INITIAL CHARACTERIZATION OF A NOVEL HUMAN RETINAL GENE ENCODING A PUTATIVE PLECKSTRIN HOMOLOGY DOMAIN PROTEIN

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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University of Toronto

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Master of Science, 1995,
Elizabeth Garami,
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ABSTRACT

The human 198L gene is abundantly expressed in retina and encodes a protein containing a putative Pleckstrin Homology Domain. Other proteins containing this domain participate in signalling or cytoskeletal function. Here I describe expression studies of the human and mouse 198L gene. The human 198L transcript was localized to the photoreceptor layer of the adult human retina as shown by in situ hybridization. In addition, the human transcript was previously shown to be expressed in the human brain and kidney by northern blot. Northern blot analysis shows that the mouse 198L transcript is abundantly expressed in mouse retina and throughout the mouse CNS, and less abundantly expressed in liver, lung and kidney. These studies suggest that the 198L gene could play an important role in the biology of the photoreceptor cell. Because of its abundant expression in photoreceptors, the human 198L gene may be a possible candidate gene for retinitis pigmentosa.
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CHAPTER 1: INTRODUCTION

This thesis reports my results on the initial characterization of the expression of the message and protein of the novel, retinal-abundant 198L gene. This chapter contains an overview of topics of retinal biology relevant to the study of 198L, and as well, I present a review of what we presently know about this gene and its products. Lastly, I include information about the pleckstrin homology domain (PH domain), a motif which appears to be present in the 198L protein. Understanding of the biology of the PH domain may help elucidate the function of 198L.

Functional anatomy of the retina

The retina is a thin layer of tissue located at the back of the vertebrate eye. Its function is the absorption and processing of light into neuronal signals that can be transmitted to the visual centers in the brain for further decoding and analysis. The retina consists of two major components: the pigment epithelium (PE) and the neuroretina (NR). The PE is formed by a monolayer of pigmented cells. Three functions can be ascribed to the PE. First, cells of the PE contain melanin granules which contribute to the enhancement of the quality and resolution of the visual image absorbing scattered light. Secondly, the PE participates in photoreceptor cell outer segment renewal by phagocytosis of the discarded apical disks. Thirdly, the PE provides the photoreceptor with metabolites and nutrients (Rowe, 1991).

The neuroretina consists of six principal classes of neurons (photoreceptor, horizontal, amacrine, bipolar, interplexiform and ganglion cells) and of glial cells (Müller cells and astrocytes) that are organized in a laminar fashion. The nuclei of the retinal neurons are disposed in three parallel nuclear layers separated by two plexiform layers, corresponding to the axons and dendrites of these neurons (Fig. 1). The arrangement of the layers of the neuroretina appears inverted to what one intuitively expects. Light has to travel through all the layers of the retina to be captured in the outer segment of the
photoreceptors (Fig. 1). The outermost layer of the retina (with respect to the center of the eye) is adjacent to the pigment epithelium and consists of the photoreceptor layer. The photoreceptor layer is divided into three parts, the outer and inner segments, and the outer nuclear layer (ONL), containing the nuclei of the photoreceptors. The outer plexiform layer (OPL) is the site where neuronal processes of the photoreceptors synapse with horizontal and bipolar cells. The inner nuclear layer (INL) contains the nuclei of the interneurons (all nuclei of horizontal, bipolar and interplexiform cells, the majority of amacrine cell nuclei and a few ganglion cell nuclei). The INL is followed by the inner plexiform layer (IPL), where synapses between interneurons and ganglion cells take place. The ganglion cell layer (GCL) contains nuclei of the majority of the ganglion cells and a few from amacrine cells. The axons of the ganglion cells collectively form the optic nerve which links the retina with the visual centers of the brain. The Müller cells span the whole retina, but their nuclei are located in the INL (Rowe, 1991).

**Photoreceptor structure/function**

Photoreceptors are highly specialized neurons which function mainly to capture and convert light into an electrical signal. The vertebrate retina contains two types of photoreceptor cells, the rod and the cones. Rods function optimally at low intensities of light. Cones function optimally at high intensity of light and they facilitate color vision and the acuity of pattern detection. Three different types of cone cells, classified on the bases of their visual pigment (green, red, blue), exist in humans. Animal cells contain only one type of rod and specific combination of cone cells (Dowling, 1987 and Hargrave and McDowell, 1992).

Rods and cones are structurally similar in that they are extremely compartmentalized and present the same general features: an outer segment, an inner segment, a cell body and a synaptic terminal (Fig. 2). The rod outer segments (ROS) are formed by more than a
Figure 1.

Schematic representation of the structure of the vertebrate retina. Abbreviations: R= rods, C= cones. The outer and inner plexiform layers are areas of complex synaptic interactions, represented in the figure as gaps between the nuclear layers. The direction that the light has to travel in order to excite the photoreceptor is indicated. Modified from Adler and Farber, 1986.
1000 individual disks surrounded by a plasma membrane. Each disk consists of a continuous lamellar membrane that encloses a small space called the intradiskal space. The edges of the disks have a hairpin shape and are referred to as the disk rim. The orderly array of the disks is maintained by cytoskeletal connections between adjacent disk rims and between disk rims and the plasma membrane (Molday and Molday, 1993). Disk formation begins at the base of the ROS where they are in continuity with the plasma membrane. Once formed they become independent from the plasma membrane and migrate from the base to the tip of the ROS. Cone outer segments tend to be shorter than those of rods and the disks of the cone outer segment are in continuity with the plasma membrane. The outer segment and the inner segment are connected by a slender nonmotile cilium characteristic of sensory cells. This cilium has nine microtubule doublets but is missing the central microtubules (Hargrave and McDowell, 1992).

The inner segment of cones in mammalian retina are larger in size relative to their outer segment giving the cones their apparent conical shape. The inner segments consist of the myoid and the ellipsoid (Fig. 2). The ellipsoid is proximal to the outer segment and is the place where the cilium arises. In addition, the ellipsoid is highly packed with mitochondria which generate the ATP required for metabolic functions of the photoreceptor, such as ion pumping and macromolecular synthesis. The major metabolic machinery is located in the myoid. This machinery includes the endoplasmic reticulum and the Golgi apparatus which are highly active in protein synthesis. Vesicles are found in the inner segment, some of which are likely to transport molecular components such as rhodopsin to the outer segment. Other vesicles are known to be endocytic vesicles. The nuclei are located within the cell body which in turns gives rise to the axon at its distal end. The axons end at the synaptic terminal. In general the cones have large endings shaped as pedicles, with multiple synaptic invaginations and basal contacts, whereas rods have spherule shaped endings with a single synaptic invagination (Hargrave and McDowell, 1992, Fisher et al, 1993 and Rowe, 1991).
Figure 2.
Structure of the vertebrate rod photoreceptor. The rod is divided into the outer segment (OS), inner segment (IS), cell body and synaptic terminal. The OS is the site of phototransduction, the IS contains the metabolic machinery, the cell body contains the nucleus and the synaptic terminal connects with the next set of neurons for further processing of the light signal. Modified from Stryer, 1988.
Signal transduction in the photoreceptors

Phototransduction is the process by which photoreceptors capture energy from light, and convert it into a neural signal. Rods and cones have very similar transduction mechanisms. Compared to cones, rods are more abundant in most species retinas and relatively easier to isolate. Therefore, the signal transduction process is described in terms of signaling in rod cells because they have been studied more extensively (Hargrave and McDowell, 1992).

Several proteins are known to participate in visual transduction. The majority have been purified and for some the cDNA has been isolated. The availability of this information has facilitated detailed structural and functional studies that have led to a very complete description of the phototransduction events.

The visual system is highly sensitive, one photon being capable of exciting the rod cell. In addition, the signal cascade generated by a single photon is highly amplified. The primary event in phototransduction is the light activated isomerization of 11-cis-retinal to 11-all trans-retinal. The isomerization process leads to the formation of several intermediate conformations of rhodopsin, all of which show different spectral properties. The key intermediate is Meta II rhodopsin (MII) that has an unprotonated Schiff base of all trans-retinal. MII binds to inactive Tαβγ and induces activation of Tα by catalyzing the exchange of GDP to GTP, and consequent dissociation of Tα from Tβγ. The first amplification step of the visual cascade is the activation of approximately five hundred transducin molecules by a single photoexcited MII. Activated Tα releases phosphodiesterase from its inhibited state by interacting with a PDEγ inhibitory subunit. The second step of amplification in the cascade is provided by the activated phosphodiesterase, a strong enzymatic catalyst which quickly hydrolyzes cGMP and decreases the cytoplasmic concentration of cGMP quite efficiently. In the dark, cGMP binds to the cation channels of the plasma membrane and maintains them open. Reduction in the amount of available cGMP results in the closure of cGMP-sensitive cation channels.
Interruption of the current flow through these channels causes hyperpolarization of the rod cell and reduction of the rate of the glutamate release from the rod synaptic terminal (Fig. 3). At the postsynaptic terminal, the response is either hyperpolarization or depolarization of the membrane in sign-inverting or sign-preserving synapses respectively (Khorana, 1992, Yau, 1994) (Fig. 3). A close examination of the biology of the proteins that participate in the visual cascade, allows to appreciate the complexity of the cascade and of regulatory mechanism of the visual events.

Rhodopsin is a member of the G protein coupled receptor family. It is the most abundant protein in the disk membrane, it has been estimated that rhodopsin represents over 95% of the protein of bovine disk membranes (McDowell, 1993). Rhodopsin, a photosensitive molecule, has a protein component called opsin and a chromophore (Stryer, 1988). The universal chromophore for both cones and rods is the 11-cis-retinal which is linked to the e-amino group of lysine-296 in the helix G of rhodopsin via a protonated Schiff base. Three structural domains have been defined for rhodopsin: 1) intradiscal, 2) membrane-embedded helical domain, and 3) cytoplasmic domain. Mutational analyses have suggested potential functions for these domains. The intradiscal domain has an important function in maintaining the structure of rhodopsin. The membrane domain contains the retinal binding pocket and is responsible for transmitting the light signal to the cytoplasmic domain where all downstream signaling events occur (Khorana, 1992).

The function of transducin is to couple the light activation from rhodopsin to the phosphodiesterase. Transducin (T) is a member of the G protein family, a heterotrimeric protein formed by the α subunit of 39 kDa, the β subunit 37 kDa and a γ subunit of 8.5 kDa. The α subunit of transducin (Tα) alternates between a GTP (active) and GDP (inactive) bound states. Two forms of Tα have been described, TαI and TαII, which are the alpha subunit of transducin in rods and cones respectively. Biochemical studies have predicted that Tα has an N terminal domain for interaction with Tβγ, a phosphodiesterase binding domain C terminal to the Tβγ binding domain and the C terminus of Tα is
responsible for binding to rhodopsin. The Tβ subunit presents 43 amino acids repeats. The C terminus of Ty is similar to the C terminus of Ras proteins. Both of these proteins, Ras and Ty, have the consensus cysteine-aliphatic-aliphatic-(serine, methionine, cysteine) that is acetylated by palmitic acid. This modification allows anchoring of the proteins to the membrane. Thus, Ty is anchored to the membrane and binds tightly, but not covalently to Tβ (Falk and Applebury, 1988).

The concentration of cytoplasmic guanosine 3', 5' cyclic monophosphate (cGMP) controls the light-sensitive conductance of the vertebrate photoreceptor plasma membrane. The net concentration of cGMP in the cytoplasm is the balance between the rate of synthesis from GTP catalyzed by calcium sensitive guanylate cyclase and the rate of hydrolysis to 5'-GMP catalyzed by cGMP-phosphodiesterase (PDE), a tetrameric enzyme formed by two catalytic subunits (α and β) and two γ inhibitory subunits (Kaplan and Palczewski, 1993 and Hargrave and McDowell, 1992). Activated Tα combines with the PDE inhibitory subunits and cause activation of PDE that hydrolyses cGMP and will lead to closure of the cation channels.

The cation channels and the Na⁺-Ca⁺² exchanger are the principal means of transport of ions in the photoreceptors. The location of these proteins is restricted to the plasma membrane. The cation channels are directly and cooperatively activated by cGMP and as well are suppressible by light. In the dark, approximately one percent of the channels are kept open by free cGMP. Only a small fraction of the cGMP is in the free form. The majority of the cGMP of the photoreceptor is bound to non-catalytic cGMP binding sites of PDE. It is likely that the channel has at least four cGMP binding sites. It has not been established how many of the sites have to be occupied in order to open the channel, but it has been suggested that the binding of cGMP to the channel is very weak. A cDNA has been cloned from human and bovine retina that codes for a 63 kDa channel protein. The primary amino acid sequence presents a consensus for a single nucleotide binding site, suggesting that the native channel is likely to be a homotetramer, although
channel activity has been observed when expressing this single subunit. The cDNA of a 30% homologous channel protein (subunit 2) has been cloned from human retina. This protein does not have channel activity unless coexpressed with the human 63 kDa subunit (subunit 1). Interestingly, the heterosubunit channel shows cycles of rapid closing and opening and is very sensitive to L-cis-diltiazem which blocks the channel. Both of these properties have been described for the native channel, suggesting that the native channel is composed of these two subunits (Yau, 1994).

Other proteins with an indirect role in the visual cascade include the exchanger and phosducin. The exchanger transports Na⁺, K⁺ and Ca²⁺ with a stoichiometry of 4Na⁺:1K⁺:1Ca²⁺, so it effectively decrease the internal concentration of calcium by utilizing the electrochemical gradients of both sodium and potassium to extrude calcium (Cook, 1993). Phosducin, also known as 33k protein, is a 245 amino acid protein found in vertebrate photoreceptors and in pinealocytes. Phosducin in its native conformation interacts with Tβγ forming a trimeric complex of 77 kD. The levels of Tβγ/phosducin are comparable to the levels of Tαβγ. The transducin complex is localized mainly in the outer segments, but the Tβγ/phosducin complexes are scattered throughout the photoreceptor cytoplasm. Although both Tα and phosducin bind to Tβγ they do not share sequence homology. Phosducin interaction with Tβγ, decreases the amount of Tβγ available to bind Tα. The binding of Tα to Tβγ is necessary for activation of Tα. Therefore, the effect of phosducin is inhibition of both activities mediated by Tα: the GTPase and PDE activation. Addition of exogenous Tβγ reverses this inhibition (Lee et al, 1993).

Phosducin can be phosphorylated at serine 73 both in vivo and in vitro by the catalytic subunit of protein kinase A. This phosphorylation site is conserved in phosducins of human, rat retina and pinealocytes, suggesting that phosphorylation could play an important role for the regulation of phosducin activity. There seems to be a correlation between phosphorylation of phosducin and its ability to bind to Tβγ; phosducin has decreased affinity for Tβγ when phosphorylated. In the dark state, phosphorylated
phosducins are more abundant (Lee et al, 1993). Upon light stimulation, phosphatase 2A dephosphorylates phosducin (Yarfitz and Hurley, 1994).

Termination of the visual cascade

Termination of the visual cascade requires inactivation of rhodopsin. Two processes cause the inactivation, phosphorylation of rhodopsin and binding of arrestin to phosphorylated rhodopsin (Fig. 3).

As for other G-protein-coupled receptors, rhodopsin desensitization occurs via two mechanisms that involve phosphorylation of the receptor and uncoupling of the cognate G protein from the receptor. At low concentration of ligand, known as heterologous desensitization, a second-messenger-regulated kinase phosphorylates the receptor. There is no discrimination between ligand-bound or ligand-free receptor, both are equally phosphorylated by these kinases. At high concentration of ligand, known as homologous desensitization, a specialized kinase phosphorylates the receptor. The kinase is a member of the G-protein-coupled receptor kinase family and only phosphorylates the receptor in the presence of ligand (Newton and Williams, 1993).

Protein kinase C (PKC) is suspected to play a role in the visual transduction cascade of the rod outer segment. First, PKC is abundantly expressed in the retina. Secondly, turnover of phosphatidylinositol biphosphate in response to light has been described. PKC mediated phosphorylation of rhodopsin in intact retina seems to be more pronounced at low levels of light. The affinity of PKC for bleached and unbleached rhodopsin is the same. The consequences of PKC mediated phosphorylation is to disrupt the light activated coupling of transducin and rhodopsin (Newton, 1993).

Enzymatic phosphorylation of rhodopsin in the presence of light is catalyzed by a member of the G protein coupled receptor kinases, rhodopsin kinase. Rhodopsin kinase (RK) can be post-translationally modified by isoprenylation and by myristoylation at its C terminus and N terminus respectively. These modifications provide a mechanism for
bringing RK to the membrane and in proximity with rhodopsin. RK phosphorylates itself both at its N and C termini. Autophosphorylated RK has decrease affinity for phosphorylated rhodopsin and dissociates from it, allowing arrestin binding. RK presents substrate specificity, it is able to phosphorylate serine and threonine residues at the C termini of photolyzed rhodopsin, but fails to phosphorylate opsin and rhodopsin. RK has also been shown to phosphorylate the β-adrenergic receptor (Palczewski, 1993 and Halgrave, 1992).

Complete inactivation of rhodopsin is achieved upon binding of arrestin. Arrestin, also known as 48K protein or S antigen, binds rapidly to phosphorylated rhodopsin and interferes with further activation of transducin by competing for binding to rhodopsin. Arrestin also blocks the phosphatase 2A catalyzed dephosphorylation of rhodopsin and of light activated rhodopsin until the complete inactivation of rhodopsin occurs. Arrestin dissociates from the rhodopsin when the photoisomerized all-trans-retinal rhodopsin decays to the inactive phosphorylated metharodopsin III (Lagnado and Baylor, 1992).

Arrestin-like proteins have been identified in cells other than the retina. These proteins play role in signal transduction analogous to their role in the retina. β-arrestin is known to interact with the β-adrenergic receptor and cause desensitization of the receptor (Buczyłko and Palczewski, 1993).

In addition, recovery to the dark state requires inactivation of transducin, an increase in calcium concentration and restoration of dark state levels of cGMP concentration. Tα is inactivated upon hydrolysis of GTP to GDP, catalyzed by its intrinsic activity. TαGDP combines with Tβγ to allow a new activation cycle (Ting et al, 1993). Reassociation of Tαβγ causes the release of the PDE inhibitory subunits which bind and inactivate PDEαβ. The concentration of calcium increases as the cation channels are reopened. Resynthesis of cGMP is catalyzed by guanylate cyclase (Hayashi et al, 1993).
Calcium in phototransduction

In the dark the concentration of calcium in the outer segment exist as a steady state. Calcium enters the outer segment through the cation channels and is extruded by the Na\(^+\)-Ca\(^{2+}\), K\(^+\) exchanger. Light causes the closure of the cation channels eliminating influx of calcium, but calcium efflux continues, resulting in a net decrease of the intracellular calcium concentration. Reduction in intracellular calcium is important for light adaptation and acceleration of recovery after light response.

The concentration of calcium and cGMP display a reciprocal relationship, increased calcium concentration reduces cGMP concentration and vice versa. This relationship can be in part explained by inhibition of guanylate cyclase by high concentration of calcium, through a calcium binding protein known as GCAP (guanylate cyclase activating protein). Light activated hydrolysis of cGMP causes reduction of intracellular calcium concentration and consequent release of inhibition of the cyclase. The calcium feedback accelerates the recovery after light and contributes to light adaptation by downregulating the sensitivity of the rod to light, by opposing the light-activated hydrolysis of cGMP. The calcium feedback is important in darkness as well, it controls random fluctuations of cGMP concentration reducing the dark noise that could interfere with the visual cascade and also avoids excess influx of sodium that could harm the rod cell (Yau, 1994 and Palczewski, 1994).

There are other targets for calcium in the visual cascade that contribute to the negative feedback. One of them is the cation channel. A calcium-calmodulin complex is known to bind to the subunit 2 of the channel and decrease the affinity of cGMP for the channel. When calcium decreases in response to light, the calcium-calmodulin complex dissociates from the channel, which in turn is able to remain opened and therefore accelerates the recovery from the light response. In addition, calcium regulates deactivation of light stimulated rhodopsin, by phosphorylation, this effect is mediated by the calcium
Figure 3.

Representation of the events in the visual cascade. A. Light activates rhodopsin (Rh). Activated rhodopsin (Rh*) catalyzes the activation of transducin (T) which in turns activates phosphodiesterase (PDE) that hydrolyzes cGMP. Reduction of the concentration of cGMP causes closure of the cation channels and hyperpolarization of the photoreceptor cell. B. Termination of the visual cascade occurs upon phosphorylation of rhodopsin by rhodopsin kinase (RK) or by protein kinase C (PKC), and binding of arrestin. Modified from Yau, 1994.
binding protein S-modulin. The catalytic activity of activated rhodopsin is calcium regulated by an unknown mechanism (Yau, 1994 and Williams, 1995).

Another minor regulatory contribution of calcium includes blockage of the cation channel. The blockage can be explain by relatively high affinity divalent cation binding sites in the channel that slow down the influx of calcium. The blockage improves light detection because it decreases the conductance of a single channel that might randomly open in the dark state resulting in a better signal-to-noise ratio (Yau, 1994).

Cloning and initial characterization of the human 198L

198L cDNA was cloned in our lab from an adult human retina cDNA library as described by Bascom et al (1992). The cloning strategy is based on the hypothesis that evolutionarily conserved transcripts that are abundantly and specifically expressed in the retina are likely to have a key role in its normal structure, function and development. Such SAC (Specific Abundant and Conserved) cDNAs constitute candidate genes for retina specific genetic diseases. Identification of these cDNAs was achieved by differential hybridization. Four hundred randomly selected clones from an adult human λgt10 retinal cDNA library (provided by J. Nathans) were plated in ordered arrays. Several nitrocellulose plaque lifts were done from the individual plates and screened at high stringency with radioactively labeled total bovine retinal single stranded-cDNA to identify evolutionarily conserved retinal clones. By using a total retinal cDNA mixture as probe, clones of abundantly expressed genes were selected, since it has been reported that a specific cDNA clone will be detected if its relative abundance in the probe is ~0.2% or greater (Crampton et al, 1980). Total human fibroblast cDNA was used to eliminate cDNA clones of housekeeping genes, and human mitochondrial DNA to exclude any cDNA clones of mitochondrial genes, because photoreceptors are rich in mitochondria.

Using a total cDNA mixture from a non-human species as a probe for screening avoids selecting false positive clones containing species-specific repetitive sequences
normally found in mammalian mRNA (Crampton, 1981). To corroborate the effectiveness of this strategy, total human genomic DNA was used to identify clones containing human repetitive sequences. Positive clones from the differential hybridization screen were analyzed by human multitissue northern blot to classify them as SAC cDNAs (expression restricted to the retina), or AC cDNAs (expression in additional tissues).

The 198L cDNA was identified in the differential hybridization screen and by RNA blotting shown to be an AC cDNA. The expression of the 198L message was found to be abundant in retina, and less abundant in kidney and brain. No expression was found in liver, fibroblast, lymphoblast or muscle. The message identified in all the expressing tissues is approximately 2 kb in length (Fig. 4). The insert of the original 198L clone was used to rescreen the human retinal cDNA library to isolate the full length 198L cDNA clone, and three overlapping cDNA clones were identified and sequenced (Fig. 5A): clone 71, clone 155 and clone 174. These clones exhibit alternative splicing (Fig. 5B), evidenced by the presence of a 105 bp region in clone 71 that is absent in both 155 and 174 cDNA clones. There is also a 284 bp fragment present in both clones 155 and 174 that is absent in clone 71.

198L has been mapped to human chromosome 11q13 by in situ hybridization and PCR of DNA from mouse-human hybrid cells (Alessandra Duncan, Queens University, personal communication). Four retinopathies have been linked to this chromosomal region by linkage analysis. The retinopathies are Best's disease (Stone et al, 1992), Usher syndrome type 1 (Kimberling et al, 1992), Autosomal Dominant Neovascular Inflammatory Vitreoretinopathy (AdNIV) (Stone et al, 1992), and Bardet-Biedl syndrome (Leppert et al, 1994). Because of the abundant expression of 198L in the retina and its chromosomal localization, 198L is a candidate gene for these retinal degeneration diseases. The gene responsible for Usher syndrome has been identified and it was found to code for myosin VIIA (Weil et al, 1995).
Figure 4.

RNA blot of 198L in multiple human tissues. The 198L transcript is abundantly expressed in retina and less abundantly expressed in brain and kidney. The size of the human 198L transcript is ~ 2 kb.
Splicing events are indicated by the broken lines. Composite 1987 CDNA and the three overlapping CDNA clones isolated. Alternative priming. Homology Domain is shaded. B. Schematic representation of the protein in the 5' UTR are underlined. The initiation methionine is underlined twice. The Nucleotide and derived amino acid sequence of the human 1987 CDNA. In-frame stop.

Figure 5.
Closer mapping of 198L to this region has excluded 198L as a candidate gene for Best disease (Ed Stone, personal communication).

Bardet-Biedl (BBS) syndrome is of particular interest because it affects retina, brain and kidney, representing organs where 198L transcript is known to be expressed. The BBS syndrome is an autosomal recessive disease characterized by retinal degeneration, mental retardation, polydactyly, obesity and hypogenitalism. Internal organs, such as kidney, are also affected. Histopathological studies suggest that the primary retinal degeneration event occurs in the photoreceptors followed by secondary events in the other retinal layers including the pigment epithelium (Beck and Rosenberg 1995). It has been suggested that the pathogenesis of the BBS might be related to mutation(s) in a gene that could regulate maturation of the fetus during the second month post-conception. Defects in such a gene could interfere with final maturation of some organs (Anadoliisika and Roussinov, 1993).

In order to analyze DNA samples of patients with these retinopathies for mutations, the genomic structure of 198L was determined. The gene has at least 10 exons, two of which are alternatively spliced (Fig. 6).

DNA samples from eight linked Bardet-Biedl syndrome families have been analyzed to identified mutation in the 198L gene and no mutation in the gene was found (Lupski and McInnes, unpublished results).

The predicted open reading frame of the two differently spliced forms of 198L is either 729 bp or 624 bp, encoding proteins of predicted mass 27 kDa or 23 kDa, respectively. The in vitro transcription/translation products are approximately 30 and 24 kDa. These represent products with and without the alternatively spliced coding exon and agree well with the predicted sizes (Fig. 7). GenBank database search using the primary amino acid sequence of the 1981 protein showed that the N terminus of 1981 has slight homology to rasGAP (GTPase activating protein), members of the rac family of serine and
Figure 6.
Structure of the human I98L gene. The gene has at least 10 exons, represented as boxes, two of which are alternatively spliced (shaded boxes). The PH domain is encoded by the exons indicated as hatched boxes. The number inside the boxes represent the sizes of the exons in bp. The sizes of the introns are indicated kb in between the boxes.
### Figure 7.

*In vitro* transcription/translation of 198L cDNA's. The arrows indicate the position of the 198L *in vitro* transcription translation products, 30 kDa (including exon 6) and 24 kDa (exon 6 spliced out) polypeptides. Lanes: 1) Negative control, 2) Rom1 positive control (~33 kDa), 3) Clone 71, 4) Clone 174, 5) Clone 174/155, 6) Clone 71/155.

<table>
<thead>
<tr>
<th>Exon</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<td>29</td>
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<tr>
<td>18</td>
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Lanes: 1) Negative control, 2) Rom1 positive control (~33 kDa), 3) Clone 71, 4) Clone 174, 5) Clone 174/155, 6) Clone 71/155.
threonine kinases (rac, akt2), OSBP (oxysterol binding protein), dynamin, and pleckstrin in a region of these proteins for which there was no described function (Fig. 8). This region was later shown to correspond to the recently described pleckstrin homology domain. Therefore the only putative protein domain in 1981 protein recognized to date is the pleckstrin homology domain (Soltyk and McInnes, unpublished results).

Pleckstrin Homology Domain

The Pleckstrin Homology Domain, or PH Domain, was first identified as two similar motifs in the major protein kinase C substrate of platelets, pleckstrin (Tyers et al, 1988). To date, more than seventy proteins are known to contain PH domains. Proteins that contain this domain are predominantly involved in signal transduction, but there are also some proteins involved in cytoskeletal function. Many of these proteins can be grouped into distinct functional categories: 1) serine/threonine kinases: Akt/Rac, βARK/Gprk, NrkA, PKC-μ, 2) Tec family of tyrosine kinases, 3) regulators of small GTP binding proteins: ras-GAP, ras-GRF, Bcr, Sos, Bem3, Dbl, Vav, cdc24, and 4) phospholipase C isoforms: β, δ and γ (Ingley et al, 1994).

In the majority of cases, proteins that contain pleckstrin homology domain have one copy of the domain, but two copies of the domain have been found in: pleckstrin, GRF, AFAP-110, Tiam-1, and syntrophin. Proteins containing more than two PH domains have not been yet identified (Gibson et al, 1994).

The primary amino acid sequence of the PH domain is loosely conserved. Proteins containing the domain only share 21 to 25% identity in the domain. Because of such a low degree of identity, recognition of putative PH domain proteins is difficult. It would not be surprising if some PH domain-containing proteins have not been detected as such (Mayer et al, 1993). The PH domain consists of approximately 100 amino acids and is not specifically positioned within the protein that contains it. The domain has been subdivided
<table>
<thead>
<tr>
<th>198L</th>
<th>N-PLECKSTRIN</th>
<th>DYNAMIN</th>
<th>β SPECTRIN</th>
<th>RAS-GAP</th>
<th>RAC β</th>
<th>OSBP</th>
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<td>AQMEQFLNKRKHEW</td>
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<td>MRHCRGTVINLATANITVEDS . . . .</td>
</tr>
<tr>
<td>LTVNLREGGR</td>
<td>FVPKITTKQK</td>
<td>HIFALFPTEQR</td>
<td>HVFKLRLNDGN</td>
<td>NCFQIVVQHFS</td>
<td>RPRPNFVIR</td>
<td>CNFIISNGGAG</td>
</tr>
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<th>DYNAMIN</th>
<th>β SPECTRIN</th>
<th>RAS-GAP</th>
<th>RAC β</th>
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<td>IFYFAGETPEQADMKGLQAPC</td>
<td>ERTFHVDSPEREEMRMAIQMVA</td>
<td>TYHLKASESSEVERQRMVTALELAK</td>
</tr>
</tbody>
</table>

**Figure 8.**

Sequence comparison of the putative PH domain of 198L with a selection of other PH domains of proteins that are similar to 198L, as identified by BLAST search (Altschul et al., 1990). The asterisk indicates β spectrin which was not retrieved by BLAST, but is included with N-pleckstrin and dynamin as structurally defined PH domains. The conserved tryptophan is underlined.
into 6 subdomains (I-VI). The only invariant amino acid in all PH domain containing proteins is a tryptophan in subdomain VI (Musacchio et al, 1993). There is variation in the sizes and in the distance between the subdomains (Fig. 9). An extreme example is PLCγ, which has a split PH domain where two SH2 and one SH3 domain separate subdomains I-III from subdomains IV-VI.

The PH domain is likely to be of functional relevance. First, although the primary amino acid conservation is very low, the overall domain is preserved in evolution, it has been described in proteins ranging from yeast to man. Secondly, some PH containing proteins are known to be potentially oncogenic, e.g., Dbl, Vav, Bcr and Rac kinase (Ingley et al, 1994). Thirdly, there is a point mutation in the PH domain of Burton's tyrosine kinase (R28C) which is known to cause xid (X-linked immunodeficiency). This mutation does not affect the expression nor the in vitro kinase activity of Btk in B cells of xid mice. Arginine 28, located within a conserved region among the members of the Btk family of kinases, is surrounded by a stretch of hydrophilic amino acids. It is likely that arginine 28 is located on an exposed surface of the protein and participates in interactions that lead to response to intracellular signals. Mutation of arginine 28 impairs the ability of Btk to confer signals required for B cell activation and maturation (Rawlings et al, 1993).

Structure of the PH domain

The structure of the N terminal PH domain of pleckstrin has been studied by NMR spectroscopy. The structure consists of 7 β strands followed by a C terminal amphiphilic helix. The β strands are arranged as two orthogonally packed antiparallel β sheets of 4 and 3 β strands respectively. The structure can be described as an up-and-down β-barrel with the C terminal helix capping one end of the barrel. The tryptophan conserved in all PH domain alignments interacts with the hydrophobic core of the barrel, therefore it seems that its role is to contribute to protein stability. The loops between strands vary greatly in length.
Figure 9.
Schematic representation of the Pleckstrin Homology Domain. Subdomains are indicated as ovals. The number of residues in each subdomain, and those separating each subdomain are indicated. The position of the conserved tryptophan is shown, as well as the position of the predicted antiparallel beta strands (represented as small arrows) and alpha helix. The insertion in the PH domain of PLCγ is located between subdomains III and IV.
among PH domains. These regions include amino acids insertions in the alignments. It is therefore expected that if the PH domains can tolerate these insertions without disruption of the structure, the 3D structure of all PH domains would be basically the same (Yoon et al, 1994).

NMR studies of the PH domain of β-spectrin have shown that the overall topology of the PH domain is conserved: two antiparallel β sheets, followed by a C terminal helix. In the case of spectrin there is a two turn helix in the loop that connects the third and fourth β strands. Multiple sequence alignments suggest that this insertion is characteristic of β-spectrin, therefore it is probable that the two turn helix only occurs in spectrin. The N and the C terminus of the structure are in close proximity. Electrostatic polarization of the molecule is evident, there is a pocket surrounded by positively and negatively charged amino acids (Macias et al, 1994).

**Function of the PH domain**

Examining the sequences containing PH domains does not obviously suggest a common function. The sequence diversity of the PH domain suggest the possibility that PH domain can have different types of ligands (Ingley, 1994).

The function of the PH domain is unknown. The domain is not likely to have a catalytic activity, because it lacks conserved residues and as well is small in size. The presence of the domain in signal transduction proteins, alone or in addition to SH2 and SH3 domains, might indicate that the PH domain could, like SH2 and SH3 domains, act as a protein module for protein-protein interactions (Mussachio et al, 1993).

Structural and biochemical studies are providing evidence as to what the function of the pleckstrin homology domain might be. The structure of the N terminal PH domain of pleckstrin is similar to that of the up-and-down β-barrel of retinol binding protein (RBP). RBP binds small lipophilic molecules in the hydrophobic core of the β barrel. Based on structural similarities, it has been suggested that a PH domain can bind lipid molecules
There are 6 lysines found at the entry of the barrel of the N terminal PH domain of the pleckstrin protein, four of which are conserved in many PH domains. These lysines could contribute to lipid binding by interacting with polar head groups. In addition, some proteins containing a PH domain, like βARK and phospholipases, are regulated by βγ subunits of G proteins which are isoprenylated. Other PH containing proteins are regulators of p21Ras, which is also lipophilically modified. Interactions of lipids with a PH domain could allow localization of a PH containing protein to the membrane (Yoon et al, 1994).

Harlan and coworkers decided to take the similarity to lipid binding proteins one step further and they study if the PH domain might function as a lipid binding module (Harlan et al., 1994). They established that the N terminal PH domain of pleckstrin binds to phosphatidilinositol-4,5-biphosphate. Phosphatidylinositol-4-phosphate is also able to bind to PH domains but with lower affinity. Lipid binding was dependent on the presence of PH domain. Proteins lacking the PH domain, such as the Shc SH2 domain, lysozyme, ribonuclease A, cytochrome C were unable to bind lipids, whereas the PH domain of pleckstrin (both N and C terminal PH domains), Ras-GAP, Tsk and βARK bound vesicles containing PIP2. Furthermore, amino acids of the PH domain responsible for the interaction with PIP2 were identified by NMR upon addition of lipid. The lipid binding site is located N terminal of the PH domain and includes residues: K13, K14, S16, V17, N19, T20, W21, K22, F35, Y36 and G46. The underlined residues are conserved in several PH domains. PH domains containing acetylated lysines were incapable of binding lipids. These findings corroborate the importance of these residues for lipid binding. Interactions between PH domains and PIP2 might be biologically relevant, representing a new mechanism for proteins that lack known membrane anchoring groups, such as hydrophobic helices or post translational lipid modifications, to associate with membranes (Harlan et al., 1994).
Another system where the role of the PH domain has been evaluated, is the insulin signal system. The insulin receptor does not appreciably bind SH2 containing proteins after tyrosine phosphorylation upon stimulation. The insulin receptor instead recruits IRS-1 (insulin receptor substrate 1) to the membrane. IRS-1 becomes extensively tyrosine phosphorylated and allows the binding of SH2 containing proteins, providing a link between the insulin receptor and downstream signaling events. IRS-1 mediates activation of PI 3'-kinase and SH-PTP2, and the stimulation of MAPK and p70s6k. IRS-1 has a PH domain which is 100% identical among rat, mouse and human IRS-1 and 62% identical to the IRS-1 homologue, IRS-2 (Myers et al, 1995 and reference therein). Deletion of the PH domain of rat IRS-1 impaired the insulin induced tyrosine phosphorylation of IRS-1, but did not affect its basal or insulin induced pattern of serine, threonine phosphorylation. The lack of tyrosine phosphorylation in the PH deletion mutant caused the absence of downstream activation events that normally occur in the wild type IRS-1 scenario. Tyrosine phosphorylation and signaling of IRS-1 $\Delta^P$H was restored by overexpression of insulin receptor in 32D cells lacking endogenous IRS proteins. These results suggest that the potential function of the PH domain of IRS-1 is to couple IRS-1 to insulin receptor (Myers et al, 1995).

Indirect evidence of the role of the PH domain has been obtained by studies to establish $\beta$ spectrin binding sites for brain membranes. These studies have led to the identification of two potential distinct binding sites in spectrin for brain membranes depleted of peripheral proteins. The N terminal binding site, which can not discriminate between $\beta$G (general isoform of $\beta$ spectrin) and $\beta$R (red blood cell isoform of $\beta$ spectrin) is sensitive to calcium and calmodulin. The C terminal binding site is specific for the $\beta$G, it fails to bind $\beta$R, is not sensitive to calcium. Interestingly, the C terminal region of $\beta$G contains a PH domain. Therefore, the PH domain of $\beta$G could contribute to the binding of $\beta$ spectrin to brain membranes (Davis et al, 1994).
Another described target for interaction with PH domain is PKC isoforms. A GST fusion protein containing the PH domain of Btk was shown to directly interact with PKC in vitro. A GST fusion containing the PH domain of Btk with xid mutation had 3-5 fold reduction of binding activity compared to wild type PH domain. By using PKC isoform specific antibodies it was shown that GST-BtkPH interacts with both calcium dependent (α, βI, βII) and calcium independent (ε and ζ) PKC isoforms. GST-BtkPH fails to bind to γ, θ, γ and δ isoforms. In the absence of PKC, Btk is autophosphorylated at tyrosine residues. PKC phosphorylates Btk at serine residues and this results in an 80 to 90% decrease in tyrosine autophosphorylation of Btk (Yao et al, 1994). Furthermore, PKC has been shown to interact with the serine/threonine protein kinases Racα and Racβ through their PH domain (Konishi et al, 1994).

The system from which the study of the potential function(s) of the PH domain has benefit the most is the deactivation of β adrenergic receptor by βARK (beta adrenergic receptor kinase). βARK requires to be localized to the plasma membrane in order to phosphorylate and deactivate the receptor. βARK has been shown to bind to βγ subunits of G proteins (Pitcher et al, 1992). The βγ binding domain is mapped to the last 125 amino acids of βARK. This region has a PH domain. In addition, GST fusion proteins containing the PH domain of: Ras-GRF, Ras-GAP, PLCγ, Atk, OSBP, IRS-1, β-spectrin, and Rac β have been shown to bind to bovine brain βγ subunits in vitro. PLCγ, Atk, and OSBP bound most efficiently to βγ. The binding of GST-PH domain fusions was mutually exclusive to the binding of Goα. Deletion constructs of the ras-GRF PH domain were made and used to show that subdomain VI of the PH domain along with amino acids C terminal were responsible for βγ binding. Supporting these findings was the observation that binding of PLCγ PH domain to βγ was achieved by only using the C terminal part of the split PLCγ PH domain (Touhara et al, 1994). The PH domain of Btk was also shown to bind to βγ subunits of G proteins both in vitro and in vivo. Fusion proteins containing subdomains V and VI of the Btk PH domain bind as efficiently as full length PH, whereas
the N terminal part of the PH domain (subdomains I to IV) fails to bind βγ both in vivo and in vitro. The conserved tryptophan was shown to be important for binding, because protein from an expression construct encoding Btk PH domain with the conserved tryptophan mutated to glycine fails to show binding to βγ (Tsukada et al, 1994).

Many proteins are known or suspected to bind βγ subunits of G proteins. βγ binding domain has been identified in some of these proteins, which include: βARK1, βARK2, the drosophila homologue GPRK1, phosducin, adenylate cyclase type II, α subunits of G proteins, PLCβ isoforms and the atrial potassium channel (GIRK1). There are no universal βγ binding sequences, but it seems that the pleckstrin homology domain could allow βγ effector binding. The PH domain overlaps with the βγ binding domain only in the case of βARK isozymes. It is known that the βγ binding domain of PLCβ is located in the N terminal two thirds of the protein which might coincide with the PH domain that is mapped to the first 100 amino acids of this region. A βγ binding domain has been assigned to the first 100 amino acids of phosducin. This region of the protein displays slight homology to subdomains IV to VI of the PH domain of βARK (Inglese et al, 1995).

βARK has been shown to bind to PIP2 (Harlan et al, 1994) and βγ subunits of G proteins (Touhara et al, 1994). Re-evaluation of the binding properties of both ligands to βARK has demonstrated that both ligands are necessary for membrane association and activation of the kinase. In addition, the specificity of lipids shown to promote membrane association of βARK is similar to the patterns of lipid binding to PH domains shown by Harlan et al, 1994. Furthermore, the C terminus of βARK, including the PH domain, expressed as a fusion can inhibit the βARK activity dependent on Gβγ and PIP2. In contrast, a fusion protein of the βARK PH domain where the conserved tryptophan has been mutated to alanine fails to inhibit the activity (Pitcher et al, 1995). Other studies that evaluate the role of lipids in the regulation of βARKs have shown that phosphatidylinerine, phosphatidic acid, phosphatidyl inositol, phosphatidylethanolamine and
phosphatidylglycerol enhance the phosphorylation of hm2 mAChRs (human m2 muscarinic acetylcholine receptor) by βARK1 by 2-3 fold as compared to phosphorylation of the receptor in the absence of lipids. A significant inhibition of βARK mediated receptor phosphorylation was observed upon addition PIP2. Other lipids show no effect on receptor phosphorylation. Phosphatidylserine and PIP2 were not only shown to modulate βARK phosphorylation activity, but they were also able to bind directly to βARK. It has been demonstrated that βγ subunits of G protein increase the rate and the extent of receptor phosphorylation by βARK (Pitcher et al, 1992). The same effect was observed for phosphatidylserine (PS). The effect was not additive in the presence of βγ subunits and PS, suggesting that βγ and PS share activation mechanism and potentially binding place. The PH domain is the binding site for both Gβγ and lipids. Because of their proximity, it is possible that there might be allosteric regulation between Gβγ and lipids in their interaction with βARK. The PH domain could represent one potential mechanism for regulation of the activity of some G protein receptor kinase (GRKs). Of all the GRKs, only βARK1 and βARK2 have a PH domain. The response of GRKs to lipids is different. For example, increased autophosphorylation of GRK5 has been demonstrated in the presence of lipids. Autophosphorylation does not seem to play a role in the regulation of βARK1 and βARK2, but evidence strongly suggests that the PH domain allows for membrane localization of these kinases as well as facilitates the potential co-regulation by Gβγ and lipids (DebBurman et al, 1995).

Further mapping of the site in Gβγ that binds the recombinant PH domain of βARK and β spectrin have shown that both PH domain containing constructs can bind strongly to the C terminal region of Gβ which only contains 5 copies of the WD40 repeat (Wang et al, 1995 and references therein). These repeats were first identified in the β subunit of G proteins as 7 tandem regions of sequence homology. They are rich in tryptophan and asparagine and have a total length of approximately 40 amino acids hence their name (Van der Voorn, 1992). TecIIa and dynamin PH domains were also shown to bind the Gβ
WD40 construct, although with different affinities. Binding to the WD40 construct suggests that PH domains might bind to other WD40 proteins. Several proteins involved in signaling or cytoskeletal function are known to contain WD40 repeats. PAHAF-45, the product of the Lis-1 gene, corresponds to the non-enzymatic subunit of the platelet activating factor acetylhydrolase. PAHAF-45 contains WD40 repeats but it functions differently than Gβ. A GST fusion protein of the Lis-1 gene containing sequences coding for most of the PAFAH-45 protein, including the 7 WD40 repeats, but lacking N terminal non WD40 sequences, bound with different affinities to the PH domains of βARK, βspectrin, TecIIa, and dynamin. Both Gβ and PAFAH-45 proteins were able to compete for binding to the PH domain, indicating that they bind to a similar region of the PH domain. These results suggest that some PH domains might bind WD40 containing proteins. Interestingly, RACK1, a protein that binds activated PKC isoforms, consists mainly of WD40 repeats. PH domain constructs of Btk and the serine kinases, Racα and Racβ, have been shown to coimmunoprecipitate with PKC. It is possible that the interaction of these PH domains is mediated by binding of the PH domain to WD40 repeats of RACK1 bound to PKC. Some of the PH domain containing proteins are known PKC substrates, others contain putative PKC phosphorylation sites. Binding of PH containing proteins to RACK1 could target these proteins to PKC and facilitate their phosphorylation (Wang et al, 1995 and references therein).

Interactions between with βγ subunits of G proteins have been shown to occur in vitro (Touhara et al, 1994). The βγ binding site has been mapped to protein sequences in the C terminus of PH domains (subdomain VI) and 30 amino acids beyond the PH domain. These results are not conflicting with the existence of a lipid binding domain within the PH domain, because the βγ binding site is located at the C terminal end of the PH domain and the PIP2 binding site maps to the N terminal of the domain. These two binding sites could complement their function, interaction of N terminal PH domain with phosphoinositol
containing membranes could position the C terminal PH in proximity to βγ subunits of G protein and facilitate their interaction (Yoon et al, 1994).

On the basis of all experimental evidence it is possible that the function of the PH domain is to target proteins to their relevant membrane compartment (Inglese et al, 1995). The five potential functions of the PH domain previously described in this section, include: interaction of PH domains with βγ subunits of G proteins, interaction with lipids, both of them located in the plasma membrane. Interaction with additional plasma membrane PH domain binding sites, as described for β spectrin and IRS-1. Lastly, interaction of PH domains with PKC isoforms.
CHAPTER 2: ORIGINAL RESEARCH

1. INTRODUCTION

The principal interest of our lab is the isolation and characterization of genes important for the biology of the retina. By definition, mutations in genes essential to the normal biology of the retina, are likely to be associated with genetic eye disease. We hypothesized that one class of important retinal genes are those represented by SAC (Specific Abundant and Conserved) or AC (Abundant and Conserved) cDNAs. Screening for SAC and AC yielded cDNAs, including those of ROM1 and CHX10, which play key roles in the retina. Novel cDNAs identified include the cDNA of ROM1, an integral membrane protein found in the disk rims of rod outer segments. The rom-1 protein is likely to be important for the maintenance of the disks morphology and as well for the formation of the ROS (Bascom et al, 1992). CHX10 encodes a homeodomain protein essential for the development of the retina (Liu et al, 1994, Burmeister et al, 1994).

The 198L cDNA was also identified as a result of the screen for SAC and AC cDNAs. The high level of 198L retinal expression and the presence of the putative PH domain suggest that 198L may have relevant functions in retinal biology. In addition, 198L maps to human chromosome 11q13, a region to which four degenerative retinopathies have been mapped, suggesting that 198L may be a candidate gene for one of these diseases. Thus, our long term goal is to understand the function of 198L in the retina. As an initial approach, I have begun to study the expression of the 198L message and protein in the adult human retina. This chapter summarizes my studies on the expression of 198L.

To examine the expression of the 198L human transcript in the retina I have done 35S in situ hybridization of human retinal sections, and shown that the expression of the 198L transcript is restricted to the photoreceptors. To investigate the expression of the 198l protein, I have raised, characterized, and purified polyclonal sera against the human 198l polypeptide. Using affinity-purified 198l specific sera, I have shown by western blot of
retinal lysates that both forms (27 kDa and 23 kDa, with and without the alternatively spliced coding exon respectively) of the 198L protein are expressed in human retina. I have extended my expression studies to the mouse, and evaluated the tissue distribution of the mouse 198L transcript by northern blot analysis. This study demonstrated that the transcript is expressed in mouse retina, brain, kidney, lung, and liver.

2. MATERIALS AND METHODS

Subcloning

DNA fragments of interest were isolated in 1% agarose gel in 1X TAE buffer and purified using QIAEX II gel extraction kit according to the manufacturer's specifications (Qiagen, CA). Purified fragments were ligated with 100 ng of vector in 1:2 molar ratio vector to insert with 1-2 units of T4 ligase (BRL) in 1X ligase buffer. Ligation reactions were incubated overnight at 16°C and then transformed to DH5α competent cells (BRL). Transformed cells were plated on LB agar containing 100 μg/ml ampicillin. M15 competent cells (prepared accordingly the manufacturer instructions, QIAGEN) were used to transform the histidine-tagged construct and then plated in LB agar containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. Plates were incubated overnight at 37°C.

Plasmid DNA from the transformants was prepared by alkaline lysis method (Sambrook et al, 1989) and positive transformants were identified by verifying the presence of the correct size insert by restriction enzyme digestions.

Subcloning included 1) a subclone of 198L cDNA into pBluescript KS (Stratagene), as an intermediate step for the GST-small construct. A Bluescript plasmid containing 198L cDNA sequences, was digested with EagI, then filled in with Klenow, and religated in the presence of EcoRI linkers, 2) subcloning of the human 198L in situ probe, 3) production of the expression constructs.
Expression constructs

a) GST fusion constructs

Three constructs were made with different portions of the 198l N terminus (Fig. 10) fused to sequences coding for Glutathione S-Transferase (GST): 1) GST-198l, a PstI-EcoRI fragment, coding for amino acids 1-243 of the full length unspliced open reading frame of 198L (Soltyk and McInnes, unpublished data), was ligated in frame to BamHI-EcoRI digested pGEX-2T (Pharmacia) in the presence of annealing oligonucleotides: 5' GATCCATGAGCCCTGCA 3' and 5' GGGCTCATG 3'; 2) GST-PH Domain, a BamHI fragment from the GST-198l construct, coding for amino acids 1-211 of 198l (Soltyk and McInnes, unpublished data), was cloned in frame to BamHI digested pGEX-2T vector; and 3) GST-Small, a MscIEcoRI fragment, coding for amino acids 1 to 105 of 198l (Soltyk and McInnes, unpublished data), was ligated in frame to EcoRI, BamHI/blunt-ended pGEX-2T. The maintenance of the correct reading frame was verified by sequence analysis of the junction of the 3' end coding sequence of GST and 5' end of the 198L cDNA fragments.

b) Hexahistidine construct

A PstI/EcoRI fragment, coding for amino acids 3 to 243 of 198l (Soltyk and McInnes, unpublished data) was ligated in frame to PstI/EcoRI digested TrcHisB expression vector (Invitrogen).

Sequencing

Sequencing reactions were performed with the CircumVent thermal cycle dideoxy DNA sequencing kit (New England Biolabs) and [35S]dATP (NEN) according to manufacturer's protocols. Reaction products were separated on 6% polyacrylamide gels (acrylamide/N'N'-bis-methylene-acrylamide ratio 36.5/1, 0.053% APS, 0.052% TEMED) at 2000 V, for 2-4 hours, gels were fixed for 20 minutes with 10% glacial acetic acid and 10% methanol solution, dried and exposed overnight to XAR film (Kodak).
Oligonucleotide primers used included: 5' GST, of sequence 5'GGGCTGGCAAGCCACGTTTGGTG3' and 198L internal primers of sequence 5'GGGATCTACCACGATG3', 5'GATCTTTATGTCACGGAC3', 5'CTGTCTCCACAGCCAGCY, 5'GCTGGCTGTGGAGACAGA3'.

Expression and purification of GST fusion proteins

Purification of the GST fusion proteins was done according to the manufacturer's instructions (Pharmacia) with slight modifications.

a) Preparation of bacterial protein lysates

Overnight cultures of bacteria transformed with correct GST fusion constructs were diluted 1:100 into 1L of LB medium containing 100 µg/ml ampicillin. The cultures were grown at 37°C until they reached OD600 of 0.6-1.0. Expression of the GST fusions was induced by addition of IPTG (isopropylthio-β galactosidase) to a final concentration of 1 mM. Incubation at 37°C was then continued for an additional 3 hours. Bacterial pellets were collected from the cultures by centrifugation at 4°C for 10 minutes at 5,500 rpm in JA-10 rotor (Beckman) with J2-21 centrifuge (Beckman). The cells were resuspended in 25 ml of ice cold PBS with added protease inhibitors: 0.1 mM PMSF (Phenylmethylsulfonyl fluoride), 20 µg/ml aprotinin, 0.5 mM EDTA. Resuspended bacterial lysates were stored at -20°C overnight, then the lysates were subjected to three cycles of freeze-thaw to aid the bacterial lysis. Lysates were sonicated by at least three bursts of sonication for 30 seconds and chilling on ice for one minute until the lysate was clear. Triton X-100 was added to the lysates in a final concentration of 1%, allowed to mix with the lysate for 10 minutes at 4°C. The lysate was clarified by two rounds of centrifugation for 10 minutes at 9,500 rpm, 4°C, in JA-20 rotor (Beckman) with J2-21 centrifuge (Beckman). The supernatant was transferred to a new tube for further purification.
b) Purification of the fusions proteins

All purification steps were done at 4°C unless otherwise indicated. The supernatant was applied to a pre-equilibrated column containing glutathione sepharose 4B (1 ml) (Pharmacia). The loaded column was washed three times with 10 bed volumes of PBS (Phosphate-Buffered Saline). Bound proteins were eluted at room temperature with five bed volumes of elution buffer (10mM reduced glutathione in 50 mM Tris-HCl pH 8.0). One ml fractions were collected. Ten μl aliquots of each of the purification steps were used for analytical gel electrophoresis.

Expression and purification of hexahistidine-tagged constructs

Large scale purification of recombinant hexahistidine-tagged protein was done under denaturing conditions following the procedure of Klima et al, 1993 with slight modifications. Overnight bacterial cultures were diluted in 500 mls of LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. The cultures were incubated at 37°C until O.D600 of 0.7-0.9. The expression of the fusion protein was induced with 3mM final concentration of IPTG. Induced cultures were incubated a minimum of 6 hours at 37°C. Bacterial pellets were recovered by centrifugation at 6,000 rpm using JA-10 rotor (Beckman) with J2-21 centrifuge (Beckman), resuspended in 10 mls of sonication buffer (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, 3% beta-mercaptoethanol, pH 8). The bacteria were lysed overnight at 4°C in sonication buffer. Next day the bacterial lysate was sonicated by three cycles of sonication for 30 seconds and chilling on ice for one minute. The sonicated lysate was cleared by centrifugation at 10,000 rpm at 4°C in JA-20 rotor (Beckman) with J2-21 centrifuge (Beckman). To shear the DNA the lysate was forced through a 22G syringe. The lysate was then added to a previously packed and equilibrated 4 ml nickel column (ProBond™ resin, Invitrogen) and allow to run through the column four times. The column was then washed using 70 mls of a solution containing 8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, 3% beta-mercaptoethanol, pH 7 followed
by 70 mls of 6 M guanidine chloride, 0.1 M NaH2PO4, 0.01 M Tris-HCl, 3% beta-
mercaptoethanol, pH 7. The column was eluted with 5 bed volumes of elution buffer (6
M guanidine chloride, 0.1 M NaH2PO4, 0.01 M Tris-HCl, 3% beta-mercaptoethanol, pH
5). Four fractions of 5 mls each were collected.

A sample of 100 μl was collected from each fraction and precipitated at -20°C
overnight with 1 ml of 99% ethanol and 1% acetic acid. Precipitant was recovered by 30
minutes centrifugation at 4°C, then washed with 70% ethanol and resuspended in 10 μl of
C/EDTA solution (8 M urea, 0.1 M NaH2PO4, 0.01 M Tris-HCl, pH 6.3 with 100 mM
EDTA). Aliquots of 10 μl of all purification steps were saved and used for analytical gel
electrophoresis.

Fractions that were identified to contain the recombinant protein were pooled and
diluted with five volumes of folding buffer A (2 mM reduced glutathione, 50 mM
Tris/HCl, pH 8 and 0.003% tween 20) and stored at 4°C overnight. The solution was then
dialysed against three changes of 1 L of folding buffer B (100 mM NaCl, 50 mM Tris
HCl, pH 8.0 and 0.003% tween 20). The solution was then lyophilized overnight and
resuspended in 1 ml of PBS containing 0.1 mM AEBSF (Calbiochem).

Production of polyclonal sera

Fifty μg of purified GST fusion proteins diluted in 250 ml of PBS were emulsified
in complete Freund’s adjuvant. The solution was injected subcutaneously into rabbits in at
least four different sites. Boosters injection of GST fusion proteins antigens emulsified in
Freund’s incomplete adjuvant were given at 15, 29 and 36 days after the first injection.
The first two boosters injection included 50 μg of fusion protein and the last injection
included 100 μg of antigen. Rabbits were bled 43 days after the first injection. (Dr Greg
Lee, personal communication).
Affinity purification of crude serum

1. Preparation of the affinity columns

Three different antigens were coupled to CNBr Activated Sepharose 4B (Pharmacia) to produce matrixes for affinity purification of sera. The antigens used were GST, GST-1981, and His6-1981 at a concentration of 1 to 2 mg of antigen/ml of gel. CNBr beads were activated by incubation with 1 mM HCl for 15 minutes at room temperature. The slurry was washed with 120 mls of 1 mM HCl by passing the solution through a sintered glass filter. In addition, the slurry was washed with cold coupling buffer (0.1 M NaHCO3 and 0.5 M NaCl pH 8.3). The antigens were diluted in 3 mls of coupling buffer and incubated with the activated beads for 3 hours at room temperature with gentle shaking. To discard uncoupled antigen, the CNBr-antigen slurry was washed with 0.1 M NaHCO3 and 0.5 M NaCl pH 8.3. To block non-reacting groups, the matrix-antigen complex was incubated for 2 hours at room temperature with 5 mls of 0.2 M glycine pH 8 with gentle shaking. Excess glycine was eliminated by washing the matrix with 25 mls 0.1 M NaHCO3 and 0.5 M NaCl pH 8.3, followed by 25 mls of PBS. The slurry was then packed into a column and washed with PBS followed by 0.1 M potassium phosphate pH 7.

2. Purification of crude serum

One to two mls of crude antiserum were added to the affinity purification column. The serum was incubated with gentle agitation of the column overnight at 4°C. Next day the column was stand at 4°C to allow the slurry to settle down. The serum passed through the column at least 3 times. The column was washed with 20 mls of potassium phosphate buffer pH 7. A total of 10 mls of 1 M glycine and 0.5 M KCl solution, pH 2.5, was used to elute antibodies from the column. One ml fractions were collected and monitored by measuring OD 280. Fractions with the highest OD280 readings were pooled, neutralized by addition of 1 M Tris pH 9.5 and dialysed overnight against 4 L of 10 mM Tris pH 7.5.

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Collected samples were then lyophilized overnight and resuspended in 1 ml of water containing 100 mg of BSA and 0.02% sodium azide.

In addition, antibodies were purified by using nitrocellulose strips containing fusion GST-1981 or His6-1981. Preparative protein gels containing 500 μg of GST-1981, His6-1981, or GST were electrophoresed, transferred to nitrocellulose and then stained with Ponceau S to visualize the proteins. The protein band of interest was cut from the nitrocellulose and rinsed briefly in TBST (20 mM Tris, 0.5 M NaCl, pH 7.2). Each purification consisted of three cycles of blocking, incubation with sera, washing, and elution. Blocking of the nitrocellulose strips was done for 1 hour at room temperature with 20% fetal calf serum (FCS) in TBST. Incubation with polyclonal serum diluted 1:10 in 10% FCS in TBST with the nitrocellulose strips was done for 1 hour at room temperature. Washing of strips was done four times for 15 minutes with TBST and the antibodies were eluted with 1 or 2 mls of 50 mM glycine pH 2.6. Purified antibodies were neutralized with 2M Tris pH 8. GST strips were used to preabsorb GST antibodies from the affinity purified GST-1981 fraction.

Preparation of retinal protein lysate

Sample buffer was prepared fresh before the dissection of the retinas. Dissected human retinas were placed immediately in 2 mls of fresh sample buffer (2% SDS, 0.1 M dithiothreitol, 60 mM Tris pH 6.8, 1 mM PMFS, 1 mM Benzamidine, 10 μg/ml aprotinin), and vortexed for one minute. The solution was boiled 5 minutes and incubated at 60°C for 20 minutes. The solution was then passed 5 times through 18 gauge syringe followed by one pass through a 22 gauge syringe to shear the DNA and decrease the viscosity of the solution. The solution was centrifuged at 10,000 rpm at 4°C for 10 minutes. The supernatant was transferred to a new tube and stored at -20 C. The protein concentration was determined using the BCA kit (Pierce).
SDS PAGE

Recombinant fusion proteins or retinal protein lysates were resolved using 5% stacking and 12% resolving, 0.75 mm thick, acrylamide gels in Bio-Rad Minigel system. The amount of retinal lysate proteins loaded per lane was 30 µg. Gels were either fixed for 1-2 minutes with 10% TCA and 50% isopropanol and stained with Coomasie blue (Bio-Rad) or the proteins were transferred to nitrocellulose for western blot analysis.

Western blots

Proteins were electrophoretically transferred to nitrocellulose. The transfer was done at 4°C overnight, at 30V using a buffer containing 25 mM Tris 192 mM glycine, 20% methanol, pH 8.3. After transfer, the nitrocellulose strips were stained using Ponseau S to confirm the efficiency and extent of the transfer. Strips were washed in Tris-buffered saline (20 mM Tris-HCl, 0.5 M NaCl, pH 7.2) with 0.05% Tween 20 (TBST). Non-specific background was blocked by 1 hour incubation at room temperature with 10% FCS diluted in TBST, followed by three 10-minutes washes at room temperature with TBST. The strips were incubated with primary antibody diluted in 10% FCS in TBST for 1 hour at room temperature with agitation. Unbound primary antibody was removed by washing 3X 10 minutes with TBST, at room temperature. The membranes were incubated with alkaline phosphatase-conjugated AffiniPure Donkey antirabbit IgG (H + L) secondary antibody (Jackson ImmunoResearch Laboratories, Inc), diluted 1:5000 in 10% FCS in TBST. Incubation was for 1 hour at room temperature with agitation. Excess secondary antibody was removed by 3 washes of 10 minutes each with TBST solution at room temperature. Staining was developed by addition of the alkaline phosphatase substrate: BCIP (5-bromo-4-chloro-3-indoly1 phosphate) and NBT (nitro blue tetrazolium) diluted in alkaline phosphate buffer (100 mM NaCl, 100 mM Tris, 5 mM MgCl₂, pH 9.5). NBT and BCIP were from BRL.
Antibodies

The primary antibodies used for western blots included: 1) Goat anti-GST antibody (Pharmacia) in 1:300 dilution, 2) preimmune and immune 198I sera in 1:100 dilution, 3) affinity purified 198I sera in ~ 2 μg/ml.

Preparation of human retina slides for in situ hybridization

The neuroretina, without the pigment epithelium, was removed from human eyes (from the Ontario Eye Bank). The tissues were rinsed once in PBS then fixed for two hours in 4% paraformaldehyde in PBS at room temperature. The tissues were dehydrated by passing them through 70%, 85%, 95% and twice through 100% ethanol and then embedding them in paraffin. Sections of approximately 7 μm of thickness were cut and placed on pretreated RNAse free slides. The slides were subsequently baked at 60°C overnight (Y. Zhou, personal communication).

In situ hybridization

A 624 bp PstI-BamHI fragment from the coding region of 198L (nt 8-632 in Fig. 5A) was subcloned into pBluescript SK (Stratagene) and used as a template to generate [α-35S]UTP labeled riboprobes. Sense probe was produced from the T7 promoter by in vitro transcription of BamHI linearized template and antisense probe was in vitro transcribed from the T3 promoter using EcoRI digested template. Riboprobes were synthesized using Stratagene in vitro transcription kit according to the instructions of the manufacturer.

Human retina tissue slides were hybridized at 55°C overnight with 150 μl of hybridization solution containing radioactive probe at a concentration of 5x10⁴ cpm/μl. The washing protocol was as follows:

a) 30 minutes at 65°C in shaking water bath with a solution containing 50% formamide, 2X SSC, 0.1% β-mercaptoethanol (βME).
b) Three washes of 10 minutes each, with 10 mM Tris (pH 7.5), 5 mM EDTA, 0.5 M NaCl solution. The first two washes were done at room temperature and the last wash at 37°C.

c) 30 minutes at 37°C with 25 μg/ml RNase diluted in 10 mM Tris (pH 7.5), 5 mM EDTA, 0.5 M NaCl solution. This wash is for the elimination of unhybridized riboprobe.

d) 15 minutes wash with 10 mM Tris (pH 7.5), 5 mM EDTA, 0.5 M NaCl solution at 37°C.

e) 30 minutes wash at 65°C in shaking water bath with a solution containing 50% formamide, 2X SSC, 0.1% βME solution.

f) Two washes of 10 minutes each at 37°C: first in 2X SSC then in 0.1X SSC.

g) The sections were dehydrated by passing them quickly through 30%, 60%, 80%, and 95% ethanol containing 0.3M ammonium acetate, and twice through 100% ethanol.

The sections were allowed to dry and then dipped in Eastman Kodak NTB-2 Nuclear emulsion. Sections were exposed at 4°C for 3 to 5 days. The sections were develop and counterstained with toluidine blue and mounted with permount. Dark field and bright field pictures were taken with Leica Leitz DMRB microscope using Kodak Ektachrome 160 film.

**Northern blot**

Total RNA from adult CD1 mouse tissues was prepared immediately after dissection using Trizol (Bethesda Research Laboratory). Two mouse northern blots were prepared: one containing thirty micrograms of total RNA of intestine, pancreas, ovary, testis, lung, heart, spleen, kidney, liver, olfactory bulb, cerebral cortex, thalamus, basal ganglia, mid brain, pons, medulla, cerebellum and spinal cord per lane (Ding Fang Bu, personal communication). The second, containing 5 micrograms of total RNA of kidney,
brain, heart, retina, lung, testis, spleen, liver (Bryan Snow, personal communication). In both cases the total RNA was electrophoresed and blotted to nitrocellulose according to Sambrook et al (1989). Membranes were deplored by three rounds of incubation, 5 minutes each, with a boiling solution containing 0.01% SDS and 0.01X SSC. An additional incubation step was carried on with the same solution in the shaking platform until the solution cooled down. The membranes were prehybridized with 30 mls of a prehybridization solution (50% formamide, 5X SSC, 1X Denhard's, 20 mM NaPO4, pH 6.8, 1% SDS and 50 μg/ml single stranded salmon sperm DNA), for at least 3 hours at 42°C. The membranes were hybridized to a 619 bp (nt 92-711 in Fig. 16) [32P]-dCTP labeled SstI/MscI fragment of the coding region of the mouse 198 Releases (Feinberg and Vogelstein, 1983). Hybridization continued overnight at 42°C in a solution identical to the prehybridization fluid except that it contained 10% w/v dextran sulfate and ~3.5 x 10⁶ cpm/ml 32P-labeled mouse 198L probe. The membranes were washed twice at room temperature for 15 minutes each with 2X SSC, 0.2% SDS, followed by two washes of 30 minutes each with 0.2X SSC and 0.2% SDS at 65°C. Membranes were exposed to XAR film (Kodak) at -70°C.

3. RESULTS

Attribution of data

Production of polyclonal sera (Dr. Greg Lee, NCE)

Preparation of mouse total RNA blots (Ding Fang Bu and Bryan Snow)

Human retina tissue slides (You Zhou)

Isolation and characterization of mouse 198L cDNA clones (Lynda Ploder)

Purification of the fusion proteins

To investigate the expression of the 1981 protein, polyclonal antibodies were raised to a series of bacterial expressed GST (Glutathione S-transferase) fusion proteins
containing different fragments of the 1981 protein (Fig. 10). Three fusion proteins were made, each of which share the N terminus but which extend to include the carboxyl terminus to different lengths. The fusion proteins were GST-1981, containing all 243 amino acids of a full length unspliced open reading frame, GST-PH containing the first 211 amino acids, and GST-Small containing the first 105 amino acids of the 1981 protein. The predicted sizes of the fusion proteins are 53 kDa, 50 kDa, and 38 kDa, respectively.

The junctions of the GST-cDNA inserts were sequenced in order to verify the in frame fusion of the cDNA constructs. Induction of a full size product was evident by comparison of bacterial protein lysates before and after induction with IPTG (Fig. 11). The relative mobility of the induced fusion proteins was compared to that of molecular mass standards and indicated masses of 55 kDa for GST-1981, 52 kDa for GST-PH, 41 kDa for GST-Small. These sizes closely correspond to the predicted protein size.

Conditions were optimized for fusion protein expression by modifying the temperature and time of induction. Purification of the predicted products was only possible for GST-Small. Protein degradation prevented the purification of significant amounts of the full length GST-1981 (55 kDa) or GST-PH (52 kDa) proteins, but from each of these proteins a 45 kDa predominant band was obtained. Coomassie blue stained gels containing fractions from the purification experiments show only faint protein bands corresponding to the expected products of both GST-1981 and GST-PH (Fig. 11).

To determine if any full length GST-1981 and GST-PH products were purified, and to confirm the 45 kDa band as a degradation product, a western blot was done using anti-GST antibodies. The anti-GST antibodies did not identify any proteins from control lysates of DH5α. Bands were identified in the lanes of purified GST-1981 and GST-PH samples corresponding to the expected sizes but a series of products ranging between 31-54 kDa, with a very prominent 45 kDa band were also observed (Fig. 12). These results suggest that the expected products are partially degraded and that the fusion proteins were purified for both the GST-1981 and GST-PH expression constructs.
To facilitate the purification of the sera obtained against the GST fusion proteins a hexahistidine-tagged 198l protein was also prepared. The hexahistidine expression construct was designed such that the first methionine of the 198l protein is deleted to avoid producing fusion protein devoid of the hexahistidine that would not be possible to affinity purify using nickel resin. The maintenance of the 198L reading frame was confirmed by sequence analysis of the junction of the TrcHis B vector and the 198L cDNA.

The His6-198l protein contains 37 vector encoded amino acids followed by amino acids 4 to 243 of the 198l protein. The His6-198l protein was found to be insoluble and therefore purification was performed under denaturing conditions. Purification of the His6-198l yielded two polypeptides of 33 kDa and 24 kDa. The 33 kDa represents the full size fusion protein and the 24 kDa is a degradation product, since both of these proteins were abundantly expressed upon induction, both bound to the nickel column, and both were recognized by 198l antibodies raised against GST-198l fusion proteins (Fig. 13C).

Production and characterization of polyclonal serum

The three purified GST-198l fusion proteins were injected into rabbits to raised polyclonal sera. Six rabbit sera were obtained, two inoculated with GST-198l, three with GST-PH and one rabbit with GST-Small protein. To obtain some indication of whether the immune sera could specifically recognize the 198l protein, western blots of human retinal lysates were done with both preimmune and immune sera. All the available sera recognized several bands on retinal protein western blot, complicating the identification of the 198l protein. In addition, no predominant specific protein was recognized by the immune sera of the expected size of the 198l protein. Furthermore, no consistent bands close to the predicted molecular sizes of 198l were recognized by any subset of the sera samples (data not shown). Because of these results and because antibodies specific to 198l could be present at very low titer, the crude sera were affinity purified using antigen coupled to CNBr sepharose.
Figure 10.

Schematic representation of the putative 1981 protein, and of the GST-1981 fusion proteins and of the hexahistidine-1981 fusion protein. The PH domain is indicated by the hatched region.
Figure 11.
Coomassie Blue stained analytical gels of bacterial lysates used for the purification of GST-1981 fusion proteins. A. Purification of GST-1981 (full length). B. Purification of GST-PH. C. Purification of GST-Small. Abbreviations: - = uninduced bacterial lysate, + = induced bacterial lysate, L = the lysate that was applied to the affinity columns, M = molecular size markers. The numbered lanes correspond to samples of the eluted fractions collected after the addition of reduced glutathione to the glutathione sepharose column. Sizes of the expected induced fusion proteins are indicated.
Figure 12.
Immunoblot of bacterial protein lysates of cells expressing various GST-198l fusion proteins. The filter was probed with anti-GST antibodies. Abbreviations: - = uninduced bacterial lysate, + = bacterial lysate induced with IPTG, pu = purified fusion protein obtained from a glutathione agarose column. Arrows indicate the position of the full length GST fusion proteins: the GST-198l protein is ~ 55 kDa and the GST-PH polypeptide is ~ 52 kDa.
In order to purify 1981-specific sera, three different antigens (GST, GST-1981, and His\textsubscript{6}-1981) were coupled to CNBr. Sera were affinity purified directly from the GST-1981 column which yielded both GST and 1981 specific antibodies. Antibodies specific to 1981 were isolated either by first eliminating GST-specific antibodies on a GST affinity column, followed by purification on the GST-1981 affinity column or by purifying 1981 antibodies from the His\textsubscript{6}-1981 affinity column. The sera were purified with at least one of these three methods and then tested on western blots. One serum raised against the GST-1981 (hereafter called serum B) construct was identified which recognizes a unique pattern of bands on retinal lysate western blots, regardless of the purification scheme used. Two proteins immunolabeled by this affinity purified serum have sizes that closely correspond to that of the two forms of the 1981 protein. Of the other five sera examined, either no bands on retinal lysates were observed, or no consistent bands were observed when comparing a single serum purified by two of these methods.

The crude sera B was also purified using three different antigens (GST, GST-1981, or His\textsubscript{6}-1981) which were electroblotted to nitrocellulose. Serum B was affinity purified directly from the GST-1981 protein strip which yielded both GST and 1981 specific antibodies (serum B1). Serum B1 was further purified by elimination of GST specific antibodies on GST protein strips (serum B2). In addition, 1981-specific antibodies were purified from His\textsubscript{6}-1981 protein strips (serum B3). The purified sera were tested on human retinal protein extracts (Fig 13A).

The affinity purified antibodies, serum B1, B2, and B3, each recognizes two bands of 27 kDa and 25 kDa. These bands were not recognized by the preimmune sera but were detected by the crude sera B although the observed bands were not very prominent. The observed sizes of these polypeptides are close to the predicted sizes of 27 and 23 kDa of the 1981 proteins with and without exon 6, respectively. In addition, serum B1 strongly immunolabels a 48 kDa band which is not detected as strongly with the affinity purified GST-1981 preabsorbed with GST, suggesting that this protein was labeled by cross-
reactivity of GST antibodies. Furthermore, the 48 kDa band was not recognized by antibodies affinity purified from His6-198l protein strips, a purification that should have eliminated antibodies to GST.

The affinity purified sera were also tested on western blots of purified fusion proteins (Fig. 13 B and 13C). Affinity purified serum B2 recognizes the His6-198l fusion protein products of 33 and 24 kDa (Fig. 13 C). Likewise, affinity purified serum B3 recognizes the GST-198l fusion protein products of 55 kDa and 45 kDa (Fig. 13 B). Taken together these data indicate that the purified antibodies are specific to 198l.

**Northern blot analysis**

In order to establish the tissue specific expression of the 198l mouse transcript, RNA from many adult mouse tissues was examined by northern blot analysis. After 22 hours of exposure hybridizing signals of comparable intensity could be detected in brain and in retina. The expression of the 198l transcript in the CNS is approximately the same for olfactory bulb, thalamus, basal ganglia, mid brain, pons, medulla. Compared to these brain regions the transcript appeared less abundantly expressed in spinal cord and even less in cerebellum (Fig. 14A). In addition, a reduced level of message was also detected in lung. After one week exposure, the mouse 198l transcript also appeared expressed in liver and kidney (Fig. 14B). In addition, additional signals became evident in the retina lane (data not shown). No transcript was detected in spleen, heart, testis, ovary, pancreas and intestine.

Expression of the mouse and human transcript was observed in retina, brain and kidney. The expression of the mouse transcript differs slightly from that of the human transcript, in that it was detected in mouse liver but not in human liver and the relative levels of transcript in the retina and the brain varied. The 198l transcript was shown to be expressed in the mouse lung. The expression of 198L message has not been examined in
Figure 13.

Figure 14.
Northem blot of 198l in adult mouse tissues. A. Nitrocellulose membrane containing 30 μg of total RNA from different adult mouse tissues and regions of the brain (provided by Ding Fang Bu). B. Nitrocellulose membrane containing 5 μg of total RNA from adult mouse tissues (provided by Bryan Snow). The 198l message is 2.4 kb and is expressed in retina and throughout the brain, lung, liver and kidney.
human lung. The size of the mouse \textit{198L} message is approximately 2.4 kb and the size is the same in all tissues that express it.

\textbf{In situ hybridization}

\textit{In situ} hybridization of human retinal sections was performed to determine if the \textit{198L} message is expressed throughout the neuroretina or if it is cell type specific. Specific hybridization of the antisense probe was observed solely in the photoreceptor layer of the retina. The signal was not detected in the outer nuclear layer where the nuclei of the photoreceptors reside, but was found in a region corresponding to the inner and outer segment of the photoreceptors (Fig. 15A). No significant labeling was found with the sense probe (Fig. 15B). That the \textit{198L} message is very abundant is suggested by the short exposure time required to detect the transcript in the \textit{in situ} hybridization experiments. These results corroborate the RNA blot analyses, and suggest that the \textit{198L} message is abundantly and specifically expressed in the photoreceptor layer. Since the rod photoreceptors comprise more than 95\% of the cells in this layer, \textit{198L} must be present in abundance in these cells, although no comment can be made about its expression in cones.
Figure 15.

*In situ* hybridization of 198L in human retina. A. Dark field picture of hybridization with the antisense probe. B. Dark field picture of hybridization with control sense probe. C. Bright field of the human retina stained with toludine blue. The 198L message is specifically expressed in the photoreceptor layer of the retina. Abbreviation: ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer.
4. DISCUSSION

The 198L message was observed to be abundantly expressed in total retina RNA by northern blot analysis but was shown to be restricted to the photoreceptor layer as shown by in situ hybridization. As the PH domain is found in proteins involved in signal transduction or cytoskeletal function, expression of the 198L message in the photoreceptors is consistent with this message coding for a putative pleckstrin homology domain containing protein. The photoreceptors are the cells of the retina where most of the transduction events leading to vision occur. In addition, the cytoskeleton of the cells themselves play an important role in the structure and function of the photoreceptor.

The function of the PH domain is presently unknown, but three potential target for interactions with PH domain has been proposed, which are, βγ subunits of G proteins, lipids, and isoforms of protein kinase C. It has been suggested that the PH domain interacts with βγ subunits of G proteins. Transducin, a member of the G protein family, is expressed in photoreceptors. Tβγ interacts with Tα when Tα is in the inactive state and as well Tβγ interacts with phosducin. Phosducin has a slight homology to subdomains IV-VI of the PH domain of βARK (Inglese et al., 1995). The PH domain of 198l was identify as sequence homology with PH containing proteins in the region of subdomains I-III. Subdomains IV-VI were predicted by visual inspection. 198l could potentially interact with Tβγ and compete for either Tα or phosducin binding. Another interesting possibility is that 198l, in conjunction with phosducin, form a full PH domain (subdomain I-VI) and both interact with Tβγ. PH domains have been shown to bind to lipids, in particular PIP2 (Harlan et al., 1994). PIP2 is an important component of the photoreceptor membranes and a phosphoinosite signaling system has been described for the photoreceptors (Udovichenko et al., 1994 and references therein). The N terminus of the PH domain is the site of lipid binding. This is the region of the 198l PH domain most easily identified on the bases of primary sequence alignment.
Another interaction target of PH domain is PKC which is expressed in the photoreceptor and plays a role in phototransduction. All the potential targets for interaction with PH domain so far described are expressed in the photoreceptors. Additional experiments are required to determine whether interactions between described PH domain targets and the PH domain of 1981 are indeed occurring in photoreceptors.

Subcellular localization is an important mechanism for controlling the activity of proteins. In general the PH domain seems to facilitate the interaction of PH domain containing proteins with membranes. Membrane localization of rhodopsin kinase is known to enhance its activity because it brings it in proximity with its substrate (Pitcher et al, 1992). The 1981 protein could serve as an adapter molecule in photoreceptors, by interacting with components of photoreceptor membranes, such as Tβγ or PIP2. Membrane localized 1981 via its PH domain could facilitate the binding of another protein or proteins that lack membrane anchoring motifs but require membrane localization for function. The two predicted protein forms of the 1981 protein contain the pleckstrin homology domain. The alternative splicing event eliminates sequences C terminal of the putative PH domain. These sequences have been suggested to form part of the βγ binding domain of PH containing proteins. Therefore the 23 kDa form of the 1981 protein may not be able to bind to βγ subunits of G proteins.

In order to immunolocalize the 1981 protein in the retina I have raised, purified and characterized polyclonal sera that will be use in immunohistochemistry studies. The antigens isolated for sera production were three different GST fusion proteins sharing the N terminus of the 1981 protein. Isolation of the full length GST fusion proteins proved to be technically difficult for GST-1981 and GST-PH proteins, because of protein degradation. Several conditions were tested in order to overcome the degradation problem, and none of them proved optimal for the isolation of the full length fusion protein. The degraded proteins could be purified, suggesting that the structural-functional integrity of the
GST moiety had not been compromised. Therefore degradation is likely to be occurring at the C termini of these fusion proteins, in sequences corresponding to 198l.

Using the degraded proteins as antigens for the production of polyclonal sera should not affect the outcome of the experiments. Depending on the experimental requirements of these fusion proteins, it might be necessary to invest additional efforts to achieve isolation of full length fusion products.

Previous quantitative RT PCR experiments showed that two 198L transcripts resulting from alternative splicing of exon 6 are expressed in the retina in equal amounts (Soltyk and McInnes unpublished results). The fusion constructs used for preparation of antigens for producing polyclonal sera did contain the sequences coding for the alternatively spliced exon. The obtained 198l polyclonal sera should recognize the products from either transcripts. Two isoforms were in fact observed in retinal immunoblots with sizes consistent with that predicted by the alternative splicing.

A polyclonal serum against the GST-198L construct serum B, has been purified by several methods and tested in western blots of human retinal lysates (Fig. 13A). This serum immunolabels proteins of 48 kDa, 28 kDa, 26 kDa and a faint band of 30 kDa when purified from GST-198l protein strips (serum B1). Interestingly, following preabsorption using GST proteins strips (serum B2), the 48 kDa protein is almost undetectable in western blot experiments. This result suggests that the 48 kDa band is recognized by GST and not 198l antibodies. Serum affinity purified with His6-198l recognizes bands of 54 kDa, 32 kDa, 30 kDa, 28 kDa, 26 kDa, but fails to recognize the 48 kDa band.

In addition to information about the expression of the protein I have obtained information about the expression of the 198l message in mouse. The tissue specific expression of the mouse 198l transcript differs from that of the human transcript. Both human and mouse transcripts are expressed in retina, brain and kidney. In addition, the mouse 198l transcript was shown to be expressed in lung and liver by probing two
independent northern blots. The expression of the human message was studied in liver but not in lung. No expression was shown in human liver. This difference in the expression could be species specific, or it might be that the quality of the human liver RNA was not optimal. Additional experiments are necessary to access whether the 198L message is expressed in the liver, and as well to determine the expression status of the message in human lung.

The size of the mouse transcript is approximately 2.4 kb and corresponds to the size of the isolated mouse cDNA clones. Ten mouse cDNA clones were isolated from a postnatal 0-3 day mouse cDNA eye library (Ploder and McInnes, unpublished results). The longest clone is 2.4 kb in size. This clone has not been fully sequenced. Only the open reading frame of the mouse 198L has been sequenced using human primers (Fig. 16 and Fig. 17). Additional sequence data from the mouse 198L cDNA clones is necessary to establish if the size of the cDNA is consistent with the size of the transcript. In addition, after prolonged exposure of the northern blot signals of approximately 25 kb, 7 kb and 3.3 kb in size, became evident (data not shown). These signals could be explained by alternative splicing of alternative transcription start or termination sites. At present there is insufficient sequence information of either the 5' or 3' UTRs that could indicate the existence of alternative lengths. Another possibility is that the additional transcripts detected are those of close homologues. Additional experiments are required to determine the identity of these hybridizing bands.
### Figure 16.
Comparison of the nucleotide sequences of human and mouse 198L cDNAs. These sequences correspond to the open reading frames of both mouse and human 198L. Regions of identity are boxed. The N indicates ambiguous sequence. The mouse and human 198L sequences are 85% identical at the nucleotide level.
Figure 17.
Comparison of the predicted amino acid sequences of human and mouse 1981. Regions of identity are boxed. The amino acid sequence of the human and the mouse 1981 proteins are 93% identical.
CHAPTER 3: FUTURE DIRECTIONS

This thesis describes initial results on the study of the expression of the human 198L message and protein. My objective was to determine the cellular and subcellular localization of 198l protein in the human retina. The 198L message has been localized to the photoreceptor layer. Additional studies including immunolocalization of 198l in human retina, identification of 198l interacting partners, functional studies in the mouse, generation of a mouse 198l mutant, and mutational analysis of patients with degenerative retinopathies, all are required to gain insight into the biological role of this protein.

Localization of 198l in the human retina

The main reason for raising antibodies to 198l was to determine the cellular and subcellular localization of the 198l protein by immunohistochemistry in human retina sections. 198l localization experiments will confirm and complement the information obtained by in situ hybridization where the expression of the 198L transcript was shown to be restricted to the photoreceptor cells. The immunohistochemistry studies will determine whether the expression in photoreceptors is general or restricted to either rods or cones. In studies so far, the abundance of the message and the restriction to the photoreceptor layer suggests that the protein is likely to be expressed in rods, because in human retina rods are 95% of the photoreceptors. Use of 198l antibodies will allow to determine specifically if the 198l protein is localized to the inner and/or outer segments of the photoreceptors. In addition, 198l-specific antibodies can be used for immunogold-labeling electron microscopy to determine 198l localization in photoreceptors at the ultrastructural level. If the 198l protein is expressed in the disks of the outer segments, it would strengthen the hypothesis that 198l is a protein involved in phototransduction.
Identification of PH domain binding targets of 198l

Studies of the PH domain can give further knowledge of the function of 198l in the retina, and contribute additional information about the domain. Evaluation of the role of the domain in the retina requires the identification of interacting partners. It has been suggested that PH domains interact with lipids, and as well with proteins that include βγ subunits of G proteins, other WD40 proteins, and PKC isoforms. These potential targets of a PH domain can be directly tested for interaction by in vitro binding using the available GST-198l fusion proteins. Additional proteins that interact with 198l can be identified by several methods. One approach is affinity chromatography, where a retinal lysate is exposed to an immobilized fusion protein containing either full length 198l protein or the PH domain only. A second method is coimmunoprecipitation of interacting proteins from a retinal lysate using 198l-specific sera. Alternatively, another strategy would employ the yeast two hybrid system, using the 198L cDNA fused to the DNA binding domain of GAL4 as a bait to identify interacting polypeptides by screening a retina library fused to the activation domain of GAL4. A final test would be to screen a T7 based retina expression library with GST-198l fusion protein. For this latter approach, it is important to point out that some interactions might not be detected because they might require a cooperative lipid binding to occur (Pitcher, 1995).

Studies of 198l function in the mouse

The studies of the function of the 198L gene should be transferred to the mouse system. A major advantage of doing such studies in the mouse is the availability of tissues for experimental use. At present, northern blot analysis of human mRNA has detected the 198L transcript in retina, brain and kidney. Sub-localization of the transcript has been studied only in human retina.

Northern blot analysis of mouse 198l expression has also been done. The transcript is found in retina, brain, kidney, liver, and lung. In situ hybridization
experiments can be performed in mouse retina to evaluate if the mouse transcript has the same localization in the retina as the human one. In addition, the localization of the transcript in brain, lung, liver and kidney can also be examined by in situ hybridization.

At present, no evidence suggests temporal regulation of the expression of 198L, but nonetheless it will be interesting to determine if the 198l transcript is developmentally regulated. Analysis of the expression of the 198l transcript during retinal development can be done by in situ hybridization of sections from different mouse developmental stages.

The 198l fragment used as a probe for mouse northern blots is a subclone of mouse 198l ORF comparable to the probe used for the human in situ hybridization experiments. Unfortunately, this fragment of the cDNA detected additional bands in mouse northern blots and therefore may not be suitable for the interpretation of experiments unless these additional transcripts are characterized further.

Immunolocalization of the 198l protein in mouse tissues can verify and complement the information obtained about the message expression identified by in situ hybridization. Immunolocalization requires the availability of highly specific sera. Polyclonal sera against the human 198l protein could be used for immunolocalization of mouse 198l protein, provided that the antibodies against the human protein specifically recognize the mouse protein. The specificity of these antibodies can be tested on western blot of protein lysates of mouse tissues. It is expected that immunolabelling of the mouse 198l protein will be observed in tissues expressing the 198l message, provided that the expression of the 198l products are not under extensive or unusual translational control. In addition, the antibodies can be tested by probing in vitro transcribed and translated mouse 198l cDNA.

Mouse model

Strains of mice mutant at a particular gene locus constitute an important resource. Analysis of a mutant mouse phenotype gives insight to normal function of a specific gene. Therefore, research on the function of the 198L gene will benefit from the analysis of mice
carrying mutations in the 198L gene. A mutation in 198L could be a naturally occurring mutation or created. The mouse 198L gene has been mapped to mouse chromosome 7 (Taylor and McInnes, unpublished data), but no obvious naturally occurring candidate phenotypes map to the same area. It will, therefore, be necessary to create a 198L null mouse by transgenic technology. In order to create the mutant mice, the genomic structure of the mouse 198L must first be obtained.

Mutations screening in human retinopathy patients

198L has been mapped to human chromosome 11q13. Because of its map location and its near specific expression in retina, 198L is a candidate gene for retinopathies linked to 11q13. These retinopathies include Bardet-Biedl (BB) syndrome and Autosomal Dominant Neovascular Inflammatory Vitreoretinopathy (ADNIV) (Stone et al 1992).

ADNIV is characterized by neovascularization of the retina and the iris, uveitis, abnormal retinal pigmentation, cystoid macular edema, vitreus hemorrhage, and traction detachment of the retina (Stone et al, 1992). The characteristics of BB syndrome have been described in the introductory chapter. Analysis of DNA samples of patients with either BBS or ADNIV is underway, but to date, no mutation in the 198L gene has been identified.

Mutation screening should be extended to include patients with retinitis pigmentosa (RP). Mutations in photoreceptor expressed genes are known to cause RP. Mutations resulting in RP have been detected in genes coding for proteins of the phototransduction cascade, such as rhodopsin and the β subunit of phosphodiesterase, and as well, in genes coding for cytoskeletal proteins such as peripherin/RDS (Bird, 1995) and ROM1 (Dryja, 1994; Bascom et al, 1995). 198L expression has been localized to the photoreceptors, and the protein has a putative PH domain, making 198L protein a possible phototransducing protein or cytoskeletal protein. Therefore, 198L could be considered as a potential RP candidate gene. 198L is apparently not retina-specific in its expression, at least as revealed
by northern blots. However, RP can occur from mutations in non-retina specific genes, such as that responsible for spinocerebellar ataxia type II (Rod McInnes, personal communication). Moreover, the RNA blot results may not be specific: the hybridizing transcripts seen in other tissues may correspond to highly similar homologues. Western blot analyses of various tissues or RT-PCR of RNA from brain, kidney and lung, followed by direct sequencing of the products would help address this possibility.
REFERENCES


