MOLECULAR CLONING, LOCALIZATION, AND HETEROLOGOUS EXPRESSION
OF BOVINE ROM-1, A PHOTORECEPTOR DISC RIM SPECIFIC PROTEIN

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ABSTRACT

Rom-1 is an integral membrane protein found in mammalian rod photoreceptor disc membranes. Much interest in rom-1 has been generated by its structural similarity to peripherin/rds, a protein implicated in a number of inherited retinal degenerative disorders. Defects in the ROM1 gene have also been associated with a digenic form of retinal degeneration, in which changes in the sequence of both rom-1 and peripherin/rds are necessary to produce the retinal degeneration phenotype.

As an essential step in studying structure-function relationships of rom-1 in photoreceptor membranes, the cDNA encoding the bovine homolog of rom-1 was cloned and sequenced. The coding sequence was obtained from a cDNA library, while upstream and promoter sequences were obtained by inverse polymerase chain reaction. The sequence is 84% identical to human rom-1 and 32% identical to bovine peripherin/rds at the amino acid level. The predicted membrane topology of bovine rom-1 and a number of short motifs found within the sequence indicate that bovine rom-1 is a member of the transmembrane 4 super family (TM4SF) group of proteins.

Short segments of bovine rom-1 were expressed in E. coli as fusion proteins, which were then used as immunogens to generate monoclonal and polyclonal antibodies to specific regions of bovine rom-1. The antibodies were used as probes to study the membrane topology, subcellular distribution and interaction of rom-1 with peripherin/rds. The results indicate that rom-1 and peripherin/rds are subunits of a membrane protein complex that is localized to the rim regions of photoreceptor outer segment disc membranes. In contrast to previous reports, rom-1 was detected in cone photoreceptors as well as rod photoreceptors. These results are discussed in terms of the role of rom-1 in disc structure and retinal degenerative diseases.

Bovine rom-1 was heterologously expressed in COS-1 cells. The heterologously expressed protein was characterized using immunocytochemistry techniques.
Heterologously expressed rom-1 is similar to rom-1 from ROS membranes in terms of glycosylation and subunit interactions with itself and with peripherin/rds. A number of mutant forms of rom-1 were also examined using the heterologous expression system in order to determine whether intracellular distribution or subunit interactions are altered relative to wild-type rom-1. Truncation of rom-1 after the fourth transmembrane domain was found to increase expression levels and did not affect interaction with peripherin/rds. The mutation romL188P was found to alter the quaternary structure of rom-1, as determined by velocity sedimentation.

These studies provide valuable new information concerning the interaction of rom-1 and peripherin/rds subunits in photoreceptor outer segment membranes. Additionally, a system is described for studying the interaction of wild-type and mutant forms of these subunits heterologously expressed in COS-1 cells. Finally, the discovery that rom-1 is present in cone photoreceptors as well as rod photoreceptors proves that the cone-dominant and rod-dominant phenotypes associated with different RDS mutations are not due to absence of the rom-1 subunit in cone photoreceptors.
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LIST OF ABBREVIATIONS

ADRP........... autosomal dominant retinitis pigmentosa
ARMD........... age related macular degeneration
ATP.............. adenosine 5'-triphosphate
BCA............. bicinchoninic acid
BME.............. β-mercaptoethanol
BSA.............. bovine serum albumin
bp................ base pairs
cDNA........... DNA reverse-transcribed from an mRNA template (coding DNA)
cGMP........... guanosine 3',5'-cyclic monophosphate
CHAPS......... 3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CNBr............ cyanogen bromide
CNG............. cyclic nucleotide-gated
DEAE........... diethyl aminoethyl
DMEM......... Dulbecco's modified Eagle medium
DNA............. deoxyribonucleic acid
dNTP........... deoxyribonucleoside tri-phosphate
DSP............. dithiobis (succinimidyl propionate)
DTT............. dithiothreitol
ECL............. enhanced chemiluminescence
EDTA.......... ethylenediamine tetraacetic acid
ERG.............. electroretinogram
FBS............. fetal bovine serum
FITC........... fluorescein isothiocyanate
GCAP........... guanylate cyclase activating protein
GCL.............. ganglion cell layer
GDP ........ guanosine 3'-diphosphate
GTP ........ guanosine 3'-triphosphate
HAT .......... IMDM media supplemented with hypoxanthine, aminopterin and thymidine
HBS .......... hepes buffered saline
HBSG ........ glucose supplemented Hepes buffered saline
HEPES ...... N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HT .......... IMDM media supplemented with hypoxanthine and aminopterin
Ig .......... immunoglobulin
IMDM .......... Iscove's modified Dulbecco's medium
INL .......... inner nuclear layer
IPL .......... inner plexiform layer
kb .......... kilobase
kDa .......... kilodalton
mRNA .......... messenger RNA
ONL .......... outer nuclear layer
OPL .......... outer plexiform layer
pA .......... picoAmpere
PAGE .......... poly acrylamide gel electrophoresis
PBS .......... phosphate buffered saline
PCR .......... polymerase chain reaction
PDE .......... phosphodiesterase
PMSF .......... phenyl methyl sulfonyl fluoride
rd .......... retinal degeneration
rds .......... retinal degeneration slow
RNA .......... ribonucleic acid
ROS .......... rod outer segment
RP .......... retinitis pigmentosa
SDS...........sodium dodecyl sulfate
RPE............retinal pigment epithelium
T.................transducin
TM4SF........transmembrane 4 superfamily
Tris...........Tris [hydroxymethyl]aminomethane
TRITC.........tetramethylrhodamine isothiocyanate
TX100.........Triton X-100 detergent
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To:

My teacher: my Father.
INTRODUCTION

1.1 The retina

The processes leading to vision are complex, and in many cases poorly understood. When defects occur in a complex system such as vision, the effects are often obvious, but the causes remain elusive. This thesis is concerned with one small element of the visual system, a protein complex called peripherin/rom-1 found in photoreceptor cells. Its function is unknown, but its absence leads to loss of vision. We have attempted to further characterize this component in order to gain further insight into its role in vision and visual dysfunction.

The structure of the vertebrate eye is illustrated in Fig 1. The eye is surrounded by a hard outer layer known as the sclera. It is filled with clear fluids, the aqueous and vitreous humours. Light enters the eye through the pupil, the opening in the iris diaphragm. The amount of light entering the eye is restricted by the iris, which dilates and contracts in response to varying light levels. Light is then focused by the lens to form an image on the retina. The retina detects the light and transmits the information to the optic nerve.

The retina is a layer of neural tissue located at the back of the eye (Fig 1). It is only a few cell layers thick and very fragile. If one were to compare the eye to a camera, the retina would be the equivalent of the film, the surface on which the image is formed and detected. The retina is far more sophisticated than a piece of film, however. Unlike film, the retina is sensitive to light over a huge range of intensities from starlight to bright sunlight. It is divided into different functional regions adapted for peripheral and central vision. Images formed on the retina are not permanent; the light detection system of the retina continually regenerates itself.

The cell layers of the retina are illustrated in Fig 2. The layer closest to the choroid is the retinal pigment epithelium (RPE). This pigmented layer of cells lies

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FIG. 1. Cross-section of an eyeball. Light enters the eye through the transparent cornea, and is focused by the lens to form an image on the retina. Photoreceptor cells in the retina detect the light, and the information is transmitted via the optic nerve to the brain. Other features of the eyeball include the hard outer sclera, and the vascular layer known as the choroid. The pigment epithelium lies between the choroid and the retina, and is closely associated with the photoreceptor cells of the retina. Figure reproduced from Dosé, 1996.
FIG. 2. The cell layers of the retina. This schematic illustrates the spatial relationships of the cells of the retina. Beginning at the outermost layer of the retina (closest to the sclera), the first layer is composed of rod and cone photoreceptor outer segments (OS) and inner segments (IS). The outer nuclear layer (ONL) is composed of the photoreceptor nuclei. The outer plexiform layer (OPL) is the site of synapse formation between the photoreceptors and the cells of the inner nuclear layer (INL), which are primarily bipolar cells, amacrine cells, and horizontal cells. The inner plexiform layer (IPL) contains synapses between the cells of the INL and the ganglion cell layer (GCL). Ganglion cell axons form the optic nerve. The labeled cells are rod (R) and cone (C) photoreceptors, midget (MB), rod (RB) and flat (FB) bipolar cells, amacrine cells (A), horizontal cells (H), midget (MG) and diffuse (DG) ganglion cells. (Modified from Dowling and Boycott, 1966)
adjacent to the layer of photoreceptor neurons. There are two types of photoreceptor neurons, rods and cones, which are responsible for detection of dim and bright light respectively. Additionally, there are three subtypes of cones in the human retina, each sensitive to different wavelengths of light, thereby permitting color vision.

Photoreceptor cell bodies make up the outer nuclear layer (ONL). Beyond the ONL lie two other layers of nuclei. The first is the inner nuclear layer (INL), and is made up of bipolar cells, amacrine cells, and horizontal cells. Between the INL and ONL is a synaptic layer known as the outer plexiform layer. The INL is followed by a second synaptic layer, the inner plexiform layer (IPL) and a third row of nuclei, the ganglion cell layer (GCL). Ganglion cell axons form the optic nerve, which leads to the brain. Many subtypes of each set of interneuron are defined by their morphology and neurotransmitters. In order to reach the photoreceptor cell layer, light must first pass through all of these interceding layers (Reviewed in Kolb, 1994, Masland 1986).

The neural circuitry of the retina includes both vertical and lateral pathways. Specialized circuits exist for detecting various visual phenomena, including dim light, dark/light contrast, movement and color contrast. As a general rule, photoreceptor neurons synapse with bipolar cells. In mammalian retinas, cone bipolar cells are functionally distinct from rod bipolar cells, and synapse directly with ganglion cells. Rod bipolar cells synapse with amacrine cells, which in turn synapse with cone bipolar cells, integrating the circuits of the two photoreceptor types. Lateral pathways which are inhibitory, modulatory, or divergent involve the horizontal cells, interplexiform cells, and amacrine cells. The cells of the INL integrate the signals from photoreceptor cells; a single ganglion cell can receive input from up to 75,000 rod photoreceptors, and a single rod photoreceptor can be involved in circuits involving as many as eight cone bipolar cells.(Kolb, 1994)
1.2 The photoreceptor cell

Rod and cone photoreceptors are specialized neurons adapted for light detection. Each photoreceptor has a synaptic terminus, a cell body, an inner segment and an outer segment (Fig 3). Rod and cone photoreceptors are named for the shape of their outer segments, specialized organelles peculiar to the photoreceptor cell where the process of visual transduction occurs. The outer segment is joined to the inner segment by a thin cilium junction. The inner segment is rich in mitochondria and is the site of protein synthesis, while the cell body contains the nucleus, and the synaptic terminus allows the cell to transmit signals to other neurons. The neurotransmitter released by photoreceptors is glutamate (Reviewed in Massey, 1995).

In most mammalian retinas the number of rod photoreceptors greatly exceeds the number of cone photoreceptors. In human retina, the ratio is 20:1 (Osterberg 1935). Biochemical investigations typically utilize the bovine retina, since large quantities of tissue can be obtained from slaughter houses. As a consequence, the biochemistry of the visual transduction process is better understood in rods than in cones.

The photoreceptor outer segment consists of a stack of thousands of membranous discs surrounded by a plasma membrane (Fig 3). The discs are oriented perpendicular to the incident light. Rod photoreceptor discs are completely sealed, whereas cone photoreceptor discs are continuous with the plasma membrane. The discs contain the photosensitive opsin pigments, rhodopsin in rods and cone opsins in cones. New discs are continuously formed at the base of the outer segment; old discs are shed at the apex and are phagocytized by the cells of the RPE.

The photoreceptor discs have a rim region that is distinguished from the central lamellar region by a number of properties. The disc rim is thicker than the lamellar region, and fibrous structures that may represent disc-disc or disc-plasma membrane connections are present (Roof and Heuser 1982, Sjostrand and Kreman 1978).
FIG. 3. The photoreceptor cell. The two major classes of photoreceptors, rods and cones, are compared in a schematic diagram. Note that the disc membranes of the cone photoreceptor are continuous with the plasma membrane, while in the rod photoreceptor, the disc membrane is separate and distinct from the plasma membrane. Bovine cone photoreceptors can be distinguished from rods under a light microscope by the shape of their inner segments and by their shorter, cone shaped outer segments. Photoreceptor cells are not drawn to scale. Modified from Dose, 1996.
number of proteins have been identified that are exclusively localized to the disc rim, including peripherin/rds, rom-1, and a 290 kDa protein. (Molday et al., 1987a; Bascom et al., 1992; Papermaster et al., 1979).

Purified preparations of rod outer segments (ROS) are relatively easy to obtain. The bovine retina can easily be dissected from the eyecup by pulling it away with tweezers. The retina separates between the RPE and the photoreceptor layer, leaving the photoreceptor outer segments exposed. Because the cilium junction is very fragile, rod outer segments can easily be isolated from the retina by gentle agitation followed by sucrose gradient ultra-centrifugation. Such preparations of rod outer segments (ROS) are the starting material for most biochemical characterizations of the visual transduction process (Papermaster and Dreyer, 1974).

Analysis of ROS by SDS-PAGE reveals that the protein composition of these organelles is fairly simple relative to membrane extracts of whole cells. The major protein present in the ROS is the visual pigment rhodopsin, which makes up approximately 90% of the photoreceptor membrane protein. Significant quantities of other proteins are present. A number of these proteins have been characterized, including the transducin subunits, the cyclic nucleotide gated channel subunits, the phosphodiesterase subunits, calmodulin, arrestin, the Na\(^+/\)Ca\(^{++}\) exchanger, and glucose-3-phosphate dehydrogenase (Molday and Molday, 1993). However, many proteins have yet to be characterized, and remain anonymous bands on gels.

1.3 Visual transduction

The cascade of reactions known as visual transduction is illustrated in Fig 4. In rod photoreceptors, the cascade (reviewed by Stryer, 1986) begins with the interaction of a photon with the 11-cis-retinal chromophore of a rhodopsin molecule. The retinal chromophore undergoes a series of isomerizations, eventually resulting in conversion to
FIG. 4. The phototransduction cascade. This schematic illustrates the cascade of reactions beginning with the photoactivation of rhodopsin and ending with the hydrolysis of cGMP and the closure of cyclic nucleotide gated Na\(^+\) channels (CNG). Activation of rhodopsin, transducin and phosphodiesterase are each illustrated. Other abbreviations: Ar-arrestin, RK-rhodopsin kinase, G.C. guanylate cyclase, PDE-phosphodiesterase, GCAP-guanylate cyclase activating protein, T-transducin. Figure modified from Dose, 1996.
all-trans-retinal and dissociation of the retinal chromophore from the opsin. The activated form of rhodopsin (R*) is an intermediate, metarhodopsin II, that is formed approximately 1 millisecond after interaction with a photon (Hagins, 1956). R* interacts with the G-protein transducin (Emeis et al., 1982). Upon interaction with R*, transducin exchanges bound GDP for GTP, generating the activated form of the protein (Stryer et al., 1981). One R* molecule can activate up to 37,000 molecules of transducin, (70% of the activatable transducin present on a disc face, (Gray-Keller et al., 1990)) before being inactivated by rhodopsin kinase catalyzed phosphorylation and binding of the protein arrestin, also known as S-antigen (Wilden et al., 1986). Rhodopsin is then recycled. The retinal chromophore is reduced to retinol by retinol dehydrogenase, resulting in the release of arrestin and retinol (Hofman et al., 1992). The phosphates are removed by protein phosphatase 2A (Palczewski et al., 1989), and the opsin is regenerated by binding of another retinal chromophore.

Transducin is composed of three subunits called Tα, Tβ and Tγ. Upon exchanging GDP for GTP, the α subunit dissociates from the βγ complex (Fung et al., 1981). Tα•GTP activates the enzyme phosphodiesterase (PDE), which catalyzes the hydrolysis of cGMP to GMP (Fung et al., 1981). There is no amplification of the cascade at this step. Tα is eventually inactivated by conversion of the bound GTP molecule to GDP, a reaction that is catalyzed by the inherent GTPase activity of transducin (Fung and Stryer, 1980). This GTPase activity is stimulated by PDE (Pages et al., 1993).

Phosphodiesterase is also a multi-subunit protein complex, composed of two catalytic subunits called PDEα and PDEβ, and two inhibitory subunits called PDEγ (Deterre et al., 1988). Activated Tα binds PDE, thereby neutralizing the inhibitory effects of PDEγ on the catalytic subunits (Hurley and Stryer, 1982). Localized concentrations of activated transducin are thought to exceed localized quantities of PDE, resulting in activation of virtually all available PDE (Gray-Keller et al., 1990). PDE can catalyze the hydrolysis of hundreds of cGMP molecules before Tα is inactivated,
resulting in further amplification of the cascade. Activation of a single rhodopsin molecule will result in hydrolysis of $4 \times 10^5$ molecules of cGMP per second (Yee and Liebman, 1978).

The final step in the cascade is the closure of cation channels located in the plasma membrane. The channels are gated by cGMP, and a decrease in cGMP concentration results in the closure of these channels (Fesenko et al., 1985; Yau and Nakatani, 1985). This produces an interruption in the dark current, the flow of ions which keeps the resting photoreceptor in a depolarized state (Fig 5). The resulting hyperpolarization of the cell leads to a decreased neurotransmitter release from the synaptic terminus, thereby communicating a neurological signal. Activation of a single rhodopsin molecule produces a decrease of 1 pA in the dark current, which normally rests at 25 pA (Baylor et al., 1979). The rod photoreceptor is therefore capable of responding with an electrical signal to interaction with a single photon.

Many of these steps of phototransduction and additional recovery steps are regulated by intracellular calcium levels (reviewed by Koch, 1995). Because the cGMP gated channel also conducts calcium ions, closure results in a decreased rate of calcium entry into the outer segment. Coupled with a steady efflux of calcium by a Na$^+$/Ca$^{++}$ exchanger, this decreases intracellular calcium. Calcium binding proteins regulate a number of key sites in the phototransduction cascade. Low levels of intracellular calcium increase the activity of guanylate cyclase, an effect mediated by calcium binding proteins known as GCAPs (guanylate cyclase activating proteins) (Gorczyca et al., 1995; Dizhoor et al., 1995). Decreased intracellular calcium also increases the apparent affinity of the cGMP gated channel for cGMP, an effect mediated by calmodulin (Hsu and Molday, 1993). Additionally, high intracellular calcium inhibits the activity of rhodopsin kinase through the calcium binding protein recoverin (Kawamura, 1993). These regulatory steps in part account for the wide range of light levels to which photoreceptors are sensitive.
FIG. 5. **The dark current.** This schematic illustrates the dark current, the current across the outer segment plasma membrane of a non-stimulated photoreceptor cell. The magnitude of the current in a single photoreceptor is about 25 pA, and primarily consists of sodium ions entering through a cGMP-gated cation channel, which also conducts calcium ions. Calcium is simultaneously conducted out of the photoreceptor by a sodium/calcium exchanger. Stimulation of the photoreceptor with light results in hydrolysis of cGMP and closure of the channels, interrupting the dark current. The sodium/calcium exchanger remains active, resulting in a decrease in intracellular calcium levels. Figure reproduced from Dose, 1996.
Phototransduction in cones follows a similar cascade of reactions, but many of the proteins of the cascade have cone specific isoforms. The three subclasses of cones in the human retina, red, green and blue, each contain an opsin sensitive to different wavelengths (Wald, 1967). Additionally, cone specific forms of transducin, PDE and the cGMP gated channel have also been identified (Lerea et al., 1986; Hurwitz et al., 1985; Bönigk et al., 1993). Cones do not respond to the extremely low levels of light that rods are capable of responding to, and cones recover much faster, though the mechanisms involved are not clearly understood.

1.4 Retinal degeneration

Retinal degeneration is a condition that is largely self-descriptive-- the retina degenerates, losing cell layers and becoming non-functional. Degenerative disorders of the retina affect thousands of people, but remain poorly understood. Few treatments are available. Retinal degeneration can be spontaneous, hereditary, or can be caused by trauma such as retinal detachment. The most common forms of retinal degeneration in humans are macular degeneration and retinitis pigmentosa.

1.4.1 Clinical features. Retinitis pigmentosa (RP) affects approximately 1 in 3700 people (Boughman et al., 1980). RP is a group of diseases that cause progressive retinal degeneration, and loss of vision (reviewed by Berson, 1993). There are many forms of RP; some are hereditary, some seem to occur spontaneously. However, most cases are believed to involve some genetic component. The various forms of RP are grouped according to their hereditary patterns and clinical descriptions of symptoms. Much study of retinal degeneration has focused on hereditary forms of RP, because identification of the underlying defect of these diseases may provide clues for treatment, and because animal models with hereditary retinal degeneration are available.
Typical retinitis pigmentosa is clinically identified by a number of criteria. These include night blindness early in life, depigmentation of the RPE, deposition of pigment in the retina, and narrowing of the retinal blood vessels. The patient begins to experience loss of visual field at twenty to thirty years of age, which progresses until the patient is completely blind at age sixty to seventy. Electrical recordings of the retinal response to light flashes made using contact lens electrodes (known as electroretinograms or ERGs), are generally abnormal in early stages of RP and show no response in advanced stages (Berson, 1993; Henkenlively, 1988).

There are a large number of classifications of RP. Genetically, RP can be divided into autosomal dominant, autosomal recessive, X-linked recessive, simplex and multiplex forms. The most common form of hereditary RP, autosomal dominant retinitis pigmentosa (ADRP), is often further divided into type 1 RP, characterized by rapid onset, and type 2 RP, which progresses more slowly. ERG recordings can also be used to distinguish between two major forms of RP. ERG recordings made using bright flashes of light on light-adapted eyes record the response of cones, whereas recordings made using dim flashes of light on dark-adapted eyes record the response of rods. These recordings reveal that some cases of RP initially affect the rod response (rod-cone dystrophy) while others initially affect the cone response (cone-rod dystrophy).

Additionally, there are forms of RP with unusual pigmentation patterns (retinitis punctata albescens) and forms of RP associated with other congenital defects such as deafness (Usher's syndrome). No one RP classification dominates the population of RP patients. Most make up less than 10% of total cases, with simplex/multiplex rod-cone degeneration and simplex/multiplex cone-rod degeneration accounting for 12.0 % and 13.5 % of RP cases respectively (Henkenlively, 1988; Daiger et al., 1995).

Macular degeneration is also a heterogeneous group of disorders affecting the retina (reviewed in Daiger et al., 1995; Zhang et al., 1995). Many more people are affected by macular degeneration than by RP. However, a large number of macular
degeneration cases are of a late onset form known as age-related macular degeneration (ARMD), which affects up to 10% of adults over 50 years of age (Bressler et al., 1988). Because of the late onset of the disease, inheritance patterns are difficult to establish; parents of patients are often deceased, and their children are often too young to express the disease phenotype. Macular degeneration begins with loss of central vision, resulting from degeneration of the macular (primarily cone) photoreceptors and associated layers of the retina. Age related macular degeneration largely follows an autosomal dominant inheritance pattern. Other, rarer forms of macular degeneration which strike earlier in life include Stargardt's macular dystrophy, fundus flavimaculatus, butterfly-shaped pattern dystrophy, and Best's vitelliform macular dystrophy. These diseases are distinguished by inheritance pattern, age of onset, and appearance of the degenerating retina (reviewed in Zhang et al., 1995; Daiger et al., 1995; Bressler et al., 1988).

1.4.2 Animal models. Because of the scarcity of tissue samples from human patients with retinal degeneration, particularly tissue samples from patients in the early stages of disease, animal models have proven to be extremely valuable in retinal degeneration research. A number of animal models exist, including the \textit{rd} (retinal degeneration) mouse, the \textit{rds} (retinal degeneration slow) mouse, the RCS (royal college of surgeons) rat, the \textit{rcdl} (rod-cone dysplasia 1) Irish setter, the \textit{rcd2} collie, the \textit{prcd} (progressive rod-cone degeneration) miniature poodle, the XLPRA (X-linked progressive retinal atrophy) Siberian husky, the \textit{rd} (retinal degeneration) chicken, and many others (Aguirre, 1995). Additionally, the advent of transgenic animal technology has resulted in the production of a large number of new animal models for human retinal diseases including retinitis pigmentosa. In a number of cases, the underlying genetic defect responsible for retinal degeneration in the animal has been identified. This is true for \textit{rd} and \textit{rds} mice, and for the \textit{rcdl} dog. Identification of the mutations responsible for animal models of retinal degeneration has prompted searches for mutations in the
equivalent genes of human patients with retinal degeneration. In some cases, these searches have been successful.

1.4.3 Genetic defects leading to retinal degeneration. The underlying mutations responsible for retinal degeneration in the \textit{rd} mouse and the \textit{rcdl} dog have been identified, and occur in the genes encoding the \(\beta\) subunit of rod phosphodiesterase (Bowes et al., 1990; Suber et al., 1993). Early in development, photoreceptors form in the retinas of these animals. The photoreceptors contain abnormally high levels of cGMP (Farber and Lolley, 1974). In \textit{rd} mice, rod photoreceptor death begins at about postnatal day 10 and continues until postnatal day 21, at which point only cones remain (Blanks et al., 1974). The cone photoreceptors eventually die as well, over the course of several months (Carter-Dawson et al., 1978). The \textit{rcdl} phenotype is similar, though photoreceptor cell death occurs over a longer timecourse (Aguirre, 1978). Photoreceptor cell death in these animals occurs through apoptosis, or programmed cell death (Chang et al., 1993).

The underlying defect in the \textit{rds} mouse has also been identified. The \textit{RDS} gene of normal mice was found to encode the photoreceptor specific protein peripherin/rds (Connell et al., 1991a). In \textit{rds} mice, the gene is disrupted by a viral insertion, resulting in a probable null allele; photoreceptor degeneration is prevented in transgenic \textit{rds} mice that express wild-type peripherin/rds (Travis et al., 1992). Peripherin/rds had been previously described as a photoreceptor disc rim specific membrane protein of unknown function (Molday et al., 1987). Unlike mice homozygous for the \textit{rd} mutation, mice homozygous for the \textit{rds} mutation fail to develop any photoreceptor outer segments (Sanyal et al., 1980); in the heterozygous mouse, outer segments initially appear but are deformed (Hawkins et al., 1985). Photoreceptors of the \textit{rds} mouse also die by the apoptotic pathway (Chang et al., 1993).
In humans, at least 40 genes are responsible for retinal degeneration and syndromes with associated retinal degeneration (Daiger et al., 1995). A number have been cloned, and their protein products have been identified; these are summarized in Table 1. The first mutations to be identified were in the rhodopsin gene (Dryja et al., 1990). Mutations in rhodopsin are thought to account for about 25% of cases of ADRP, and have also been associated with autosomal recessive RP (Berson, 1993). Almost 80 different mutations in the rhodopsin gene have been associated with retinal degeneration (Daiger et al., 1995). Some examples of disease causing mutations are rhoP23H, rhoP347S, rhoK296E, and rhoQ64Stop. Each of these mutations is thought to affect rhodopsin differently. RhoK296E disrupts the binding site of the 11-cis-retinal, resulting in rhodopsin that can constitutively activate transducin in vitro; the mutation is associated with a severe dominant RP phenotype (Robinson et al., 1992; Keen et al., 1991). RhoQ64Stop is probably a null allele with little or no protein produced and a dominant RP phenotype (Macke et al., 1993). RhoP23H and rhoP347S (Dryja et al., 1990a; Dryja et al., 1990b) may disrupt transport signals resulting in inability of the photoreceptor inner segment to export these rhodopsins to the outer segment after synthesis (Sung et al., 1994). Transgenic mouse models for retinal degeneration due to the rhodopsin mutation rhoP347S have been produced. These mice show phenotypes similar to the rd mouse, with the major pathway for photoreceptor death being apoptosis (Chang et al., 1993).

Mutations in the RDS gene responsible for retinal degeneration in humans have also been reported. At least 30 different mutations in the human peripherin/rds gene leading to retinal degeneration have been described (reviewed in Molday, 1995); these mutations have been linked to a large number of phenotypes, including ADRP, macular degeneration, butterfly-shaped pigment dystrophy, cone-rod dystrophy, and retinitis punctata albescens. In at least two cases, a mutation in peripherin/rds manifests itself differently in different family members. Weleber et al. (1993) reported that perΔK153 is
Table 1: Genes associated with retinal degenerations and their protein products.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Associated disease phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHO</td>
<td>a rhodopsin</td>
<td>autosomal dominant RP, autosomal recessive RP</td>
</tr>
<tr>
<td>RDS</td>
<td>a peripherin/rds</td>
<td>autosomal dominant RP, macular degeneration, digenic RP, butterfly dystrophy</td>
</tr>
<tr>
<td>ROM1</td>
<td>a rom-1</td>
<td>digenic RP</td>
</tr>
<tr>
<td>CNCG</td>
<td>b cGMP-gated channel α subunit</td>
<td>autosomal recessive RP</td>
</tr>
<tr>
<td>SFD</td>
<td>c TIMP-3</td>
<td>Sorsby's fundus dystrophy</td>
</tr>
<tr>
<td>PDEA</td>
<td>d PDE α subunit</td>
<td>autosomal recessive RP</td>
</tr>
<tr>
<td>PDEB</td>
<td>e PDE β subunit</td>
<td>autosomal recessive RP</td>
</tr>
<tr>
<td>RP3</td>
<td>f RPGR</td>
<td>X-linked RP</td>
</tr>
<tr>
<td>CHM</td>
<td>g geranylgeranyl transferase A</td>
<td>choroideremia (X-linked)</td>
</tr>
<tr>
<td>ABL</td>
<td>h microsomal triglyceride transfer protein</td>
<td>abetalipoproteinaemia (syndrome RP)</td>
</tr>
<tr>
<td>OAT</td>
<td>i ornithine aminotransferase</td>
<td>dominant gyrate atrophy (syndrome RP)</td>
</tr>
<tr>
<td>USH1B</td>
<td>j myosin VIIA</td>
<td>Usher's syndrome (syndrome RP)</td>
</tr>
<tr>
<td></td>
<td>(mitochondrial) k mitochondrial ATPase 6</td>
<td>NARP syndrome (syndrome RP)</td>
</tr>
</tbody>
</table>

Review sources:
- Dryja and Li, 1995; Shastry, 1994; Molday, 1995.
- Dryja et al., 1995b; Weber et al., 1994; Huang et al., 1995; McLaughlin, 1993; Meindl et al., 1996; Seabra et al., 1993; Narcisi et al., 1995; Ramesh et al., 1988; Weil et al., 1995; Holt et al., 1990.

associated with RP with onset at 63 years in a mother, pattern dystrophy (macular degeneration) with onset at 31 years in her daughter, and fundus flavimaculatus (macular degeneration) with onset at 38 and 44 years in another daughter and son. Apfelsted-
Sylla et al. (1995) report that perR46Stop causes progressive cone-rod degeneration in a 58-year-old father and retinal pattern dystrophy in his 40-year-old son. (Possible explanations for this phenotypic diversity are discussed in section 1.5.3.)

Retinitis pigmentosa with an unusual inheritance pattern has been associated with the concerted effects of the mutations perL185P in the \textit{RDS} gene and romL114(1-bp ins) or romG80(1 bp ins) in the \textit{ROM1} gene. \textit{ROM1} encodes the rom-1 protein, which is highly homologous to peripherin/rds. One family tree reported by Kajiwara et al. (1994) with this form of inheritance (known as digenic) is illustrated in Fig 6. The mutations in both \textit{RDS} and \textit{ROM1} are required in order for the retinal degeneration phenotype to appear, though some carriers had ERG amplitudes slightly below normal. Strangely, another group has reported that the romL114(1 bp ins) is associated with retinitis pigmentosa without a mutation in \textit{RDS} (Sakuma et al., 1995). However, this is a case of simplex RP with a limited family tree, and therefore linkage cannot be proven.

Additionally, mutations causing retinal degeneration in humans have been found in the genes encoding rhodopsin, PDE\textit{\alpha}, PDE\textit{\beta}, the cGMP gated channel \textit{\alpha} subunit, tissue inhibitor of metalloprotease-3 (TIMP-3) and a number of other genes. These are summarized in Table 1.

\section*{1.5 Rom-1 and peripherin/rds}

Because of their involvement in retinal degenerative diseases, peripherin/rds and rom-1 have been the focus of much research, yet the function of these proteins still remains enigmatic. Some clues to their function are provided by animal models like the \textit{rds} mouse, and by the recent discovery that they are distantly related members of the transmembrane 4 superfamily (TM4SF); in general, most evidence suggests peripherin/rds and rom-1 have a structural role, though other functions are also possible.
FIG. 6. Family tree illustrating digenic inheritance due to mutations in both RDS and ROM1. This family tree, previously reported by Kajiwara et al. (1994), illustrates digenic inheritance of retinitis pigmentosa due to mutations at both the peripherin/rds and rom-1 loci. Affected individuals (shaded circles/squares) have inherited both a mutation in one RDS allele and a mutation in one ROM1 allele. Individuals who inherit only one of the mutations are essentially unaffected, though upon close examination their ERG responses may be slightly below normal. In this family, the RDS mutation is perL185P, and the ROM1 mutation is romL114(1-bp ins). The combination of perL185P and romG80(1-bp ins) is also reported to cause digenic RP.
1.5.1 Initial characterization of peripherin/rds. Peripherin was originally detected as a 35 kDa integral membrane protein present in bovine rod outer segments. Peripherin migrates on SDS-PAGE gels with approximately the same mobility as rhodopsin, which obscures the peripherin band on coomassie blue and silver stained gels. Though peripherin makes up a significant percentage of total ROS membrane protein (approximately 2%, Goldberg and Molday, 1996), it was undetected until two monoclonal antibodies were serendipitously generated that detected peripherin in ROS by western blot (Molday, Hicks and Molday, 1987).

Peripherin was named for its unusual subcellular localization. It is localized to the rims of outer segment discs (Fig 7), unlike proteins such as rhodopsin and transducin that are distributed throughout the disc plane. A similar localization had been previously demonstrated for a 290 kDa protein from frog photoreceptors (Papermaster et al., 1979). It should be noted that the subcellular distribution of peripherin has been disputed by other authors, who claim that peripherin is distributed throughout the disc (Travis et al., 1991).

Further biochemical characterization of peripherin revealed that it migrates on SDS-PAGE gels under non-reducing conditions as a disulfide-linked dimer, and that it is glycosylated. Coomassie stained SDS-PAGE gels showed that purified peripherin migrates as a doublet (Connell et al., 1991b).

The cDNA encoding bovine peripherin was cloned by Connell et al. (1990), using an expression cloning procedure in which a λgt11 library was screened with an anti-peripherin antibody. The cDNA showed no homology to any existing sequences in the EMBL database. The cDNA encodes a protein of 346 amino acids. Hydropathy profiles predict four transmembrane domains, with the third and fourth transmembrane domain separated by a large 141 amino acid loop with an intradiscal orientation (Fig 8). The fourth transmembrane domain is followed by a cytoplasmic C-terminal region of about
FIG. 7. Peripherin/rds distribution in ROS membranes. Peripherin/rds distribution in photoreceptor cells and disc membranes is shown by immunogold labeling. Panel a: Anti-peripherin monoclonal antibody per2B6 labeling of retina sections shows that peripherin/rds distribution is confined to the rod outer segment. Cone labeling is also observed. This indicates localization to either the rims of the discs or the plasma membrane. Panel b: per2B6 labeling of a lysed ROS preparation. Labeling is confined to the rims of discs, demonstrating that peripherin/rds is a disc rim-specific protein. The larger gold particles (arrows) are a plasma membrane marker. Reproduced from Molday 1994. (Magnification = 25,000 X (a) and 70,000 X (b))
FIG. 8. **Features conserved between peripherin/rds and rom-1.** All peripherin/rds and rom-1 sequences have similar predicted membrane topology, with four transmembrane domains and a large intradiscal loop, as illustrated above. Several short sequences, noted on the illustration, are highly conserved between rom-1 and peripherin/rds molecules. There are also seven invariant cysteine residues found within the intradiscal loop region. Residues within these sequence motifs that are highly conserved among all members of the TM4SF group of proteins are illustrated with a bold outline.
60 amino acids. A consensus sequence for N-linked glycosylation is present within the intradiscal loop at N229.

The predicted topology of peripherin was confirmed in part by pre-embedding immunogold labeling for electron microscopy using the monoclonal antibody per2B6. The epitope of this antibody was mapped to the C-terminus of peripherin. Intact discs could be labeled using this antibody, thereby confirming the cytoplasmic orientation of the C-terminus (Connell and Molday, 1990).

At about the same time that the peripherin cDNA was cloned by Connell and Molday, Travis et al. (1989) reported the sequence of the mouse RDS gene transcript. A defect in the RDS gene is responsible for retinal degeneration in the rds mouse. The sequences are extremely similar (92.5% identical), suggesting that the RDS gene encodes the mouse homologue of peripherin. Subsequent to reports that rds and peripherin were the same protein, many investigators have taken to calling the gene product peripherin/rds or rds/peripherin.

Using immunocytochemical techniques, peripherin/rds was found in the outer segments of both rods and cones (Arikawa et al., 1992). While these techniques do not distinguish between closely related proteins, the existence of a cone-specific form of peripherin/rds is unlikely because both rods and cones fail to develop outer segments in the rds mouse (Sanyal et al., 1980). The presence of peripherin/rds in cones has been confirmed by in situ hybridization (Uehara et al., 1993).

1.5.2 Initial characterization of rom-1. Rom-1 was first identified by Bascom et al. (1992) using a differential screening approach designed to detect evolutionarily conserved retina specific sequences. A large number of clones from a human retinal cDNA library were spotted onto filters and probed with bovine retina cDNA, human fibroblast cDNA, and human mitochondrial DNA. Hybridization to the bovine cDNA probe indicated evolutionary conservation, and lack of hybridization to human fibroblast
cDNA or mitochondrial DNA indicated retina tissue specificity. Clones which showed a hybridization pattern suggesting evolutionary conservation and retinal specificity were sequenced. The rom-1 cDNA was among the novel sequences identified.

The rom-1 sequence was found to be homologous to peripherin/rds, with an identity of 35% at the amino acid level. The cDNA encoded a protein of 351 amino acids. The predicted topology of rom-1 based on hydropathy profiles is very similar to that of peripherin/rds, with four transmembrane domains, the third and fourth separated by a large intradiscal loop. In contrast to peripherin/rds, there is no consensus sequence for N-linked glycosylation within this loop. Additionally, the intradiscal loop region of rom-1 is substantially more acidic than that of peripherin/rds (pI 5.2 vs. 8.2) (Bascom et al., 1993).

The identification of a sequence further removed evolutionarily from the previously reported peripherin/rds sequences enabled the identification of features conserved between the two proteins. Apart from the overall topology and length of peripherin/rds and rom-1, specific regions with a high degree of conservation are present (Fig 8). Of the twelve cysteine residues present in human peripherin/rds, seven are conserved in the human rom-1 sequence. Sequences at the start of the first transmembrane domains (LAQGLWL) and at the end of the fourth transmembrane domain (GLRYL) are conserved between the two proteins. These may be transport signal sequences responsible for the localization of peripherin/rds and rom-1. Additionally, the entire intradiscal loop region is of above average conservation (47%), with two stretches of sequence RYLD and DGVPFSCCNPXSPRPC 100% conserved between the two proteins.

Bascom et al. (1992) were able to generate a polyclonal antibody to rom-1, and they used this antibody to localize rom-1 within the retina. By electron microscopy, rom-1 was found to have a localization similar to peripherin/rds. Labeling was confined to the rims of the rod outer segment discs. However, by immunofluorescence
microscopy no rom-1 labeling was seen in cone outer segments, suggesting that rom-1 is not present in cones.

Rom-1 was found to co-purify with peripherin/rds on a per2B6 antibody-Sepharose column, though the per2B6 epitope is not present in rom-1. This provides an explanation for the doublet seen on coomassie stained SDS-PAGE gels of purified peripherin/rds. Bascom et al. (1992) demonstrated that the association between peripherin/rds and rom-1 was non-covalent; in two-dimensional SDS gel electrophoresis, disulfide linked peripherin/rds and rom-1 homodimers were present, but disulfide linked peripherin-rom-1 dimers were not detected.

1.5.3 Peripherin/rds and rom-1 participation in retinal degeneration. As mentioned above, since the identification of peripherin/rds as the protein product of the RDS gene of mice, a number of alterations in the human peripherin/rds sequence have been associated with retinal degeneration. The mutations and their respective phenotypes are summarized in several recent reviews (Daiger et al., 1995; Molday, 1994). Mutations in RDS are capable of causing a large number of autosomal dominant inherited retinal degenerations, including retinitis pigmentosa and macular degeneration.

The mutations found in peripherin/rds and rom-1 are of three types. First, some mutations result in insertion of a stop codon or frameshift causing premature termination early in the transcript sequence. These mutations are thought to represent null alleles; protein products of these transcripts would be recognized by proteolytic mechanisms of the photoreceptor cell and degraded. Examples of such mutations are perR46Stop, which causes progressive cone-rod degeneration, retinal pattern dystrophy and ADRP, and the viral insertion responsible for retinal degeneration in the rds mouse, which causes a frameshift of the normal sequence at codon perN230, followed by the addition of 35 additional amino acids before premature termination (Ma et al., 1995). Secondly, some mutations result in an alteration in the amino acid sequence without frameshift and
premature termination. These mutations generally involve small deletions or amino acid substitutions, and are usually located at conserved points in the sequence. These mutations may lead to structurally abnormal proteins that are quickly cleared by proteolysis, or they may result in proteins that are translated and transported to the outer segment, but are functionally incompetent (loss of function). They could also result in proteins that are translated and transported normally, but then deleteriously affect outer segment function by aberrant behaviors, such as inappropriate interaction with other proteins (gain of function). Examples of this type of mutation are perL185P, which causes retinal degeneration with digenic inheritance, and perS212G, which causes ADRP. The third class of disease causing mutation involves insertion of a stop codon or frameshift followed by premature termination late in the transcript sequence, beyond the fourth transmembrane domain. This class of mutation results in proteins with truncated C-termini. The mechanisms by which these mutations lead to disease are unclear; the resulting proteins may be misfolded and degraded, they may be incorrectly transported, or they may be loss or gain of function mutants.

Most of the disease-causing mutations in the \textit{RDS} gene reported thus far are of the codon substitution or deletion type. The majority of these mutations result in changes in the intradiscal loop region of peripherin, the most evolutionarily conserved region of the protein. These mutations result in a wide range of phenotypes, including both rod-cone and cone-rod dystrophies such as retinitis pigmentosa and macular degeneration, and both severe and mild forms of retinal degeneration. One question that has intrigued researchers is the mechanism by which mutations in a single gene sequence could affect primarily rods or primarily cones.

One explanation for this phenotypic discrepancy is that some of these mutations affect regions of peripherin/rds that function specifically in either rods or cones. For example, peripherin/rds was shown to interact with rom-1, and rom-1 was not found in cones. Mutations affecting the interaction of peripherin/rds with rom-1 might therefore
affect only rods, leaving cones relatively untouched. Other mutations that affect
primarily cones might disrupt cone-specific functions of peripherin/rds, such as
interaction with a cone homologue of rom-1.

Similarly, the difference between severe and mild phenotypes could be explained
by invoking gain of function versus loss of function mutations. In the case of loss of
function mutations and null alleles, one copy of the normal RDS gene remains, and
approximately half the normal quantity of peripherin/rds is made to meet the
photoreceptor's requirements, resulting in a mild phenotype. However, a gain of
function mutant could deleteriously affect the photoreceptor to a more severe extent, for
example by interacting with normal peripherin/rds and rom-1 and sequestering them in an
inactive form.

Unfortunately, these models are unsuccessful in explaining the phenotypic
variation seen in patients with RDS mutations. In fact, as previously noted above, one
mutation can produce different phenotypes in different patients, and supposedly null
alleles result in a variety of phenotypes. One possible explanation for this variation is
that the effects of mutations in the RDS gene are strongly influenced by genetic context.
Thus, polymorphisms that do not initiate disease on their own, located in RDS or other
genes, influence the phenotype caused by an RDS mutation.

The most extreme example of genetic context affecting phenotype is the digenic
inheritance pattern described above (Fig 6), where the RDS mutation perL185P shows
no phenotype at all unless paired with an otherwise innocuous mutation in ROM1. Cases
could be imagined where phenotypic differences are less extreme, for example a mutation
in RDS that produces a mild RP phenotype could become severe when coupled with a
mutation in ROM1.

It is interesting that disease-causing mutations in ROM1 have been largely
unreported, despite the fact that rom-1 and peripherin/rds are so similar. This is not for
want of trying-- investigators have searched for such mutations but have failed to find
any unambiguous cases. (Nichols et al., 1994; Sakuma et al., 1995; Bascom et al., 1993b). It is also particularly interesting that null mutations in \textit{RDS} cause disease, while null mutations in \textit{ROM1} do not. Perhaps peripherin/rds serves a more important function than rom-1, or possibly peripherin/rds is able to function in place of rom-1, while rom-1 is unable to function in place of peripherin/rds.

Bascom et al. (1992) have determined the chromosomal location of \textit{ROM1}. This gene is located at 11q13 in the human chromosome map, very close to the location of the gene responsible for Best's disease. However, subsequent extensive analysis of \textit{ROM1} in patients with Best's disease failed to find any mutations that segregated with the disease. Further refinement of the chromosomal location of \textit{ROM1} by the same authors has excluded \textit{ROM1} from any involvement in this disorder.

\textbf{1.5.4 Relationship of peripherin/rds and rom-1 to the TM4SF group of proteins.} While it was originally thought that peripherin/rds and rom-1 constituted a protein family composed of only two members, it has become clear that they are members of a recently discovered protein family known as the transmembrane 4 superfamily (TM4SF), also known as the tetraspan family. Members of this family (tetraspannins) are characterized by hydropathy profiles that predict four membrane spanning domains, with overall topology similar to that of peripherin/rds and rom-1. There is also some limited sequence homology between membrane spanning domains, and three very short sequence motifs involving cysteine residues conserved within the intradiscal (cytoplasmic for other tetraspannins) loop region. Many of the original members of the TM4SF family were first described as leukocyte cell surface markers (hence "Cluster of Differentiation" or CD) and tumor antigens.

The TM4SF group of proteins was originally defined by homology between four proteins, Sm23, CD81, CD63 and CD37. In general, the roles of these proteins are at least as mysterious, if not more so, than the roles of peripherin/rds and rom-1. Sm23 is a
23kDa antigen found in *Schistosoma mansoni* flatworms (Wright et al., 1990). CD81, also known as TAPA-1, is a cell surface marker found in some leukemia and lymphoma cell lines, and it is present on B and T cells. Anti-CD81 antibodies have an anti-proliferative effect on some lymphoma cell lines (Oren et al., 1990). CD81 is part of a signal transduction complex with a role in B-cell activation (reviewed in Tedder et al., 1994). CD37 is also associated with this complex (Angelisova et al., 1994). CD63 is a tumor antigen detected in the early stages of melanoma, and is also found on normal macrophages (Atkinson et al., 1984). In general, TM4SF family members are ascribed roles in cell adhesion or signal transduction. Ion channel functions have been suggested but are unproven (Wright and Tomlinson, 1994).

One recent example of a tetraspannin with a unique and fairly well characterized function is the *latebloomer* protein from *Drosophila* (Kopczynski et al., 1996). The nervous systems of fruit flies that lack this protein develop more slowly. It appears that *latebloomer*, which is expressed at the tips of developing motor neuron axons in a region known as the growth cone, is involved in forming contacts between the axons and their efferent muscles. However, absence of *latebloomer* does not preclude the formation of these contacts; they just develop more slowly.

Peripherin/rds and rom-1 are unique amongst known members of the TM4SF in that they possess long cytoplasmic C-terminal domains that are absent in other tetraspannins. Nevertheless, their membership in this group seems clear, as sequence motifs present in the intradiscal/extracellular loop regions are conserved between tetraspannins and peripherin/rom-1. These motifs include the sequences CCG, PXSCC, and GC, located in the intradiscal loop regions (Fig 8), as well as limited homology found in the transmembrane domains. An evolutionary tree showing the relationship of peripherin/rds and rom-1 to a number of tetraspannins is shown in Fig 9.

Assuming that peripherin/rds and rom-1 have some properties in common with other members of this group of proteins, one could impute a role in adhesion or signal
FIG. 9. Dendrogram showing sequence similarity of peripherin/rds and rom-1 to other members of the TM4SF group of proteins. The dendrogram was constructed using the CLUSTAL program of pgene (IntelliGenetics), which uses the method of Higgins and Sharp (1988). The sequences CD20, CD81, CD37, and CD63 are human, rom-1 and peripherin/rds sequences are bovine, and latebloomer sequence is from Drosophila. References are in the text. The sequence homology between members of this protein family is extremely limited, and cannot be reliably detected between all members of this group using alignment programs. Members of the TM4SF group are identified primarily by membrane topology and the presence of short sequence motifs (Fig 8 and text). The highest degree of similarity is seen between rom-1 and peripherin/rds, suggesting that they may have diverged from a common ancestral disc rim protein relatively recently. Rhodopsin is included as an unrelated transmembrane protein of approximately the same size.
transduction to peripherin/rds and rom-1. However, it should be noted that the cytoplasmic C-terminal region is absent in other tetraspannins, and that it is the intradiscal region that is conserved. Thus, it is adhesion within the disc that is implied by models based on TM4SF homology. Similarly, the implied signal transduction would proceed from within the disc to the cytoplasm. While this is not inconceivable, it seems inconsistent with what is currently known about visual transduction in photoreceptors. Adhesion within the disc is compatible with models that suggest peripherin/rds and rom-1 are involved in maintaining disc rim curvature (Fig 10). It is possible that peripherin/rom-1 complexes form contacts across the disc rim region, thereby stabilizing the disc rim. The cytoplasmic C-terminal region could serve a function not found in other tetraspannins; it could promote adhesion between adjacent discs, or between discs and the plasma membrane.

1.6 Thesis investigations

Peripherin/rds and rom-1 are crucial photoreceptor proteins whose function is poorly understood. At the time the investigations described here were initiated, the study of their biochemistry was hampered by the fact that the amino acid sequence of bovine rom-1 was not known. No antibodies were available that recognized bovine rom-1, and consequently, in the absence of any known function, there was no way to assay its presence.

Therefore, the aims of the investigations described here were to clone the cDNA encoding the bovine homologue of rom-1, and then utilize the sequence information to further characterize the protein using biochemical and immunochemical methods. Initially this involved generating antibodies, thus providing a means of detecting and assaying bovine rom-1. Subsequently, the antibodies were used to further characterize the protein from bovine ROS in terms of cellular and subcellular localization within the
FIG. 10. A schematic diagram of a rod or cone outer segment disc membrane. The rom-1/peripherin complex is confined to the rim regions, while the central lamellar region contains primarily rhodopsin. Cone rom-1 may be identical to rod rom-1, or may be a cone-specific form. The arrangement of rom-1 and peripherin/rds depicted is purely speculative, and rom-1 and peripherin homodimers have been omitted for clarity; each peripherin/rom-1 complex is composed of four subunits. It has been suggested that peripherin/rds and rom-1 may act to stabilize the highly curved disc rim region via interactions of their intradiscal regions.
retina, and membrane topology. The same antibodies were also used to examine the localization of rom-1 in the human retina. Additionally, the antibodies were used to determine the extent of the interaction of peripherin/rds and rom-1, i.e., to determine whether any peripherin/rds is not complexed with rom-1, or whether any rom-1 is not complexed with peripherin/rds.

The cloned cDNA and anti-rom-1 antibodies were then used to examine rom-1 and its interaction with peripherin/rds in a heterologous system. The intracellular localization of rom-1 expressed in COS-1 cells was examined. Rom-1 and peripherin/rds were found to be capable of interacting when expressed in the heterologous COS-1 cell system, and the nature of this interaction was examined. The heterologous COS-1 cell system was also used to examine the interaction of wild-type rom-1 and peripherin/rds with various mutant forms of these proteins. For these studies, mutant cDNAs were generated by site-directed mutagenesis or by other methods. The mutants studied include romL185P, the rom-1 equivalent of perL185P, and various mutant forms of peripherin/rds and rom-1 with truncated C-termini.
MATERIALS AND METHODS

2.1 Cloning procedures

The molecular biology procedures used in cDNA cloning have been widely described in readily available laboratory manuals and will not be reviewed in detail here. Except where noted, general procedures such as agarose gel electrophoresis were essentially as described by Sambrook et al. (1989). In cases where commercially available kits or enzymes were employed, the protocols used were as recommended by the manufacturer. Exceptions are noted below. The sequences of synthetic oligonucleotides used in these protocols, as well as the recipes for commonly used buffers, are provided at the end of the chapter (2.13).

2.1.1 Library screening. A λgt10 human retinal cDNA library kindly provided by Dr. Jeremy Nathans was screened using a $^{32}$P end-labeled oligonucleotide probe based on the previously published human rom-1 cDNA sequence (Bascom et al., 1992). The probe sequence was 5'GCCACCCGGGCAAGTCTGAATGCAGCTCTAS'. A 1222-bp clone was isolated from the library using this probe, and confirmed as a cDNA encoding bases 116 to 1337 of the human rom-1 sequence by sequencing the 5' and 3' ends of the clone. A $^{32}$P probe was then prepared from this clone using a nick-translation protocol. The probe was used to screen a λZAP bovine retinal cDNA library.

A 999-bp clone with a high degree of homology to the human rom-1 cDNA sequence was isolated on the first screening of this library. The clone was named Brom1A. A 667-bp BamHI/HindIII fragment of this clone was then used as a probe for a second screening of the library. A number of longer clones were isolated, the longest of which (1340 bp) was named Brom1G. Brom1A and Brom1G were sequenced in both directions.
2.1.2 Inverse polymerase chain reaction. The clone Brom1G was found to be an incomplete cDNA, as it contained no start ATG codon. Despite repeated screenings of the cDNA library, no longer clones were found. To obtain the remainder of the sequence, inverse polymerase chain reaction (inverse PCR) was carried out on bovine genomic DNA, using a protocol based on that of Silver et al. (1989). Genomic DNA isolated from bovine liver was digested with MboI. A southern blot of the digested DNA probed with the 5' BamHI fragment of Brom1G labeled an approximately 940-bp fragment. (Note: A BamHI site (GGATCC) contains an MboI site (GATC)). MboI digested genomic DNA was then diluted and circularized at a concentration of 5 µg/ml in a final volume of 400 µl using 20 U of T4 ligase, conditions calculated to give 93% circularization of 940 bp fragments (Collins and Weissman, 1984). The circularized DNA was then extracted with phenol/chloroform and ethanol precipitated. PCR was carried out on this template using a touch-down protocol. An initial 5-minute denaturation at 94°C was followed by 50 cycles of a 15-second denaturation at 94°C, a 30-second annealing, and a 1-minute extension at 72°C, over which the annealing temperature was dropped from 68°C to 55°C at a rate of 1°C per cycle and maintained at 55°C. The primers were INV1 and INV2. The reactions were carried out in a 100 µl volume using a buffer containing 67 mM Tris pH 8.8, 17 mM ammonium sulfate, 1 mM magnesium sulfate, 10 mM β-mercaptoethanol (BME), 0.2 mM of each deoxyribonucleotide tri-phosphate (dNTP), 20 pmol of each primer, 100 ng of template and 2.5 units of Taq polymerase (BRL) overlaid with 2 drops of mineral oil. No product was seen in control reactions containing no template or in reactions using a control template prepared without ligase. The size of the inverse PCR product was 859 bp, very close to the size predicted by a southern blot of MboI digested genomic DNA probed with the BamHI fragment of Brom1G. Reaction products were cloned into the TA cloning vector (Invitrogen), and one clone (BrINV1) was sequenced in both directions.
2.1.3 Conventional polymerase chain reaction. Sequence obtained from inverse PCR products was confirmed by sequencing products obtained by conventional PCR. Regular PCR was also performed on 100 ng of bovine genomic DNA using identical buffer conditions and a similar amplification protocol. The primers used were INV3 and INV2. Products were cloned into the TA cloning vector (Invitrogen), and two clones (BrPCR1 and BrPCR2) were sequenced completely in one direction.

2.1.4 DNA sequencing. DNA sequencing was carried out using a variety of methods. For λgt10 clones, inserts were subcloned into pBluescript II SK+ (Stratagene). For λZAP clones, in vivo excision of the pBluescript I plasmid containing the inserts was carried out as described by Short et al. (1988). PCR products were cloned into the TA cloning vector for sequencing. Both single-stranded and double-stranded sequencing protocols were employed. Double stranded DNA was prepared using Magic Mini-preps (Promega), or as described by Berghammer and Auer (1993). Single stranded DNA was prepared using the M13K107 helper phage as described by Sambrook et al. (1989). DNA was sequenced using the Sequenase version 2.0 kit as recommended by United States Biochemical. For λZAP clones, internal sequence was obtained by subcloning restriction fragments into pBluescript II and sequencing either single or double stranded DNA, and by generating nested deletions of clones as described by Henikoff (1987) and sequencing double stranded DNA. Finally, some internal sequence was obtained by using specific primers.

2.1.5 RNA isolation and Northern blot. Total RNA was isolated from retinas using the method described by Chirgwin et al. (1979) involving solubilization in guanidinium isothiocyanate followed by ultracentrifugation through a dense cushion of cesium chloride. Northern blots were carried out essentially as described by Ausuhel et al.
(1994), using formaldehyde denaturing agarose gels, 10 μg of total RNA per lane, and capillary transfer onto nylon membranes (Hybond-N, Amersham). For bovine rom-1 Northern blots, a 32P-labeled random-primed probe was made from the EcoRI/HindIII fragment of the Brom1G clone.

2.1.6 Genomic DNA isolation and Southern blot. To determine a suitable restriction enzyme for use in inverse PCR, a Southern blot was necessary. Genomic DNA was isolated from liver as follows. A fresh piece of bovine liver was obtained from a slaughter house and quick-frozen in liquid nitrogen. Approximately 0.5 g of tissue was broken off and pulverized to a fine powder using a mortar and pestle cooled with liquid nitrogen. The powdered tissue was suspended in 25 ml of NE (150 mM NaCl, 25 mM EDTA pH 8.0) and centrifuged at 12,000 x g for 5 minutes at 4°C in a Sorvall centrifuge. The supernatant was decanted, and the pellet was washed again in NE. Finally, the pellet was resuspended in 5 ml of NE. Proteinase K (500 μg) was added and mixed in by vortexing gently. SDS was added to 0.1% and mixed in gently. Digestion was continued overnight at 50°C. The next day, an equal volume of buffer saturated phenol (BRL) was added and the mixture was extracted for 30 minutes using an electric rotating device. The phenol was removed and discarded, after which the aqueous phase was again extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Finally, the mixture was extracted with an equal volume of chloroform/isoamyl alcohol (24:1). The DNA was precipitated by adding 5M NaCl to a final concentration of 0.4M, followed by 2 volumes of 100% ethanol. The DNA was gently re-dissolved in dH₂O, and the phenol/chloroform extraction procedures were repeated. Finally the DNA was precipitated by adding one half volume of 7.5 M ammonium acetate and 2.5 volumes of 100% ethanol. The precipitate was washed with 70% ethanol and resuspended in 2 ml of dH₂O. The final concentration of DNA was 300 μg/ml, as determined by A₂₆₀, and the A₂₆₀/A₂₈₀ ratio was 2.0. (The same
genomic DNA was used for inverse PCR, described above.) Genomic DNA (30 µg) was
digested with 15 units of restriction enzyme for 1.5 hours, after which another aliquot of
restriction enzyme was added, and digestion was continued for 1.5 hours. The reactions
were phenol/chloroform extracted, ethanol precipitated, and resuspended in 25 µl of
dH2O. Digested samples were mixed with agarose gel loading buffer and loaded onto
TBE/agarose gels containing no ethidium bromide. Gels were run at 55 V. DNA
fragments were transferred from the gel onto Hybond-N membrane (Amersham) as
described by Sambrook et al. (1989). A digoxigenin-labeled probe was made from the
BamHI fragment of Brom1G, and hybridization and detection were carried out using the
Dig DNA labeling and detection kit (Boehringer Mannheim) as described by the
manufacturer.

2.2 Generation of antibodies using fusion proteins

A number of anti-rom-1 and anti-peripherin/rds antibodies, both monoclonal and
polyclonal, were generated. The general scheme for antibody production was to express
portions of the protein of interest fused to an affinity tag, glutathione-S-transferase, in E.
coli. These fusion proteins were used as immunogens. Use of purified fusion proteins
insured that immunogens would not be contaminated by other ROS proteins, and
allowed antibodies to be generated to specific regions of proteins.

2.2.1 Generation of fusion proteins. A number of GST-rom-1 and GST-peripherin/rds
fusion proteins were generated for use as immunogens. The fusion proteins were
generated using the pGEX system of plasmids, and were affinity purified on glutathione
agarose as described by Smith et al. (1988). Table 2 lists the fusion proteins, the vectors
employed for their production, the protein sequences from which they were derived, and
the antibodies that resulted from their use in immunizations. RomS1L6 was constructed
by subcloning a PvuII/AluI fragment of the bovine rom-1 cDNA into SmaI cut pGEX3X. RomCterm23 was constructed by subcloning a MseI/RsaI restriction fragment of BromlA into SmaI cut pGEX1. Insert orientation was verified by sequencing. PerT2 and perB1 were constructed by amplifying short sections of the peripherin/rds cDNA by PCR, then cloning the fragments into BamHI/EcoRI cut pGEX2T. Constructs based on PCR-amplified DNA were sequenced to ensure no mutations were present. The primers used for perT2 were PL1 and PL3. The primers used for perB1 were PL1 and PL2.

<table>
<thead>
<tr>
<th>Protein sequence</th>
<th>Construct name</th>
<th>Vector</th>
<th>Resulting Antibodies[^a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>romL289-A351</td>
<td>romCterm23</td>
<td>pGEX1</td>
<td>romC2 (p), rom1D5 (m),</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gst9A6 (m), fp11A11 (m)</td>
</tr>
<tr>
<td>romR165-Q229</td>
<td>romSlL6</td>
<td>pGEX3X</td>
<td>romL4 (p), rom1C6 (m)</td>
</tr>
<tr>
<td>perR183-S212</td>
<td>perT2</td>
<td>pGEX2T</td>
<td>perL1 (p)</td>
</tr>
<tr>
<td>perR183-E242</td>
<td>perB1</td>
<td>pGEX2T</td>
<td>none</td>
</tr>
</tbody>
</table>

[^a]: polyclonal antibodies are indicated by (p), monoclonal antibodies are indicated by (m).

2.2.2 Generation of polyclonal antibodies. Female New Zealand White rabbits were immunized with approximately 100 µg of protein, estimated by absorbance at 280 nm. Immunizations were given in a volume of 1 ml, consisting of 500 µl of fusion protein in PBS and 500 µl Freund's adjuvant. For initial immunizations, complete Freund's adjuvant was used, and incomplete Freund's adjuvant was used for boosts. Boosts were given at least 10 days apart. Blood serum was collected 10 days after boosts.

2.2.3 Purification of polyclonal antibodies. For electron microscopy and immunofluorescence applications romL4 and romC2 polyclonal antibodies were affinity
puriﬁed. Afﬁnity columns were made by coupling fusion proteins to CNBr activated Sepharose beads at approximately 1 mg protein/ml beads. CNBr activated beads were prepared as previously described (Cuatrecasas, 1970), and the coupling reaction was performed overnight in 20 mM borate pH 8.4. Afﬁnity columns were made by sequentially passing the serum over Sepharose-GST and Sepharose-GST-fusion protein columns. The columns were washed with ten volumes of PBS, and the puriﬁed antibodies were eluted from the Sepharose-GST-fusion protein matrix with 500 mM NaCl in 100 mM formic acid. Peak fractions containing antibody as determined by $A_{280}$ reading were quickly pooled and dialyzed against three changes of 500 ml phosphate buffered saline (PBS). Glycerol (20% v/v) was added prior to storage at 4°C. The ﬁnal concentration of puriﬁed romL4 and romC2 antibodies was 0.2 mg/ml.

2.2.4 Generation of monoclonal antibodies. Female C57Bl mice were immunized with approximately 10 µg of protein, estimated by absorbance at 280 nm. Immunizations were given in a volume of 100 µl, consisting of 50 µl of fusion protein in PBS and 50 µl Freund's adjuvant. For initial immunizations, complete Freund's adjuvant was used, and incomplete Freund's adjuvant was used for boosts. Boosts were given 10 days apart. After three immunizations, if an immune response was obtained to the desired protein (as determined by western blot of ROS proteins), the mouse was sacriﬁced, and its splenocytes cells were collected and fused to NS-1 cells (ATCC). Monoclonal antibodies were identiﬁed by a primary screening using dot blots of ROS proteins. Initial positives were then tested by western blots of ROS proteins and western blots of the heterologously expressed protein of interest. It should be noted that Balb/C mice were found to give extremely poor immune responses to these GST fusion proteins; whether this is a problem common to all GST fusion proteins is not clear. Balb/C mice did show an immune response to the GST portion of the fusion proteins, and anti-GST antibody
gst9A6 was raised in Balb/C mice, as well as antibody fp11A11, which recognizes the romCterm23 fusion protein, but not rom-1 from ROS.

2.2.5 *Fusion and screening protocols.* The following procedure was used to fuse splenocytes and myeloma cells for generation of monoclonal antibodies. All centrifugations were done in 50 ml Falcon tubes in a clinical centrifuge. NS-1 myeloma cells were grown in petri dishes in IMDM 10% FBS (fusion grade, Gibco). Each plate of cells was visually assessed for health based on apparent viability and absence of aggregation. For a single fusion, 6 x 10⁶ cells were collected by centrifugation at 400 x g. The cells were resuspended in 50 ml serum free IMDM and counted. The cells were then spun down again and washed one more time in IMDM and resuspended in 20 ml IMDM. Meanwhile, to obtain feeder cells, a mouse was killed by cervical dislocation. The spleen was removed and washed in serum free IMDM. Two 1 ml syringes equipped with 26 gauge needles were used to perforate the spleen and wash out the splenocytes by forcing in IMDM. The spleen was discarded and the cells were spun down and resuspended in 80 ml of HAT medium at 37°C. The immunized mouse was then treated similarly, except that the cells were resuspended in serum-free IMDM. These cells were washed once, resuspended in 20 ml serum-free IMDM, and combined with the myeloma cells. The pooled cells were centrifuged at 400 x g for 5 minutes, and the supernatant was aspirated off. The pellet was loosened by tapping on a benchtop a few times. The tube was placed in a beaker of 37°C water, and 800 μl of 50% PEG was added while simultaneously swirling the tube gently for one minute. The cells were allowed to sit for one minute, after which 1 ml of serum-free IMDM was added dropwise over one minute. Finally, 20 ml of serum-free IMDM was added over 5 minutes with occasional swirling. The cells were centrifuged for 5 minutes at 400 x g, and resuspended in 80 ml of HAT medium containing the feeder cells. The cell suspension was then plated into 8 to 10 96-well plates. The cells were re-fed with 100 μl of HAT the next day and with
HT after 5-7 days when hybridomas became visible. As a control to ensure that the aminopterin selection was effective, a few wells of feeder cells were plated prior to the addition of the fused cells. The author gratefully acknowledges Theresa Hii for perfecting and carrying out the fusion protocols.

The following procedure was used for primary screening. ROS were isolated from 100 frozen cattle eye retinas as previously described. The ROS were solubilized at 0.1 mg/ml in 1% SDS, 0.2% TX100 in 10 mM Tris pH 7.5. Immobilon-P nylon membranes (Millipore) were marked with 0.5 cm pencil grids, and soaked in methanol, then transferred to the solubilized ROS for 30 minutes with agitation. The membranes were rinsed with distilled water and dried. Membranes were stored for further use (up to several months). For screening, a section of membrane was wetted with methanol, rinsed with PBST and blocked in PBST containing 0.5% milk powder for 30 minutes. The membrane was rinsed with PBST and placed on two sheets of Whatman 3MM paper soaked in PBST. One μl of cell culture supernatant was spotted into the center of each grid square. After 20 minutes, the filter was rinsed 3 times for 5 minutes with PBST, then transferred to PBST containing 0.05% milk powder and a 1:5000 dilution of the secondary antibody (sheep anti(mouse Ig - horseradish peroxidase Fab' fragment, Amersham) for 30 minutes. The filter was again rinsed 3 times for 5 minutes with PBST and developed using the ECL (enhanced chemiluminescence) detection kit (Amersham). Primary screening positives showed a signal significantly higher than the background level of labeling obtained with the majority of clones tested. Positives from the primary screening were then tested on western blots of total ROS proteins and western blots of the heterologously expressed ROS protein.

2.2.6 Purification of monoclonal antibodies. For preparation of fluorescently labeled monoclonal antibodies and production of immunoaffinity matrices, monoclonal antibodies were purified from ascites fluid by NH₄SO₄ precipitation followed by DEAE
chromatography as previously described (Goding, 1986). For rom1C6 and rom1D5, ascites fluid was raised in pristane-primed mice that were the F1 generation of a cross between C57Bl and Balb/C mice. For per2B6 and rho1D4, ascites fluid was raised in Balb/C mice. Antibody concentrations in tissue culture supernatants were found to be sufficiently high for all other purposes.

2.2.7 Production of immunoaffinity matrices. Immunoaffinity matrices were made by coupling purified antibodies to CNBr activated Sepharose beads at approximately 1 mg protein/ml beads. CNBr activated beads were prepared as previously described (Cuatrecasas, 1970), and the coupling reaction was performed overnight in 20 mM borate pH 8.4.

2.2.8 Production of fluorescently labeled monoclonal antibodies. Tetramethylrhodamine isothiocyanate (TRITC) and fluorescein isothiocyanate (FITC) labeled monoclonal antibodies were used for some fluorescence microscopy applications. These antibodies were prepared as previously described (Harlow and Lane, 1988). Briefly, 5μl of TRITC or FITC (1 mg/ml in DMSO) was added to 1 ml of the monoclonal antibody to be labeled (2 mg/ml in 100 mM carbonate buffer pH 9.0) and incubated overnight at 4°C with gentle agitation. The reaction was quenched by addition of NH4Cl to 50 mM and a further 2 hour incubation. The labeled antibody was separated from unincorporated TRITC or FITC on a Sephadex G-50 column eluted with PBS. Peak fractions as judged by visual inspection were pooled, and glycerol was added to 20% v/v. The labeled antibodies were stored at -20°C for later use.

2.2.9 Epitope mapping. The epitope of antibody rom1D5 was determined by assessing rom1D5 binding to a sequence of overlapping 9 amino acid peptides encompassing the portion of rom-1 used as an immunogen. The overlap between adjacent peptides was 7
amino acids, i.e. each peptide represented a 2 amino acid shift. The peptides were synthesized using the Pin Technology System (Cambridge Research Biochemicals). The epitope was defined as the stretch of sequence common to all peptides bound by rom1D5.

2.3 Cell expression systems

Plasmids for heterologous expression of bovine rom-1, peripherin/rds and rhodopsin in mammalian cell lines were constructed and used in both transient transfections and for generating permanent cell lines.

2.3.1 Transient transfections. For transient transfections, plasmid constructs were based on the pcDNA1/AMP vector (Invitrogen). The bovine peripherin/rds cDNA was subcloned into the BamHI site of pcDNA1/AMP by Dr. AFX Goldberg. A synthetic bovine rhodopsin cDNA, kindly provided by Dr. Dan Oprian, was subcloned into the pcDNA1/AMP vector by Dr. AFX Goldberg. The Kasl/HindIII fragment of Brom1G was cloned into the EcoRI/NotI sites of the pcDNA1/AMP vector using two synthetic linkers, HN1 and EK1. The linkers were made from four oligos, Hind1, Not1, Eco1 and Kas1. The linker EK1 inserts a Kozak consensus sequence (Kozak, 1987) for translation initiation.

The following protocol, based on that of Chen et al. (1987), was used for transient transfection. Stock solutions CA (2.5 M CaCl2) and BBS (50 mM BES, 280 mM NaCl, 1.5 mM Na2HPO4 pH 6.95) were prepared and stored at -20°C. Prior to use, aliquots were thawed and filter-sterilized. Plasmid DNA (12 μg in TE or dH2O) was mixed with 16.5 μl of CA, and the volume was made up to 165 μl with sterile dH2O. This mixture was slowly added to 165 μl of BBS with gentle pipetting, avoiding the introduction of air bubbles. After sitting at room temperature for 20 minutes, the mixture was dripped
onto a petri dish in which $2 \times 10^5$ COS-1 cells had been plated the previous day in DMEM medium with 10% FBS. The plate was then placed in a 4% CO$_2$ incubator at 35°C overnight. The next day the media was changed, and the dishes were transferred to a 5% CO$_2$ incubator at 37°C. Cells were harvested 48-72 hours after transfection. Typically, the transfections were done in 49 mM dishes. However, when larger quantities of protein were desired, the transfections were scaled up 3 fold for 87 mM dishes. For immunofluorescence experiments, cells were plated in 24 well plates at about 2,000-10,000 cells per well, depending on the degree of confluence desired, with a round glass coverslip in each well. A standard CaPO$_4$ precipitation was split into 4 and added to each well. For co-transfection experiments, half the normal quantities of each plasmid were mixed, then treated as described above.

2.3.2 Permanent cell lines. For permanent cell lines, plasmid constructs were based on the pcDNA3 vector (Invitrogen). The peripherin/rds cDNA was subcloned into the BamHI site of pcDNA3 by Dr. AFX Goldberg. The KasI/AflII fragment of Brom1G was cloned into the EcoRI/NotI sites of the pcDNA3 vector using two linkers, EK2 and AN1. The linkers were made from four oligonucleotides, Eco2, Kas2, Afl1 and Not2:. The linker EK2 inserts a Kozak consensus sequence for translation initiation (Kozak, 1984). The linker AN1 incorporates an additional SmaI site.

The following protocol was used to establish permanent cell lines expressing either peripherin/rds or rom-1. CHO-K1 cells were transfected by electroporation. Cells were grown in F12 media with 10% FBS. Approximately $1 \times 10^6$ cells were resuspended by light trypsinization in HBSG. The cells were spun down and resuspended in 500 µl of HBSG containing 10 µg of plasmid DNA. This mixture was then transferred to a 0.5 cm electroporation cuvette (Invitrogen) and electroporation was carried out in an ElectroPorator (Invitrogen) electroporator at 350 V using a 250 µF capacitor and an infinite parallel load. The cells were immediately plated in a single 87 mm petri dish in
F12 medium with 10% FBS. After 24 hours of post-transfection growth, the medium was changed and supplemented with 500 μg/ml G418 (BRL). After 14 days of growth, during which the medium was replaced every third day, the cells were trypsinized and cloned twice by limiting dilution in medium containing 500 μg/ml G418. Rom-1 and peripherin/rds expressing clones were detected by Western blot analysis on cell extracts as follows: Clones were expanded until they could be split into two wells of a 24-well plate. 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) detergent extracts of the cell lines were prepared by washing one well of 90% confluent cells with PBS followed by solubilization in 100 μl of PBS containing 1% (w/v) CHAPS and 100 μg/ml phenylmethylsulfonylfluoride (PMSF). The cell extract was scraped off the plates and centrifuged for 5 minutes at maximum speed in a microfuge. The supernatant was analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis and Western blotting.

2.4 Protein assay

Protein assays were performed using the BCA (bicinchoninic acid) system (Pierce). The procedure was modified slightly from that given in the protocol handbook. Assays were performed in standard 96-well plates. 10 μl samples were placed in the wells, and 200 μl of detection reagent was added. The plate was agitated gently by tapping for one minute, then placed in a 37°C incubator for 30 minutes. After color development, the plate was read in an ELISA plate reader (Bio-Tek instruments) at 540 nm.

2.5 SDS polyacrylamide gel electrophoresis and Western blot

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out essentially as described by Laemmli et al. (1970) using a slab gel apparatus.
manufactured by Hoeffer. Typically, samples for SDS-PAGE were made up in an equal volume of SDS-PAGE loading buffer (4% SDS, 40% sucrose, 8% BME, 50 mM Tris buffer, pH 7.4). For gels run under non-reducing conditions, β-mercaptoethanol was omitted from the loading buffer. Gels were stained with either coomassie blue or silver according to the method of Wray et al. (1981). For Western blots proteins were transferred onto Immobilon-P (Millipore) using a semi-dry transfer apparatus (Biorad). Western blots were blocked in PBST containing 0.5% powdered milk and labeled with antiserum or hybridoma cell culture supernatant diluted in PBST containing 0.05% powdered milk. Secondary antibodies were goat anti-rabbit Ig-Fab' fragments-horseradish peroxidase or sheep anti-mouse Ig-Fab' fragments-horseradish peroxidase (Amersham) diluted 1:5000 in PBST with 0.05% powdered milk. Blots were labeled for 30 minutes, and washed between labeling steps 3 times for 5 minutes with PBST. Antibody labeling was detected using the ECL detection system (Amersham).

2.6 Preparation and proteolytic digestion of osmotically intact discs

For proteolytic digestion, ROS membranes were prepared by flotation on Ficoll gradients using the method of Smith et al. (1975). This preparation produces osmotically intact disc membranes associated with plasma membrane vesicles. Samples of membranes (300 μg protein) were digested at 37°C with 50 μg/ml tosyl-phenylalanine chloromethyl ketone-treated (TPCK-treated) trypsin (Sigma) in 1 ml of 50 mM Tris buffer, pH 7.0, containing 20 mM CaCl₂ in the presence or absence of 0.1% Triton X-100. At specified times up to 20 hours, 100 μl aliquots were removed and added to 100 μl SDS-PAGE loading buffer containing 100 μg/ml PMSF and boiled for 10 min. Control samples containing no trypsin were incubated 20 hours and treated identically. The samples were then analyzed by Western blotting.
2.7 Immunoperoxidase labeling.

Cross-reactivity of antibodies was assessed by immunoperoxidase labeling of fixed CHO-K1 cells expressing bovine rom-1 and peripherin/rds. CHO-K1 cell lines were grown on glass coverslips, and after the desired degree of confluency had been reached, the cells were rinsed 3X with PBS containing 0.2% bovine serum albumin (BSA). The cells were fixed with 3% paraformaldehyde in PBS for 10 minutes at room temperature. The cells were again washed with PBS 0.2% BSA and incubated in PBS containing 20 mM glycine for 10 minutes. The cells were blocked and permeabilized by incubating in PBS containing 1% goat serum and 0.05% Triton X-100 (TX100) for 30 minutes. Antibodies were bound in PBS + 0.1% goat serum + 0.05% TX100. Horseradish peroxidase conjugated secondary antibody (goat anti-rabbit Ig - horseradish peroxidase Fab' fragment (Amersham)) was diluted 50X. Cells were incubated with the primary antibody for 45 minutes, and with the secondary antibody for 30 minutes. Between incubations cells were washed with PBS 0.2% BSA. Peroxidase labeling was detected using metal-enhanced DAB (3,3' diamino benzidine tetrahydrochloride) substrate (Pierce) as recommended by the manufacturer. Development of staining was monitored using a microscope. When staining reached satisfactory levels, coverslips of control and expressing cells were simultaneously transferred to PBS buffer.

2.8 Immunofluorescence microscopy

2.8.1 Immunofluorescence microscopy of cultured cells. Transiently transfected cells were prepared and fixed as described in sections 2.3.1 and 2.7. Secondary antibody was CY3-labeled goat anti-mouse Ig or anti-rabbit Ig (Jackson), which was applied at 200X dilution in PBS containing 0.1% goat serum and 0.05% TX100. For
immunofluorescence microscopy of non-permeabilized cells, TX100 was left out of the blocking and antibody-binding steps.

2.8.2 Immunofluorescence microscopy of retinal tissue cryosections. Bovine eyes were obtained fresh from the slaughter house and fixed on the premises. Human eyes were obtained from the UBC eye-bank. The corneae and lenses of the eyes were removed, and the vitreous humor was gently drawn off with a pipette. The eye-cups were placed in fixative composed of 4% paraformaldehyde 1% sucrose 100 mM phosphate pH 7.2 for 1 hour. The retina was gently detached and further fixed in the same solution for another hour. Small sections of retina were sliced off and embedded by quick-freezing in O.C.T. embedding compound (Tissue-Tek). Thin sections were cut from O.C.T. blocks containing retina slices using a cryostat. Sections were labeled using a protocol essentially identical to that used on transiently transfected cells. For double labeling experiments using fluorescently labeled antibodies, both antibodies were applied in a single labeling step, and washed with PBS 0.2% BSA.

2.9 Immunoelectron microscopy

Two different protocols were used for immunoelectron microscopy labeling: pre-embedding and post-embedding. In pre-embedding experiments the tissue is labeled prior to embedding and sectioning, and antibodies only have access to epitopes on exposed membrane surfaces. In post-embedding labeling, the tissue is labeled after embedding and sectioning, and the antibodies have access to all regions of the sections.

2.9.1 Post-embedding labeling. Bovine eyes were obtained from a slaughter house and fixed as described above (2.8.2). Tissue was embedded and sectioned using a modification of the method of Erikson et al. (1987). Small fragments of retina were
washed with 0.5% sucrose 137 mM phosphate pH 7.2 and then incubated in 100 mM glycine, 0.5% sucrose, 137 mM phosphate pH 7.2 for 5 minutes. After washing again in phosphate/sucrose, the buffer was changed to 0.5% sucrose, 2% maleic acid pH 5.1 using 5 X 5 minute washes. Finally, the buffer was changed to 2% maleic acid pH 6.0 with 2% uranyl acetate (filtered) and the tissue was incubated for one hour with 2 buffer changes. The tissue was then dehydrated and post-fixed with uranyl acetate by passing it through a graded series of increasing methanol concentrations containing 2% uranyl acetate (15%, 30%, 50%, 70%, 85%, 95% and 100% methanol, 10 minutes each). Finally, the tissue was washed in 100% methanol without uranyl acetate for 2 X 20 minutes. The tissue was then placed in a 1:1 mixture of LR white resin and methanol overnight at 4°C. The next day the tissue was transferred to 100% LR white resin at 4°C for 2 hours and transferred to fresh resin in a gelatin capsule for 4 hours at room temperature. The LR white resin was allowed to polymerize in the sealed capsules for 2-3 days at 52-57°C. Small pieces of LR white resin containing the embedded tissue fragments were cut out of the LR white blocks and re-oriented for sectioning in Epon/Araldite resin, which is stronger than LR white and has better sectioning properties. Thin sections were cut using a microtome equipped with a diamond knife, and were collected on nickel grids.

Treatment of lysed ROS for post-embedding labeling was similar. Lysed ROS were prepared by incubating ROS at a concentration of 1 mg/ml in 10 mM Tris pH 7.5 for 20 minutes on ice. One volume of 10% sucrose, 20 mM Tris pH 7.5 was added and the discs were gently spun down onto plastic Thermanox coverslips (Nunc). Coverslips were fixed and dehydrated as described above for retinas, then placed disc-surface down on rubber wells filled with LR white resin. The resin was allowed to polymerize as above, and the surface of the block was cooled in liquid nitrogen. This allowed the plastic coverslip to be easily pulled off, after which ultra-thin sections were sliced off the surface of the block and collected on nickel grids.
Sections were immuno-gold labeled by incubating the grids inverted on small drops of the following solutions:

- 100 mM Tris pH 7.5, 5 minutes
- 4% BSA, 100 mM Tris pH 7.5, 10 minutes
- 20 mM Glycine, 100 mM Tris pH 7.5, 15 minutes
- Primary antibody (3X dil.) in 1% BSA, 100 mM Tris pH 7.5, 30 minutes
- 100 mM Tris pH 7.5, 6 X 1 minute
- Secondary antibody (gold conjugate) 5X dilution in 1% BSA, 100 mM Tris pH 7.5 30 minutes
- 100 mM Tris pH 7.5, 6 X 1 minute
- 100 mM phosphate pH 7.5, 6 X 1 minute
- 2% glutaraldehyde in 100 mM phosphate pH 7.5, 20 minutes
- dH₂O, 6 X 1 minute
- Saturated uranyl acetate, 1 minute
- dH₂O, 6 X 1 minute
- Reynold's lead citrate stain, 1 minute
- dH₂O, 6 X 1 minute
- air dry overnight

2.9.2 Pre-embedding labeling. Hypotonically lysed ROS membranes were prepared from ROS isolated from fresh eyes and maintained at 1 mg/ml in 10 mM Tris pH 7.5 for 20 minutes on ice. One volume of 10% sucrose, 20 mM Tris pH 7.5 was added and the discs were gently spun down onto plastic Thermanox coverslips. The coverslips were washed with a solution of 2% sucrose, 20 mM Tris pH 7.5 (TW) and then blocked in 5% sucrose, 4% BSA, 20 mM Tris pH 7.5 (BIB) for 30 minutes at room temperature. The primary antibody was applied in the same solution. Purified polyclonal antibodies were used at 3X dilution, and labeling was carried out for 2 hours at 4°C. Coverslips were again washed in WB and incubated with goat anti-rabbit Ig secondary antibody conjugated to 10 nm gold particles (British Biocell) diluted 5X in BIB for 1 hour at 4°C. Finally, the secondary antibody was washed off with TW.
The coverslips were fixed by washing in 2% sucrose 20 mM cacodylic acid pH 7.5 (CW) followed by incubation in Karnovsky's fix (Karnovsky, 1965) for 1 hour at room temperature. The coverslips were washed again in CW and then in 0.2% sucrose, 100 mM cacodylic acid pH 7.5. The coverslips were post-fixed in a 1:1 mixture of OsO₄ and 0.4% sucrose, 200 mM cacodylic acid pH 7.5 for one hour at 4°C and dehydrated by passing through a graded series of ethanols (50%, 70%, 80%, 90%, 95%, 5 minutes each) followed by 100% ethanol for 30 minutes. The coverslips were then placed disc side down on rubber wells filled with Epon/Araldite resin and left 2-3 days to polymerize at 52-57°C. The coverslips were removed and resin blocks were sectioned and stained with saturated uranyl acetate and Reynold's lead citrate as described above (2.9.1).

2.10 Immunoprecipitation

2.10.1 Immunoprecipitation from ROS membranes. For immunoprecipitation studies on ROS, 250 µg ROS membranes (Molday and Molday, 1993) were solubilized in 5.0 ml PBS containing 1% CHAPS. Typically, 1 ml of solubilized ROS was added to 150 µl of rom1D5-Sepharose or per2B6-Sepharose (2.2.7) and the mixture was incubated overnight at 4°C. The beads were washed 4 times with 1 ml of PBS containing 1% CHAPS and the bound protein was eluted with 200 µl SDS-PAGE loading buffer without BME to which 200 µl of PBS containing 1% CHAPS and 8% BME was then added. Supernatants were concentrated 5X in a Centricon 10 (Amicon) before adding an equal volume of SDS-PAGE loading buffer.

2.10.2 Immunoprecipitation from COS-1 cell extracts. For immunoprecipitation studies on COS-1 cell extracts, cells were transiently transfected as described in section (2.3.1). TX100 or CHAPS detergent extracts of the cells were prepared by washing plates of 90% confluent cells with PBS, followed by solubilization in 0.5 ml of PBS
containing 1% (w/v) CHAPS or TX100 and 100 μg/ml PMSF. Cell extracts were scraped off the plates and centrifuged 5 minutes in a microfuge at maximum speed to obtain a supernatant fraction. Ten μl of per2B6 or rom1D5 Sepharose (2.2.7) were added and mixed gently at 4°C for 1 hour. The beads were washed with 1% detergent in PBS using an Ultrafree-MC Centrifugal Filtration unit (Millipore). Bound protein was eluted from the antibody-Sepharose using either 1 mg/ml of an epitope-specific peptide in the same buffer, or SDS-PAGE loading buffer containing no BME.

2.11 Velocity sedimentation

Velocity sedimentation analysis was carried out on heterologously expressed wild-type rom-1, wild-type peripherin/rds, and rom-1 mutants using a modification of the method of Clarke and Smigel (1989) as described by Goldberg et al. (1995). Protein was harvested from transiently transfected cells as described above (2.3.1, 2.10.2), except that cells were extracted with 1% TX100 in PBS with 100 μg/ml PMSF and 1 mM dithiothreitol (DTT), and extract supernatants were obtained by ultra-centrifugation at 50,000 rpm in a TLA 100.4 rotor (Beckman) instead of centrifugation in a microfuge. For co-transfection experiments, protein was first purified by immunoprecipitation and elution with a specific peptide as described above (2.10.2) with 1 mM DTT included in the wash and elution buffers.

Sucrose gradients (5-20%) were poured by sequentially adding 0.5 ml of 20%, 15%, 10% and 5% sucrose 0.1% TX100 1 mM DTT in PBS to an Ultraclear centrifuge tube (Beckman). The gradients were allowed to diffuse for 1 hour at room temperature, then placed on ice for 30 minutes. Samples were loaded onto the gradient in a volume of 100 μl and centrifuged at 50,000 rpm in a TLS-55 rotor (Beckman) for 16 hours at 4°C. After centrifugation, gradients were collected by puncturing the bottom of the tube.
and collecting 4-drop fractions. Protein peaks were detected by SDS-PAGE followed by western blotting (2.4). Elution profiles were determined by laser-densitometry.

Sedimentation coefficients at 20°C in dH₂O (S₂₀,w) were calculated using a Q-basic computer program written by Dr. AFX Goldberg (see appendix 1). The computer program calculates the S₂₀,w value for peaks obtained under the conditions described above (tubes and rotors) based on the following formulae:

(1) \[ S_{T,m} = \frac{\nu}{\omega^2 r} \]

This is the basic formula that describes the sedimentation coefficient (Sₜₘ) at the measured temperature and medium, where \( \nu \) is the instantaneous velocity of the macromolecule, \( r \) is the distance of the macromolecule from the axis of rotation, and \( \omega \) is the angular velocity of the rotor. The expression \( \nu \) can be replaced by the average velocity (\( \nu_{avg} \)), determined by dividing the distance traveled through the gradient by the time of the spin, \( t \). Here, \( r_f \) is the final radial position of the macromolecule, and \( r_0 \) is the starting radial position:

(2) \[ \nu_{avg} = \frac{(r_f - r_0)}{t} \]

Substitution of (2) into (1) allows the calculation of the sedimentation coefficient of the macromolecule at the experimental temperature (T) and medium (m) at the average radial position (\( r_{avg} \)).

(3) \[ S_{T,m} = \frac{[(r_f - r_0)/t]}{\omega^2 r_{avg}} \]
Finally, by determining the viscosity and density of the medium at \( r_{\text{avg}} \), the sedimentation coefficient at 20°C in water \( (S_{20,w}) \) can be calculated using the following formula:

\[
S_{20,w} = S_{T,m}(\eta_{T,m}/\eta_{20,w})[(1 - \nu \rho_{20,w})/(1 - \nu \rho_{T,m})]
\]

\( \eta_{T,m} \) and \( \eta_{20,w} \) are the viscosities of the medium at temperature \( T \) and the viscosity of water at 20°C. \( \rho_{T,m} \) and \( \rho_{20,w} \) are the densities of the medium at the temperature \( T \) and the density of water at 20°C. The value \( \nu \) is the partial specific volume of the protein/detergent complex. The adjusted value \( S_{20,w} \) is more useful for comparison to previously published values. The following assumptions are made in these calculations:

- The temperature of the centrifuge tube is a constant 4°C throughout its length. Thus, all densities and viscosities are calculated for the medium at 4°C.

- The partial specific volume (\( \nu \)) of bovine rom-1 is 0.83 ml/g, the same as the partial specific volume reported for the peripherin/rom-1 complex by Goldberg and Molday, (1996).

- The sucrose gradients are linear. Thus, the sucrose concentration of the fractions is determined by assuming a linear gradient from 5% to 20% sucrose. This assumption was found to be valid on a number of occasions by determining sucrose concentrations of fractions using a refractometer.

2.12 Generation of rom-1 and peripherin/rds mutants
2.12.1 Generation of rom-1 deletion mutants by exonuclease digestion. A number of mutant bovine rom-1 cDNAs encoding proteins with truncated C-termini were constructed using a modification of the unidirectional exonuclease digestion protocol of Henikoff et al. (1987), summarized in Fig 11. To create a target for exonuclease digestion, the EcoRI/NotI fragment of the pcDNA3 construct described above (2.3.1) was cloned into EcoRI/NotI cut pBluescript II SK+. This target construct (romTarg) was cut with SacI and SmaI to generate exonuclease resistant and exonuclease sensitive sites. The linearized plasmid was digested unidirectionally with exonuclease III, and blunt ends were generated with S1 nuclease followed by treatment with the Klenow fragment of DNA polymerase 1. The ends of the linearized plasmid were dephosphorylated, and the plasmid was circularized in the presence of a phosphorylated palindromic synthetic oligonucleotide (NOTSTOP). This oligonucleotide contained stop codons in all three reading frames, as well as a NotI site. Clones that contained truncated inserts were identified by digestion with AflIII, and clones that contained the NOTSTOP oligonucleotide were identified by NotI digestion (The AflIII site is just upstream of the SmaI site, and is deleted almost immediately). Clones that contained both a truncated insert and a NOTSTOP oligonucleotide were sequenced.

2.12.2 Generation of the mutants romL188P, perY258Stop and perL307(1bp del) by PCR mutagenesis. A PCR-based mutagenesis protocol was used to introduce single base changes in the bovine rom-1 and peripherin/rds cDNAs. The mutants constructed via this protocol were romL188P, perY258Stop and perL307(1bp del). For rom-1 mutagenesis, the construct romTarg described above (2.12.1) was used as a template. For peripherin/rds mutagenesis, the peripherin/rds cDNA was cloned into pBluescript II SK+ cut with BamHI, with the upstream end of the cDNA adjacent to the T3 promoter. This construct was called perT3, and was provided by Dr. AFX Goldberg. Three different mutagenic primers were synthesized for construction of these mutants, L188P,
**FIG. 11. Construction of rom-1 deletion mutants.** This flow chart illustrates the methodology used to create truncated rom-1 cDNAs. The rom-1 cDNA was cloned into pBluescript, then cut with restriction enzymes to generate exonuclease III sensitive and resistant ends. After controlled digestion with exonuclease III, the ends were re-ligated in the presence of an phosphorylated oligonucleotide (NOTSTOP) that encodes stop codons and a NotI site. Inserts were then transferred to the expression vector pcDNA1/AMP as EcoRI/NotI fragments.
L307(1bp del), and Y258Stop, and these were used in conjunction with three additional primers, primer B, primer C, and primer D. Primer B and primer C bind to opposite strands of pBluescript, and primer D binds to a sequence in primer B that is not found in pBluescript.

The protocol for PCR mutagenesis was based on that of Nelson and Long, (1989), and is illustrated in Fig 12. Initially, the mutagenic primer was used in a standard PCR reaction with primer B and the relevant template. The product of this reaction was purified on an agarose gel, and then used as a "mega-primer" in a second round of PCR, using the same template. Only the strand originating from primer B can prime synthesis, as the 3' end of the opposite strand does not bind the template. After 3 rounds of amplification, primers C and D were added. Primer D binds to sequence found only in primer B, and will not prime synthesis from pBluescript. Primer C will only prime synthesis from pBluescript derived sequences. Thus, all products amplified from this reaction must originate from extension of the original PCR product containing the mutation on the pBluescript template. The product of this reaction should be equal in length to the pBluescript insert plus some pBluescript multiple cloning site-derived sequence.

Fragments of these full length PCR mutagenesis products containing the mutations were sequenced and subcloned back into the rom-1 and peripherin/rds expression constructs. The methodology varied from construct to construct. The rom-1 mutant romL188P was assembled in pBluescript in order to take advantage of restriction sites unavailable in pcDNA1/AMP. The romL188P PCR product was cut with EcoRI and BamHI, and the 607 bp fragment was cloned into pBluescript II SK- for single-stranded sequencing. Six of the six inserts sequenced were found to contain the desired mutation. However, some inserts contained additional PCR-induced mutations, and these clones were discarded. Next, the wild-type rom-1 cDNA was transferred from pcDNA1/AMP to pBluescript II SK+ as an EcoRI/NotI fragment. This construct was called
FIG. 12. **PCR mutagenesis.** The protocol used for PCR mutagenesis is summarized in the flow diagram above. The protocol is based on the method of Nelson and Long (1989). The protocol employs one mutagenic primer and three other invariant primers. Initially, PCR is carried out with the mutagenic primer used in conjunction with a vector specific primer B to generate a "mega-primer." The mega-primer is then extended on the same template. Finally, the mutant sequence is amplified using vector specific primer and a primer B- specific primer (primers C and D.) The final amplified sequence consists of the entire mutant insert flanked by vector multiple cloning site sequences.
romAssembl. A 346 bp region containing the L188 codon of the rom-1 cDNA was excised from romAssembl by digestion with Nhel/BamHI, and the sequence was replaced with the Nhel/BamHI fragment of a PCR clone with the desired mutation. The presence of the mutation in the assembled cDNA was confirmed by restriction analysis of the construct with Nhel/BamHI as compared to Nhel/BamHI digestion of romAssembl. (The introduction of the romL188P mutation deletes a BamHI site, resulting in a larger band on an agarose gel.). The entire romL188P cDNA was then transferred back to the pcDNA1/AMP expression vector as an EcoRI/NotI fragment.

The perL307(1bp del) PCR product was cut with EcoRI/BamHI, and cloned into EcoRI/BamHI cut pBluescript II SK+ for sequencing. Four of four clones sequenced contained the desired mutation. To assemble the complete cDNA, a three-way ligation was set up between the XhoI/Ball fragment of wild-type peripherin/rds (from the pcDNA1/AMP expression construct) the Ball/BamHI fragment of a sequenced PCR product, and XhoI/BamHI cut pcDNA1/AMP. Clones containing full length cDNAs were identified by BamHI digestion.

The perY258Stop PCR clones were sequenced by cutting the PCR product with SacII/BamHI and cloning the mutation-containing fragment into pBluescript II SK+. Four out of four clones sequenced carried the mutation. The full length construct was assembled in pcDNA1/AMP as follows. A three-way ligation was set up containing XhoI/BamHI cut pcDNA1/AMP, the XhoI/SacII fragment of wild-type peripherin/rds (from the pcDNA1/AMP expression construct), and the SacII/BamHI mutation-containing fragment from a sequenced PCR clone. Clones containing full length cDNAs were identified by BamHI digestion.

2.12.3 Generation of the mutants perW306, perM293+VSE and perP296+VSE using synthetic oligonucleotide linkers. A number of truncated peripherin/rds cDNAs were constructed as follows. The peripherin/rds construct perT7 was provided by Dr. AFX
Goldberg. This construct is similar to the perT3 construct (2.12.2), but the peripherin cDNA is cloned in the opposite orientation. The perT7 construct was cut with Ball and NotI. Ball cuts the peripherin/rds cDNA at codon perA294, and NotI cuts the multiple cloning site of pBluescript II SK+. A synthetic linker composed of two oligonucleotides was ligated to the Ball/NotI cut plasmid. The oligonucleotides used were PD and PC. The linker encodes an alternate truncated peripherin/rds C-terminal region containing a silent mutation that inserts an AvaI site. The translated sequence is the same as wild-type up to codon perW306, after which the sequence terminates. Additionally, the NOTSTOP linker (2.12.1) was ligated into Ball cut perT7. The resulting cDNA encodes a translated sequence identical to wild-type peripherin up to codon M293, followed by three additional amino acids, VSE, after which the sequence terminates. Finally, a third mutant was generated by cutting the perW306 cDNA with AvaI. The ends of the plasmid were made blunt using a Klenow fill-in protocol, and dephosphorylated. The NOTSTOP linker was then ligated to the blunt-ended plasmid, generating a cDNA encoding a sequence identical to wild-type peripherin/rds up to codon perP296, followed by three additional amino acids, VSE, after which the sequence terminates. All of these constructs were sequenced, and the BamHI/NotI cDNA inserts were transferred to pcDNA1/AMP.

2.13 Oligonucleotide sequences and reagent recipes. The sequences and applications of synthetic oligonucleotides used in this study are listed in Table 3.
The following reagents are referred to throughout the text:

**PBS**: phosphate buffered saline: 8.0 g NaCl, 0.2 g KCl, 0.2 g KH$_2$PO$_4$, 1.2 g Na$_2$HPO$_4$, dH$_2$O to 1 liter.

**PBST**: phosphate buffered saline with 0.05% Tween-20 detergent

**HBS**: hepes buffered saline: 21 M HEPES pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na$_2$HPO$_4$

**HBSG**: hepes buffered saline with glucose: HBS containing 6 mM glucose.

<table>
<thead>
<tr>
<th>Table 3: Synthetic oligonucleotide sequences and applications</th>
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<tbody>
<tr>
<td><strong>Name</strong></td>
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<tr>
<td>INVI</td>
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<tr>
<td>INV2</td>
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<tr>
<td>INV3</td>
</tr>
<tr>
<td>PL1</td>
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<tr>
<td>PL2</td>
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<tr>
<td>PL3</td>
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<td>Hind1</td>
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<tr>
<td>Not1</td>
</tr>
<tr>
<td><strong>Eco1</strong></td>
</tr>
<tr>
<td><strong>Kasl</strong></td>
</tr>
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<td><strong>EK1 linker for 5' end of pcDNA1/AMP construct</strong></td>
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<tr>
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<tr>
<td><strong>EK2 linker for 5' end of pcDNA3 construct with Kozak sequence</strong></td>
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<tr>
<td><strong>Kas2</strong></td>
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<tr>
<td><strong>Afl1</strong></td>
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<tr>
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<tr>
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<tr>
<td><strong>NOTSTOP</strong></td>
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<tr>
<td><strong>L188P</strong></td>
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<td><strong>L3071bpdel</strong></td>
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<tr>
<td><strong>Y258Stop</strong></td>
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<td><strong>mutagenesis: perY258Stop</strong></td>
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<td><strong>Primer B</strong></td>
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<td><strong>PCR mutagenesis</strong></td>
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<td><strong>Primer C</strong></td>
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<tr>
<td><strong>PCR mutagenesis</strong></td>
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<tr>
<td><strong>Primer D</strong></td>
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<td><strong>Linker encoding truncated peripherin/rds C-terminus and Ava1 site.</strong></td>
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<tr>
<td><strong>PCR mutagenesis</strong></td>
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<td><strong>PC</strong></td>
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</table>
RESULTS

3.1 The primary structure of bovine rom-1

3.1.1 Cloning and sequencing of the bovine rom-1 cDNA. The cDNA sequence of bovine rom-1 was obtained from two partial cDNA clones and four PCR fragments amplified from genomic DNA (Fig 13, Fig 14). Initially, a partial bovine cDNA clone (brom1A) was isolated by screening a bovine retinal expression library with a human rom-1 cDNA fragment. This clone was then used to re-screen the library for longer clones. A cDNA clone (brom1G) was obtained that contained the complete coding sequence except for A of the ATG initiation codon. Inverse PCR was used to obtain the initiation codon and part of the promoter sequence.

The open reading frame codes for a protein of 351 amino acids (Fig 14, Fig 15) that is identical in length and 84% and 86% identical in sequence to human and mouse rom-1, respectively (Bascom et al., 1992; Bascom et al., 1993). Sequence differences are noted in Fig 14. Bovine rom-1 is also 32% identical in sequence to bovine peripherin/rds (Connell and Molday, 1990). A hydropathy profile of bovine rom-1 predicts a four transmembrane topological model (Fig 15) with a large hydrophilic loop between transmembrane segments M3 and M4 as previously predicted for human rom-1 and peripherin/rds (Molday, 1994; Bascom et al., 1992; Connell and Molday, 1990). Other features of bovine rom-1 that are also present in human and mouse rom-1 include nine conserved cysteine residues, seven of which are localized within the large loop, and the absence of a consensus sequence for N-linked glycosylation. Like other rom-1 sequences, bovine rom-1 contains a conserved cysteine present in the first transmembrane domain, and another in the first small intradiscal loop region that are not found in peripherin/rds sequences. Similarly, there are no cysteines present in the second and third transmembrane regions, or in the small cytoplasmic loop region, the site of
Fig. 13. **Positional relationship and sequence overlap of bovine rom-1 clones.**
The sizes and overlap of the various rom-1 clones obtained are compared in this scale
drawing. The coding region is highlighted, and the first base of the coding region is
numbered +1. The sequences Brom1A and Brom1G are clones obtained from a cDNA
library, and are therefore derived from mRNA. The sequences BrINV1, BrPCR1 and
BrPCR2 are the products of inverse PCR and regular PCR protocols carried out on
genomic DNA. The sequence of the inverse PCR product has been represented such
that it aligns with the other sequences, though the -374 and +568 positions are actually
joined by the Mbo1 site.
FIG. 14. Nucleic acid sequence and deduced amino acid sequence of the bovine rom-1 cDNA and promoter. The predicted transcription start site is indicated by an asterisk (*) and is based on homology with the initiation sites and promoters of the human and mouse ROM1 genes (Bascom et al. 1993a). Start positions of the clones Brom1G and Brom1A are underlined. Annealing sites of primers used for inverse and regular polymerase chain reaction are indicated INV1, INV2 and INV3. Amino acid differences between bovine rom-1 and human and mouse rom-1 are indicated. (Bascom et al. 1992, 1993a)

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**Fig. 15. Topological model of bovine rom-1.** Predicted topology of bovine rom-1 in the disc membrane is shown in the form of a two-dimensional model. The model is based on hydropathy profiles predicting four transmembrane segments and the binding of C-terminal-directed antibodies on the cytoplasmic side of disc membranes. Heavy circles indicate amino acid residues conserved between bovine rom-1 and bovine peripherin/rds. The C-terminal GST-peptide used to generate the antibodies romC2 and rom1D5 and the intradiscal GST-peptide used to generate the antibodies romL4 and rom1C6 are shown as shaded regions. The epitope for rom1D5 antibody FPAGLKD is indicated.
three single cysteine residues and a cysteine pair in peripherin/rds. The predicted pI of bovine rom-1 is much lower than the pI of bovine peripherin/rds (pI 5.77 vs. 7.97), a feature also seen in other rom-1 sequences. Unlike other rom-1 and peripherin/rds sequences, bovine rom-1 contains an additional cysteine residue in the C-terminal region (C347).

The promoter sequence obtained by inverse PCR has a high degree of homology to previously reported rom-1 promoters (Bascom et al., 1993a), but no discernible homology to the peripherin/rds promoter (Ma et al., 1995). The sequences of known rom-1 promoters are aligned in Fig 16. The rom-1 promoter is somewhat unconventional in that it does not contain a TATA box (Bascom et al., 1993a). However, a TATA-like sequence is present and conserved between known rom-1 promoters, but this sequence is too far removed from the transcription start site to constitute a true TATA-box (Bucher, 1990). Promoter activity is thought to be mediated by CpG islands and GC boxes. Like other rom-1 promoter sequences, the bovine rom-1 promoter contains a number of potential GC boxes. The highest scoring GC box based on the weight matrix of Bucher (1990) is marked in Fig 16 and is conserved between the three known rom-1 promoters. Similar weight matrices did not identify any potential CCAAT boxes. Insufficient promoter sequence was obtained to identify a CpG islands. However, the GC content of the promoter sequence does increase towards the upstream end, suggesting a CpG island may be present.

3.1.2 Bovine rom-1 Southern blot. A Southern blot of MboI digested bovine genomic DNA labeled with a probe derived from the 5' BamHI fragment of Brom1G labeled a 940 bp band (Fig 17). This southern blot was used to predict the size of the inverse PCR product. As the primers were separated by 79 bp, the predicted size of the inverse PCR product was therefore 861 bp. This compared favorably with the size of the 859 bp fragment obtained by inverse PCR. EcoRI digestion proved unsuitable for inverse
FIG. 16. Alignment of the bovine, human and mouse promoters. The sequence of the bovine ROM1 promoter obtained by inverse PCR is compared to the sequences of the previously published human and mouse ROM1 promoters (Bascom et al. 1993a) using the alignment program CLUSTAL (PCGENE, Intelligenetics). Identical bases are indicated by (*) and similar bases are indicated by (.). The bovine promoter is 82% identical to the human promoter and 76% identical to the mouse promoter. The site of transcript initiation (!) was determined for the human ROM1 promoter by primer extension (Bascom et al. 1993a) and is highly conserved between the three sequences. A number of possible GC boxes are found in the sequence. The highest scoring box in all three sequences is underlined. A TATA-like sequence (double underlined) is also highly conserved between the three sequences, but lies too far from the transcription start site to be a TATA box.
FIG. 17. Southern blots of bovine genomic DNA probed with rom-1 cDNA. Bovine genomic DNA (30 ug) was digested with either EcoRI or Mbol, and run on a 0.8% (EcoRI) or 2.0% (Mbol) agarose gel. The DNA was transferred to nylon membranes and probed with the BamHI fragment of Brom1G. The probe labeled a single 5.6 kb band in EcoRI digested genomic DNA, and a 940 bp band in Mbol digested DNA, suggesting that bovine ROM1 is a single-copy gene. (Size standards are 1kb ladder (BRL), and are given in base pairs). Mbol-digested genomic DNA was circularized and used for inverse PCR. Based on this Southern blot, the predicted size of the inverse PCR product was 861 bp, as the primers were separated by 79 bp (Figure 14). This compares well with the size of the product obtained, 859 bp.
PCR, since the size of the genomic fragment was too large (5.6 kb). A single band was labeled on Southern blots of both MboI and EcoRI digested bovine genomic DNA. This suggests that bovine ROM1 is a single copy gene.

3.1.3 Bovine rom-1 Northern blot. A Northern blot of bovine retina total RNA probed with the EcoRI/HindIII fragment of Brom1G (Fig 18) showed labeling of a 1.35 kb band. This was in agreement with the predicted transcript size based on the cloned cDNAs and inverse PCR products, indicating that the complete cDNA sequence has been cloned.

3.2 Generation and characterization of polyclonal and monoclonal antibodies

In order to aid in the study of the bovine rom-1 protein, antibodies were generated against selected segments of bovine rom-1 expressed in E. coli as glutathione-S-transferase fusion proteins. Polyclonal antibody romC2 and monoclonal antibody rom1D5 were raised against a GST fusion protein containing the C-terminal 63 amino acids of bovine rom-1, while polyclonal antibody romL4 and monoclonal antibody rom1C6 were raised against a fusion protein containing a 65 amino acid segment found within the proposed intralumenal loop (Fig 15). Additionally, a polyclonal peripherin/rds antibody, perL1, was generated against a fusion protein incorporating a 30 amino acid segment of the peripherin/rds intradiscal loop.

The antigens used to generate these antibodies share some sequence identity between bovine rom-1 and peripherin/rds. To determine if the rom-1 polyclonal antibodies cross-react with peripherin/rds and vice versa, stably transfected CHO-K1 cell lines expressing either bovine peripherin/rds or bovine rom-1 were produced and cell extracts were analyzed for antibody cross-reactivity by Western blotting. As shown in Fig 19, the anti-rom-1 polyclonal antibodies labeled a single 35 kDa polypeptide in both
FIG. 18. Northern blot of bovine retina total RNA probed with rom-1 cDNA. Bovine retina total RNA (30 µg) was run on a 1.4% formaldehyde-agarose gel, transferred to a nylon membrane, and probed with the EcoR1/HindIII fragment of Brom1G. Size standards are RNA ladder (BRL), given in kilobases. The size of the labeled fragment is 1.35 kb, which agrees well with the size of the cDNA obtained (1356 bp plus a poly-A tail). Thus it is likely that the complete coding sequence was obtained.
FIG. 19. **Cross-reactivity of polyclonal antibodies romL4 and romC2 determined by western blot.** CHAPS-solubilized bovine rod outer segments (lane a = 0.3 μg protein) and CHAPS extracts of a rom-1 expressing CHO-K1 cell line (RomG) (lane b = 6.0 μg protein), a peripherin/rds expressing CHO-K1 cell line (ChoPer) (lane c = 6.0 μg protein), and a control CHO-K1 cell line (ControlB) (lane d = 6.0 μg protein) were separated on an 8% SDS-PAGE gel, transferred to Immobilon-P membranes and labeled with anti-rom-1 polyclonal antibodies romC2, romL4 or anti-peripherin/rds monoclonal antibody per2B6 followed by secondary antibody-horseradish peroxidase conjugates for detection by ECL. The romC2 and romL4 antibodies label rom-1 in rod outer segment membranes and in rom-1 expressing CHO-K1 cells, but they do not cross-react with peripherin/rds expressing CHO-K1 cells. The mobility of rom-1 from both sources is identical, indicating that the complete coding sequence of bovine rom-1 was obtained. The broad peripherin/rds band is due to non-uniform glycosylation of peripherin/rds by the CHO cells.
bovine ROS and extracts of CHO-K1 cells expressing rom-1. No labeling was observed in extracts of CHO-K1 cells expressing peripherin/rds. Thus, there is no cross-reactivity of these antibodies on Western blots. Additionally, these studies show that the bovine rom-1 cDNA clone encodes the full-length rom-1 protein as found in native rod outer segment membranes.

Many antibodies exhibit differential labeling of fixed and non-fixed antigens. Because the anti-rom-1 antibodies were used for microscopy applications where fixed tissue was labeled, the specificity of the rom-1 antibodies was confirmed by immunoperoxidase labeling for light microscopy. In this study the rom-1 antibodies labeled paraformaldehyde-fixed COS-1 cells that transiently express bovine rom-1, but not cells that express peripherin/rds (Fig 20). These results indicate that the rom-1 antibodies do not cross-react with peripherin/rds in fixed tissue.

Monoclonal antibody rom1D5 was tested for cross-reactivity with peripherin/rds on western blots as described above. The rom1D5 epitope was mapped using a series of synthetic peptides. Peptides were 9 amino acids long and overlapped by seven amino acids. Rom1D5 bound the peptide sequences YLFPAGLKD and FPAGLKDMML, but not AGLKDMMLKT or QGYLFPAGL. The epitope for the rom1D5 monoclonal antibody is therefore contained within the seven amino acid segment FPAGLKD near the C-terminus of rom-1 (Fig 15). The absence of this sequence in peripherin/rds is consistent with the lack of cross-reactivity of the rom1D5 antibody with peripherin/rds.

The species cross-reactivity of the various antibodies produced was tested on Western blots of ROS isolated from bovine, human, mouse, rat, dog, pig, chicken and frog retinas. Antibodies rom1D5 and romC2 were found to have fairly broad specificity, while romL4 did not cross-react with any species tested, and rom1C6 cross-reacted only with mouse rom-1. Because of the high degree of homology between the bovine rom-1 sequence used as an immunogen to produce rom1C6 and the corresponding regions of human and mouse rom-1 (Bascom et al., 1993), the rom1C6 epitope could be further
FIG. 20. **Cross reactivity of romC2 antibody by immunoperoxidase labeling.**

COS-1 cells transfected with either rom-1 expression plasmid (rom), peripherin/rds expression plasmid (per) or pcDNA1/AMP control plasmid were fixed with 2% paraformaldehyde, permeabilized with TX100, and labeled with romC2 or per2B6 antibodies. Labeling was detected using a secondary antibody conjugated to horseradish peroxidase and DAB peroxidase substrate. The romC2 antibody showed no cross-reactivity with peripherin/rds. The antibodies romL4 and rom1D5 were also tested using an identical protocol, and also showed no cross-reactivity with peripherin/rds. (Magnification = 100 X)
narrowed down based on its cross-reactivity. It is likely that rom1C6 binds somewhere in the region between romR186 to romR198. The names of the various antibodies, their corresponding epitopes, species cross-reactivity (where known) and applications for which they have been tested, are summarized in Table 4.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Epitope</th>
<th>Applications*</th>
<th>Cross-reactivity*</th>
</tr>
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<tr>
<td>romC2</td>
<td>romL289 to romA351</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>romL4</td>
<td>romR165 to romQ229</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>rom1C6</td>
<td>perR183 to perR212</td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Applications are Western blot, Immunofluorescence microscopy, Enzyme-linked immunosorbent assay, and immunoelectron microscopy.

| Abbreviations are B-bovine, H-human, D-dog, M-mouse, C-cat, P-pig |

3.3 Distribution of rom-1 in rod and cone cells

3.3.1 Localization of rom-1 in the bovine retina by immunoelectron microscopy. The distribution of rom-1 in retinal photoreceptors was examined using the anti-rom-1 antibodies and immunogold labeling techniques. Both the romC2 and romL4 antibodies were found to label both rod and cone outer segments by post-embedding labeling methods (Fig 21A & C). The labeling density of the romL4 antibody was significantly greater than that of the romC2 antibody, presumably due to the greater accessibility of the romL4 epitope in resin-embedded samples. Immunogold labeling was generally confined to the periphery of cone outer segments and to both the periphery and incisures
FIG. 21. Immunogold localization of rom-1 to the rim regions of bovine rod and cone photoreceptor outer segment disc membranes using polyclonal antibodies romC2 and romL4. Immunogold localization of rom-1 to the rim regions of rod and cone photoreceptor outer segment disc membranes. (A-C) Electron micrographs showing rod and cone outer segments of bovine retina embedded in LR white resin and labeled with anti-rom-1 antibodies and immunogold markers (10 nm dia.). (A) romL4 antibody labeling showing peripheral labeling of cone outer segments (COS) and peripheral and incisure (arrow) labeling of rod outer segments (ROS); (B) romL4 antibody labeling in the presence of competing GST-fusion peptide used to generate the romL4 antibody; only random labeling is observed in this control sample; and (C) romC2 antibody labeling showing peripheral labeling although less intense than that of the romL4 antibody. (D) Electron micrograph of hypotonically lysed rod outer segments labeled with the romC2 antibody and immunogold particles (10 nm dia.) and subsequently embedded in Epon-Araldite resin. Immunogold particles are observed on the rims of discs (arrows), but not on the lamellar region. (Bar = 500 nm)
of ROS. Labeling was not observed to any significant degree within the inner segment of photoreceptor cells or in other retinal cells. Localization of rom-1 to the outer segment layer of retina tissue was also observed by immunofluorescence microscopy (data not shown). The specificity of labeling was confirmed in control experiments in which pre-incubation of the antibodies with their respective fusion protein antigens abolished the peripheral labeling of the outer segments (Fig 21B).

Labeling of cone outer segments was confirmed by double labeling. Post-embedding labeling was performed as described above, using two labeling steps. First, the sections were labeled with romL4 and anti-rabbit-Ig secondary antibody conjugated to 10 nm gold particles. Then the sections were labeled with anti-rhodopsin monoclonal antibody rho4D2, (which labels ROS but not cone outer segments) and anti-mouse-Ig secondary antibody conjugated to 5 nm gold particles. As seen in Fig 22, romL4 labels outer segments that have 5 nm gold labeling (rods) and outer segments that do not have 5 nm gold labeling (cones).

3.3.2 Localization of rom-1 in the human retina by immunofluorescence microscopy.
The distribution of rom-1 was also examined in the human retina using immunofluorescence microscopy. In order to differentiate rods and cones in this procedure, a double labeling protocol was used. Retinas were labeled with both anti-rom-1 antibody rom1D5 conjugated to TRITC and anti-rhodopsin antibody rho1D4 conjugated to FITC. The rho1D4 antibody has previously been shown to label ROS but not cone outer segments (Hicks and Molday, 1986).

Double labeling of human retinas with rom1D5 and rho1D4 reveals that rom-1 is also present in human cones (Fig 23). This contradicts the results of Bascom et al. (1992) who did not find rom-1 in human cones using a polyclonal antibody raised against a human rom-1 fusion protein. The reasons for this discrepancy are not clear. Possible explanations include a closely related cone homolog of rom-1, post-translational
Fig. 22. Immunogold localization of rom-1 to the rim regions of rod and cone photoreceptor outer segment disc membranes: double labeling. Electron micrograph showing sections of rod and cone outer segments of bovine retina embedded in LR white resin and labeled with both anti-rom-1 antibody romL4 (rabbit) and anti-rhodopsin antibody rho4D2 (mouse), which labels rod outer segments but not cone outer segments. The anti-mouse secondary antibody was conjugated to 5 nm gold particles and the anti-rabbit secondary antibody was conjugated to 10 nm gold particles. The absence of 5 nm label on the photoreceptor on the left of the micrograph indicates that it is a cone, while the photoreceptor on the right is a rod. Both photoreceptors are labeled with the anti-rom-1 antibody, verifying that rom-1 is present in both rod and cone photoreceptors. (Magnification = 40,000 X)
FIG. 23. Immunofluorescence localization of rom-1 to human rod and cone photoreceptor outer segments: double labeling using rom1D5 and rho1D4. Two sections of human retina are shown double labeled with TRITC-rom1D5 and FITC rho1D4. Top row: rom1D5 labeling. Middle row: rho1D4 labeling. Bottom row: Double exposure. Rom1D5 labels outer segments of both rods and cones. Labeling with rho1D4 verifies that rods have not been mistaken for cones, as rho1D4 does not label rods. (Arrows indicate cone outer segments). (Magnification = 1000 X)
modification or alternate splicing of the rom-1 transcript in cones, or masking of the antibody epitope in cones.

3.3.3 Localization of rom-1 in preparations of lysed ROS by immunoelectron microscopy. Pre-embedding and post-embedding labeling of lysed ROS, in which isolated discs are exposed, were used to more precisely localize rom-1 on ROS membranes and to obtain insight into the accessibility of epitopes on the disc membrane surface. Pre-embedding labeling showed that immunogold particles were preferentially localized to the edges of exposed discs in hypotonically lysed ROS labeled with the romC2 antibody (Fig 21D). This pattern of labeling is consistent with the localization of rom-1 to the rim region of disc membranes and further indicates that the C-terminus of rom-1 is exposed on the cytoplasmic surface of discs. No labeling of lysed ROS was observed with the romL4 antibody, presumably due to the intradiscal localization of the antigen for this antibody. However, post-embedding labeling of lysed ROS with the romL4 antibody revealed a labeling pattern identical to that seen with the romC2 antibody in the pre-embedding experiment (Fig 24). This again confirms the disc-rim localization of the rom-1 protein. It is unlikely that differential labeling of the disc rims is due to epitope masking of rom-1 in the lamellar region of the discs, as two different antibodies with completely different epitopes were employed.

3.4 Topological analysis of rom-1 by proteolytic digestion

Proteolysis studies on discs were carried out to further define the topological organization of rom-1 in disc membranes. In this study intact discs obtained from hypotonically lysed ROS were treated with trypsin in the presence and absence of 0.1% TX100, and the resulting products were analyzed with the romC2 and romL4 antibodies by Western blotting. As shown in Fig 25 the romC2 antibody epitope was rapidly
FIG. 24. Post-embedding labeling of isolated discs using romL4. Lysed ROS were embedded in LR white resin, sectioned, and labeled with polyclonal antibody romL4. The labeling is confined to the rims of the discs, a pattern identical to that seen in Fig 21d using romC2. As both romL4 and romC2 label the rim regions, it seems unlikely that the rim-specific labeling is an artifact caused by epitope masking of rom-1 in the lamellar regions of the discs as argued by Travis et al. (1991). (Magnification = 100,000 X)
**Fig. 25. Proteolysis of rom-1 in rod outer segment disc membranes.** Hypotonically lysed ROS membranes (0.3 mg/ml protein) were incubated with trypsin in the presence or absence of 0.1% Triton X-100 for up to 20 hours. Aliquots were removed at the times indicated (hours) and protease digestion was terminated by boiling for 10 min in the presence of SDS-PAGE loading buffer containing 100 μg/ml PMSF. The digests were separated on a 15% SDS-PAGE gel and Western blots were labeled with either the romC2 (top panels) or romL4 (bottom panels) polyclonal antibodies. While the romC2 epitope was rapidly degraded in the presence or absence of detergent, the romL4 epitope was degraded only in the presence of detergent. The 20 hour time points marked (*) are controls containing no trypsin.
cleaved in both non-permeabilized and detergent-permeabilized disc preparations. In contrast the romL4 antibody epitope was preserved within a 30 kDa fragment that was slowly further degraded to a 22 kDa fragment in non-permeabilized preparations. In the presence of a small quantity of detergent, however, the romL4 epitope was rapidly digested by trypsin. These results are consistent with the topological model for bovine rom-1 depicted in Fig 15, in which the C-terminal romC2 epitope is accessible on the cytoplasmic surface of discs, and the romL4 epitope is contained within a large loop region accessible on the lumen surface of the disc membrane.

Analysis of the primary sequence indicates that there are 18 sites for trypsin cleavage in bovine rom-1 (Table 5). Ten of these are found within the region encompassing the intradiscal loop, and 5 sites are found within the region encompassing the romL4 antigen. In the absence of detergent, the predicted trypsin cleavage sites closest to the romL4 binding sites are at R97 in the predicted second cytoplasmic region, and R287 in the predicted cytoplasmic C-terminal region. Cleavage at these sites would release a 21 kDa peptide. The second cytoplasmic region of the protein is very small, and access of trypsin to sites in this region could be inhibited by its close proximity to the membrane, or by a masking effect of the longer C-terminal region. Therefore, the cleavage pattern observed in non-permeabilized discs as detected by romL4 could be explained by initial cleavage at R287 and possibly another site such as R16 close to the N-terminus, followed by slower cleavage at R85 and/or R97.

3.5 Co-immunoprecipitation of rom-1 and peripherin/rds from solubilized ROS

In order to determine if the total complement of rom-1 is complexed with peripherin/rds and vice versa, rom-1 and peripherin/rds were immunoprecipitated from CHAPS solubilized ROS with either rom-1 or peripherin/rds specific antibodies and the precipitated (bound) and supernatant (unbound) fractions were analyzed for rom-1 and
peripherin/rds by Western blotting. As shown in Fig 26, rom1D5 antibody-Sepharose quantitatively immunoprecipitated all detectable rom-1 and peripherin/rds (lanes b and c). Similarly, per2B6 antibody-Sepharose also quantitatively immunoprecipitated

<table>
<thead>
<tr>
<th>Site of cleavage</th>
<th>Predicted topological orientation</th>
<th>Predicted peptide size (permeabilized)</th>
<th>Predicted peptide size (Non-permeabilized)</th>
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<tbody>
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**FIG. 26. Immunoprecipitation of rom-1 and peripherin/rds from detergent-solubilized rod outer segment membranes.** Membranes of hypotonically lysed rod outer segments were solubilized in 1% CHAPS were immunoprecipitated with rom1D5-Sepharose or per2B6-Sepharose. The bound and unbound fractions were subjected to SDS-PAGE and the gels were either silver stained or transferred to Immobilon-P membranes for labeling with monoclonal antibodies rom 1D5 (rom-1) or per2B6 (peripherin/rds) for detection by ECL. Lane a: detergent-solubilized rod outer segment membranes; lane b: unbound fraction of per2B6-Sepharose precipitated rod outer segment membranes; lane c: bound fraction of per2B6-Sepharose precipitated rod outer segment membranes; lane d: unbound fraction of rom1D5-Sepharose precipitated rod outer segment membranes; and lane e: bound fraction of rom1D5-Sepharose precipitated rod outer segment membranes. Rom-1 and peripherin/rds co-precipitate with both antibodies. No detectable quantities of either protein remain in the supernatants.
peripherin/rds and rom-1 (lanes d & e). These results indicate that all the rom-1 and peripherin/rds in ROS disc membranes interacts to form an oligomeric complex. Furthermore, these results confirm the immunolocalization previously described for peripherin/rds (Molday, Hicks and Molday. 1987), as peripherin/rds must be localized to the same region of the membrane as rom-1.

3.6 Expression of bovine rom-1 and peripherin/rds in stable cell lines.

In order to provide a ready source of rom-1 free from peripherin contamination, two cell lines were established that stably express full length bovine rom-1. These were named RomA and RomG. Additionally, a cell line that expresses bovine peripherin/rds and a G418 resistant control cell line (transfected with pcDNA3 plasmid containing no insert) were established. As seen in Fig 19, the rom-1 protein expressed in these cell lines is the same size as rom-1 found in ROS membrane, indicating that the complete coding sequence of the bovine rom-1 cDNA was obtained. Of the two cell lines, the expression level of RomG was slightly higher, as judged by SDS-PAGE and western blot analysis. The cell line RomA was found to have an unusual elongated morphology, but this was not likely due to rom-1 expression; RomG expresses a higher level of rom-1 and does not have an unusual morphology. Comparison of coomassie blue stained extracts of the RomA cell line, the RomG cell line and normal CHO-K1 cells show a major protein band is altered in size in RomA cells. It is likely that integration of the expression vector disrupted the gene encoding this protein, causing the unusual RomA morphology.

3.7 Mutagenesis of bovine rom-1 and peripherin/rds cDNAs.
A number of peripherin/rds mutations that result in truncated proteins have been reported to cause retinal degeneration, including the \textit{rds} mutation, perY258Stop, and perL307(1bp del) (Ma et al., 1995; Wells et al., 1993; Apfelstedt-Sylla et al., 1995). We were interested to determine whether truncated proteins of this type could be expressed. We also wished to examine the intracellular distribution of mutants lacking the C-terminal region, and to determine whether they were capable of forming multi-subunit complexes.

A number of mutant cDNAs of both bovine rom-1 and peripherin/rds were constructed that encoded proteins with partially deleted C-termini. Because of the methodology involved in generating the mutants (2.11.1, Fig 11), the sequences terminate prematurely at a particular codon, with the addition of zero to three additional amino acid codons, followed by a stop codon. Therefore, these mutants are named according to the last correct codon of the cDNA, in the form perW306. If additional amino acids are present, they are listed after a plus sign, in the form romG298+E. The following rom-1 and peripherin/rds deletion mutants were prepared and transferred to the pcDNA1/AMP expression vector: RomE341, romT337, romE336, romH330+E, romE303, romG298+E, romQ200, perW306, perM293+VSE and perP296+VSE. The positions of several of these mutations relative to topological models of rom-1 and peripherin/rds are illustrated in Figure 27.

Additionally, PCR-based site-directed mutagenesis was used to introduce single base changes into the cDNA sequences. Using PCR mutagenesis, the following mutations were introduced into the respective cDNAs and transferred to the pcDNA1/AMP expression vector: romL188P, perY258Stop and perL307(1bp del). The mutant romL188P is equivalent to the peripherin/rds mutation perL185P, which was reported by Kajiwara et al. (1994) to participate in digenic retinitis pigmentosa. The perY258Stop and perL307(1bp del) were reported by Wells et al. (1993) and Apfelstedt-Sylla et al. (1995) to cause macular dystrophy and ADRP respectively.
FIG. 27. **Positions of rom-1 and peripherin/rds deletion mutants relative to the predicted topology of the proteins.** The positions of the various deletion mutants relative to the predicted topology of rom-1 and peripherin/rds are illustrated. Rom1 mutant Q200 was found to express poorly, and showed a high degree of proteolytic degradation. RomE303 and romG298+E expressed well and could interact with wild-type peripherin/rds. The mutant romE341 also expressed poorly, but proteolytic degradation was not observed. The mutants perY258Stop and perL307(lbp del) were generated by PCR mutagenesis and have been associated with macular dystrophy and autosomal dominant retinitis pigmentosa respectively. The frameshift at L307 causes the addition of twelve extra amino acids before termination; the mutant perW306 terminates at the point that the frameshift causes sequence divergence to occur. Due to delays encountered in making an antibody suitable for analysis of the peripherin/rds mutants, they have not yet been characterized.
3.8 Localization of heterologously expressed bovine rom-1

3.8.1 Localization of wild type rom-1 in COS-1 cells. Both rom-1 and rhodopsin are vectorally transported to ROS where rom-1 is expressed at the rim regions of discs (Molday 1994, Molday Hicks and Molday 1987) and rhodopsin is distributed to both the plasma membrane and the lamellar faces of the disc membranes (Molday Hicks and Molday 1987, Hicks and Molday 1986, Nir and Papermaster 1983). It was of interest, therefore, to compare the cellular distribution of these proteins in a mammalian cell expression system. Figure 28A and 28B show immunofluorescence labeling of COS-1 cells expressing rom-1 and labeled with the rom1D5 and romL4 antibodies. When cells were permeabilized with detergent, a punctate distribution of rom-1 label was observed that was primarily restricted to a perinuclear region (Fig 28A). Cells that had not been permeabilized with detergent were not labeled (Fig 28B). Similar labeling was seen in the CHO-K1 cell line expressing rom-1. In contrast, permeabilized COS-1 cells expressing bovine rhodopsin showed diffuse labeling at the perimeter of the cells and a punctate labeling pattern within the cells when labeled with anti-rhodopsin monoclonal antibody rho4D2 directed against the N-terminus of rhodopsin (Fig 28C). Cells that were not permeabilized showed only diffuse surface labeling (Fig 28D). Thus, rhodopsin is translocated from the endoplasmic reticulum to the cell surface in mammalian culture cells as previously reported (Oprian et al., 1987), whereas rom-1 is retained inside the cells and is not transported to the plasma membrane. The localization of rom-1 is the same as that reported for peripherin/rds (Goldberg et al., 1995).

3.8.2 Localization of rom-1 deletion mutants in COS-1 cells. Because the C-terminal regions of proteins can contain localization signals, (Munro and Pelham, 1987; Sung et al., 1994), it was of interest to determine whether deletion of the C-terminal region of rom-1 would alter its distribution in COS-1 cells. The intracellular localization of the
FIG. 28. Immunofluorescence labeling of bovine rom-1 and rhodopsin in transiently transfected COS-1 cells. Immunofluorescence labeling of bovine rom-1 and rhodopsin in transiently transfected COS-1 cells. COS-1 cells transiently transfected with bovine rom-1 or bovine rhodopsin cDNAs were fixed with paraformaldehyde and labeled with anti-rom-1 and anti-rhodopsin antibodies in the presence or absence of permeabilizing detergent. (A) Rom-1 transfected COS-1 cells permeabilized and labeled with rom1D5. Intracellular vesicle labeling is observed; no labeling is observed on the cell surface. RomC2 and romL4 antibodies gave similar labeling patterns for permeabilized cells. (B) Rom-1 transfected COS-1 cells labeled with romL4 antibody in the absence of detergent. No intracellular or surface labeling is apparent even over long exposure times. (C) Rhodopsin transfected COS-1 cells permeabilized and labeled with N-terminal monoclonal antibody rho4D2. Both intracellular vesicle and surface labeling is present. (D) Rhodopsin transfected COS-1 cells labeled with rho4D2 without detergent. Only surface labeling is observed. (Magnification = 1000 X)
rom-1 mutants romE303 and romG298+E, which lack the cytoplasmic C-terminal region, was examined by immunofluorescence microscopy (Fig 29). These mutants were confined to intracellular membranes and vesicular structures. The distribution of these mutants as observed by immunofluorescence microscopy was indistinguishable from that of wild type rom-1.

3.9 Rom-1 expressed in COS-1 cells is not N-glycosylated

In ROS membranes, rom-1 is not N-glycosylated, while peripherin/rds is. Peripherin/rds expressed in COS-1 cells is N-glycosylated (Goldberg et al., 1995). In order to determine whether rom-1 expressed in COS-1 cells is N-glycosylated, detergent extracts were prepared from COS-1 cells transfected with rom-1 and peripherin/rds cDNAs. Samples were incubated in the presence or absence of EndoH endoglycosidase, and then subjected to analysis by SDS-PAGE and Western blot (Fig 30). Peripherin/rds from COS-1 cells shows a characteristic shift in mobility associated with deglycosylation. Rom-1 from COS-1 cells does not show this shift. Thus, rom-1 expressed in COS-1 cells is similar to the protein found in ROS membranes in that it is not N-glycosylated.

3.10 Disulfide-linked homodimer formation of rom-1 expressed in COS-1 cells.

3.10.1 Wild-type rom-1 forms disulfide-linked homodimers in COS-1 cells. Rom-1 and peripherin/rds in ROS membranes form disulfide-linked homodimers (Bascom et al., 1992), and peripherin/rds expressed in COS-1 cells forms disulfide-linked homodimers (Connell et al., 1991b). In order to determine whether rom-1 expressed in COS-1 cells forms disulfide linked homodimers similar to those found in ROS, detergent extracts of ROS and COS-1 cells transfected with rom-1 and peripherin/rds cDNAs were subjected to SDS-PAGE and Western blot analysis in the presence and absence of a disulfide
FIG. 29. Immunofluorescence labeling of bovine rom-1 deletion mutants in transiently transfected COS-1 cells. The intracellular localization of rom-1 mutants romE303 and rom G298+E was examined by immunofluorescence. These mutants are missing the cytoplasmic C-terminal region. COS-1 cells transiently transfected with bovine rom-1 wild-type or mutant cDNAs were fixed with paraformaldehyde and permeabilized with 0.05% TX-100. Cells were labeled with polyclonal antibody romL4 and CY3 conjugated secondary antibody. Labeling was confined exclusively to internal membranes and vesicular structures in all cases. Labeling is more intense in cells transfected with romG298+E and romE303, in keeping with the higher expression levels observed for these mutants. No labeling is seen in the absence of permeablizing detergent or in mock-transfected cells (data not shown). (Magnification = 1000 X)
FIG. 30. **Heterologously expressed rom-1 is not N-glycosylated and forms disulfide-linked homodimers.** In order to determine the glycosylation state of bovine rom-1 expressed in COS-1 cells, TX100 extracts of COS-1 cells transfected with bovine rom-1 cDNA (C) were treated with endoglycosidase H (EndoH), and compared to similarly treated samples of solubilized ROS (R). The digested samples were run on a 9% SDS-PAGE gel, transferred to Immobilon-P membrane and blotted with either romC2 or per2B6 antibodies. Digests were performed at 0.7 mg/ml protein, but ROS samples were diluted 30X prior to loading on gel. No shift in mobility was detected in rom-1 from ROS or COS-1 cells, indicating that rom-1 is not glycosylated in either of these systems. However, a mobility shift was seen in peripherin/rds from ROS (per2B6 lanes), indicating that the experimental conditions used were suitable for detection of glycosylation. Additionally, some samples were run in the absence of BME reducing agent to determine whether rom-1 forms disulfide-linked homodimers in the COS-1 cell system. Disulfide linked dimers (70 kDa) are present in samples of rom-1 from both ROS and COS-1 cells.
reducing agent. As seen in Fig 30, rom-1 from ROS membranes migrates as a 35 kDa polypeptide in the presence of reducing agent and as a 70 kDa polypeptide in the absence of reducing agent, indicating the presence of disulfide linked homodimers. The same effect is observed when TX100 extracts of COS-1 cells expressing rom-1 are examined, indicating that rom-1 expressed in COS-1 cells forms disulfide-linked homodimers.

3.10.2 Rom-1 mutants form disulfide-linked homodimers in COS-1 cells. A number of rom-1 mutants were also tested for homodimer formation in COS-1 cells using an identical protocol. As seen in Fig 31, the mutant romL188P migrates as a 70 kDa band in the absence of reducing agent, indicating that disulfide-linked homodimers are formed. In Fig 31, a large quantity of both wild-type and mutant 35 kDa monomer is seen in the non-reduced lanes. Variable amounts of rom-1 monomers were often seen in samples of non-reduced extracts of both ROS and COS-1 cells. This may indicate reduction of disulfide-linked dimers by small quantities of reductant present in the detergent extracts or by contaminating metal ions.

The mutants romG298+E, and romE303 were also tested for homodimer formation using the same protocol. Fig 32 shows typical results obtained with these mutants. While these mutants may form disulfide-linked homodimers (bands slightly below 70 kDa) the most prominent feature of these gels is the large smear extending from the putative homodimer band upwards. Apparently, when the mutants romE303 and romG298+E are analyzed in the absence of BME, a large amount of material is recruited into larger aggregates of non-uniform size. It is not clear whether these aggregates form before or after solubilization.

Fig 32 also demonstrates the very low expression levels obtained with the mutant romQ200. The level of expression appears to be further decreased by proteolytic degradation. This is interesting in light of the fact that similar mutants resulting in truncation before the fourth transmembrane domain are thought to represent null alleles.
Fig. 31. Disulfide-linked homodimer formation in the heterologously expressed mutant romL188P. The mutant romL188P was tested to determine whether it forms disulfide linked homodimers in the COS-1 cell system. Extracts of COS-1 cells (10 μg of total protein per lane) transfected with rom-1 wild-type (a) or romL188P (b) cDNAs were run on a 9% SDS-PAGE gel under reducing (+BME) and non-reducing (-BME) conditions and transferred to Immobilon-P membrane. Rom-1 was detected with monoclonal antibody rom1C6. The mutant romL188P forms disulfide-linked homodimers similar to those seen in wild-type rom-1 (band at 70 kDa). The expression level of romL188P is significantly lower than that of wild-type rom-1.
FIG. 32. Disulfide-linked homodimer formation in heterologously expressed rom-1 deletion mutants. The mutants romE341, E303, G298+E and rom Q200 were tested to determine whether they form disulfide linked homodimers in the COS-1 cell system. Extracts of COS-1 cells (10 ug of total protein per lane) transfected with the various cDNAs were run on a 9% SDS-PAGE gel under reducing (+BME) and non-reducing (-BME) conditions and transferred to Immobilon-P membrane. Rom-1 was detected with monoclonal antibody rom1C6. The mutants romE341 and romQ200 express poorly—romQ200 appears to be substantially degraded by proteolysis. The mutants romE303 and romG298+E may form disulfide-linked homodimers similar to those seen in wild-type rom-1 (bands below 70 kDa). However, the majority of these mutant proteins were recruited into larger aggregates of non-uniform size.
It is possible that romQ200 is recognized by the COS cells as misformed and is therefore destroyed by proteolysis. The expression level of romQ200 was too low to determine whether disulfide-linked homodimers are formed.

3.11 Interaction of heterologously expressed rom-1 and peripherin/rds

3.11.1 Co-immunoprecipitation of wild-type rom-1 and peripherin/rds. Rom-1 and peripherin/rds co-purify from bovine ROS membranes (Moritz and Molday 1996, and 3.5, above). In order to determine whether rom-1 and peripherin/rds expressed in COS-1 cells also co-purify, extracts were prepared from COS-1 cells co-transfected with rom-1 and peripherin/rds cDNAs. Peripherin/rds was immunoprecipitated from the extracts using per2B6, and the pellets were analyzed by SDS-PAGE and western blot using romC2 to detect co-purifying rom-1 (Fig 33). Rom-1 was found to co-purify with peripherin/rds from extracts of co-transfected cells, but not from the combined extracts of singly transfected cells. Thus, the rom-1 and peripherin/rds proteins are specifically assembled into a complex by the COS-1 cells. It should be noted that the specificity of this interaction was further verified by Dr. AFX Goldberg (Goldberg et al., 1995). Total cell protein was labeled by growing the cells in media supplemented with $^{35}$S-methionine and cysteine, and immunoprecipitations were performed as described above. Per2B6 immunoprecipitates were examined by SDS-PAGE followed by autoradiography. No proteins other than rom-1 and peripherin/rds were seen in the immunoprecipitates, indicating the interaction of these proteins in the COS-1 cell system is highly specific, and similar to the interaction seen in ROS membranes (Moritz and Molday, 1996; 3.5 above).

3.11.2 Co-immunoprecipitation of rom-1 mutants and wild-type peripherin/rds. A number of rom-1 mutants were examined to determine whether they will assemble with
FIG. 33. **Co-precipitation of rom-1 and peripherin/rds from co-transfected COS-1 cell extracts.** In order to determine whether rom-1 and peripherin/rds could be assembled into complexes in the COS-1 cell system, peripherin/rds and rom-1 cDNAs were co-transfected into COS-1 cells. CHAPS extracts of the transfected cells (1 mg/ml total protein, 1 ml extract per lane) were prepared, and peripherin/rds was immunoprecipitated from the extracts using the per2B6 antibody. The immunoprecipitates were eluted with SDS-PAGE loading buffer, separated on a 9% SDS PAGE gel and transferred to Immobilon-P membrane. The blot was then labeled with romC2, in order to detect associated rom-1. No rom-1 was precipitated from extracts of cells singly transfected with peripherin/rds cDNA (a) or rom-1 cDNA (b). However, rom-1 was precipitated from cells transfected with both peripherin/rds and rom-1 cDNAs (c). This precipitation could be inhibited by including 1 mg/ml of peptide per4B, which contains the sequence of the per2B6 epitope (d). No rom-1 was precipitated from cells transfected with control pcDNA plasmid (e). No rom-1 was immunoprecipitated when extracts of cells singly transfected with the peripherin/rds cDNA and rom-1 cDNA were mixed and incubated on ice 2 hours (f), or from the mixed extracts incubated with per4B peptide (g). The high and low molecular weight bands seen throughout the blot are the immunoglobulin light and heavy chains.
peripherin/rds in a manner similar to wild-type rom-1 (Fig 34, Fig 35). The mutants tested were romL188P, romE303 and romG298+E. All of these mutants co-precipitated with peripherin/rds, indicating that these mutations do not disrupt the peripherin-rom-1 interaction.

3.11.3 Co-immunoprecipitation of romL188P and perL185P. The mutants perL185P and romL188P have disrupted quaternary structures in COS-1 cells. (Goldberg et al., in press, and 3.12 below). These mutants were tested to determine whether they were capable of interacting with each other in the COS-1 cell system. As seen in Fig. 34, romL188P and perL185P co-purify from membrane extracts of co-transfected COS-1 cells, but not from combined extracts of singly transfected cells. Thus, these mutant proteins are specifically assembled into a complex by the COS-1 cells.

3.12 Characterization of bovine rom-1 by velocity sedimentation.

Velocity sedimentation was used to examine the tertiary and quaternary structure of bovine rom-1 expressed in COS-1 cells, as well as mutants of bovine rom-1 and peripherin/rds. The results of these studies are summarized in Table 6. Wild-type bovine rom-1 has an $S_{20,w}$ value of $5.6 \pm 0.1$. This value is similar to values reported for the peripherin/rds complex from ROS membranes and COS-1 cells (Goldberg et al., 1996; Goldberg et al., 1995). Therefore, it is likely that peripherin/rds and rom-1 adopt similar quaternary structures when expressed in COS-1 cell membranes. The $S_{20,w}$ value of peripherin/rds expressed in COS-1 cell membranes is consistent with a homotetrmeric quaternary structure (Goldberg and Molday, 1996). It is therefore likely that rom-1 also forms similar homotetrmeric structures. Mutant rom-1 proteins were found to have similar $S_{20,w}$ values, (Table 6), except for the mutant romL188P. For this mutant, the $S_{20,w}$ value was significantly lower, at $3.9 \pm 0.3$ ($n = 3$). Typical
FIG. 34. Co-precipitation of romL188P, peripherin/rds, and perL185P from co-transfected COS-1 cell extracts. The mutant romL188P was tested to determine whether it could be assembled into complexes with peripherin/rds in the COS-1 cell system in a manner similar to wild type rom-1. Assembly with the equivalent peripherin/rds mutant perL185P was also tested. RomL188P or rom-1 wild-type cDNAs were co-transfected into COS-1 cells with peripherin/rds wild-type or perL185P cDNAs. TX100 extracts of the transfected cells (1 mg/ml total protein, 0.1 ml extract per lane) were prepared, and peripherin/rds was immuno-precipitated from the extracts using the per2B6 antibody. The immunoprecipitates were eluted with SDS-PAGE loading buffer, separated on a 9% SDS PAGE gel and transferred to Immobilon-P membrane. The blot was labeled with rom1C6, in order to detect associated rom-1. No rom-1 was precipitated from extracts of cells singly transfected with wild-type rom-1 (a) wild-type peripherin/rds (b) romL188P (c) or perL185P (d). Rom-1 was precipitated from extracts of cells co-transfected with wild-type rom-1 and wild-type peripherin/rds (e), romL188P and wild-type peripherin/rds (f), and romL188P and perL185P (g) indicating that, like wild type rom-1, the mutant romL188P can be assembled into complexes with peripherin/rds and perL185P. No rom-1 was precipitated from combined extracts of cells singly transfected with wild-type rom-1 and wild-type peripherin/rds (h) or romL188P and perL185P (i).
FIG. 35. Co-precipitation of rom-1 deletion mutants and peripherin/rds from co-transfected COS-1 cell extracts. The mutants romE303 and romG298+E were tested to determine whether they would assemble into complexes with peripherin/rds in the COS-1 cell system in a manner similar to wild type rom-1. Mutant or wild-type rom-1 cDNAs were co-transfected into COS-1 cells with wild-type peripherin/rds cDNA. TX100 extracts of the transfected cells (1 mg/ml total protein, 0.1 ml extract per lane) were prepared, and peripherin/rds was immuno-precipitated from the extracts using the per2B6 antibody. The immunoprecipitates were eluted with SDS-PAGE loading buffer and the cell extracts (extracts) and eluted immunoprecipitates (pellets) were separated on a 10% SDS PAGE gel and transferred to Immobilon-P membrane. The blot was labeled with rom1C6, in order to detect associated rom-1. No rom-1 was precipitated from extracts of cells singly transfected with wild-type rom-1 (romWT) or mutant rom-1 cDNAs, wild-type peripherin/rds (per), or control pcDNA plasmid. Rom-1 was precipitated from extracts of cells co-transfected with wild-type or mutant rom-1 cDNAs and wild-type peripherin/rds, indicating that, like wild type rom-1, the mutants can be assembled into complexes with peripherin/rds.
sedimentation profiles are shown for wild-type rom-1 and romL188P in Fig 36. The altered $S_{20,w}$ value for romL188P suggests that the protein exists in COS-1 cell membranes as homodimers. (Dimers are suggested by disulfide analysis, described in 3.10.2.)

<table>
<thead>
<tr>
<th>Species Analyzed</th>
<th>Sedimentation coefficient ($S_{20,w}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>peripherin/rom-1 wild type complex</td>
<td>6.0 (n = 2)</td>
</tr>
<tr>
<td>peripherin/romG298+E complex</td>
<td>6.3 ± 0.2 (n = 3)</td>
</tr>
<tr>
<td>rom-1 wild type (singly transfected)</td>
<td>5.6 ± 0.1 (n = 3)</td>
</tr>
<tr>
<td>romG298+E (singly transfected)</td>
<td>5.9 ± 0.1 (n = 3)</td>
</tr>
<tr>
<td>romE303 (singly transfected)</td>
<td>5.5 ± 0.1 (n = 3)</td>
</tr>
<tr>
<td>romL188P (singly transfected)</td>
<td>3.9 ± 0.3 (n = 3)</td>
</tr>
</tbody>
</table>

Sedimentation analysis of the complex formed between romL188P and wild-type peripherin/rds was attempted on several occasions. Results showed essentially linear sedimentation profiles—there appears to be some romL188P associated with all of the fractions. As a result of this large dilution of the applied sample, it was necessary to expose these blots for very long periods of time (>2 hours). Accordingly, the level of confidence in these results was somewhat lower, as there is a chance that these bands represent some form of background labeling. Nevertheless, it seems likely that the conformation adopted by romL188P and wild-type peripherin/rds is not the same as the conformation adopted by the wild-type complex, but instead is non-uniform, possibly indicating a denatured state induced by association with peripherin/rds. This is in contrast to the mutant perL185P, which adopts a wild-type conformation when complexed with wild-type rom-1 (Goldberg et al., in press).
FIG. 36. Typical sedimentation profiles of wild-type rom-1 and romL188P. Sedimentation profiles of heterologously expressed wild-type rom-1 and romL188P were obtained using 5-20% sucrose gradients with 0.1% TX100 as described in (2.11). The percentage of protein eluted in each fraction (normalized to 100% total) is plotted as a function of sucrose concentration. In order to demonstrate the reproducibility of the procedure, four sedimentation profiles are shown, two for wild type rom-1 (circles, squares) and two for romL188P (triangles). The sedimentation coefficient of romL188P is significantly lower than the sedimentation coefficient of wild-type rom-1 ($S_{20,w} = 3.9 \pm 0.3$ vs. $S_{20,w} = 5.6 \pm 0.1$), indicating that this mutant is likely a dimer and not a tetramer like wild-type rom-1.
3.13. Expression of bovine peripherin/rds mutants

Because of difficulties in obtaining an antibody that binds to a region of peripherin/rds other than the C-terminus, peripherin/rds mutants have not yet been extensively characterized. However, an antibody has recently been obtained that recognizes intradiscal loop region of peripherin/rds (perL1). This antibody has been successfully used to detect the expression of wild-type peripherin/rds in COS-1 cell extracts, as well as the various peripherin/rds mutants described above.
DISCUSSION

4.1 The primary structure of bovine rom-1

Bovine ROS are the primary source of material to study biochemical properties of proteins involved in phototransduction, outer segment metabolic reactions and other cellular processes. In this study we have cloned the cDNA for bovine rom-1 as a means to study the structural features, cellular distribution and protein-protein interactions of rom-1 in both native photoreceptor membranes and heterologous cell expression systems, and to begin to define the role of rom-1 in retinal degenerative disorders. Although the 5'-end of the cDNA could not be obtained by conventional library screening techniques, the remainder of the open reading frame and part of the upstream promoter were obtained by inverse PCR. Because the rom-1 protein encoded by this open reading frame is identical in length to human and mouse rom-1 (Bascom et al., 1993a), and because this protein expressed in mammalian cells migrates by SDS-PAGE with the same apparent molecular mass as rom-1 from bovine ROS membranes (Fig 19), we conclude that we have obtained the complete coding sequence of bovine rom-1. The difficulty in cloning the 5' end of the cDNA combined with Northern blot analysis (Fig 18) and sequence comparisons with previously cloned rom-1 upstream sequences (Fig 16) suggests that the mRNA encoding bovine rom-1 has a very short 5' non-coding region (15 bp) as previously reported for the human rom-1 mRNA by Bascom et al. (1993a).

4.1.1 Structural features of the bovine rom-1 coding sequence. Bovine rom-1 exhibits several structural features common to human rom-1 and bovine peripherin/rds. These include similar hydropathy profiles and the absence of a consensus sequence for N-linked glycosylation. Sequence analysis suggests a topological model consisting of four
transmembrane spanning segments, a large intradiscal region and cytoplasmic N and C-termini, as had been earlier proposed for peripherin/rds (Connell and Molday, 1990; Travis et al., 1991) and human rom-1 (Bascom et al., 1992). The sequences CCG and PFSCC present in the predicted loop region of bovine rom-1 reveal that this protein is a member of the TM4SF superfamily of proteins (Fig 8).

The predicted pI of bovine rom-1 is 5.77, similar to that of human rom-1 (5.59). The predicted pI of bovine rom-1 is considerably lower than that of bovine peripherin/rds at 7.97. The pI of these proteins differs most notably in the loop regions (Bascom et al., 1993a). The difference in pI between peripherin/rds and rom-1 may contribute to the strong interaction observed between these two proteins.

The bovine rom-1 sequence contains seven cysteine residues within the intradiscal loop region that are completely conserved between all peripherin/rds and rom-1 sequences. Additionally, there are cysteine residues present in the first transmembrane region and the first intradiscal region that are conserved between all rom-1 sequences but are not found in any peripherin/rds sequences. Unlike previously cloned members of this group of proteins, bovine rom-1 has a tenth cysteine near its C-terminus at position 347.

4.1.2 Structural features of the bovine rom-1 promoter. The bovine rom-1 promoter has a number of unusual features, which it shares with other reported rom-1 promoters (Bascom et al., 1993a). The human rom-1 promoter is associated with a large (~1 kb) CpG island. CpG islands are GC rich regions that contain an abnormally high content of the dinucleotide CpG, which is underrepresented in vertebrate genomes. The CpG island is thought to be a promoter element associated with both housekeeping and tissue-specific genes (Gardiner-Garden and Frommer, 1987). However, tissue-specific genes associated with upstream CpG islands usually have a TATA-box in the promoter region (Larsen, 1992). The bovine rom-1 promoter, like the previously described human and mouse rom-1 promoters, is lacking a true TATA box, although a TATA-like sequence is
present at position -128 (Fig 16). (The maximum range of position for a true TATA box is -36 to -20 (Bucher, 1990)). Similarly, the sequence contains no CCAAT box. Insufficient promoter sequence was obtained to determine whether a CpG island is also present in the bovine rom-1 promoter. However, the GC content of the bovine rom-1 promoter does increase towards the upstream end. Like other rom-1 promoters, the sequence contains a number of potential GC boxes, which may act as SP1 binding sites, and which are often associated with CpG islands.

4.2 Localization of bovine and human rom-1

4.2.1 Localization of bovine and human rom-1 to both rod and cone photoreceptors.
Although peripherin/rds monoclonal antibodies have been used to localize peripherin/rds to both rod and cone outer segments (Molday, 1994; Arikawa et al., 1992), a human rom-1 specific polyclonal antibody has been reported to label rod, but not cone, outer segments by immunofluorescence (Bascom et al., 1992). This has led to the suggestion that the absence of rom-1 in cone cells may in part account for the difference in morphology of cone and rod outer segments. Similarly, absence of rom-1 in cone cells could account for the phenotypic variation seen with mutations in peripherin/rds: some mutations in peripherin/rds lead to autosomal dominant retinitis pigmentosa phenotypes (rod degeneration), whereas other mutations lead to macular dystrophy phenotypes (cone degeneration) (Wells et al., 1993).

We have re-investigated the presence of rom-1 in cone outer segments by post-embedding immunogold labeling techniques for electron microscopy and by immunofluorescence microscopy. Our results using both C-terminal and intradiscal loop directed antibodies indicate that a form of rom-1 is indeed present in bovine cone outer segments as well as rod outer segments (Fig 21). To ensure that the presence of rom-1 in cones is not unique to the bovine retina, we examined the distribution of rom-1 in the
human retina by immunofluorescence microscopy, using a double labeling technique to ensure that rods were not mistaken for cones (Fig 23). The results show that a form of rom-1 is also present in human cones.

Such antibody labeling studies do not distinguish between immunochemically related proteins and identical proteins; therefore it is possible that cone and rod rom-1 may be similar, but not identical. However, it is interesting that the loop specific antibody (romL4) shows very low cross-species reactivity. In particular, this antibody does not cross-react with rom-1 in rat, mouse, pig, dog or human rod outer segments. Since homologues of rod outer segment proteins of different species are in general more closely related to each other than rod and cone variants within the same species, (Chiu et al., 1994; Okano et al., 1992; Bonigk et al., 1993; Pittler et al., 1992) it is likely that the rom-1 in bovine cones is very similar if not identical to the rom-1 present in rods. No evidence of a closely related gene was found by Southern blot (Fig 18), and computer analysis of the bovine cDNA sequence and the human genomic sequence did not identify any possible splice junctions that could result in alternate C-termini.

Another possible explanation for the discrepancy in the observed labeling patterns is that the epitope for the antibody used by Bascom et al. (1992) could be post-translationally modified in cones, or could be involved in interaction with a cone specific protein, thus masking the epitope in cones. However, no signals for common post-translational modifications are seen in the rom-1 C-terminal region. Further studies are needed to conclusively determine whether rod and cone rom-1 are encoded by the same or different genes.

4.2.2 Localization of bovine rom-1 to the rims of discs. The immunogold labeling studies reported here indicate that rom-1 is localized to the rim region of photoreceptor disc membranes. Post-embedding labeling of retina sections shows a peripheral distribution of rom-1, indicating localization to either the plasma membrane or disc rims.
Localization to the disc rims was confirmed by labeling of lysed ROS (Fig 21, Fig 22). Co-localization of rom-1 and peripherin/rds to the disc rims is expected on the basis of the co-immunoprecipitation studies reported here (3.5). Therefore, these studies also provide additional support for the exclusive rim localization of peripherin/rds and argue against the uniform disc distribution of peripherin/rds previously reported by Travis et al. (1991).

4.3 Membrane topology of bovine rom-1.

Membrane topology models for bovine rom-1 and peripherin/rds, as well as other members of the TM4SF group of proteins, have previously been proposed (Connell and Molday, 1990; Bascom et al., 1992; Wright and Tomlinson, 1994). The predicted membrane topology of bovine rom-1 is similar (Fig 15). We have obtained experimental support for this model from immunolabeling studies and proteolytic digestion of rom-1 in ROS disc membranes (Fig 21, 25). Pre-embedding immunogold labeling using a C-terminal directed antibody clearly indicates that the C-terminus of rom-1 is exposed on the cytoplasmic surface of disc membranes. The model is further supported by the finding that the C-terminus of rom-1 in sealed disc membranes is readily digested by trypsin. In contrast, a large segment containing the romL4 epitope is not accessible to the romL4 antibody, and is resistant to trypsin digestion unless the disc membrane is first permeabilized with a low concentration of TX100. These results are consistent with a large loop between membrane spanning regions 3 and 4 being exposed on the lumen side of the disc membrane. These studies provide the first direct experimental evidence in support of the proposed topological model for rom-1 obtained using rom-1 in its native membrane system, the ROS disc.
4.4 Rom-1 and peripherin/rds are subunits of a membrane protein complex.

Previous studies by Bascom et al. (1992) have shown that at least some rom-1 is associated with peripherin/rds. We have further investigated the extent to which these proteins interact to form a complex. Using rom-1 and peripherin/rds specific antibodies in conjunction with immunoprecipitation and Western blotting, we have found that all detectable rom-1 is immunoprecipitated from detergent-solubilized ROS membranes with a peripherin/rds specific monoclonal antibody, and all peripherin/rds is immunoprecipitated with a rom-1 specific monoclonal antibody (Fig 26). These results indicate that rom-1 and peripherin/rds are not only structurally related proteins, but in fact are subunits of a disc membrane protein complex. This complex appears to consist of one disulfide linked peripherin/rds dimer non-covalently associated with one disulfide-linked rom-1 dimer (Bascom et al., 1992; Goldberg et al., 1995; Goldberg and Molday, 1996; this study).

4.5 Heterologous expression of bovine rom-1.

4.5.1 Heterologously expressed bovine rom-1 is similar to rom-1 from ROS membranes. We have studied the expression of rom-1 in mammalian cells transfected with the rom-1 cDNA. In contrast to rhodopsin, rom-1 is not translocated to the plasma membrane of these cells, but instead is retained in intracellular vesicles (Fig 28). This pattern of expression is not unexpected when one considers that whereas rhodopsin is translocated to both the plasma membrane and disc lamellar membranes of rod photoreceptor outer segments, rom-1 and peripherin/rds are targeted specifically to the rim region. Thus, rom-1 and peripherin/rds may contain signal sequences or conformations that prevent their targeting to plasma membrane. Alternatively, rom-1
could be improperly folded in the absence of peripherin/rds and therefore be retained in the endoplasmic reticulum and intracellular vesicles.

Further characterization of heterologously expressed rom-1 revealed additional similarities to rom-1 from ROS membranes. Heterologously expressed rom-1 is not glycosylated (Fig 30), though heterologously expressed peripherin/rds is (Goldberg et al., 1995). Furthermore, heterologously expressed rom-1 forms disulfide linked homodimers (Fig 30). In ROS membranes, rom-1 is part of a tetrameric protein complex formed from two rom-1 subunits and two peripherin/rds subunits (Goldberg and Molday, 1996). Heterologously expressed rom-1, in the absence of peripherin/rds, assembles into a complex with similar hydrodynamic properties, which is most likely also a tetramer (Table 6). In the presence of peripherin/rds, a heterotetrameric complex similar to that found in ROS membranes is formed. Thus, heterologously expressed rom-1 shares many of the properties of rom-1 from ROS membranes. Therefore, it seems unlikely that heterologously expressed rom-1 is retained intracellularly because of improper folding.

4.5.2 Heterologously expressed bovine rom-1 and peripherin/rds interact in COS-1 cell membranes. We have developed a system for studying complex formation between peripherin/rds and rom-1. The system is illustrated schematically in Fig 37. The first step is co-transfection of peripherin/rds and rom-1 expression constructs into COS-1 cells. Association of the two heterologously expressed proteins is detected by immunoprecipitation of one of the two subunits using a specific antibody, followed by Western blot analysis of the immunoprecipitates using an antibody to the other subunit. Some feature of the COS-1 cell system is necessary for the assembly of the complexes, as complexes are not formed when extracts of singly transfected cells are mixed (Fig 33). Using this system, interaction between wild-type subunits has been demonstrated, as well
**Fig. 37. Assay for assembly of rom-1 and peripherin/rds.** Peripherin/rds and rom-1 can be assembled into a complex similar to that found in ROS membranes in the heterologous cell system. Formation of the complex can be detected by immunoprecipitation of one of the subunits from COS-1 cell extracts using a monoclonal antibody (e.g., per2B6) followed by western blot analysis of the immunoprecipitate using an antibody to the other subunit (e.g., rom1C6). Co-transfection of both cDNAs is required to obtain a band on the western blot; combining extracts of singly transfected cells does not result in assembly of complexes.
as interaction between mutant subunits and wild-type subunits. (This study; Goldberg et al., 1995; Goldberg and Molday, in press).

4.6 Heterologous expression of bovine rom-1 and peripherin/rds mutants

4.6.1 The perL185P mutant. Mutations in the peripherin/rom-1 complex have been associated with retinal degeneration. Using site-directed mutagenesis techniques, we can reconstruct these mutations in the bovine cDNA, or introduce novel mutations of our own design. Using the system we have developed for studying the interaction of these subunits, we can examine the mutant proteins to see if assembly of peripherin/rom-1 complexes is affected. Additionally, other properties of mutant and wild-type proteins can be compared, such as disulfide bond formation, intracellular localization, and sedimentation velocity.

Dr. AFX Goldberg has applied this system to the study of the mutant perL185P, which is responsible for digenic retinitis pigmentosa when combined with a romL114(1 bp ins) or romG80(1 bp ins) (Kajiwara et al., 1994). The perL185P mutant is capable of forming complexes with wild-type rom-1. Sedimentation analysis of this mutant complex reveals that it has a $S_{20,w}$ value similar to that of wild-type peripherin/rom-1 complex. However, sedimentation analysis of protein from singly transfected cells reveals a dramatic difference between the mutant and wild-type forms. In the absence of rom-1, wild-type peripherin/rds sediments as a homotetrameric complex made up of two disulfide linked dimers (Goldberg and Molday, 1996). However, the perL185P complex sediments as a disulfide linked dimer, not a tetramer (Goldberg and Molday, in press).

This provides a possible explanation for the digenic inheritance of retinitis pigmentosa associated with this mutant (Fig 38). If functional peripherin/rom-1 complex is a heterotetramer, absence of a rom-1 allele will result in less heterotetrameric complex being formed, and an excess of peripherin/rds subunits. In the case of wild-type
FIG. 38. Model for digenic inheritance of retinitis pigmentosa. The fact that the mutant perL185P can form heterotetrameric complexes with wild-type rom-1, but is unable to form homotetrameric complexes, suggests a model for digenic inheritance of retinitis pigmentosa. a) Wild type alleles result in heterotetrameric peripherin/rom-1 complexes. b) Rom-1 alleles such as romG80(1 bp ins) (which are likely null) result in excess peripherin/rds subunits. In the absence of sufficient rom-1 these form homotetramers. c) The mutant perL185P forms heterotetramers with rom-1 when a full complement of rom-1 is present. d) When a perL185P allele and a rom-1 null allele are both present, excess perL185P is unable to form homotetrameric complexes (perL185P/rom-1 complexes and wild-type peripherin/rds homotetramers may also be present but are omitted for clarity). Either the presence of perL185P dimers or the absence of tetrameric complexes eventually leads to retinal degeneration.
peripherin/rds, this excess could assemble into homotetrameric complexes, which may possess some of the functional properties of the heterotetrameric complexes. However, perL185P cannot form these homotetrameric complexes, and therefore the quantity of functional complex drops below a critical level, and retinal degeneration ensues. Alternatively, perL185P dimers may somehow stress the photoreceptor cell, eventually leading to photoreceptor death. A full complement of two normal rom-1 alleles prevents the accumulation of these dimers, but when a rom-1 allele is absent, dimer formation occurs.

4.6.2 The romL188P mutant. We have constructed a rom-1 mutant, romL188P, which is equivalent to the perL185P mutant. This mutant behaves similarly to perL185P, in that it does not form homotetrameric complexes (Fig 36). However, the mutant phenotype could not be "rescued" by co-expression of peripherin/rds. The results obtained with this mutant illustrate a number of important points.

First, the fact that this mutant forms disulfide linked dimers indicates that its structure is minimally a dimer. The fact that it sediments at a slower rate than wild-type rom-1 suggests that wild-type rom-1 has a higher order structure than this mutant, minimally a tetramer. Thus, this provides further evidence that the quaternary structure of wild-type rom-1 heterologously expressed in COS-1 cells is tetrameric, like the structure of heterologously expressed peripherin/rds.

Second, these results show that the quaternary structure of heterologously expressed rom-1 is similar to the quaternary structure of heterologously expressed peripherin/rds. It seems likely that the formation of rom-1 homotetramers and peripherin/rds homotetramers are mediated by similar interactions. The fact that the quaternary structure of these molecules is disrupted in a similar way by a single mutation is proof of this hypothesis.
Finally, we have shown that interaction of the mutants perL185P and romL188P can be detected with this system (Fig 34). This was unexpected, as the mutants do not form homotetrameric complexes when singly transfected. There are a number of possible explanations for this observation. First, the interaction of peripherin/rds and rom-1 to form a heterotetrameric complex may be structurally different from the interactions that form homotetrameric complexes. Thus, interaction to form homotetramers is prevented by perL185P-like mutations, but interaction to form heterotetramers is not. Second, this result may reveal a problem with the methodology we have chosen. Because our system does not differentiate between varying degrees of interaction, it is possible that only a relatively small amount of the mutant proteins interact to form complexes, yet this population is specifically selected by our protocol. When extracts of singly transfected cells are examined by sedimentation, no selection for complexes is employed--the entire population of rom-1 or peripherin/rds in the extracts is examined. Therefore, small quantities of romL188P or perL185P homotetramers could be present, but are overlooked due to the overwhelming signal obtained from homodimers. However, even extremely long exposures of western blots failed to detect any homotetramers in these cell extracts. Unfortunately, due to the variability of transfection and co-transfection, our system can not easily be adapted to allow quantitation and comparison of all of the various species present in the various extracts in these experiments. Finally, a third possibility is that the association between romL188P and perL185P represents disulfide linked heterodimers. While heterodimers of peripherin/rds and rom-1 are not detected in ROS membranes by 2D-gel electrophoresis (Bascom et al., 1992), it is possible that small quantities of these heterodimers are formed, or that in COS-1 cells homodimers are not formed exclusively.

4.6.3 Bovine rom-1 deletion mutants. A number of cases have been reported in which mutations causing truncation of peripherin/rds have been associated with retinal
degeneration, including the mutations perL307(1bp del) (Apfelstedt-Sylla et al., 1995) and per299(2 bp del) (Nichols et al., 1993) that result in a frameshift and premature termination of the C-terminal region, and the mutations perY258Stop (Wells et al., 1993), and perY140(4 bp ins) (Keen et al., 1994) that result in truncation before the fourth transmembrane domain. Truncations that occur before the fourth transmembrane domain are thought to represent null alleles, however, there is no experimental proof for this. Several different phenotypes are reported to be associated with these "null" alleles, suggesting that they may not be null. (perY258Stop is associated with macular degeneration, perY140(4 bp ins) is associated with retinal pattern dystrophy). We have constructed and characterized a number of mutant rom-1 and peripherin/rds cDNAs with varying degrees of deletion of the C-terminus, in an attempt to further understand the nature of the protein products of similar alleles.

Although mutations in rom-1 of this type have not been reported to be associated with disease phenotypes except in the case of digenic retinitis pigmentosa, we feel that we can extrapolate some of the findings with rom-1 mutants to peripherin/rds, due to the high degree of similarity between these proteins. Nevertheless, it will be interesting to determine whether similar results are obtained with peripherin/rds mutants.

The introduction of C-terminal deletions in the rom-1 cDNA was found to affect expression levels to a large extent (Fig 32). Short truncations of the C-terminus, for example romE341, were found to result in decreased expression levels. (Similar results were also found for the mutants romE336 and romT337, data not shown). In contrast, the larger deletions romG298+E and romE303 were found to have increased expression levels. Unfortunately, the results of these experiments are difficult to extrapolate to the photoreceptor system. Changes in expression levels could easily be due to artifacts of the expression system used, for example altered stability of the truncated RNAs. However, it is interesting to note that unlike mutants such as romE341, the deletion romQ200 exhibits a large amount of proteolytic degradation in addition to a low
expression level, suggesting that the protein product may be recognized by the COS-1 cells as malformed and selected for degradation. This lends support to the theory that deletions of this type are likely to represent null alleles.

The mutants romE303 and romG298+E were characterized using a number of techniques to see if any abnormalities (aside from expression level) could be found that would distinguish them from wild-type rom-1. It was found that the truncated proteins formed disulfide linked homodimers like wild-type rom-1 (Fig 32). However, they had a tendency to aggregate under non-reducing conditions. The aggregates could be eliminated with reducing agents such as DTT and BME. It is not clear at what point the aggregates form: in the COS-1 cells, after extraction with TX100, or after addition of SDS. It is possible that this tendency to aggregate could affect the normal tetrameric structure of the peripherin/rom-1 complex and cause photoreceptor degeneration.

The mutants romE303 and romG298+E were also characterized by velocity sedimentation (Table 6). The sedimentation coefficients obtained for these proteins are almost identical to wild-type rom-1, suggesting that these mutations do not result in any gross abnormalities in tertiary or quaternary structure. Furthermore, it is interesting to note that these sedimentations were performed in the presence of non-denaturing detergent and 1 mM DTT, and the proteins migrated as a single band in the gradients. Thus, a small concentration of reducing agent under non-denaturing conditions is sufficient to eliminate the high-molecular weight disulfide-linked aggregates. Again, while it is unclear at what point these aggregates form, it seems likely that the aggregates do not represent denatured protein formed within the COS-1 cells.

Finally, the mutants romE303 and romG298+E were characterized in terms of their ability to assemble into complexes with wild-type peripherin/rds. Both mutants were capable of assembling into complexes with peripherin/rds in the heterologous COS-1 cell system (Fig 35), and mutant complexes were found to have normal sedimentation profiles (Table 6). Thus, the C-terminal region of the rom-1 protein does not appear to
be essential for assembly of peripherin/rom-1 complexes. It is possible, however, that the highly specific assembly of peripherin/rds and rom-1 into exclusively heterotetrameric complexes that occurs in the photoreceptor cell requires signals found in the C-terminal regions of these proteins.

Thus, apart from elevated expression levels and an increased tendency to aggregate under non-reducing conditions, the mutants romE303 and romG298+E appear to behave like wild-type rom-1 in the heterologous COS-1 cell system. Although equivalent RP-associated peripherin/rds mutants have not yet been characterized, it seems likely that similar results will be obtained. While the observed changes in expression level and tendency to aggregate could potentially explain why such mutations would be associated with retinal degeneration, it seems likely that other as yet uncharacterized properties of these mutants are likely to be responsible. The C-terminal region of peripherin/rds and rom-1 is exposed to the cytoplasm of the ROS, and is in close proximity to the plasma membrane. It is possible that the C-terminal regions of peripherin/rds and rom-1 participate in low avidity interactions with other proteins of the cytoplasm, plasma membrane, or adjacent disc rims, and that these interactions are disrupted by commonly employed solubilization conditions. Accordingly, it may be worthwhile to search for possible interaction partners for the rom-1 and peripherin/rds C-termini using techniques such as cross-linking or use of a yeast two-hybrid system.

4.6.4 Peripherin/rds mutants. We have constructed a number of mutations resulting in truncation of the bovine peripherin/rds cDNA that correspond to disease-associated mutations found in the human peripherin/rds sequence. These mutations include perY258Stop and perL307(1bp del), which are associated with macular dystrophy and ADRP (Apfelstedt Sylla et al., 1995; Wells et al., 1993). Because the frameshift mutation perL307(1 bp del) adds 16 additional abnormal amino acids before termination (Gruning et al., 1994), the mutation perW306 was also constructed that simply
terminates at the point of sequence divergence. Properties of perL307 (1 bp del) associated with the 16 amino acid tail can therefore be differentiated from properties associated with deletion of the normal C-terminus. The intent was to characterize these mutants using similar techniques described above for rom-1 deletion mutants. The investigations have been hampered by lack of a suitable antibody— one that binds to a region of peripherin/rds other than the C-terminus. However, we have now developed a suitable antibody (perL1), and these experiments can proceed.

4.7 Summary and future directions.

A large number of studies of peripherin/rds and rom-1 have emphasized a genetic approach, in which mutations in the gene sequences are identified and the associated phenotypes are described. While peripherin/rds has been characterized biochemically in the bovine system, little is known about it's interaction partner rom-1. The experiments described in this thesis provide valuable biochemical information concerning bovine rom-1 and its interaction with peripherin/rds. The results indicate that peripherin/rds and rom-1 are subunits of a membrane protein complex localized to the rims of photoreceptor outer segments. Rom-1 was detected in cone photoreceptors; this is a key piece of information regarding RDS-associated disease phenotypes that show differential effects on rods and cones. A system for heterologous expression of the peripherin/rom-1 complex was developed that provides new insight on the structure and function of wild-type and mutant forms of this complex. Additionally, the C-terminal region of rom-1 was found to be non-essential for heterologous assembly of the peripherin/rom-1 complex. Finally, the various antibody reagents, wild-type and mutant cDNAs developed for use in these experiments will hopefully prove to be invaluable in further studies.
However, a large number of unanswered questions remain. In my opinion, the two key (and interrelated) questions that need to be addressed are firstly, "How do mutations in the peripherin/rom-1 complex result in such a large array of phenotypes?" and secondly, "Can we identify more precisely the structural role of the peripherin/rom-1 complex, or identify other potential roles?"

In order to address the first question, the identity of rom-1 in cones needs to be determined. One possible line of biochemical research that could conclusively determine whether cone rom-1 is encoded by a separate gene is to examine retinas of transgenic mice with a disrupted ROM1 gene. If rom-1 is found to be absent from both rods and cones of these mice, cone rom-1 must be encoded by the same gene as rod rom-1. If this is indeed the case, cone-specific and rod-specific interactions of the peripherin/rom-1 complex could explain the variation in phenotype. Genetic context or environmental factors may also play a role, and could potentially be identified by closer analysis of phenotypic variation within affected families.

In order to further characterize the structural role, or other roles of the peripherin/rom-1 complex, it will likely prove useful to study interactions of the complex with other proteins. No other proteins co-purify with the peripherin/rom-1 complex under non-denaturing conditions. However, interactions with other proteins need not be highly stable in order to be functionally important. In order to identify other proteins that interact with the peripherin/rom-1 complex, it may be possible to preserve low avidity interactions by employing membrane-permeable sulphhydryl cleavable crosslinking reagents such as DSP prior to solubilization of the ROS organelle. After solubilization, associated proteins could be detected by purifying the peripherin/rom-1 complex and examining the samples by SDS-PAGE in the presence of reducing reagent. Alternatively, potential partners for interaction could be identified by screening a yeast two-hybrid library using peripherin/rds and rom-1 sequences as "bait."
4.8 Concluding remarks.

At present the function of rom-1 and peripherin/rds is not known. The combination of their unique localization to the disc rims, the presence of large intradiscal segments of these proteins, and the lack of outer segments in the rds mouse (Sanyal and Jansen, 1981) has led to the proposal that the peripherin/rds-rom-1 complex is an important structural component that serves to establish and maintain the unique rim region of rod and cone outer segment disc membranes through homotropic or heterotropic protein-protein interactions. In this regard, the importance of the intradiscal loop of peripherin/rds is illustrated by the fact that most point mutations in these proteins associated with autosomal dominant retinitis pigmentosa or macular degeneration occur in the large intradiscal loop of peripherin/rds (Kajiwara et al., 1991; Farrar et al., 1991; Nichols et al., 1993). Interestingly, except for digenic forms of retinitis pigmentosa that require mutations in both components, no mutations in rom-1 have yet been conclusively linked to retinal degeneration. Perhaps peripherin/rds is the more easily disrupted component (by both changes in protein sequence and non-functional alleles) or simply the more functionally important component of this complex. Clearly, many questions concerning the function of the peripherin/rom-1 complex in the normal and diseased states remain to be answered.
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APPENDIX

The following Q-Basic computer program, written by Dr. AFX Goldberg, was used to calculate $S_{20,w}$ values:

HIT "F5" TO RUN THIS PROGRAM

OR SELECT "Exit" FROM THE [File] MENU

REM PROGRAM TO CALCULATE S-VALUES, OCT 95, AFXG
COLOR 7, 1
CLS
REM THE USER INTRODUCTION
PRINT
PRINT
COLOR 14, 1
PRINT "  *****************************************************"
PRINT "  »»»»»»»»»»» SVEDBERG'S BEST FRIEND"
PRINT "  »»»»»»»»»»»"""
PRINT "  *****************************************************"
PRINT
PRINT "This program will calculate sedimentation coefficients based upon radial"
PRINT "distance traveled in a TLS-55 rotor containing a linear sucrose gradient of "
PRINT "5 - 20% (w/w) at 4 degrees C. Fractions are counted from the tube"
PRINT "BOTTOM."
PRINT
PRINT "--------------------- Oct 95 / A.F.X.G ---------------------"
begin:
COLOR 7, 1
PRINT
PRINT
PRINT
PRINT
PRINT "(TYPICAL RUN PARAMETERS: 0.83 ml/g, 50000 rpm, 16 hours)"
PRINT
PRINT
REM COLLECT THE "RUN" PARAMETERS
INPUT " What molecule/species is being analyzed"; id$
INPUT " What is its partial specific volume (ml/g)"; newbar
INPUT " What was the rotor speed (rpm)"; rpm
INPUT " What was the run length (hrs)"; run.length
INPUT " How many gradients do you wish to analyze"; ngrads
LET counter = 0
FOR counter = 1 TO ngrads
PRINT
PRINT " GRADIENT #"; counter
INPUT " How many fractions were collected in total"; total.fractions(counter)
INPUT " Which fraction contained the peak of interest"; peak.fraction(counter)
REM DEFINE THE ROTOR (TLS-55) CONSTANTS
LET r.min = .0422
LET tube.length = .0343

REM CALCULATION OF S FOR GIVEN GRADIENT AND TEMP
LET distance.traveled(counter) = (tube.length / total.fractions(counter)) * ((total.fractions(counter) - peak.fraction(counter)) + .5)
LET time = (run.length * 3600)
LET omega = rpm * .10472
LET r.avg = ((r.min * 2) + distance.traveled(counter)) / 2
LET exp.s.value(counter) = (distance.traveled(counter) / time / omega ^ 2 / r.avg) * 10 ^ 13

REM COMPUTE PEAK. SUCROSE BASED UPON POSITION
LET peak.sucrose(counter) = ((15 / (1 - total.fractions(counter))) * peak.fraction(counter)) + (20 - (15 / (1 - total.fractions(counter))))

REM CONVERSION TO S,20,w VALUES
avg.sucrose(counter) = (peak.sucrose(counter) + 5) / 2!
avg.exp.density(counter) = ((.000394) * (avg.sucrose(counter))) + 1.0004
avg.exp.viscosity(counter) = (.000047138# * avg.sucrose(counter) ^ 3) + (.00103341# * avg.sucrose(counter) ^ 2) + (.0427446 * avg.sucrose(counter)) + 1.56405
LET s.20.w(counter) = (exp.s.value(counter)) * (avg.exp.viscosity(counter) / 1.004) * ((1! - (newbar * .9988)) / (1! - (newbar * avg.exp.density(counter))))

REM Round and Truncate to 3 places
LET avg.sucrose(counter) = (CINT(avg.sucrose(counter) * 1000)) / 1000
LET avg.exp.viscosity(counter) = (CINT(avg.exp.viscosity(counter) * 1000)) / 1000
LET avg.exp.density(counter) = (CINT(avg.exp.density(counter) * 1000)) / 1000
LET s.20.w(counter) = (CINT(s.20.w(counter) * 1000)) / 1000

NEXT counter

REM THE OUTPUT STATEMENTS
PRINT
PRINT
PRINT
PRINT
PRINT
PRINT
PRINT
PRINT
PRINT
PRINT
PRINT
PRINT
PRINT
PRINT
PRINT
"***************************************************************************************************************
*
PRINT
PRINT "This sedimentation velocity analysis has been performed for:"
PRINT
COLOR 14, 1
PRINT "  » "; id$; "  «"
COLOR 7, 1
PRINT
PRINT "Grad #", "peak/tot", "Avg Sucr/Rho/Eta", "S20,w"
PRINT " ----", " ----", " ---------", " ----"
PRINT
LET count2 = 0
FOR count2 = 1 TO ngrads
PRINT count2, peak.fraction(count2);"/"; total.fractions(count2), avg.sucrose(count2);"/"; avg.exp.density(count2);"/"; avg.exp.viscosity(count2), s.20.w(count2)
NEXT count2
PRINT
PRINT
PRINT "Be sure the printer is online if you desire a hardcopy of your data"
INPUT "What's the magic word (or <enter>)"; hardcopy$
IF hardcopy$ <> "please" THEN GOTO nohardcopy
LPRINT
LPRINT
LPRINT
LPRINT
LPRINT
LPRINT
LPRINT
LPRINT
LPRINT
PRINT
PRINT
"***************************************************************************************************************
*
PRINT
PRINT
PRINT "This sedimentation velocity analysis has been performed for:"
PRINT
PRINT "  » "; id$; "  «"
PRINT
LPRINT
LPRINT
LPRINT
LPRINT
LPRINT
LPRINT
LPRINT
LPRINT
LPRINT
LPRINT "Grad #", "peak/tot", " Avg Sucr/Rho/Eta", "S20,w"
LPRINT " ----", " ----", " --------", " ----"
LPRINT
LET count3 = 0
FOR count3 = 1 TO ngrads
LPRINT count3, peak.fraction(count3); "/"; total.fractions(count3),
avg.sucrose(count3); "/"; avg.exp.density(count3); "/"; avg.exp.viscosity(count3),
s.20.w(count3)
NEXT count3
LPRINT
LPRINT
"********************************************************************
*
LPRINT
LPRINT
LPRINT
BEEP
PRINT "Take the printer off-line and hit 'form-feed'"
nohardcopy:
PRINT
PRINT
INPUT "Another calculation (y/n)"; another$
PRINT
PRINT
PRINT
IF another$ = "y" THEN GOTO begin
END