsomal membranes on the binding of 4B2 monoclonal antibody to Triton X-100 solubilized ROS was studied by using RIA competitive inhibition assay previously described (Molday & MacKenzie, 1983). Briefly, 25 μL of varying concentrations of 0.1% Triton X-100 treated bovine ROS membranes, bovine brain microsomal membranes, or bovine RBC ghosts in RIA buffer was incubated at 23 °C with 25 μL of 4B2 hybridoma culture fluid diluted to a concentration that gave 80–90% saturation of binding by solid-phase RIA. After a 60-min incubation at 23 °C, 25 μL of the mixture was removed and screened for remaining antibody activity by solid-phase RIA employing Triton X-100 solubilized ROS as the immobilized antigen and ^{125}I-labeled goat anti-mouse Ig as a tracer second antibody.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis and Gel Transfer. Samples were solubilized in an equal volume of denaturing solution containing 5% $NaDodSO_4$, 40% sucrose, 10 mM Tris, pH 6.8, 10% 2-mercaptoethanol, and 4% bromophenol blue. Samples (10 μL) were applied to a 6% polyacrylamide minislab gel (0.75-mm thickness $\times$ 3.0-cm length) and electrophoresis was carried out by using the buffer system of Laemmli (1970). Gel slices were either stained with Coomassie blue (Fairbanks et al., 1971) or silver (Wray et al., 1981) or subjected to electrophoretic transfer. In the latter procedure unstained $NaDodSO_4$–polyacrylamide gels were washed over 20 min with two 50-mL changes of transfer buffer: 20 mM Tris–acetate, pH 7.4, containing 2 mM NaEDTA and 0.01% $NaDodSO_4$. Proteins were electrophoretically transferred from $NaDodSO_4$–polyacrylamide gels to nitrocellulose paper (Towbin et al., 1979) in transfer buffer at 600 mA for 6–12 h in a Bio-Rad Transblot apparatus. After gel transfer, nitrocellulose paper was stained with 0.025% Amido Black in 22.5% EtOH and 7.5% HOAc in order to detect transferred proteins. The nitrocellulose paper was then incubated at 23 °C for 1 h in immunoblot buffer: 0.15M NaCl, 10 mM sodium phosphate, 1 mM NaEDTA, 1 mM NaN_3, 0.2% Triton X-100, and 2% BSA. The quenched paper was incubated with either 5–10 mL of hybridoma culture fluid or rabbit anti-human RBC spectrin antibody for 30 min at 23 °C and then rinsed 5 times with immunoblot buffer (without BSA), once with 2 M urea, 0.1 M glycine, and 1% Triton X 100, and once with PBS. After being washed, the paper was incubated with 5 mL of ^{125}I-labeled goat anti-mouse

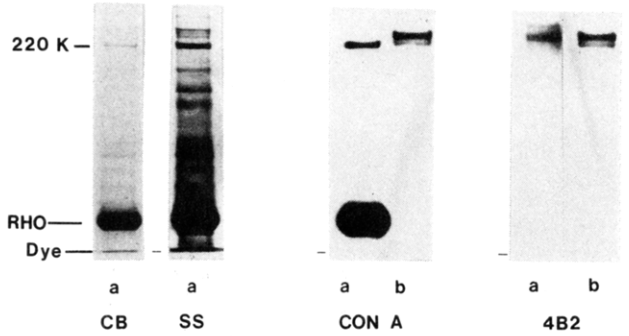

FIGURE 1: $NaDodSO_4$ gel electrophoresis and immunoblots of ROS disk membranes. $NaDodSO_4$-solubilized ROS disk membranes (20 μg/well) and ^{125}I-labeled bovine RBC spectrin (6 μg/well) were subjected to $NaDodSO_4$ gel electrophoresis on a 6% polyacrylamide slab gel. Gels were either stained with Coomassie blue (CB) or silver stain (SS) or transferred to nitrocellulose paper. Transfer papers were either directly labeled with ^{125}I-labeled ConA or indirectly labeled with undiluted 4B2-antibody culture fluid and ^{125}I-labeled goat anti-mouse Ig for autoradiography. ROS membrane proteins are in gel a, and ^{125}I-labeled spectrin used as an external molecular weight marker for autoradiography is in gel b.

Ig (1.8×10^6 dpm/μg; 2.3 μg/mL) or ^{125}I-labeled protein A (0.8×10^5 dpm/μg) in immunoblot buffer for 30 min at 23 °C. Finally, the paper was washed as described above and dried for autoradiography. For most experiments, transfer papers were exposed to X-ray film for 1 day. In the case of ^{125}I-labeled protein A binding to ROS membranes, exposure time was increased to 7 days. In some experiments, quenched nitrocellulose papers were labeled with ^{125}I-labeled ConA (sp act. 7.9×10^5 dpm/μg).

Lowicryl Thin Section Labeling. Bovine retina was fixed in 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and 0.2 M sucrose for 30–60 min at 23 °C. After washing in the same buffer for 1 h, the tissue was cut into 1-mm^2 pieces and embedded in Lowicryl resin according to the method of Roth et al. (1981). Sections were cut, collected on clean copper grids, and preincubated in 50 μL of PBS with 0.1% BSA for 10 min to quench nonspecific binding sites. The grids were then incubated in 50 μL of 4B2 hybridoma culture fluid for 30 min at 23 °C, followed by extensive washing in PBS with 0.1% BSA. Finally, the grids were incubated in 50 μL of goat anti-mouse Ig gold–dextran (Hicks & Molday, 1986) for 30 min at 23 °C. The grids were washed extensively in PBS, stained with saturated uranyl acetate and lead citrate, and viewed under a Philips 200 electron microscope.

Urea Extraction of ROS. ROS (6 mg of protein), prepared from fresh retinas as described above, were washed twice in 20 mM Tris buffer, pH 7.4, by centrifugation at 25 000 rpm in a SW27 rotor for 1 h. The ROS pellet was resuspended with 1 mL of 6 M ultrapure urea and left for 24 h at 4 °C. The mixture was then centrifuged at 25 000 rpm in a SW 27 rotor for 1 h. Following centrifugation, the supernatant and the resuspended disk pellet were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis and immunoblot analysis as described above.

RESULTS

High Molecular Weight Polypeptides of ROS Membranes. When ROS membranes were subjected to $NaDodSO_4$ gel electrophoresis and stained with Coomassie blue, rhodopsin having an apparent M_r 34 000 was observed as the major band and ROS 1.2 of apparent M_r 220 000 was the second most intense band (Figure 1). Bands faintly visible by Coomassie blue staining were intensified by using silver staining and several bands in the molecular weight range of 200 000–

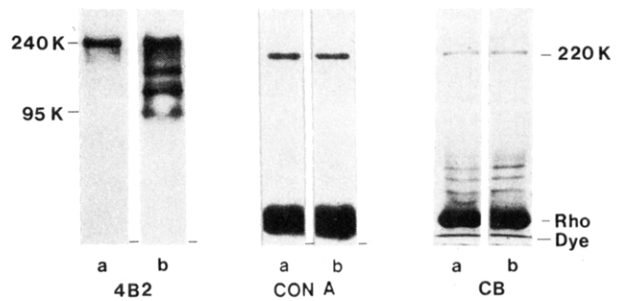

FIGURE 2: Proteolytic degradation of the 4B2-binding protein of ROS by endogenous proteases. ROS membranes prepared from freshly dissected retinas were either directly subjected to $NaDodSO_4$ gel electrophoresis (gel a) or stored at 4 °C for 24 h prior to $NaDodSO_4$ gel electrophoresis (gel b). The gels were either stained with Coomassie blue (CB) or transferred to nitrocellulose paper and labeled with undiluted 4B2-antibody culture fluid and ^{125}I-labeled goat anti-mouse Ig or with ^{125}I-labeled ConA.

240 000 were seen. The binding specificity of ConA and 4B2, an IgM monoclonal antibody, for ROS polypeptides was determined by treating polypeptides transferred to nitrocellulose paper directly with ^{125}I-labeled ConA or indirectly with 4B2 antibody followed by ^{125}I-labeled goat anti-mouse Ig. With ^{125}I-labeled spectrin as an external molecular weight reference, ^{125}I-labeled ConA was found to label rhodopsin and ROS 1.2 at 220 000 as previously shown (MacKenzie & Molday, 1982). The 4B2 antibody, however, labeled a polypeptide chain of a slightly higher molecular weight (Figure 1). This band had the same electrophoretic mobility as the α-subunit of RBC spectrin and an apparent M_r 240 000. A faintly labeled band at M_r 220 000 was also seen upon prolonged autoradiographic exposure. In some preparations, bands in the molecular weight range of 95 000–150 000 were also labeled with the 4B2 antibody. These bands appear to represent proteolytic fragments of the M_r 240 000 polypeptide (see below).

Degradation of 4B2-Specific Protein by an Endogenous Protease. The sensitivity of the 4B2-specific protein and ConA-specific glycoproteins to degradation by an endogenous protease in ROS preparations was detected by immunoblotting analysis. As shown in Figure 2, the 4B2 antibody labeled only one major polypeptide of M_r 240 000 in freshly prepared ROS membranes. When the ROS membranes were maintained at 4 °C for 24 h, partial degradation of the M_r 240 000 polypeptide to polypeptides of apparent M_r 150 000, 120 000, and 95 000 was observed. In contrast, no degradation of rhodopsin or ROS 1.2 was detected by ConA labeling or by Coomassie blue staining. ROS disk membranes prepared by hypotonic lysis of freshly prepared ROS and floatation on 5% Ficoll were less prone to degradation under these conditions. Significant degradation of the 4B2-specific polypeptide was generally observed when ROS were prepared from frozen retina.

Urea Extraction of the 4B2-Specific Protein. The extractability of the 4B2-specific protein and ConA-specific membrane glycoproteins with urea was studied to determine their interaction with the lipid bilayer. ROS disk membranes were treated with 6 M urea overnight and subsequently separated into a pellet and supernatant fraction by high-speed centrifugation. Analysis of these fractions by $NaDodSO_4$ gel electrophoresis and immunoblotting indicated that a small quantity (5–10%) of the 4B2-specific M_r 240 000 polypeptide and proteolytic fragments was extracted from the membrane (Figure 3). Extraction of the 4B2-binding protein by urea was reproducibly observed in three separate experiments. However, under these same conditions, no extraction of rhodopsin or ROS 1.2 into the supernatant was detected by ^{125}I-labeled ConA blotting or Coomassie blue staining.

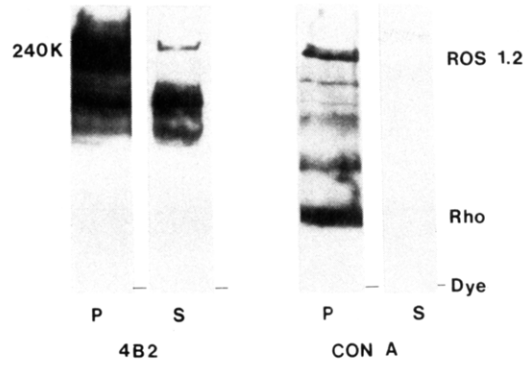

FIGURE 3: Extraction of the 4B2-binding protein from ROS membranes. ROS membranes were treated with 6 M urea at 4 °C for 24 h. The membranes were then sedimented at 25 000 rpm for 1 h. The supernatant and pellets were solubilized in $NaDodSO_4$ and subjected to $NaDodSO_4$ gel electrophoresis on 6% polyacrylamide gels and to electrophoretic transfer. The supernatant (gel s) and the pellet (gel p) were labeled with either 4B2 antibody and ^{125}I-labeled goat anti-mouse Ig or ^{125}I-labeled ConA.

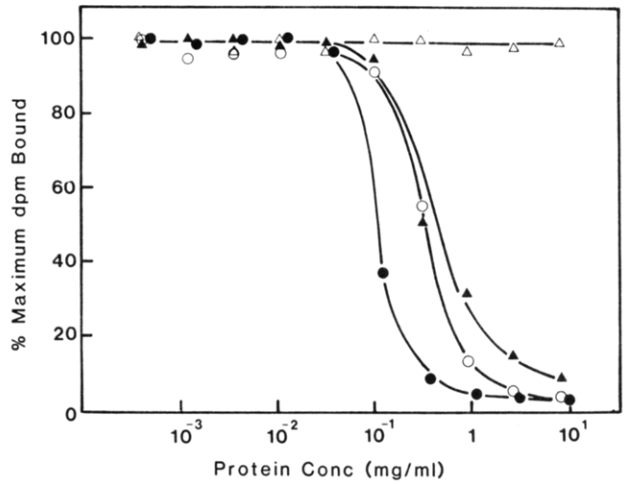

FIGURE 4: Inhibition of 4B2 antibody binding to Triton X-100 solubilized, immobilized ROS proteins by Triton X-100 solubilized bovine ROS (●), Triton X-100 solubilized bovine brain microsomal membranes (○), Triton X-100 solubilized bovine RBC membrane ghosts (▲), and bovine serum albumin (△).

Immunological Cross-Reactivity of Monoclonal Antibody 4B2 with Proteins from Other Cell Types. The presence of 4B2-binding proteins in membrane preparations of RBC and brain tissue was investigated by RIA competitive inhibition assays. As shown in Figure 4, both Triton X-100 solubilized bovine RBC ghosts and bovine brain microsomal membranes as well as bovine ROS were able to inhibit the binding of the 4B2 antibody to Triton X-100 solubilized disk membranes immobilized on microtiter plates. Fifty percent inhibition was attained at about the same total protein concentration (30 μg/mL) for brain membranes and RBC ghosts. ROS was a more effective inhibitor, requiring a 3-fold lower concentration to achieve 50% inhibition. Accurate quantitative analysis of cross-reactivity, however, could not be made since the quantity of cross-reacting antigen in these preparations was not known. Bovine serum albumin serving as a control did not inhibit 4B2 antibody binding. Competitive inhibition studies also indicated that human RBC ghosts bound the 4B2 antibody, but with a lower affinity than bovine RBC ghosts.

Identification of Spectrin as the RBC Antigen for the 4B2 Monoclonal Antibody. Solid-phase radioimmune assays and immunoblot analysis were used to identify spectrin as the 4B2-binding protein of RBC. Figure 5 shows the effect of 4B2 antibody and rho 1D4 antibody (anti-rhodopsin monoclonal

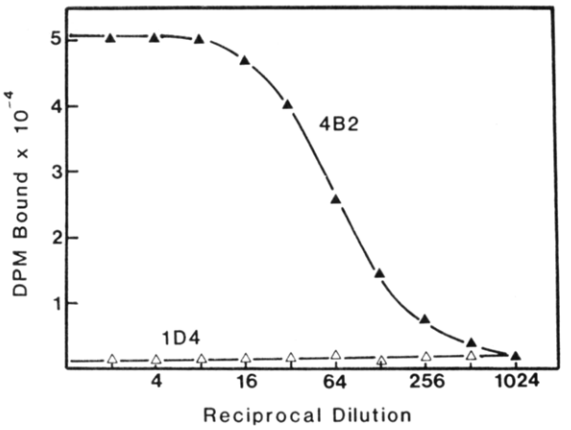

FIGURE 5: The effect of 4B2 and rho 1D4 antibody dilution on antibody binding to bovine spectrin. Triton X-100 treated bovine RBC spectrin immobilized in microtiter wells was incubated with serial dilutions of culture fluid from either 4B2 or rho 1D4 hybridoma cells, rinsed, and treated with ^{125}I-labeled goat anti-mouse Ig.

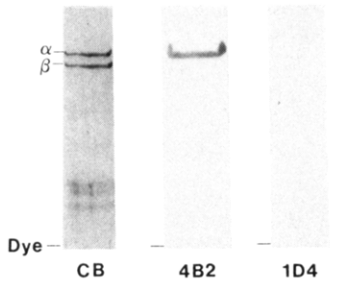

FIGURE 6: $NaDodSO_4$ gel electrophoresis and immunoblots of bovine RBC spectrin. Bovine spectrin (2 μg/well) was subjected to electrophoresis on a 6% $NaDodSO_4$–polyacrylamide slab gel and either stained with Coomassie blue (CB) or transferred to nitrocellulose paper. The transfer papers were treated with either 4B2 or rho 1D4 antibody and ^{125}I-labeled goat anti-mouse Ig and were subjected to autoradiography. The α- and β-subunits of spectrin are indicated.

antibody) dilution on the binding of these antibodies to purified bovine RBC spectrin immobilized on microtiter plates. The 4B2 antibody exhibited significant saturable binding, whereas rho 1D4 did not bind even at high antibody concentrations.

The subunit of bovine RBC spectrin that binds the 4B2 antibody was determined by sequentially labeling spectrin subunits separated by $NaDodSO_4$ gel electrophoresis with the 4B2 antibody and ^{125}I-labeled goat anti-mouse Ig. As shown in Figure 6, the 4B2 antibody predominantly labeled the α-subunit of bovine spectrin having an apparent M_r 240 000 (Branton et al., 1981). With prolonged exposure conditions (not shown), the 4B2 antibody was found to weakly react with a band at M_r 220 000.

Binding of Anti-Spectrin Antibodies to ROS Proteins. The binding of polyclonal rabbit anti-human RBC spectrin to bovine RBC spectrin and bovine ROS was determined by the solid-phase radioimmune assay using ^{125}I-labeled protein A for detection. As shown in Figure 7, a linear increase in binding of the anti-spectrin antibody was observed when increasing concentrations of bovine spectrin or ROS membranes were dried onto the assay plates. This indicated that neither the primary antibody nor the ^{125}I-labeled protein A was limiting under the conditions of this assay. For a given amount of protein, the anti-spectrin antibody was found to bind over 100 times greater to purified spectrin compared to total ROS membrane protein. This suggests that the ROS spectrin makes up less than 1% of the ROS membrane protein.

The immunoblotting technique was used to identify the polypeptides serving as antigens for the anti-spectrin antibody.

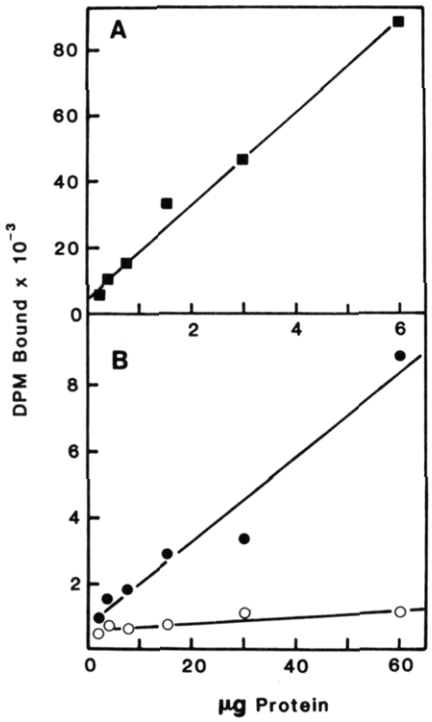

FIGURE 7: The binding of rabbit anti-human RBC spectrin antibodies to purified bovine spectrin or ROS membrane proteins. (A) Variable amounts of RBC spectrin (■) and (B) variable amounts of ROS membrane protein (●) or BSA (○) were dried onto microtiter plates and sequentially labeled with rabbit anti-spectrin antibodies and ^{125}I-labeled protein A.

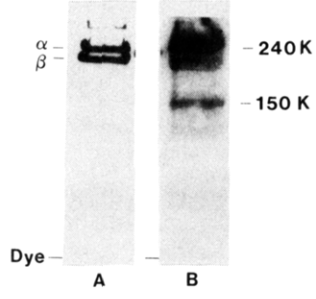

FIGURE 8: Immunoblots of bovine RBC spectrin and ROS membrane proteins labeled with polyclonal anti-spectrin antibodies. Purified bovine RBC spectrin (6 μg) and ROS membrane proteins (30 μg) were subjected to $NaDodSO_4$ gel electrophoresis on 6% gels and transferred to nitrocellulose paper. The papers were sequentially labeled with rabbit anti-human RBC spectrin antibodies and ^{125}I-labeled protein A. (Gel A) Autoradiograph of bovine RBC spectrin exposed for 5 h. (Gel B) Autoradiograph of ROS membrane protein exposed for 7 days.

As illustrated in Figure 8, the anti-spectrin antibody labeled with equal intensity the α and β bands of bovine spectrin. Faint labeling of a band near the top of the gel could also be seen. This may be undissociated α–β dimer. In ROS membranes, the anti-spectrin antibody predominantly labeled a M_r 240 000 polypeptide having the same mobility as the α-chain of spectrin. Two less intense bands were observed in the molecular weight range of 210 000–220 000. In addition, a band at M_r 150 000 was also labeled with the anti-spectrin antibody. This same band is also labeled when ROS membranes are labeled with the 4B2 antibody and most likely represents the major proteolytic fragment of the M_r 240 000 polypeptide.

Immunocytochemical Labeling of Rod Cells with the 4B2 Antibody and *Immunogold–Dextran Markers*. Localization of the 4B2-specific protein in ROS was determined by sequentially labeling Lowicryl thin sections of bovine retina tissue

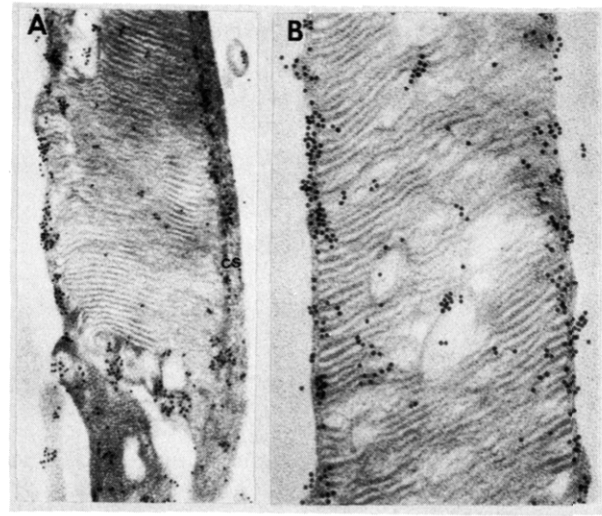

FIGURE 9: Transmission electron micrographs of ROS labeled with immunogold–dextran markers. Thin sections of Lowicryl-embedded ROS of bovine retina were sequentially labeled with 4B2 antibody and goat anti-mouse Ig–gold dextran particles (diameter of gold, 15 nm). Gold particles are distributed along the periphery of the ROS where the rims of the disks come in close proximity to the plasma membrane and ciliary spine (CS). (A) 25 000×; (B) 42 500×.

with the 4B2 antibody and goat anti-mouse Ig-gold–dextran conjugates. As shown in Figure 9, the gold particles were preferentially distributed along the periphery of the outer segments where the rims of the disks are adjacent to the plasma membrane. Gold particles were often observed to extend inward from the plasma membrane up to 50 nm. In the basal portion of the rod outer segment, gold particles were also observed to line up in the region where the rims of the disks are adjacent to the ciliary spine extending into the outer segment from the connecting cilium (Hicks & Molday, 1985). A few gold particles can be found in the central area of the ROS. It is not clear if this represents a low concentration of spectrin in this region or, alternatively, residual nonspecific binding of the 4B2 antibody, an IgM immunoglobulin. In contrast, outer segments labeled with anti-rhodopsin monoclonal antibodies were densely and uniformly distributed over the entire ROS (Hicks & Molday, 1986). No labeling was observed when a nonreactive monoclonal antibody was used in the first labeling step.

Discussion

Results of this study indicate that bovine ROS contain a polypeptide of apparent M_r 240K that is distinct from the previously identified high molecular weight glycoprotein ROS 1.2. The 240K polypeptide specifically binds the 4B2 monoclonal antibody, but not ConA, comigrates with the α-chain of bovine RBC spectrin on 6% polyacrylamide gels, is highly susceptible to degradation by endogenous proteases, and can be extracted from the membrane, although to a limited degree, with urea in the absence of detergent. In contrast, ROS 1.2 binds ConA but not the 4B2 antibody, migrates with a slightly greater mobility indicative of an apparent M_r 210–220K, is not readily degraded by endogenous proteases, and, like rhodopsin, is not extracted by urea, but requires detergent for solubilization. The 240K polypeptide is not simply a contaminant of the ROS preparation since immunocytochemical labeling studies using immunogold–dextran markers clearly show that this protein is localized within the ROS organelle.

The 4B2-binding protein of bovine ROS appears to be related to spectrin of RBC. The apparent molecular weight of the 4B2-binding polypeptide is identical with that of the α-subunit of bovine RBC spectrin. More importantly, the 4B2

monoclonal antibody cross-reacts with purified bovine RBC spectrin and specifically with its α-subunit as shown by RIA and immunoblotting studies. It also cross-reacts with brain microsomal membranes that are known to contain a spectrin-related protein called fodrin (Levine & Willard, 1981). Like the 4B2-binding protein, fodrin is known to be highly susceptible to degradation by proteases during its purification (Burridge et al., 1982). Finally, polyclonal anti-RBC spectrin antibodies cross-react with polypeptides of ROS having a molecular weight equivalent to the α- and β-chains of RBC spectrin.

Spectrin from RBC (Marchesi et al., 1976) and spectrin-related proteins from other cells types (Glenney & Glenney, 1983) are nonintegral, membrane-associated proteins consisting of an elongated α–β heterodimer having a length of about 100 nm (Branton et al., 1981; Shotton, et al., 1979). Two heterodimers further associate end to end to form a flexible 200-nm-long chain as part of a fibrous skeletal network lining the cytoplasmic surface of the plasma membrane. In the case of RBC this fibrous network interacts with the anion transport protein "band 3" primarily through interaction with ankyrin (Bennett & Davis, 1982). This membrane skeletal system appears to stabilize the plasma membrane and maintain the shape of RBC. ROS spectrin appears to be also a nonintegral, membrane-associated protein that can be partially extracted from ROS membranes with urea under conditions in which the integral ROS membrane proteins are not extracted. A strong association of ROS spectrin with disk and plasma membranes exists, however, since relatively small amounts of this protein are extracted under the conditions used in this study. In this respect ROS spectrin is different from RBC spectrin, which can be partially extracted in low ionic strength buffer in the presence of chelating agents. The α-subunit of M_r 240 000 appears to be the major unit of ROS spectrin detected in this study. However, several bands in the molecular weight range of 215 000–220 000 have been observed with polyclonal anti-spectrin antibody. These polypeptides may represent variants of the β-subunit of spectrin (Nelson & Lazarides, 1983) that are weakly cross-reactive, more susceptible to proteolytic degradation, or present in lower quantities than the α-subunit in ROS.

The molecular structure and function of ROS spectrin is not yet known. Immunocytochemical labeling studies, however, indicate that this protein is localized along the periphery of the ROS organelle in the region where the disks come in close contact to the plasma membrane and ciliary spine. Filamentous structures linking disks to the plasma membrane have been seen in frog ROS under the electron microscope by Usukura and Yamada (1981), using freeze–deep etched replica techniques. Roof and Heuser (1982) have also observed specialized filaments connecting the disk rims to each other and different filaments linking the disks to the plasma membranes. Filaments connecting the disks to each other are more numerous and have been suggested to be composed of the rim protein first characterized by Papermaster et al. (1978) in frog ROS. This protein may be related to ROS 1.2 glycoprotein of bovine ROS (Molday & Molday, 1979) although direct evidence for this is lacking. The molecular composition of the less numerous filaments connecting the disks to the plasma membrane that may be related to the filamentous network observed by Usukura and Yamada (1981) is not known. Our results indicate that the M_r 240 000 spectrin-like protein is present in significantly lower amounts than the M_r 220 000 glycoprotein ROS 1.2 as observed by Coomassie blue and silver staining of ROS proteins separated by $NaDodSO_4$ gel electrophoresis (Figure 1). RIA studies comparing the binding of anti-spectrin antibodies to RBC spectrin and ROS further suggest that ROS contains a protein that is immunochemically related to spectrin, and this spectrin-like protein constitutes less than 1% of the ROS protein (Figure 8). It would appear that the 240K polypeptides and fragments recognized by polyclonal anti-RBC antibodies are identical with those recognized by the 4B2 monoclonal antibody. This can be tested by immunoprecipitating ROS proteins with the polyclonal antibodies and measuring binding of monoclonal antibodies to the complex. On the basis of these studies and analogy with RBC spectrin, we suggest that the ROS spectrin protein is a major constituent of the filamentous network lining the cytoplasmic surface of the ROS plasma membrane as observed by Usukura and Yamada (1981) and, more specifically, may comprise the filaments extending from the disks to the plasma membrane observed by Roof and Heuser (1982). This filamentous network, which probably interacts with integral membrane proteins, would serve to maintain a constant distance between the plasma membrane and disks as seen by electron microscopy and would generally stabilize the ROS structure. The observation that gold labeling with the 4B2 antibody extends inward from the plasma membrane up to 50 nm may indicate that the spectrin network interdigitates between the rims of the disks. Further studies are now in progress to determine in more detail the molecular structure and properties of ROS spectrin, its interaction with other ROS proteins, and its possible function in the maintenance of ROS structure, phototransduction, and other specialized processes that occur in the ROS organelle.

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External Anions Regulate Stilbene-Sensitive Proton Transport in Placental Brush Border Vesicles†

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ABSTRACT: The mechanism for HCO_3^--independent proton permeability in microvillus membrane vesicles (MVV) isolated from human placenta was examined by using the entrapped pH indicator 6-carboxyfluorescein (6CF). Proton fluxes (J_H) across MVV were determined in response to induced pH and anion gradients from the time course of 6CF fluorescence, the MVV buffer capacity, and the 6CF vs. pH calibration. In the absence of anions, J_H was 12 ± 2 nequiv s^{-1} (mg of protein)$^{-1}$ (pH_{in} 7.4, pH_{out} 6.0, MVV voltage-clamped with K^+/valinomycin, 23 °C), corresponding to a proton permeability coefficient of 0.02 cm/s, with an activation energy of 9.1 ± 0.3 kcal/mol. J_H was inhibited 20% by dihydro-4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (H_2DIDS) with K_I = 8 μM ($[Cl^-]_{out}$ = 0 mM). For a 0.5-unit pH gradient J_H increased from 1.5 to 4.6 nequiv s^{-1} (mg of protein)$^{-1}$ as the internal MVV pH was increased (5.5-7.5). External Cl^-, Br^-, and I^- (but not SO_4^{2-} and PO_4^-) increased J_H 1.3-2.5-fold for both inwardly and outwardly directed pH gradients with K_D = 1.0 ± 0.4 mM (Br^-) and >100 mM (Cl^-). This increase was blocked by 100 μM H_2DIDS but not by amiloride or furosemide. Internal Cl^- did not alter J_H induced by pH gradients nor were proton fluxes induced by anion gradients in the absence of a pH gradient. Experiments in which J_H was driven by membrane potentials (induced by valinomycin and K^+ gradients) indicated that proton transport was voltage-sensitive. These experiments demonstrate a stilbene-sensitive electrogenic proton transport mechanism in MVV that is regulated allosterically by anions at an external binding site.

Passive and ion-coupled proton permeabilities in biological membranes are important for regulation of cell pH and for the net transepithelial transport of ions and proton equivalents. In pure lipid bilayers the mechanism of electrogenic proton transport is not well understood; models involving proton transport along strands of hydrogen-bonded water and proton transport facilitated by endogenous mobile carriers have been proposed to explain the anomalously high permeabilities for protons as compared to monovalent ions and the lack of significant dependence of proton conductance on pH (Cafiso & Hubbell, 1983; Guknecht, 1984; Nichols & Deamer, 1980). In biological membranes, passive proton transport in HCO_3^--free media occurs primarily by electrogenic passive

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