CHARACTERIZATION OF A HIGH-MOLECULAR WEIGHT GLYCOPROTEIN IN BOVINE ROD PHOTORECEPTOR OUTER SEGMENT BY A MONOCLONAL ANTIBODY

By

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ABSTRACT

A monoclonal antibody designated as 4B2 was used to investigate the molecular properties and structural organization of a 220,000 M₀ glycoprotein, referred to as ROS 1.2, in bovine retinal rod photoreceptor outer segment disk membranes. The approaches taken were first to produce, characterize, and purify the 4B2 antibody, then to use the purified 4B2 antibody as a ligand for the purification of ROS 1.2 by affinity chromatography, and finally to determine the amino acid composition of ROS 1.2 by amino acid analysis of purified ROS 1.2.

The 4B2 antibody was produced by growing the 4B2 hybridoma cell line as ascites tumors in mice or as mass culture. The former method proved to be easier and more economical. Also, the antibody concentration in ascites fluid was shown by solid-phase radioimmune assay to be 30-100 fold greater than in culture supernatant. The 4B2 antibody was shown by radioimmune assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be an IgM molecule with μ heavy chains of apparent M₀=55,000 and κ light chains of apparent M₀=22,000. The 4B2 antibody was purified from ascites fluid by DEAE-Sephacel ion exchange chromatography. The yield of this 4B2 antibody was low (0.5 mg/ml ascites fluid) compared to the yield of an anti-rhodopsin antibody (7 mg/ml ascites fluid) obtained from 1D4 hybridoma cell
ROS 1.2 could only be partially purified by affinity chromatography on a 4B2 antibody-agarose column. As shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a residual amount of rhodopsin, the major rod outer segment (ROS) protein, copurified with ROS 1.2. Therefore, the amino acid composition of ROS 1.2 could not be determined.

The 4B2 antibody was used to probe the structural and functional properties of ROS 1.2 by determining whether immunological cross-reactivity exists between ROS 1.2 and proteins of similar $M_r$ in other cell types, namely, myosin in muscle, spectrin in red blood cells, and brain spectrin (fodrin, or calspectin) in brain cells. The 4B2 monoclonal antibody was shown by radioimmune competition assays to cross-react with rabbit skeletal muscle myosin, bovine brain homogenate, and human red blood cell membranes. Radioimmune labeling studies indicated that the cross-reactivity was due to a 160,000 $M_r$ protein in bovine brain homogenate. Similar radioimmune labeling studies were unable to demonstrate which protein in red blood cell membranes cross-react with the 4B2 monoclonal antibody. These studies also indicated that the degraded fragments of ROS 1.2 and myosin with apparent $M_r$ of 140,000 retained 4B2 antigenic sites. In addition, actin was shown by radioimmune assay to be present in rod outer segment preparations.
On the basis of these and other results, it is concluded that ROS 1.2 is a myosin-like protein, and in conjunction with other cytoskeletal elements such as actin, serves to link ROS disks to each other or to ROS plasma membrane for the maintenance of the highly-ordered ROS structure. It is speculated that the loss of this ordered structure during the process of disk renewal involves the proteolytic degradation of ROS 1.2.
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<tr>
<td>ATP</td>
<td>adenosine 5' triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CNBr</td>
<td>cyanogen bromide</td>
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<tr>
<td>Con A</td>
<td>concanavalin A</td>
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<tr>
<td>CTAB</td>
<td>cetyltrimethyl ammonium bromide</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
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<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis(β-aminoethyl ethyl)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>M_r</td>
<td>molecular weight</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>RIA</td>
<td>radioimmune assay</td>
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<td>ROS</td>
<td>rod outer segment</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl) aminomethane</td>
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INTRODUCTION

1. Vertebrate retina

The vertebrate retina has been the object of considerable investigation for two main reasons. It is a readily-accessible portion of the central nervous system which facilitates the study of neurons, and it is the receptor tissue which initiates the complex process of vision.

The retina is the innermost layer of the eyeball. It is a thin layer of neural tissue consisting of pigment epithelial cells, photoreceptor cells, higher-order neurons, and glial cells. These retinal elements are organized into distinct layers of cell bodies and processes (Fig. 1). The retina has two functions. The first, performed by the photoreceptors, is to transform light energy into nerve impulses. The second, performed by the intricate array of higher-order neurons, is to integrate the large number of photoreceptor signals and transmit the visual information on to the brain via the optic nerve.

2. Photoreceptors

The photoreceptor cells are closely packed in the retina, and axially oriented to the incident light. Light must pass through almost the entire thickness of the retina before impinging
Figure 1. Diagram to illustrate the layers of the retina (1) pigment epithelium (2) photoreceptors (3) external limiting membrane (4) outer nuclear layer (5) outer plexiform layer (6) inner nuclear layer (7) inner plexiform layer (8) ganglion cell layer (9) optic nerve fiber layer (10) internal limiting membrane. Arrow indicates the direction of incident light (as taken from reference 78)
upon the photoreceptor cells. Light, not detected by the photoreceptors, is absorbed by the pigment particles of the adjacent pigment epithelium.

There are two types of photoreceptor cells. They are called rods and cones because of their shapes. Cones function in bright light and are responsible for color vision. Rods function in dim light and give achromatic vision. The number of rods and cones, and their distribution in a retina varies from species to species. In humans, there are about three million cones and about a billion rods distributed unequally throughout the retina. The highest concentration of cones is found in the center, whereas the highest concentration of rods is found away from the center of the retina. No rods are found in the fovea region, and neither rods nor cones are found in the optic disc of the retina.

3. Rods

The greater size and abundance of rods compared to cones dictate their use in many biochemical studies. Rods are slender and elongated, and occur in a variety of shapes and sizes. For example, frog rods are typically 6-7μ in diameter and 100-120μ in length whereas bovine rods are typically 1-2μ in diameter and 40-50μ in length. However, rods have a basic structure which is similar in all vertebrate species. Each rod cell is composed of an outer segment connected to an inner segment by a thin cilium, a nucleus, and a synaptic body (Fig. 2).
Figure 2. Schematic diagram of a vertebrate rod photoreceptor. The outer segment is connected to the inner segment by a thin cilium. Ellipsoid and myoid are characteristic regions of the inner segment. The nucleus and synaptic termination are also shown (as taken from reference 79).
The inner segment contains all the metabolic and synthetic machinery of the cell. It generates ATP at a very rapid rate and is highly active in synthesizing proteins. It consists of two distinct regions called ellipsoid and myoid. The ellipsoid is characterized by a very dense accumulation of mitochondria. The myoid is characterized by free ribosomes, rough endoplasmic reticulum, Golgi apparatus, and contractile proteins.

The synaptic body is the site of information transfer to the higher-order neurons. It is characterized by the presence of many synaptic vesicles, and the formation of a chemical synapse with a bipolar cell.

4. **Rod outer segment**

The outer segment is the site of phototransduction. It is a highly-ordered structure consisting of hundreds of stacked photoreceptor disk membranes that are oriented perpendicular to its long axis, and enclosed by a plasma membrane. Rod outer segments have been the subject of numerous studies dealing with the structure of biological membranes and the molecular basis of visual excitation.

(a) **Structure**

Both X-ray diffraction measurements on excised retinas (1) and freeze-fracture studies (2) indicate that the center-to-center
distance between adjacent disks has a constant value of about 300 Å. The internal membrane surfaces of a disk are separated by a 20 Å-wide aqueous space (3), which is maintained over the entire extent of the disk (Fig.3). These specialized disk membranes occupy a relatively compact volume, but provide a large surface area for embedding a large number of photoreceptor molecules, which is required for the high sensitivity of rods (4). The mechanisms for the biogenesis of the rod outer segment and the maintenance of its highly-ordered structure are not fully understood.

(b) Disk renewal

Rod outer segment (ROS) disks have been found to be continuously renewed (5,6). Autoradiographic studies have shown that the new disk membrane components are synthesized in the inner segment at one or more sites, then transported to the base of the outer segment where final membrane assembly takes place (7,8). The disks are formed by the invagination of the ROS plasma membrane near the ROS base, but are pinched off as they are displaced apically. Consequently, all the disks except those close to the ROS base are closed, intracellular vesicles, as indicated by electron microscopy (9) and electrophysiological observations (10,11). The rate of disk synthesis varies from species to species. For frogs, the rate is 1.2 disks per hour, and all 1,700 disks in a frog ROS are renewed in two months (12).
Figure 3. Schematic representation of the membrane topology of rod outer segment. Disks are formed by the infolding of the plasma membrane at the basal end of the outer segment, and eventually pinch off the cell membrane and form isolated vesicles. Thickness of each disk and the repeat distance between disks are shown (as taken from reference 4).
For humans, all the disks in a ROS are renewed in about 10 days (13).

To maintain the ROS at a constant length, the tips are periodically sloughed off to be phagocytized and degraded by the pigment epithelium. This shedding process has been found to follow a diurnal pattern in a large variety of species (14). The orderly process of disk synthesis and destruction appears to be extremely crucial to photoreceptor function. If the pigment epithelium is incapable of phagocytizing the shed disks, the rods eventually die off (15). The mechanism of disk renewal remains to be determined.

(c) Chemical composition

The structure and function of ROS components have been investigated extensively since ROS can be obtained in high purity. Intact ROS membranes with enclosed cytoplasmic proteins and small molecules can be released from the retina by gentle homogenization and purified by flotation on a sucrose gradient. The disk membranes are isolated by osmotically shocking the plasma membrane and sealed disks are purified by flotation on 5% Ficoll (16).

The disk membranes are composed of lipids and proteins in a bilayer configuration. Lipid to protein ratio, on a dry weight basis, is about one. Phospholipids represent more than 80% of the total lipid content; cholesterol accounts for only 3% (17). The major phospholipids are phosphatidylethanolamine,
phosphatidylcholine, and phosphatidylserine; 44, 36, and 15% lipid phosphorous respectively (18). Polyunsaturation in more than 50% of the total fatty acid composition indicates the highly fluid nature of these disk membranes. The existence of a highly fluid lipid core in disk membranes is postulated to serve the purposes of phototransduction and ionic translocation, and allow rhodopsin to be renewed rapidly (18).

SDS-polyacrylamide gel electrophoresis of bovine ROS disk membrane proteins indicate the presence of a major component, rhodopsin, with an apparent $M_r = 34,000$, a high molecular weight protein with an apparent $M_r = 220,000$, and several other minor components ranging in $M_r$ from 36,000 to 300,000 (16,19,20).

5. **Visual excitation**

In the dark, the sodium ion permeability of ROS plasma membrane is high, a billion sodium ions per second (21,22). Sodium ions rapidly flow into the outer segment because of the existence of a large sodium concentration gradient across the plasma membrane, then diffuse to the inner segment, and are extruded by $\text{Na}^+\text{-K}^+\text{-ATPase}$ pumps in the inner segment plasma membrane to maintain the sodium concentration gradient.

Absorption of a photon causes the chromophore of rhodopsin, 11-cis-retinal, to undergo a series of transformations that result in the dissociation of all-trans-retinal from the protein to yield bleached rhodopsin or opsin (23-25). This conformational
transition in a single rhodopsin molecule blocks the entry of a million sodium ions into the ROS (26). The resulting hyperpolarization of the plasma membrane is transmitted to the synapse at the other end of the rod and communicated to other cells of the retina.

It is generally accepted that an internal transmitter is essential to communicate between rhodopsin molecules in the disk membranes and the sodium channels of the plasma membrane. Two attractive transmitter candidates are Ca$^{2+}$ and cGMP.

6. Proposed mechanisms for visual excitation

(a) Ca$^{2+}$ hypothesis

According to the Ca$^{2+}$ hypothesis (27), Ca$^{2+}$ ions are stored in the intradiskal space in the dark. When induced by light, Ca$^{2+}$ ions are released into the cytoplasmic space, and diffuse to the plasma membrane to block sodium channels (Fig.4). Evidence in favor of a Ca$^{2+}$ transmitter comes from electrophysiological experiments where the addition of Ca$^{2+}$ to the retina mimics the effect of light (28-30) and the addition of calcium chelators such as EGTA makes the rod less sensitive to light (30,31). Furthermore, many Ca$^{2+}$ ions are extruded from an illuminated ROS following a light pulse (32,33), and sufficient Ca$^{2+}$ is present in ROS and in the disks for it to be available as a transmitter (34,35). However, there is controversy as to whether the number of Ca$^{2+}$ released from disks into the cytosol
Figure 4. Schematic representation of the Ca\textsuperscript{2+} hypothesis for the mechanism of visual excitation (as taken from reference 80).

Upper: In the dark, sodium channels on the plasma membrane are open and calcium ions are stored inside the disks.

Lower: Light activation of rhodopsin releases calcium ions from the disks into the cytosol. Calcium ions then diffuse to plasma membrane where they block sodium channels.
on illumination is sufficient for transmitter action. Also, it has not been established that, in the dark, disks actively take up Ca\textsuperscript{2+}.

(b) cGMP hypothesis

According to the cGMP hypothesis (26), a high cGMP concentration in the cytosol is required to maintain the opening of sodium channels in the dark. When induced by light, cGMP concentration falls, resulting in the closure of sodium channels (Fig. 5). Evidence in favor of a cGMP transmitter comes from biochemical and electrophysiological findings. ROS contain about 30 \( \mu \text{M} \) of cGMP, an unusually high concentration of cGMP (36). This cGMP level decreases markedly on illumination as more than \( 10^5 \) cGMP molecules are hydrolyzed per second per photolyzed rhodopsin molecule (37). This high rate of hydrolysis of cGMP is accounted for by the cyclic interactions of transducin and cGMP phosphodiesterase, two peripheral enzymes found in ROS, with photolyzed rhodopsin (Fig. 6). Furthermore, cGMP depolarizes the ROS plasma membrane within milliseconds of its being injected into the cell, and increases the latency of the light-induced hyperpolarization (38). However, it is unknown as to how cGMP concentration might regulate sodium channels.
Figure 5. Schematic representation of the cGMP hypothesis for the mechanism of visual excitation (as taken from reference 80).

Upper: In the dark, sodium channels on the plasma membrane are open due to a high concentration of cGMP in the cytosol.

Lower: Light activation of rhodopsin causes a decrease in cytosolic cGMP concentration, resulting in the closure of sodium channels.
Figure 6. Proposed light-activated amplification cycle. Information flows from photolyzed rhodopsin (R*) to tranducin (T-GTP) and then to the phosphodiesterase (PDE*) (as taken from reference 37).
(c) **Extended Ca\(^{2+}\) hypothesis with a role for cGMP**

According to this hypothesis (39), Ca\(^{2+}\) ions are stored in the intradiskal space in the dark. When induced by light, Ca\(^{2+}\) ions are released into the cytoplasm via pores composed of multimeric rhodopsin, and diffuse to the plasma membrane to block sodium channels. To restore the intradiskal Ca\(^{2+}\) level present in the dark, cytoplasmic Ca\(^{2+}\) ions are removed by a Ca\(^{2+}\)-binding protein whose binding capacity is increased by the decrease of cGMP which follows rhodopsin bleaching. Ca\(^{2+}\) ions are then transported to the intradiskal space by Ca\(^{2+}\)-ATPase pumps (Fig.7).

Essential features of this model, not included in the Ca\(^{2+}\) hypothesis, are that multimeric rhodopsin form pores for the release of intradiskally stored Ca\(^{2+}\), that cytoplasmic Ca\(^{2+}\) ions are removed by a Ca\(^{2+}\)-binding protein, and that cGMP regulates cytoplasmic Ca\(^{2+}\) by affecting the binding capacity of the Ca\(^{2+}\)-binding protein. These features are not well established yet. For example, rhodopsin has not been shown to be an ionic pore. However, rhodopsin is an integral protein spanning the ROS disk membrane with 7 α-helical segments (40) consisting of mostly nonpolar amino acids (39). For it to be an ionic pore, several rhodopsin molecules are required to provide a sufficient number of polar side-groups to establish a hydrophilic region penetrating the membrane. Calmodulin, a Ca\(^{2+}\)-binding protein, has been reported to be present in bovine
Figure 7. Schematic representation of the extended Ca\(^{2+}\) hypothesis with a role for cGMP for the mechanism of visual excitation. Action of Ca\(^{2+}\) as intracellular transmitter is shown in the section on the right (A). Photo-activation of cGMP phosphodiesterase is shown in the middle section (B). cGMP-controlled Ca\(^{2+}\) binding and ATP-driven Ca\(^{2+}\)-pump activity are shown in the section of the left (C). Short double-headed arrows indicate protein-protein interaction. Other active sites on proteins are indicated as follows: (○) site of reversible Ca\(^{2+}\) binding; (□) site of reversible cGMP binding; (●) site of GDP and GTP binding and of GTP hydrolysis; (■) site of cGMP hydrolysis; (●) site of ATP hydrolysis (as taken from reference 39).
ROS (41), but there is no evidence for the interaction of cGMP with calmodulin in ROS. Also, cGMP has been reported to stimulate Ca\(^{2+}\) uptake by ROS disks suspended in media containing high-energy phosphate esters and electrolytes approximating the cytoplasmic composition of live rod cells (42), but the mechanism of control of Ca\(^{2+}\) in ROS by cGMP remains to be clarified.

7. ROS proteins

Rhodopsin has been the focus of research on visual excitation, since it is the initial site of interaction with light. It represents 80-90% of the total protein content of ROS (18). It is a transmembranous glycoprotein with its N-terminus facing the intradiskal space and C-terminus facing the cytoplasmic space (53), and transverses the disk membranes with 7 helices (43). The complete amino acid sequence of rhodopsin has been elucidated, but whether it is a photosensitive channel or a modulator of the cGMP level in ROS or both remains to be determined (43).

Several recently-identified bovine ROS minor protein components have been studied in order to clarify the mechanism of visual excitation. These include cGMP phosphodiesterase (44-46), transducin (37) or G-protein (47), or guanine nucleotide-binding protein (48), opsin kinase (49,50), and a high molecular weight protein referred to as ROS 1.2 (20).
8. **Initial characterization of ROS 1.2**

ROS 1.2 constitutes about 1-3% of the total protein content in ROS. It has been shown to be a transmembranous glycoprotein which binds the lectins Con A and WGA (20,51). Lectin binding studies on sealed disks (52,53) and Con A labeling studies for electron microscopic analysis (53-56) indicate that lectin-specific oligosaccharide chains are oriented toward the intradiskal space. Protease-sensitive segments (20,53) and antigenic sites of ROS 1.2 (57) are exposed on the cytoplasmic surface of sealed disks.

A monoclonal antibody referred to as 4B2 has been shown to be specific for ROS 1.2 (57). Biochemical, immunological and electron microscopic labeling studies using 4B2 monoclonal antibody have shown that the 4B2 antigenic site of ROS 1.2 is readily-accessible on the cytoplasmic side of sealed disks. Also, ROS 1.2 can be cleaved with trypsin into a $M_r=140,000$ fragment which binds 4B2 monoclonal antibody and a $M_r=120,000$ fragment which binds Con A (57).

9. **Thesis investigation**

The aim of this present project is to further characterize ROS 1.2 using the 4B2 monoclonal antibody and determine its role in the ROS. This thesis reports the results of the production,
characterization, and purification of the 4B2 monoclonal antibody, the immunological cross-reactivities of the 4B2 monoclonal antibody to muscle myosin, red blood cell membranes, and brain homogenate, the detection of actin in ROS preparations, and the purification of ROS 1.2. The significance of a cytoskeletal network in the ROS, and the possible role of ROS 1.2 in maintaining the highly-ordered ROS structure are discussed.
MATERIALS AND METHODS

1. Chemical reagents

All general laboratory chemicals of reagent grade were obtained from either Fisher Scientific, Sigma Chemical, or BDH Chemicals.

2. Protein assay

Protein concentrations were determined by the method of Lowry et al. (58), using BSA as a standard.

3. Tissue culture

All tissue culture media, sera, antibiotics, and accessories were obtained from Grand Island Biological Company.

(a) Maintenance of hybridoma cell lines

Hybridoma cell lines were grown in a RPMI 1640 culture medium supplemented with 2mM L-glutamine, 24mM sodium bicarbonate, 2mM sodium pyruvate, 10% fetal calf serum, 5% heat-inactivated horse serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1.25 μg/ml fungizone. The cells were grown at 37°C in an atmosphere
of 10% CO$_2$, 90% air in a humidified incubator. Growing cultures were maintained in 10-ml culture dishes at concentrations ranging from 4-10$\times$10$^5$ cells/ml. The concentration of viable cells was determined by staining cell cultures with equal volume of 0.4% trypan blue and counting unstained cells with a hemacytometer.

Storage of hybridoma cells was done by centrifuging 10 ml of a growing culture at 1,500 rpm for 5 min. Cell pellet was resuspended in 5 ml of 10% DMSO in supplemented RPMI 1640 culture medium. Cells were then transferred to freezing vials and immediately placed in a box insulated with styrofoam. The box was left in a -70°C freezer. The vials were finally placed in a liquid nitrogen freezer after 24h.

Frozen cells were retrieved by quickly thawing and washing the cells with 10 ml of non-supplemented RPMI 1640 culture medium. The cell pellet was then resuspended in 10 ml of supplemented RPMI 1640 culture medium.

(b) Recloning of established antibody-secreting 4B2 hybridoma cell line

Antibody-secreting 4B2 hybridoma cell line was recloned by limiting dilution. About 230 live 4B2 hybridoma cells were suspended in 4.6 ml of a cloning medium consisting of 10$^7$ BALB/c mouse thymocytes per ml of supplemented RPMI 1640 culture medium. Aliquots (100 µl) of this suspension were used to plate 36 wells of a 96-well culture plate, such that an average of 5 cells were seeded in each well. To the remaining 1 ml of cell
suspension, 4 ml of cloning medium were added. Aliquots (100 µl) of this suspension were used to plate another 36 wells, giving an average of one cell per well.

At day 5 and again at day 12, aliquots (100 µl) of the cloning medium were added to each well of the cloning plate. The growth of cloned cells was monitored under a light microscope. Wells with cell growth were then tested for antibody activity. Positive clones were expanded and stored as described above.

4. Production of 4B2 monoclonal antibody

BALB/c mice were obtained from the Animal Care Unit of the University of British Columbia. Pristane was obtained from Aldrich.

(a) Mass culture

The 4B2 hybridoma cells were grown and expanded continuously for a 2-week period. Culture supernatants were collected and stored in 0.1% NaN₃.
(b) Ascites tumors

The 4B2 hybridoma cells were grown as ascites tumors in BALB/c mice that had been injected intraperitoneally with 0.5 ml of pristane 7 days prior to injection of 5-10x10^6 cells. When ascites tumors became noticeable, tumor-bearing mice were bled. Ascites fluid was collected from the blood by centrifugation at 12,000 rpm for 20 min and stored in a -70°C freezer until used.

5. Characterization of 4B2 monoclonal antibody

The Ig subtype of 4B2 monoclonal antibody was identified using a Mouse Ig Subtype Identification Kit (Boehringer Mannheim Biochemicals) and the indirect solid-phase radioimmune assay described in 6(c) except the following: After incubation with 4B2 hybridoma culture fluid, the wells were incubated with 25 μl of rabbit anti-mouse Ig subtype-specific antibodies or preimmune serum in RIA buffer for 60 min at 23°C. After washing in PBS, the wells were incubated with 25 μl of 125I-labeled goat-anti-rabbit Ig (15-40 μg/ml; 1-2x10^6 dpm/μg) in RIA buffer for 30 min at 23°C.

The molecular weights of the heavy and light chains of 4B2 antibody were estimated from their mobilities in SDS-polyacrylamide gel after gel electrophoresis.
6. **Purification of 4B2 monoclonal antibody**

DEAE-Sephacel and Sephacryl-S-500 beads were obtained from Pharmacia. DEAE-Affi-Gel-Blue beads were obtained from Bio-Rad.

(a) **Chromatography of 4B2 ascites fluid and culture supernatant on a DEAE-Sephacel column**

One volume of 4B2 ascites fluid or culture supernatant was diluted with three volumes of PBS and centrifuged at 10,000 rpm for 10 min. The supernatant was precipitated with an equal volume of saturated ammonium sulfate solution at 4°C, for at least an hour. It was then centrifuged at 10,000 rpm for 10 min. The pellet was resuspended in 20mM Tris-acetate, pH 7.9 (Buffer A), containing 40mM NaCl, and dialyzed against Buffer A containing 20mM NaCl, overnight at 4°C. The dialyzate was applied to a DEAE-Sephacel column (5-6 mg protein/ml column) pre-equilibrated with Buffer A containing 20mM NaCl. Antibody molecules were eluted with a continuous NaCl concentration gradient (20-400mM) in Buffer A. The NaCl concentration of each fraction was determined by conductivity measurement. Fractions were tested for antibody activity by the standard RIA procedure described below, and assayed for absorbance at 280 nm. Protein composition of fractions was analyzed by SDS-polyacrylamide gel electrophoresis as described below. Fractions containing active 4B2 monoclonal antibody were pooled and lyophilized. In some experiments, antibody molecules were eluted with 100mM NaCl in Buffer A. The remaining proteins were eluted with 1M NaCl in
Buffer A.

(b) **Chromatography of 4B2 ascites fluid on a Sephacryl-S-500 column**

One volume of 4B2 ascites fluid was diluted with three volumes of PBS and centrifuged at 10,000 rpm for 10 min. Supernatant was applied to a Sephacryl-S-500 column pre-equilibrated with PBS. Fractions (1 ml) were treated as in 4(a), except that the pooled fractions were not lyophilized, but stored in 0.1% NaN₃ at 4°C.

(c) **Chromatography of 4B2 ascites fluid on a DEAE-Affi-Gel-Blue column**

One ml of 4B2 ascites fluid was applied to a DEAE-Affi-Gel-Blue column (6 ml) pre-equilibrated with 0.02M K₂HPO₄, pH 8.0. Antibody molecules were obtained in the first fractions to flow through the column, and the remaining proteins were eluted with 1.4M NaCl. Fractions (1 ml) were treated as described in 4(b).

(d) **Comparison between rho-1D4 and 4B2 hybridoma cell lines**

The 4B2 and rho-1D4 antibodies were purified from equal volumes of 4B2 and rho-1D4 ascites fluids by chromatography on a DEAE-Sephacel column as described in 4(a). The apparent $M_r$ of the heavy and light chains of both antibodies, as well as the amount of each antibody purified were compared.
7. **Standard solid-phase radioimmune assay**

Microtiter wells were obtained from Falcon. Chloramine-T was obtained from Eastman Organic Chemicals and carrier-free $^{125}$I was obtained from Amersham.

Phosphate-buffered saline contains 8g of NaCl, 0.2g of KCl, 0.2g $\text{KH}_2\text{PO}_4$, and 2.1g of $\text{Na}_2\text{HPO}_4*7\text{H}_2\text{O}$ per liter of distilled H$_2$O.

(a) **Preparation of antigens**

Intact ROS and sealed ROS disks were isolated from frozen bovine retinas (Hormel) using the method of Smith et al. (16). Red blood cell ghosts were prepared from human red blood cells using the method of Dodge et al. (59). Brain homogenate was prepared from frozen bovine brains using the method of Burridge et al. (60). Purified rabbit skeletal muscle myosin and actin were obtained from Sigma Chemicals.

(b) **Preparation of radiolabeled goat anti-mouse and goat anti-rabbit antibody reagents**

Goat anti-mouse Ig was partially purified from goat anti-mouse antisera (Antibodies Incorporated) by ion exchange chromatography on a DEAE-Sephacel column. Goat anti-rabbit Ig was purified by affinity chromatography on a rabbit Ig-Sepharose 4B column. Goat anti-mouse Ig and goat anti-rabbit Ig were labeled
with $^{125}$I (specific activity of $1-2 \times 10^6$ dpm/μg) using the Chloramine-T method (61). Free $^{125}$I was separated from either $^{125}$I-labeled goat anti-mouse Ig or $^{125}$I-labeled goat anti-rabbit Ig by gel filtration chromatography on a Bio-Gel P-6DG column (Bio-Rad).

(c) Standard RIA procedure

Antibody-secreting hybridomas were detected by an indirect solid-phase radioimmune assay procedure of Molday & MacKenzie (57). Bleached ROS or ROS disk membranes (2.5 mg/ml protein) were solubilized with 1% (w/v) Triton X-100 and diluted to 0.25 mg/ml with distilled H$_2$O. Aliquots (25 μl) were dried on flex vinyl microtiter wells at 60°C for 2h. The wells were then rinsed with distilled H$_2$O and non-specific binding sites were quenched with RIA buffer consisting of 1% BSA (Sigma), 1% FCS, and 0.1% NaN$_3$ in PBS. After the RIA buffer was removed, the wells were incubated with 25 μl of hybridoma culture fluid or ascites fluid for 60 min at 23°C, washed in PBS, and then incubated with 25 μl of $^{125}$I-labeled goat anti-mouse Ig (15-40 μg/ml; 1-2x10$^6$ dpm/μg) in RIA buffer for 30-60 min at 23°C. The plates were then rinsed extensively in PBS, cut into individual wells and counted in a Beckman 8000 gamma counter.

In some experiments, other antigens such as actin, myosin, brain homogenate, and red blood cell preparation were immobilized on microtiter wells, and antibody binding was assayed as
described above.

8. Solid-phase radioimmune competition assay

Competition assays were used to study the cross reactivities of the 4B2 monoclonal antibody, and to detect the different concentrations of 4B2 antigen in ROS and ROS disks. Briefly, 25 μl of varying concentrations of rabbit muscle myosin, bovine ROS membranes, ROS disk membranes, bovine brain homogenate, or human red blood cell ghost preparations, in RIA buffer was incubated at 23°C with 25 μl of hybridoma culture fluid diluted to a concentration which gives 80-90% saturation of binding by the standard solid-phase RIA. After 1h, 25 μl of the mixture was removed and screened for remaining antibody activity by the standard solid-phase RIA as described in 6(c).

9. SDS-polyacrylamide gel electrophoresis and gel transfer

All chemicals and apparatuses for SDS-polyacrylamide gel electrophoresis and gel transfer were obtained from Bio-Rad, except cyanogen bromide (Fisher Scientific) and β-mercaptoethanol (Eastman Organic Chemicals).

Samples with varying concentrations (0.3-8.0 mg/ml protein) were solubilized in an equal-volume mixture containing 5% SDS, 40%
sucrose, 10mM Tris, pH 6.8, 10% β-mercaptoethanol, and 4% bromophenol blue. Samples (20-25 µl) were applied to a 6%, 7%, 8%, 9%, or 10% polyacrylamide slab gel (0.15x5.0 cm or 0.15x12 cm) and electrophoresis was carried out according to the procedure of Laemmli (62). Gel slices were either stained with Coomassie blue (63) or subjected to electrophoretic transfer as adapted from the blotting transfer procedure of Bhullar et al. (64).

Unstained SDS gels were washed with three 100-ml changes of 0.1M sodium phosphate, pH 7.4, containing 0.1% SDS over 20 min and two 100-ml changes of 20mM sodium phosphate, pH 7.4, over a 10-min period. The washed gel was sandwiched against a wet sheet of CNBr-activated paper, prepared by the method of Clarke et al. (65), and placed in a Transblot apparatus. Electrophoretic transfer was carried out at 35V and 1.5A at 4°C in 20mM sodium phosphate, pH 7.4, for 8-12h. Following transfers, the remaining reactive groups on the CNBr-activated paper were quenched by incubation in Tris buffer, pH 9.0, containing 60mM glycine and 1% BSA, overnight at 23°C. The paper containing the transferred proteins was rinsed in distilled H₂O, air-dried, and stored in a desiccator at 4°C until used.

Recently, gel slices were subjected to electrophoretic transfer as adapted from the blotting transfer procedure of Towbin et al. (66). SDS gels were washed with two 100-ml changes of 20mM Tris-acetate, pH 7.4, containing 2mM EDTA and 0.01% SDS
over 20 min. The washed gel was sandwiched against a wet sheet of nitrocellulose paper (Bio-Rad) and placed in a Transblot apparatus. Electrophoretic transfer was carried out at 35V and 1.5A at 4°C in 20mM Tris-acetate, pH 7.4, containing 2mM EDTA and 0.01% SDS for 8-12h. Following transfers, the remaining binding sites on the nitrocellulose paper were quenched by incubation in immunoblot buffer, pH 7.4, containing 0.15M NaCl, 10mM sodium phosphate, 1mM EDTA, 1mM NaN₃, and 0.2% Triton X-100, with 4% BSA, overnight at 23°C. The paper containing the transferred proteins was rinsed in distilled H₂O and used immediately.

10. Study on the electrotransfer of ¹²⁵I-labeled proteins

Myosin (200K), BSA (66K), and prolactin (23K) were labeled with ¹²⁵I (specific activity of 1-2x10⁶ dpm/µg) using the Chloramine-T method (61). Samples of ¹²⁵I-labeled myosin (1.5x10⁵ dpm/well), ¹²⁵I-labeled BSA (2.2x10⁶ dpm/well), and ¹²⁵I-labeled prolactin (2.0x10⁵ dpm/well) were applied to 8% SDS-polyacrylamide slab gels and electrophoresis was carried out as described previously. After electrophoresis, unstained gel slices were subjected to electrophoretic transfer to nitrocellulose papers using the blotting transfer procedure of Towbin et al. (66). At different times after electrophoretic transfer, corresponding strips of gel and papers were removed, air-dried, and exposed to X-ray film as described below. The
polypeptide bands detected by autoradiography were excised and counted in a Beckman 8000 gamma counter.

Similar studies were done using 6% and 9% SDS-polyacrylamide slab gels for the electrotransfer of $^{125}$I-labeled myosin and $^{125}$I-labeled prolactin, respectively.

11. Detection of polypeptides which bind monoclonal antibodies

Transfer CNBr-activated papers were rinsed in RIA buffer and incubated with 10-ml of hybridoma culture fluid containing mouse monoclonal antibody for 1h at 23°C. The papers were then washed with several changes of PBS containing 0.4% N-lauroylsarcosine over a period of 1-2h and subsequently incubated with 10-15 ml of $^{125}$I-labeled goat anti-mouse Ig (1-2x10$^6$ dpm/ml) in RIA buffer for 1h at 23°C. Finally, the papers were washed extensively with PBS containing 0.4% N-lauroylsarcosine over 1-2h, air-dried, and subjected to autoradiography on Kodak Royal X-Omat film with an X-ray intensifying screen for 6-48h at 23°C.

Transfer nitrocellulose papers were treated as described above except that the papers were washed with five 50-ml changes of immunoblot buffer, pH 7.4, containing 0.15M NaCl, 10mM sodium phosphate, 1mM EDTA, 1mM NaN$_3$, and 0.2% Triton X-100. This was followed by one washing with 0.1M glycine buffer containing 1% Triton X-100, and 2M urea.
12. **Purification of ROS 1.2**

Sephacryl-S-200 beads were obtained from Pharmacia and REACTI-GEL (6X), a highly activated imidazolyl-carbamate matrix of 6% cross-linked agarose beads was obtained from Pierce.

(a) **Gel filtration chromatography of ROS proteins on a Sephacryl-S-200 column**

ROS membranes (9 mg/ml protein) were solubilized with 3-5% SDS, and applied to a Sephacryl-S-200 column (2.0x48.0 cm) pre-equilibrated with PBS containing 0.1% SDS and 1mM EDTA. The column was eluted with PBS containing 0.1% SDS and 1mM EDTA and 30-drop fractions were collected. Fractions were assayed for absorbance at 280 nm and analyzed by SDS-polyacrylamide gel electrophoresis as previously described.

(b) **Affinity chromatography of ROS proteins on a 4B2 antibody-agarose column**

Lyophilized and partially-purified 4B2 antibody was resuspended in 0.1M sodium borate buffer, pH 9.0, and coupled to REACTI-GEL (6X) by the method of Bethell et al. (67).

ROS membranes (9 mg/ml protein) were solubilized with 10mM CTAB in 0.1M sodium phosphate buffer, pH 7.4, and applied to a 4B2 antibody-agarose column (0.5 mg antibody/ml column) pre-equilibrated with the same buffer. The column was washed with the same buffer and eluted with 50mM diethylamine in the same buffer. Fractions (1 ml) were collected and assayed for absorbance at 280 nm and analyzed by SDS-polyacrylamide gel
electrophoresis as previously described.
RESULTS

1. Production of 4B2 monoclonal antibody

The 4B2 monoclonal antibody was obtained by two conventional methods: mass culture of 4B2 hybridoma cell line and induction of ascites tumors in mice. Culture supernatant (300 ml) and ascites fluid (50 ml) containing 4B2 monoclonal antibody were collected after 2 and 5 weeks, respectively. The volume of ascites fluid obtained from a tumor-bearing mouse varied from 2 to 15 ml.

Both the cell culture supernatant and ascites fluid were analyzed for their antibody-binding properties. The effect of dilution of 4B2 cell culture supernatant and ascites fluid on the binding of antibody to Triton X-100-solubilized disks using an indirect solid-phase radioimmune assay is shown in Fig. 8. Both the cell culture supernatant and ascites fluid contained active antibody specific for disk membranes. However, half-maximum binding occurred at a reciprocal dilution of 16 for culture supernatant and 512 for ascites fluid. Nonspecific cell culture supernatant did not contain any antibody specific for disk membranes.

2. Specificity of 4B2 monoclonal antibody from recloned hybridoma cell line

The once cloned 4B2 antibody-secreting hybridoma cell line
Reciprocal Dilution

Figure 8. Titration of 4B2 hybridoma cell ascites fluid (●), 4B2 hybridoma cell culture supernatant (■), and nonspecific culture supernatant (▲) against Triton X-100-solubilized ROS membranes. Solubilized ROS membranes immobilized in microtiter wells were treated with serial dilutions of culture supernatants and ascites fluid, rinsed, and subsequently treated with $^{125}$I-labeled goat anti-mouse Ig.
was recloned. Antibody from culture supernatant of recloned cells was analyzed for its specificity against ROS membranes.

CNBr-activated paper with transferred proteins, sequentially labeled with culture supernatant of recloned cells and $^{125}$I-labeled goat anti-mouse Ig is shown in Fig. 9. Antibody secreted by the recloned 4B2 cell culture labeled a polypeptide of apparent $M_r=220,000$ and a smaller fragment of apparent $M_r=140,000$, as previously reported for the once cloned 4B2 hybridoma cell line (57). Antibody secreted by rho-1D4 cell culture labeled rhodopsin, the rhodopsin dimer, and a large aggregate of rhodopsin. Nonspecific supernatant did not label any ROS polypeptides.

3. Characterization of 4B2 monoclonal antibody

The Ig subtype of the 4B2 monoclonal antibody was determined by binding rabbit anti-mouse Ig subtype-specific antibodies to 4B2 antibody already bound to immobilized and Triton X-100-solubilized ROS disk membranes. As shown in Table I, rabbit anti-mouse $\mu$ and $\kappa$ chain antibodies bind to the 4B2 antibody 15-20 fold more than the control which contained preimmune serum. Other Ig subtype-specific antibodies bind at most 2 fold more than the control.
Figure 9. Analysis of ROS polypeptides which bind monoclonal antibody secreted by recloned 4B2 hybridoma cell line. ROS membranes were subjected to SDS-polyacrylamide gel electrophoresis (20 μg protein/gel). Proteins were electrophoretically transferred from SDS gels to CNBr-activated papers. CNBr-activated papers containing transferred proteins were labeled with 4B2, 1D4, and nonspecific culture supernatants, followed by $^{125}$I-labeled goat anti-mouse Ig.
TABLE I. Identification of Ig subtype of 4B2 monoclonal antibody

<table>
<thead>
<tr>
<th>Rabbit anti-mouse Ig-subtype specific antibody binding to 4B2 monoclonal antibody</th>
<th>anti-$(\alpha$)</th>
<th>$\gamma_1$</th>
<th>$\gamma_2^a$</th>
<th>$\gamma_2^b$</th>
<th>$\gamma_3$</th>
<th>$(\mu)$</th>
<th>$(\kappa)$</th>
<th>$(\lambda)$</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPM bound $\times 10^{-3}$</td>
<td>2.4</td>
<td>5.5</td>
<td>3.0</td>
<td>1.6</td>
<td>1.4</td>
<td>60.1</td>
<td>45.9</td>
<td>6.4</td>
<td>3.0</td>
</tr>
</tbody>
</table>
4. Purification of 4B2 monoclonal antibody by ion exchange chromatography on DEAE-Sephacel

The 4B2 monoclonal antibody from culture supernatant and ascites fluid was partially purified by ion exchange chromatography on a DEAE-Sephacel column. The elution profile for the purification of 4B2 antibody from ascites fluid and SDS gels of elutants are shown in Fig. 10. Fractions eluted with 80-120mM NaCl concentration contained antibody specific for ROS membranes as determined by RIA. SDS gels of these fractions showed three polypeptide bands. Two of which have apparent M_r=55,000 and 22,000. They are presumably the heavy and light chains of 4B2 antibody since the corresponding chains of standard IgG molecule have apparent M_r=50,000 and M_r=25,000. The other protein that copurified with 4B2 antibody has apparent M_r=85,000. Fractions eluted with higher NaCl concentration contained no active antibody specific for ROS membranes. These fractions contained many polypeptides, with serum albumin of apparent M_r=66,000 being the major protein as shown by SDS gels.

The elution profile for the purification of 4B2 antibody from 4B2 culture supernatant and SDS gels of elutants are shown in Fig. 11. Fractions eluted with 0.1M NaCl concentration contained antibody activity specific for ROS membranes as determined by RIA. However, these fractions contained two major polypeptides with apparent M_r identical to the heavy and light chains of standard IgG molecule as shown by SDS gels. This
Figure 10. (a) Chromatography of 4B2 ascites fluid on a DEAE-Sephacel column. One ml of 4B2 ascites fluid was diluted with 3 ml of PBS, and precipitated with 4 ml of saturated ammonium sulfate. Precipitated proteins were redissolved and dialyzed against 20mM Tris-acetate, pH 7.9, containing 20mM NaCl. Dialyzate was applied to a DEAE-Sephacel column (1.0x40cm), pre-equilibrated with the same buffer. The column was eluted with a linear gradient of NaCl concentration (---) at a flow rate of 0.5 ml/min and 30-drop fractions were collected. Fractions were assayed for absorbance at 280 nm (●) and antibody activity specific for ROS membranes (○). Fractions numbered 29 to 31, and 54 were analyzed by SDS-polyacrylamide gel electrophoresis. Fractions containing antibody activity were pooled and lyophilized. (b) Electrophoretic analysis of Ig purified from 4B2 ascites fluid by DEAE-Sephacel column chromatography. Equal volume (20 µl) of each fraction indicated in (a) was applied to a 8% SDS-polyacrylamide slab gel. Lane 1. standard mouse IgG. Lane 2. fraction #29. Lane 3. fraction #30. Lane 4. fraction #31. Lane 5. fraction #54.
Figure 11. (a) Chromatography of 4B2 culture supernatant on a DEAE-Sephacel column. One hundred ml of 4B2 culture fluid was diluted with 300 ml of PBS, and precipitated with 400 ml of saturated ammonium sulfate. Precipitated proteins were redissolved and dialyzed against 20mM Tris-acetate, pH 7.9, containing 20mM NaCl. Dialyzate was applied to a DEAE-Sephacel column (1.0x40 cm). The column was first eluted with 0.1M NaCl, then with 1M NaCl in 20mM Tris-acetate buffer, pH 7.9, at a flow rate of 0.5 ml/min, and 30-drop fractions were collected. Fractions were assayed for absorbance at 280 nm (●) and antibody activity specific for ROS membranes (○). Fractions numbered 68, 70, and 131 were analyzed by SDS-polyacrylamide gel electrophoresis. Fractions containing antibody activity were pooled and lyophilized. (b) Electrophoretic analysis of Ig purified from 4B2 culture supernatant by DEAE-Sephacel column chromatography. Equal volume (20 μl) of each fraction indicated in (a) was applied to a 10% SDS-polyacrylamide slab gel. Lane 1. standard mouse IgG. Lane 2. fraction #68. Lane 3. fraction #70. Lane 4. fraction #131.
indicates that the low salt-eluted 4B2 antibody was contaminated by nonspecific IgG molecules in the culture supernatant, since the 4B2 antibody was determined to be an IgM molecule. The horse serum in the culture supernatant might be the source of these nonspecific IgG molecules. Fractions eluted with 1M NaCl concentration contained no active antibody specific for ROS membranes. These fractions contained many polypeptides, with serum albumin being the major protein as shown by SDS gels.

In order to improve the yield of purified 4B2 antibody, gel filtration chromatography on a Sephacryl-S-500 column and ion exchange chromatography on a DEAE-Affi-Gel-Blue column were also used to purify 4B2 antibody from ascites fluid.

5. Purification of 4B2 monoclonal antibody by gel filtration chromatography on Sephacryl-S-500

The 4B2 monoclonal antibody was also partially purified from ascites fluid by gel filtration chromatography on a Sephacryl-S-500 column. The elution profile for the purification of 4B2 antibody from ascites fluid and SDS gels of elutants are shown in Fig. 12. There was only one $A_{280}$ peak but two antibody activity peaks. The first antibody activity peak was attributed to the 4B2 monoclonal antibody in its pentameric IgM form ($M_r=900,000$), since it eluted in the void volume of the column having an exclusion limit of 500,000. The second antibody peak was attributed to the dissociated forms of the 4B2 antibody, since it eluted in the included volume. Fractions eluted in the
Figure 12. (a) Chromatography of 4B2 ascites fluid on a Sephacryl-S-500 column. One ml of 4B2 ascites fluid was diluted with 3 ml of PBS and centrifuged at 10,000 rpm for 10 min. Supernatant was applied to a Sephacryl-S-500 column (1.0x40 cm) pre-equilibrated with PBS. The column was eluted with PBS at a flow rate of 0.5 ml/min and 30-drop fractions were collected. Void volume of the column was determined by the elution volume of Blue Dextran. Fractions were assayed for absorbance at 280 nm (●) and antibody activity specific for ROS membranes (■). Fractions numbered 22 to 24, and 29 to 31 were analyzed by SDS-polyacrylamide gel electrophoresis. Fractions containing antibody activity were pooled and stored in 0.1% NaN₃ at 4°C. (b) Electrophoretic analysis of Ig purified from 4B2 ascites fluid by Sephacryl-S-500 column chromatography. Equal volume (20 μl) of each fraction indicated in (a) was applied to a 8% SDS-polyacrylamide slab gel. Lane 1. standard proteins: phosphorylase b (92K), bovine serum albumin (66K), ovalbumin (45K). Lane 2. fraction #22. Lane 3. fraction #23. Lane 4. fraction #24. Lane 5. standard mouse IgG. Lane 6. fraction #29. Lane 7. fraction #30. Lane 8. fraction #31.
void and included volume contained polypeptides other than the heavy and light chains of the 4B2 antibody as shown by SDS gels.

6. **Purification of 4B2 monoclonal antibody by ion exchange Chromatography on DEAE-Affi-Gel-Blue**

The 4B2 monoclonal antibody was purified from ascites fluid by ion exchange chromatography on a DEAE-Affi-Gel-Blue column. SDS gels of elutants are shown in Fig. 13. Fractions eluted with 0.1M NaCl contained two polypeptides of apparent $M_r$ identical to the heavy and light chains of the 4B2 antibody. Fractions eluted with 1M NaCl contained a major polypeptide of apparent $M_r$ identical to serum albumin. Only low salt-eluted fractions contained antibody activity specific for ROS membranes (not shown).

7. **Comparison between rho-1D4 and 4B2 hybridoma cell lines in their ability to produce monoclonal antibody**

The Ig subtype and the yield of 4B2 monoclonal antibody from 4B2 ascites fluid were compared to that of rho-1D4 monoclonal antibody from rho-1D4 ascites fluid. As shown in Fig. 14, rho-1D4 antibody was an IgG molecule since the two major polypeptides, purified from low salt elution, have identical $M_r$ as the heavy and light chains of standard IgG molecule. Also, these fractions contained antibody activity specific for rhodopsin in ROS membranes (shown in Fig. 21). SDS gels also indicated that much more rho-1D4 antibody than 4B2 antibody was
Figure 13. Electrophoretic analysis of Ig purified from 4B2 ascites fluid by DEAE-Affi-Gel-Blue chromatography. Equal volumes (20 μl) of low salt-eluted fractions numbered 22 to 24 and one high salt-eluted fraction numbered 51 were applied to a 8% SDS-polyacrylamide slab gel. Lane 1. standard mouse IgG. Lane 2. fraction #22. Lane 3. fraction #23. Lane 4. fraction #24. Lane 5. fraction #51.
Figure 14. Electrophoretic analysis of Ig molecules purified from rho-1D4 and 4B2 ascites fluids by DEAE-Sephacel column chromatography. Equal volumes of 4B2 and rho-1D4 low salt-eluted Ig fractions were applied to 9% SDS-polyacrylamide slab gels. Gel A: Lane 1. standard proteins containing phosphorylase b (92K), bovine serum albumin (66K), ovalbumin (45K), carbonic anhydrase (31K), and soybean trypsin inhibitor (21K). Lane 2. standard mouse IgG. Lanes 3-5. low salt-eluted fractions containing rho-1D4 antibody as determined by RIA. Gel B: Lane 1. standard mouse IgG. Lanes 2-4. low salt-eluted fractions containing 4B2 antibody as determined by RIA.
purified. The yield of 4B2 antibody (0.5 mg/ml) was 8 fold less than the yield of rho-1D4 antibody.

8. Activity of partially-purified 4B2 monoclonal antibody

Fractions containing 4B2 monoclonal antibody, from the chromatography of 4B2 ascites fluid on DEAE-Sephacel, were pooled, lyophilized, and resuspended in PBS. The effect of dilution of this partially-purified 4B2 antibody on the binding of antibody to Triton X-100-solubilized ROS membranes is shown in Fig. 15. Partially-purified 4B2 antibody was active against ROS membranes. Half-maximum binding occurred at protein concentration of 0.2 mg/ml. As shown in Table II, half-maximum binding for ascites fluid occurred at protein concentration of 0.05 mg/ml.

9. Detection of actin in ROS

The effect of dilution of an anti-actin monoclonal antibody, referred to as 2C2, on the binding of the antibody to Triton X-100-solubilized ROS membrane preparation and purified actin is shown in Fig. 16. The 2C2 monoclonal antibody was active against actin and solubilized ROS membrane preparation. There was a higher degree of binding of 2C2 antibody to actin than to ROS membrane preparation. In control studies, two monoclonal
Figure 15. Titration of lyophilized and partially-purified 4B2 antibody against Triton X-100-solubilized ROS membranes. Solubilized ROS membranes immobilized in microtiter wells were treated with serial dilutions of 4B2 antibody partially purified by DEAE-Sephacel column chromatography, rinsed, and subsequently treated with $^{125}$I-labeled goat anti-mouse Ig.
TABLE II. Purification of 4B2 monoclonal antibody from ascites fluid by DEAE-Sephacel column chromatography

<table>
<thead>
<tr>
<th>Sample</th>
<th>*B&lt;sub&gt;50&lt;/sub&gt; (mg/ml)</th>
<th>**B&lt;sub&gt;50&lt;/sub&gt; ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>4B2 ascites fluid</td>
<td>0.05</td>
<td>1.0</td>
</tr>
<tr>
<td>0.1M NaCl-eluted Ig fractions</td>
<td>0.20</td>
<td>4.0</td>
</tr>
</tbody>
</table>

* Protein concentration of sample at which half-maximum antibody binding occurred.

** Ratio of B<sub>50</sub> (sample):B<sub>50</sub> (4B2 ascites fluid).

*** These fractions were pooled, lyophilized, and resuspended in PBS, before being assayed for antibody activity.
Figure 16. Titration of 2C2, 4B2, and rho-1D4 hybridoma cell culture supernatants against Triton X-100-treated rabbit actin (●) and 2C2 hybridoma cell culture supernatant against Triton X-100-solubilized ROS membranes ( ■ ). Solubilized ROS membranes and actin immobilized in microtiter wells were treated with serial dilutions of cell culture supernatant, rinsed, and subsequently treated with $^{125}\text{I}$-labeled goat anti-mouse Ig.
antibodies (rho-1D4 and 4B2), specific for rhodopsin and ROS 1.2 respectively, were inactive against actin.

10. Cross-reactivities of 4B2 monoclonal antibody

(a) Myosin

The effect of dilution of 4B2 ascites fluid and rho-1D4 culture supernatant on the binding of the antibodies to Triton X-100-treated rabbit skeletal muscle myosin is shown in Fig. 17 (upper). The 4B2 ascites fluid was active against purified myosin, but the rho-1D4 cell culture supernatant was not. In control studies, both the 4B2 ascites fluid and the rho-1D4 culture supernatant were active against Triton X-100-solubilized ROS membranes as shown in Fig. 17 (lower).

The relative degree of cross-reactivity of 4B2 antibody to myosin was determined by an indirect solid-phase radioimmune competition assay. As shown in Fig. 18, both Triton X-100-solubilized ROS membranes and myosin were able to inhibit the binding of 4B2 antibody to Triton X-100-solubilized ROS membranes.

(b) Red blood cell membranes and brain homogenate

The effect of dilution of culture supernatants (4B2 and
Figure 17. Titration of 4B2 ascites fluid (○) and rho-1D4 hybridoma cell culture supernatant (■) against Triton X-100-treated rabbit myosin (upper) and against Triton X-100-solubilized ROS membranes (lower). Solubilized ROS membranes and myosin immobilized in microtiter wells were treated with serial dilutions of culture supernatant, rinsed, and subsequently treated with $^{125}$I-labeled goat anti-mouse Ig.
Figure 18. Inhibition of 4B2 antibody binding to Triton X-100-solubilized ROS membranes by Triton X-100-solubilized ROS membranes (◊) and Triton X-100-treated rabbit myosin (●) using the solid-phase indirect radioimmune competition assay. Aliquots (25 µl) of serial dilutions of Triton X-100-treated rabbit myosin (0.1 mg/ml) or ROS membranes (0.1 mg/ml) were incubated with 25 µl of 4B2 hybridoma culture fluid diluted to a concentration which gives 80-90% saturation of antibody binding by radioimmune assay. After 1 h, 25 µl of the mixture was removed and screened for remaining antibody by radioimmune assay.
rho-1D4) on the binding of the antibodies to Triton X-100-solubilized human red blood cells (RBC) and bovine brain homogenate (BBH) is shown in Fig. 19. The 4B2 cell culture supernatant was active against both preparations, but rho-1D4 was not. There was a 5 fold higher degree of 4B2 antibody binding to BBH than RBC membranes.

The relative degree of cross-reactivity of 4B2 antibody to BBH and RBC membranes was again determined by competition assay. As shown in Fig. 20, Triton X-100-solubilized ROS, RBC, and BBH membranes were also able to inhibit the binding of 4B2 antibody to Triton X-100-solubilized ROS membranes. As shown in Table III, the relative cross-reactivity was in a decreasing order of ROS, myosin, BBH, and RBC membranes. However, the true relative cross-reactivity can only be determined by using purified competing antigens.

In control studies, nonspecific protein such as BSA was unable to inhibit the binding of 4B2 antibody to Triton X-100-solubilized ROS membranes over the concentrations tested. Similarly, RBC and BBH membranes were unable to inhibit the binding of rho-1D4 antibody to Triton X-100-solubilized ROS membranes (not shown).

In order to determine which protein in BBH and RBC membranes binds the 4B2 antibody, ROS, myosin, RBC and BBH proteins were electrophoretically transferred to nitrocellulose paper following gel electrophoresis. Nitrocellulose paper with transferred
Figure 19. Titration of hybridoma cell culture supernatants 4B2 (•) and rho-1D4 (■) against Triton X-100-solubilized human red blood cell membranes (upper) and bovine brain homogenate (lower). Solubilized red blood cell membranes and brain homogenate immobilized in microtiter wells were treated with serial dilutions of culture supernatant, rinsed, and subsequently treated with $^{125}$I-labeled goat anti-mouse Ig.
Figure 20. Inhibition of 4B2 antibody binding to Triton X-100-solubilized rod outer segment membranes (ROS) by Triton X-100-solubilized ROS membranes, bovine brain homogenate (BBH), human red blood cell membranes (RBC), and bovine serum albumin (BSA) using the solid-phase radioimmune competition assay as described previously.
TABLE III. Cross-reactivities of 4B2 monoclonal antibody

<table>
<thead>
<tr>
<th>Competing antigen</th>
<th>$I_{50}$ (μg/ml)</th>
<th>** $I_{50}$ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine ROS membranes</td>
<td>58</td>
<td>1.0</td>
</tr>
<tr>
<td>Rabbit skeletal muscle myosin</td>
<td>68</td>
<td>1.2</td>
</tr>
<tr>
<td>Bovine brain homogenate</td>
<td>316</td>
<td>9.0</td>
</tr>
<tr>
<td>Human red blood cell ghosts</td>
<td>1995</td>
<td>57.0</td>
</tr>
</tbody>
</table>

* Protein concentration of competing antigen required to inhibit 50% of 4B2 antibody binding to Triton X-100 solubilized ROS membranes.

** Ratio of $I_{50}$ (competing antigen): $I_{50}$ (bovine ROS membranes)
proteins, sequentially labeled with 4B2 antibody and 
\[^{125}\text{I}\text{-labeled goat anti-mouse Ig}\] is shown in Fig. 21. ROS 1.2, myosin, and a polypeptide with apparent \(M_r = 160,000\) in BBH membranes were labeled, but no polypeptide in RBC membranes was labeled. In control studies using rho-1D4 antibody, only rhodopsin, the rhodopsin dimer, and a large aggregate of rhodopsin in ROS membranes were labeled. In order to determine whether the electrophoretic transfers of RBC spectrin and brain spectrin in BBH were efficient enough for radioimmune labeling with 4B2 antibody, the electrotransfer of \(^{125}\text{I}\text{-labeled proteins}\) was studied.

11. **Electrotransfer of \(^{125}\text{I}\text{-labeled proteins}\)**

(a) **Effect of protein molecular weight**

\(^{125}\text{I}\text{-labeled proteins}\) of different molecular weights, myosin (200K), BSA (66K), and prolactin (23K), were applied to a 8% SDS-polyacrylamide slab gel. Following gel electrophoresis, these proteins were electrophoretically transferred to nitrocellulose papers. Time course of the electrotransfer of each of these proteins is shown in Fig. 22. The rate of transfer was inversely proportional to polypeptide size. After 1h, transfers of \(^{125}\text{I}\text{-labeled prolactin}\) and BSA were almost completed, but only 20% of \(^{125}\text{I}\text{-myosin}\) was transferred. The
Figure 21. Radioimmune labeling of ROS membranes, myosin, brain homogenate, and red blood cell membranes with 4B2 antibody. ROS membranes (20 μg/gel), rabbit skeletal muscle myosin (25 μg/gel), bovine brain homogenate (14 μg/gel), and human red blood cell membranes (13 μg/gel) were applied to a 8% SDS-polyacrylamide slab gel. Following gel electrophoresis, the proteins were electrophoretically transferred to CNBr-activated papers. CNBr-activated papers containing transferred proteins were treated with 4B2 and rho-1D4 culture supernatants, followed by 125I-labeled goat anti-mouse Ig. Lane 1. ROS membranes. Lane 2. myosin. Lane 3. bovine brain homogenate. Lane 4. red blood cell membranes.
Figure 22. Time courses for the electrotransfer of $^{125}$I-labeled myosin (▲), $^{125}$I-labeled BSA (■), and $^{125}$I-labeled prolactin (●). $^{125}$I-labeled proteins were applied to 8% SDS-polyacrylamide slab gels and electrophoresis was carried out. Following electrophoresis, gel slices were subjected to electrophoretic transfer to nitrocellulose papers. At different times after electrophoretic transfer, corresponding strips of gel and papers were removed and subjected to autoradiography. The polypeptide bands detected by autoradiography were excised and counted in a Beckman 8000 gamma counter.
time required for the transfer of about 70% of each of these 125I-labeled proteins is shown in Table IV.

(b) Effect of gel pore size

The effect of gel pore size on the time course of electrophoretic transfer of 125I-labeled myosin is shown in Fig. 23. The rate of transfer was proportional to gel pore size. The rate of transfer of 125I-labeled myosin increased with increasing gel pore size. Similar studies indicated the rate of transfer of 125I-labeled prolactin decreased with decreasing gel pore size (not shown).

12. Cytoplasmic pool of 4B2 antigen in ROS

The difference in the concentrations of 4B2 antigen between ROS and ROS disk membranes was detected by solid-phase radioimmune assays and was quantitated by competition assays.

The effect of dilution of culture supernatants (4B2 and rho-1D4) on the binding of antibodies to Triton X-100-solubilized ROS and ROS disk membranes is shown in Fig. 24. The degree of 4B2 antibody binding to ROS was about 2 fold higher than ROS disk membranes, but the degree of rho-1D4 antibody binding was about the same for both the ROS and ROS disk membranes.

The degree of inhibition of rho-1D4 and 4B2 antibodies
TABLE IV. Electrotransfer of \(^{125}\text{I}\)-labeled proteins

<table>
<thead>
<tr>
<th>(^{125}\text{I})-protein</th>
<th>(M_r)</th>
<th>% SDS-polyacrylamide gel</th>
<th>Transfer time (h)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin</td>
<td>200K</td>
<td>6</td>
<td>1-2</td>
</tr>
<tr>
<td>BSA</td>
<td>66K</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>Prolactin</td>
<td>23K</td>
<td>9</td>
<td>-</td>
</tr>
</tbody>
</table>

* The time required for the transfer of
70% of \(^{125}\text{I}\)-labeled proteins
Figure 23. Time courses for the electrotransfer of $^{125}$I-labeled myosin from 6% and 8% SDS-polyacrylamide slab gels. $^{125}$I-labeled myosin was applied to 6% (●) and 8% (▲) SDS-polyacrylamide slab gels and electrophoresis was carried out. Following electrophoresis, gel slices were subjected to electrophoretic transfer to nitrocellulose papers. At different times after electrophoretic transfer, corresponding strips of gel and papers were removed and subjected to autoradiography. The polypeptide bands detected by autoradiography were excised and counted in a Beckman 8000 gamma counter.
Figure 24. Titration of rho-1D4 (upper) and 4B2 (lower) hybridoma cell culture supernatants against Triton X-100-solubilized ROS membranes (●) and ROS disk membranes (○). Solubilized ROS and ROS disk membranes immobilized in microtiter wells were treated with serial dilutions of culture supernatant, rinsed, and subsequently treated with $^{125}$I-labeled goat anti-mouse Ig.
binding to Triton X-100-solubilized ROS membranes by Triton X-100-solubilized ROS and ROS disk membranes is shown in Fig. 25. Half-maximum inhibition of rho-1D4 antibody binding occurred at 0.5 μg/ml for both ROS and ROS disk membranes, but half-maximum inhibition of 4B2 antibody binding occurred at 60 μg/ml for ROS and at 110 μg/ml for ROS disk membranes.

13. Degradation and aggregation of ROS 1.2

Molecular properties of ROS 1.2 were observed in some radioimmune labeling studies. Heat-treated ROS membrane proteins were electrophoretically transferred to CNBr-activated paper following gel electrophoresis. CNBr-activated paper with transferred ROS proteins were sequentially labeled with 4B2 antibody and ¹²⁵I-labeled goat anti-mouse Ig is shown in Fig. 26. Only a 140,000 Mᵣ polypeptide was labeled.

ROS membrane proteins prepared in the presence of leupeptin (an inhibitor of Ca²⁺-activated proteases) were electrophoretically transferred to nitrocellulose paper following gel electrophoresis. Nitrocellulose paper with transferred ROS proteins were sequentially labeled with 4B2 antibody and ¹²⁵I-labeled goat anti-mouse Ig is shown in Fig. 27. ROS 1.2 and two higher Mᵣ polypeptides were labeled.
Figure 25. Inhibition of rho-1D4 antibody (upper) and 4B2 antibody (lower) binding to Triton X-100-solubilized ROS membranes by Triton X-100-solubilized ROS membranes (●) and ROS disk membranes (○) using the solid-phase indirect radioimmune competition assay as described previously.
Figure 26. Radioimmune labeling of heat-treated ROS with 4B2 antibody. ROS membrane preparation (8 mg/ml) was diluted with equal volume of sample cocktail containing 5% SDS, 40% sucrose, 10mM Tris, pH 6.8, 10% β-mercaptoethanol, and 4% bromophenol blue, and heated at 80°C for 5 min. Sample was applied to a 8% SDS-polyacrylamide slab gel and subjected to gel electrophoresis. Following gel electrophoresis, proteins were electrophoretically transferred to CNBr-activated papers. CNBr-activated papers with transferred proteins was labeled with 4B2 (a) and non-specific (b) culture supernatants, followed by $^{125}$I-labeled goat anti-mouse Ig.
Figure 27. Radioimmune labeling of ROS prepared in the presence of leupeptin with 4B2 antibody. ROS membranes (8 mg/ml) prepared in the presence of leupeptin, an inhibitor of Ca$^{2+}$-activated proteases, were mixed with equal volume of sample cocktail containing 5% SDS, 40% sucrose, 10mM Tris, pH 6.8, 10% β-mercaptoethanol, and 4% bromophenol blue and applied to a 8% SDS-polyacrylamide slab gel. Following gel electrophoresis, the proteins were electrophoretically transferred to nitrocellulose paper. Nitrocellulose paper containing transferred proteins was sequentially treated with 4B2 culture supernatant and $^{125}$I-labeled goat anti-mouse Ig.
14. **Purification of ROS 1.2 by gel filtration chromatography on a Sephacryl-S-200 column**

ROS 1.2 was partially purified by gel filtration chromatography on a Sephacryl-S-200 column. SDS-polyacrylamide gels of elutants in the void volume and in the included volume of the column were shown in Fig. 28. Elutants in the void volume contained ROS 1.2, other ROS minor proteins, and a residual amount of rhodopsin whereas elutants in the included volume contained mainly rhodopsin.

15. **Purification of ROS 1.2 by affinity chromatography on a 4B2 antibody-agarose column**

ROS 1.2 was partially purified by affinity chromatography on a 4B2 antibody-agarose column. As shown in Fig. 29, the washed fractions contained no ROS 1.2 and specifically-eluted fractions contained ROS 1.2 and a small amount of rhodopsin.
Figure 28. Chromatography of ROS proteins on a Sephacryl-S-200 column. ROS membrane preparation (4.5 mg protein) was solubilized with 3% SDS, and applied to a Sephacryl-S-200 column (2.0x48.0 cm) pre-equilibrated with PBS containing 0.1% SDS and 1mM EDTA. Void volume of the column was determined by the elution volume of Blue Dextran. Protein compositions of a fraction in the void volume (lane 2) and two fractions in the included volume (lanes 3 and 4) were analyzed by gel electrophoresis on a 8% SDS-polyacrylamide slab gel. Lane 1 contained SDS-treated ROS proteins not applied to the column. ROS 1.2 and rhodopsin are indicated.
**Figure 29. Chromatography of ROS proteins on a 4B2 antibody-agarose column.** ROS membrane preparation (4.5 mg protein) was solubilized with 1% CTAB in 0.1M sodium phosphate buffer, pH 7.4, and applied to a 2 ml 4B2 antibody-agarose column (0.5 mg antibody/ml column) pre-equilibrated with the 0.1M sodium phosphate buffer, pH 7.4, containing 10mM CTAB. The column was washed with 8 ml of the same buffer containing 10 mM CTAB and eluted with 50mM diethylamine, pH 11.5, in the same buffer containing 10 mM CTAB. Protein compositions of the buffer-washed fractions (lanes 2 and 3) and the diethylamine-eluted fractions (lanes 4 and 5) were determined by gel electrophoresis on a 8% SDS-polyacrylamide slab gel. Lane 1 contained standard proteins: phosphorylase b (92K), BSA (66K), ovalbumin (45K), carbonic anhydrase (31K), soybean trypsin inhibitor (21K), and lysozyme (14K).
DISCUSSION

The method of ascites tumors induction in mice for the production of 4B2 monoclonal antibody was found to be easier and more economical than the mass culture method. Also, ascites fluid contained a much higher concentration of 4B2 antibody than culture supernatant as determined by solid-phase RIA, and in contrast to culture supernatant it contained no nonspecific IgG molecules detectable by SDS-polyacrylamide gel electrophoresis. However, ascites tumors induction was not always successful and the volume of ascites fluid obtained from a tumor-bearing mouse varied considerably.

The monoclonal characteristic of 4B2 antibody was supported by the fact that recloning of 4B2 hybridoma cell line did not change the specificity of the 4B2 antibody, as indicated by radioimmune labeling of ROS proteins with 4B2 antibody secreted by recloned 4B2 hybridoma cell line. The identification of Ig subtype of 4B2 antibody was necessary for its use in biochemical studies and its purification. The 4B2 antibody was identified, by solid-phase RIA and SDS-polyacrylamide gel electrophoretic analysis, to be an IgM molecule with \( \mu \) heavy chains of apparent mass \( M_r = 55,000 \) and \( k \) light chains of apparent mass \( M_r = 22,000 \).

The 4B2 antibody was partially purified from ascites fluid by DEAE-Sephacel ion exchange column chromatography. The partially-purified 4B2 antibody was shown by RIA to be active
against ROS membranes. However, the concentration of partially-purified 4B2 antibody required for half-maximum antibody binding to ROS membranes was 4 fold higher than that of the original 4B2 ascites fluid. This indicated that some of the 4B2 antibodies was denatured during the purification process. Subsequently, the two purification steps likely to cause denaturation of 4B2 antibody, lyophilization and ammonium sulfate precipitation, were eliminated from the purification procedure. In addition, gel filtration chromatography on a Sephacryl-S-500 column and ion exchange chromatography on a DEAE-Affi-Gel-Blue column were used to purify the 4B2 antibody from ascites fluid.

Immunologically active 4B2 monoclonal antibody was obtained by ion exchange chromatography on a DEAE-Sephacel or a DEAE-Affi-Gel-Blue column, and gel exclusion chromatography on a Sephacryl-S-500 column. As determined by SDS-polyacrylamide gel electrophoresis, 4B2 antibody of higher purity was obtained using the former method, but the latter method provided the separation of pentameric and dissociated forms of 4B2 antibody. The difference between DEAE-Sephacel and DEAE-Affi-Gel-Blue media was that a polypeptide of apparent $M_r = 85,000$ always copurified with 4B2 antibody, only when the former medium was used. The identity of this polypeptide was not determined. It may be transferrin, since transferrin is a serum protein of similar $M_r$, and has been found to copurify with Ig molecules (68). The advantage of using DEAE-Sephacel over
DEAE-Affi-Gel-Blue medium for the purification of 4B2 antibody is the high reproducibility of the results. The yield of 4B2 antibody from 4B2 ascites fluid was much lower than the yield of rho-lD4 antibody from equal volume of rho-lD4 ascites fluid. The difference may be due to the different intrinsic properties of IgM-secreting hybridoma (4B2) and IgG-secreting hybridoma (rho-lD4) cell lines.

A 4B2 antibody-agarose affinity column and a Sephacryl-S-500 gel filtration column were only able to purify ROS 1.2 to 30-40% homogeneity, as determined by SDS-polyacrylamide gel electrophoretic analysis. Whether the coelution of a residual amount of rhodopsin and ROS 1.2 was due to the incomplete solubilization of ROS membranes or to the strong protein-protein interactions between ROS 1.2 and rhodopsin remains to be determined. Since ROS 1.2 was not purified to homogeneity, amino acid composition of ROS 1.2 could not be determined.

The molecular properties of ROS 1.2 were probed by using the 4B2 antibody to determine whether cross-reactivity exists between ROS 1.2 and some other large proteins in cell types other than the rod cell, namely, myosin in muscle, spectrin in red blood cells, and brain spectrin in brain cells. These proteins were tested for cross-reactivity with the 4B2 antibody because they not only have similar $M_r$, but also have similar structural and functional properties. For example, all three proteins interact with actin, a cytoskeletal element, found
present in ROS membrane preparations by RIA using an anti-actin monoclonal antibody. Solid-phase RIA and competition assays have shown that 4B2 antibody cross-reacted with rabbit skeletal muscle myosin, human red blood cell membranes, and bovine brain homogenate to differing degrees. Immunoblot analysis has shown that myosin and a $M_r = 160,000$ polypeptide in bovine brain homogenate cross-react with the 4B2 antibody. This analysis, however, failed to demonstrate which polypeptide in red blood cell membranes cross-react with the 4B2 antibody. The identity of the $M_r = 160,000$ polypeptide in bovine brain homogenate has not been determined, but it is believed to be the proteolytic breakdown product of brain spectrin. Burridge et al. (60) have found that partial digestion of native brain spectrin with Staphylococcal V8 protease generated a polypeptide with an apparent $M_r = 160,000$. This polypeptide cross-reacted with anti-brain spectrin antibodies. The immunological cross-reactivity of native brain spectrin and red blood cell spectrin with the 4B2 antibody was not detected by immunoblot analysis. This may be due to the inefficient electrophoretic transfer of these large proteins and/or their weak cross-reactivities with the 4B2 antibody. The $\alpha$ subunits in both proteins have $M_r = 240,000$. The $\beta$ subunit of brain spectrin has $M_r = 235,000$ and $\beta$ subunit of red blood cell spectrin has $M_r = 220,000$ (69). As shown in the study on the electrotransfer of $^{125}$I-labeled proteins, the electrophoretic transfer of small polypeptides such as BSA and
prolactin, with apparent $M_r = 66,000$ and 23,000 respectively, was completed within 30 min whereas large polypeptides such as myosin, with an apparent $M_r = 200,000$ required 18-20h. Thus the amount of erythrocyte and brain spectrins transferred to nitrocellulose papers might have been less than adequate for immunoblot detection of cross-reactivities of these proteins with the 4B2 antibody. Their weak cross-reactivities with the 4B2 antibody are due to the different species and cell type origins of these 4B2 antigens. This is not surprising since initial immunological evidence presented was against the existence of spectrin in other cell types (68), yet recent findings support the idea that erythrocyte spectrin, brain spectrin, and its immunologically related equivalents in other cell types all belong to a family of related proteins called the spectrins (60). Whether ROS 1.2 belong to the spectrin family remains to be determined.

The role of spectrin in the submembranous cytoskeleton of mammalian erythrocytes is to mediate the coupling of actin filaments to the plasma membrane and to maintain the shape and deformability of the cell. A parallel function of actin attachment to membranes in other cells containing nonerythroid spectrin is supported by the localization of nonerythroid spectrin close to the plasma membrane of many cell types (71). Similarly, ROS 1.2 is localized close to the ROS plasma membrane over the entire
length of the outer segment (72). Thus, the role of ROS 1.2 is suggested to be similar to that of spectrin in maintaining cell shape. The maintenance of ROS structure must be accomplished by two types of connections: disk-to-disk connections, since it has been known that disks do hold together even in the absence of the plasma membrane (73), disk incisures are somehow kept in precise register over the entire length of the ROS, and the disks are separated from each other by a constant 100 Å space along the rod axis (74); and disk-to-plasma membrane connections, since disks adhered strongly to the plasma membrane when rods are disrupted (73). Roof & Heuser (74) have indicated that filamentlike specializations associated exclusively with disk rims are responsible for connecting disk rims to each other and to plasma membrane. The molecular nature of these filaments is unknown. However, it has been suggested to be the 290,000 dalton "rim protein" in frog ROS described by Papermaster et al. (75) for two reasons. The location of rim protein and filaments is similarly confined to rims and incisures, and the size of rim protein and filaments is comparable. ROS 1.2 is proposed to be the "rim protein" in bovine ROS because it is slightly smaller than the rim protein in frog ROS, it extends some distance from the disk membrane (57), and it is localized in disk rims (72). The structural role of ROS 1.2 in linking disks to disks and disks to plasma membrane is schematically illustrated in Fig. 30.
Figure 30. Proposed structural role of ROS 1.2. ROS 1.2 is a myosin-like protein localized in the rim regions of disks and in conjunction with other cytoskeletal elements serves to link disks to each other and to plasma membrane for the maintenance of the highly-ordered ROS structure.
Radioimmune labeling of heat-treated ROS membranes and ROS membranes prepared in the presence of leupeptin, with the 4B2 antibody revealed two properties of ROS 1.2: ROS 1.2 can be degraded to produce a $M_r=140,000$ fragment that retains 4B2 antigenic site and ROS 1.2 is able to aggregate with each other to form multimers that retain 4B2 antigenic site. Although no control studies were done concomitantly, the degradation of ROS 1.2 was likely induced by heat since similar intense labeling of the $M_r=140,000$ polypeptide was not detected in many radioimmune labeling of ROS membranes experiments. The aggregation of ROS 1.2 was likely induced by the presence of leupeptin during the preparation of ROS membranes since no labeling of polypeptides with $M_r$ greater than ROS 1.2 has ever been detected previously using ROS membranes prepared in the absence of leupeptin. In this regard, it should be noted that leupeptin has been used to show the accumulation of neural filaments in axon terminals (76), because it inhibits Ca$^{2+}$-activated proteases which are responsible for the disaggregation of neural filaments. However, Ca$^{2+}$-activated proteases have not been found in ROS.

The ability of ROS 1.2 to self-associate may be important for its localization in disk rims, because large aggregates of ROS 1.2 can restrict its mobility in highly fluid disk membranes. The degradation of ROS 1.2 may be important in the disk renewal process. The disruption of disk-to-disk and/or disk-to-plasma
membrane connections is most likely an early event in the renewal process. Proteolytic degradation of ROS 1.2 is a way of disrupting these connections. Also, if the degraded product of ROS 1.2 is released into the cytoplasmic space, and retains 4B2 antigenic site, then there would be a cytoplasmic pool of 4B2 antigen. A cytoplasmic pool of 4B2 antigen is suggested by a 2-fold difference in the concentration of 4B2 antigen between ROS and ROS disk membranes, as indicated by RIA and radioimmune competition assays. In contrast, there was no difference in the concentration of rhodopsin between ROS and ROS disk membranes.

These findings lead to a working model on the structural and functional roles of ROS 1.2 in bovine ROS. In this model, ROS 1.2 self-associate to form disk rim regions of high curvature during disk assembly, and in conjunction with cytoskeletal elements such as actin serves to anchor disk rims in close proximity of the plasma membrane. In addition, protein-protein interactions between ROS 1.2 of adjacent disks serve to separate disks 100 Å apart. The disk-to-disk and disk-to-plasma membrane connections are removed during disk renewal by the proteolytic degradation of ROS 1.2. The activation of protease specific for ROS 1.2 may be dependent on the extent of photobleaching of disks, since only 8-10 disks are shed at a time. The existence of cross-reactivity between ROS 1.2 with myosin and polypeptides in brain homogenate and red blood cell membranes indicates that ROS 1.2 belongs to a
family of immunologically related proteins, perhaps the spectrin family, because of their similar $M_r$ and location close to the plasma membrane. In this view, it is of interest to note that members of the spectrin family have been found, in many cell types, to be high molecular weight actin- and calmodulin-binding proteins which are both structurally and functionally homologous to erythrocyte spectrin (77). Since actin and calmodulin have been found to be present in bovine ROS, ROS 1.2 is a potential candidate for binding actin and calmodulin. In mammalian erythrocytes, spectrin binds to other cytoskeletal proteins including actin filaments, Band 2.1 (ankyrin, syndeins) and Band 4.1, and through the latter two proteins, it is attached to the cytoplasmic surface of the erythrocyte membrane. Spectrin binds calmodulin in a $Ca^{2+}$-dependent manner, but it remains unknown as to whether spectrin-calmodulin interaction affects the binding of spectrin to actin, Band 2.1, and Band 4.1. It will be interesting to see if an analogous cytoskeletal network exists in bovine ROS since the role of $Ca^{2+}$ ions appears to be important in the event of visual excitation in ROS.

Thus in future work, the localization of cytoskeletal molecules close to the ROS plasma membrane and the determination of the function of ROS 1.2 may provide new insights into the mechanisms of the maintenance of the highly ordered ROS structure, disk renewal, and visual excitation. Future research will be directed toward 1) obtaining an IgG anti-ROS 1.2
monoclonal antibody in order to obtain a larger quantity of immunologically active antibody from ascites fluid, 2) improving the purification of ROS 1.2 by using a combination of gel filtration chromatography on a Sephacryl-S-200 column (since rhodopsin can be partially separated from ROS 1.2 using this method) and affinity chromatography on an anti-ROS 1.2 antibody column (since other minor proteins can be separated from ROS 1.2 using this method), and 3) investigating the structure and function of ROS 1.2 by amino acid sequencing and reconstitution studies.
REFERENCES


72. Molday, R.S., Hicks, D., and Wong, S. (manuscript in preparation).


