Optimedin: a novel olfactomedin-related protein that interacts with myocilin

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Mutations in the MYOC gene may lead to juvenile open-angle glaucoma with high intraocular pressure, and are detected in about 4% of people with adult onset glaucoma. Most of these mutations are found in the third exon of the gene encoding the olfactomedin-like domain located at the C terminus of the protein. Another olfactomedin-related protein, known as noelin or pancortin, is involved in the generation of neural crest cells. Here we describe the identification of a novel olfactomedin-related gene, named optimedin, located on chromosome 1p21 in humans. Optimedin and noelin are both expressed in brain and retina. However, unlike noelin, rat optimedin is also highly expressed in the epithelial cells of the iris and the ciliary body in close proximity to the sites of Myoc expression. In the human eye, optimedin is expressed in the retina and the trabecular meshwork. Both optimedin and myocilin are localized in Golgi and are secreted proteins. The presence of mutant myocilin interferes with secretion of optimedin in transfected cells. Optimedin and myocilin interact with each other in vitro as judged by the GST pulldown, co-immunoprecipitation and far-western binding assays. The C-terminal olfactomedin domains are essential for interaction between optimedin and myocilin, while the N-terminal domains of both proteins are involved in the formation of protein homodimers. We suggest that optimedin may be a candidate gene for disorders involving the anterior segment of the eye and the retina.

INTRODUCTION

Glaucoma is a group of neurodegenerative disorders characterized by the death of retinal ganglion cells and by a specific deformation of the optic nerve head, known as glaucomatous cupping. Glaucoma is the second leading cause of blindness in the USA, and affects 1–2% of people over the age of 40 years (1). Primary open-angle glaucoma, the most common form, is often, but not always, associated with elevated intraocular pressure. In many glaucoma cases, elevated intraocular pressure develops as a result of abnormally high resistance to the outflow of aqueous humor. The eye's outflow system is located at the junction of the cornea and iris, and includes the trabecular meshwork and Schlemm's canal, which leads to the episcleral venous system. The molecular mechanisms underlying neuronal damage associated with elevated intraocular pressure are not understood.

It is now well established that family history is an important risk factor in glaucoma. Mutations in several genes have been linked to the disease. In particular, mutations in the MYOC gene (also known as TIGR, GLC1A and MYOC/TIGR) may account for 2.6–4.3% of adult forms of open-angle glaucoma cases, and may lead to autosomal dominant juvenile open-angle glaucoma, which is often characterized by high intraocular pressure (2–6). MYOC is expressed in several ocular and non-ocular tissues (6–10), but mutations in MYOC apparently do not lead to any other pathological changes besides glaucoma. The MYOC gene encodes myocilin (also referred to as TIGR), a secreted protein that contains a coiled-coil domain at the N terminus and an olfactomedin-related domain at the C terminus (4,6–8,11,12). Olfactomedin was originally discovered in the mucus layer of the olfactory neuroepithelium of frogs (13), and genes related to olfactomedin were later identified in other species (14,15). One olfactomedin-related gene, also known as noelin or pancortin (16–18), is highly expressed in brain and might be involved in generation of the neural crest cells (18) and in neurogenesis (19). Another protein encoded by an olfactomedin-related gene, hGC-1, may be an effector of myeloid differentiation (15).

Most glaucoma-causing mutations in the MYOC gene are located in the olfactomedin domain (5). The disease-associated mutations reduce Triton solubility of myocilin (20), and may

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reduce secretion of the protein (21,22). Moreover, the presence of the mutated myocilin appears to suppress secretion of the normal protein (21,22). Recent data indicate that hemizygosity (23) or null mutation of MYOC in humans (24) and mice (25) do not lead to an elevation in intraocular pressure or to glaucoma. Thus, it has been suggested that glaucoma-causing mutations in MYOC may act through a gain-of-function mechanism (25).

Glaucoma-causing mutations in the MYOC gene may compromise trabecular meshwork cell function (11) owing to congestion of the trabecular meshwork pathway (22) or may interfere with myocilin interactions with other proteins. In the current study, we identified a new olfactomedin-related gene, which we have named optimedin. In the rat eye, both Myoc and optimedin genes are expressed in close proximity in the tissues of the eye angle and encode secreted proteins that may interact with each other. Like MYOC, optimedin is expressed in the human trabecular meshwork and in the retina, and is a candidate for glaucoma pathology.

RESULTS

Isolation of optimedin

cDNA clone tmgw11g07 encoding a novel olfactomedin-related protein was identified in a cDNA library prepared from rat eye tissues involved in aqueous humor production and outflow (see Materials and Methods). The gene corresponding to this cDNA was named optimedin, because of its high level of expression in the retina and in tissues of the eye angle (see Fig. 2). Comparison of the optimedin sequence with the GenBank database revealed two rat EST clones expressed in brain, and several homologous mouse and human optimedin clones expressed mainly in embryonic and adult brain. Three mouse genomic BAC clones were isolated and characterized as described in Materials and Methods. The exon-intron boundaries in the mouse optimedin gene were determined by direct sequencing of two BAC clones, 26567 and 26568, neither of which included the first exon of the gene. Sequence of exon 1 and partial sequence of intron 1 were obtained by 5′-RACE as described in Materials and Methods. The sequence of the mouse optimedin gene was identified in the proprietary Celera database, and the human optimedin sequence was found in a draft of the human genome (accession no. NT_004623.5).

The human optimedin gene is different from the previously described human olfactomedin-related OlfA–OlfD sequence (14,15). Figure 1A shows a schematic diagram of the mouse optimedin gene and alternatively spliced mRNA products. The mouse and human optimedin genes have lengths of about 215 and 206.5 kb, respectively, and are located on mouse chromosome 3 and human chromosome 1p21. The mouse optimedin gene contains at least eight exons. The first intron in the mouse optimedin gene is 133.7 kb long and comprises 62% of the total gene length. Analysis of different optimedin mRNA variants amplified by PCR identified two main forms of optimedin in rodents. Exons 2 and 3 were not present in one form (optimedin-A), which corresponds to the AMZ form of noelin (16,18). The second form (optimedin-B) did not contain exons 1 and 2. Thus, exon 3 is the first exon found in optimedin-B, and corresponds to the BMZ form of noelin (16,18). There are stop codons located 27 and 24 nucleotides upstream in frame with the initiator Met codons in exons 1 and 3, respectively. A minor form of optimedin (optimedin-C) was identified in mouse 15-day embryo cDNA by 3′-RACE. In this form, exon 1 is followed by a short exon 2 containing the stop codon. Mouse and rat optimedins differ by only one amino acid. They show 99% identity to human optimedin. The A and B forms of optimedin are 458 and 478 amino acids long, respectively, corresponding to calculated molecular masses of 52.8 and 54.9 kDa. The C form of optimedin is 26 amino acids long if it is translated. Figure 1B shows a comparison of optimedin with OlfA/noelin and myocilin. The A and B forms of optimedin show 65% and 68% identity and 73–76% similarity to the AMZ and BMZ forms of noelin, respectively. Optimedin and noelin also have very similar exon–intron structures (Fig. 1B). Optimedin shows 29% identity and 40% similarity to myocilin throughout the sequence and 39% identity and 51% similarity in the olfactomedin domain. Both the A and B forms of optimedin may contain potential signal peptides at their N termini as predicted by different algorithms (Fig. 1B). Two coiled-coil regions often involved in protein–protein interactions are located at positions 78–141 and 170–217 (Fig. 1B).

Optimedin expression pattern

The expression pattern of optimedin in adult rat tissues was compared with that of Myoc and noelin by northern blot analysis. Rat optimedin was expressed in the combined tissues of the eye angle, retina, brain and at lower level in the lens (Fig. 2A). The most abundant optimedin transcript had a length of about 4 kb. The nature of the minor component with a length of about 5.6 kb is not clear. As expected, noelin was expressed mainly in the retina and brain, while Myoc was expressed in the tissues of the angle, sclera, cornea and retina and less prominently in skeletal muscle. Expression of two main forms of optimedin was investigated by semiquantitative RT–PCR. Optimedin-A was preferentially expressed in adult retina and brain, while optimedin-B was preferentially expressed in the tissues of the eye angle (Fig. 2B).

The distribution of optimedin and Myoc mRNAs in the intact rat eye was studied by in situ hybridization. The probe used for in situ hybridization recognized both forms of optimedin. Optimedin mRNA was most highly expressed in epithelial cells of the posterior iris and ciliary body, and tapered to lower levels in the trabecular meshwork (Fig. 3C,F). In the retina, the optimedin gene was mainly expressed in the ganglion cell layer and in the amacrine cell subregion of the inner nuclear layer (Fig. 3I). Myoc expression was very strong in the trabecular meshwork region, and was reduced toward the ciliary body (Fig. 3B,E). Sclera was another site of strong Myoc gene expression (Fig. 3B,H). Myoc expression was also detected in the retinal pigmented epithelium/choroid area (Fig. 3H). Although the regions of strongest optimedin and Myoc expression are not identical, expression of these genes partially overlaps in the rat eye angle.

In the human eye, optimedin was expressed in the neural retina and the trabecular meshwork, as has been demonstrated by semiquantitative RT–PCR (Fig. 2C). Optimedin expression...
Figure 1. Structural characterization of the optimedin gene. (A) The exon–intron structure of the mouse optimedin gene. The upper diagram shows the mouse optimedin gene drawn approximately to scale with the exception of introns 1 and 2. The two rightward arrows depict transcription initiation sites. The three alternatively spliced mRNAs are shown below. (B) Comparison of the mouse optimedin sequence with those of noelin and myocilin. The optimedin sequence is shown in full. Only differing amino acids are shown for other sequences. Positions of introns are indicated by black triangles. Potential cleavage sites are marked by vertical arrows. OPTA (green) and OPTB (blue) indicate the different N-terminal sequences found in optimedin-A and optimedin-B encoded by exons 1 and 3, respectively; OPTC (yellow) is the C-terminal part of the minor form of optimedin encoded by exon 2; NOEL, noelin; MYOC, myocilin. The olfactomedin domain is shown in rose and the initiator methionines are marked in red.
in the retina was significantly higher than in the trabecular meshwork as judged by this assay. Hybridization of optimedin cDNA with a Clontech MTE blot containing RNA from adult human non-ocular tissues demonstrated that optimedin is also expressed in different regions of the brain and in lung (not shown). Optimedin is expressed early in the course of mouse embryonic development, and its expression was detected at all embryonic stages analyzed, starting from embryonic day 7 (not shown).

Intracellular and extracellular localization of optimedin and myocilin

The intracellular distribution of optimedin was studied in COS-7 cells transfected with optimedin constructs tagged with either red fluorescent protein or Myc. Optimedin-A and optimedin-B co-localized in transfected cells (Fig. 4A-C), where they were preferentially detected in the perinuclear region. Staining with antibodies against the Golgi-specific protein β-COP indicated that optimedin might be located in this compartment (Fig. 4D-F). Localization within the Golgi was confirmed by treating transfected cells with 10 μM of nocodazole or with 5 μg/ml of brefeldin A for 30 minutes. Nocodazole treatment leads to depolymerization of microtubules, causing Golgi disaggregation into vesicles (26), while brefeldin disrupts the structure of the Golgi apparatus, causing the dispersion of Golgi membrane components (27,28). Figure 4G-H shows that optimedin staining was indeed dispersed into numerous vesicles throughout the cell body after nocodazole treatment, while brefeldin treatment led to little or no distinct perinuclear staining.

Figure 2. Northern blot analysis of optimedin, myocilin and noelin expression in rat tissues. Two micrograms of total RNA were loaded per lane. Loaded RNA was visualized by staining with ethidium bromide (not shown). Full-size 32P-labeled rat myocilin, optimedin, and olfactomedin cDNAs were used as probes in these experiments. 1, cornea; 2, combined trabecular meshwork, iris and ciliary body; 3, sclera; 4, retina; 5, lens; 6, skeletal muscles; 7, heart; 8, brain; 9, liver; 10, kidney; 11, lung; 12, spleen. (B) Expression of optimedin-A and optimedin-B in different tissues, as determined by semiquantitative RT-PCR. Primers 5'-TGCTCAGCAGGGCAGATGA-3' and 5'-CTTTTCCAGTGCTGCGAG-3' amplified cDNA containing the third exon (optimedin-B), while primers 5'-GGGGAAGGGTCCCGCGAGTGAGTGAAG-3' and 5'-CTTTTCCAGTGCTGCGAG-3' amplified cDNA containing the first exon (optimedin-A). cDNAs from indicated tissues were used as templates. (C) Expression of human optimedin in the trabecular meshwork and neural retina as judged by semiquantitative RT-PCR. Primers 5'-CTGGAAACAGTGACCAAGACATGC-3' and 5'-CTGGAGTAGATAACTGCTGAGTAC-3' are located in exons 6 and 8 of the human optimedin gene, respectively. Primers 5'-AGGGTACAGCCAATGCCGGA-3' and 5'-CTAGTAGTTCTGAAATAAGGTTCC-3' were used to amplify the ribosomal protein RPL19 sequence for normalization of the amount of synthesized cDNA.
The presence of potential signal sequences at the N termini of the A and B forms of optimedin indicated that they might be secreted. To test this possibility, COS-7 cells were transfected with plasmids encoding optimedin tagged with the Myc epitope at the C terminus. Both forms of optimedin were detected in the extracts of transfected cells and in the incubation media (Fig. 5A). The mobility of optimedin-A was the same in the cell extracts and in the incubation media, while the mobility of optimedin-B in the incubation media was reduced compared with that in cell extracts, indicating that the secreted optimedin-B might be post-translationally modified.

Human and mouse myocilins tagged with the Flag epitope at the C terminus were also secreted by transfected COS-7 cells (Fig. 5B). In agreement with published results (22), the mobility of secreted human myocilin was slightly increased compared with the mobility of the intracellular form (compare lanes 1 and 2 in Fig. 5B). Intracellular and secreted forms of mouse myocilin showed similar mobility (compare lanes 3 and 4 in Fig. 5B). Mouse and human myocilins were also secreted when expressed in Xenopus oocytes (not shown, S.I. Tomarev, M. Andreazzoli and M. Rebert, unpublished).

Since glaucoma-causing mutations in MYOC interfere with the secretion of both wild-type and mutated myocilin (21,22), we tested whether or not the presence of mutant forms of myocilin disrupts the secretion of optimedin. COS-7 cells were co-transfected with optimedin-B and either wild-type myocilin or the I477N myocilin mutant. It has been previously demonstrated that this mutation leads to a severe glaucoma phenotype and the corresponding protein is not efficiently secreted from cells after transfection (22). Consistent with these observations, the I477N mutant was detected in cell extracts but not in the incubation media, while normal myocilin was...
efficiently secreted and detected mainly in the incubation media (Fig. 5C, lower panel). In the presence of normal myocilin, optimedin was detected in both cell extracts and incubation media, while the presence of mutated myocilin inhibited secretion of optimedin (Fig. 5C, upper panel).

Taken together, these data strongly suggest that both forms of optimedin are associated with Golgi, that optimedin as well as mouse and human myocilins are secreted proteins, and that the presence of mutant myocilin may reduce secretion of optimedin.

Optimedin interacts with myocilin

Myocilin and noelin have been shown to be able to form homodimers (11,29–31). Our data on localization and secretion of optimedin and myocilin suggested that they might interact with each other. This suggestion was tested by several techniques. Since optimedin-B was preferentially expressed in the iris and ciliary body in close proximity to the major site of myocilin expression, most of these experiments were done with optimedin-B. Co-immunoprecipitation experiments were
used to demonstrate the interaction of optimedin and myocilin. Translated in vitro optimedin-B–MYC and myocilin–Flag were mixed together and protein complexes were precipitated with either MYC or Flag antibodies. Optimedin and myocilin but not control luciferase were efficiently co-immunoprecipitated together (Fig. 6A).

GST–pull down and far-western techniques were used to confirm interactions between myocilin and optimedin and localize protein domains essential for this interaction. The N- and C-terminal domains of optimedin-B and of myocilin fused to GST were incubated with full-length 35S-labeled optimedin or myocilin. The N-terminal domains of both human and mouse myocilins efficiently pulled down myocilin (Fig. 6B, lanes 2 and 4, lower panel) but not optimedin (lanes 2 and 4, upper panel). The C-terminal domain of myocilin was less efficient than the N-terminal domain in pulling down myocilin (Fig. 6B, compare lanes 2 and 3 or lanes 4 and 5, lower panel). However, the C-terminal domains of myocilin efficiently pulled down optimedin (Fig. 6B, lanes 3 and 5, upper panel). Consistent with these results, the N-terminal domain of optimedin pulled down optimedin but not myocilin (Fig. 6B, lane 6, upper and lower panels), while the C-terminal domain pulled down both myocilin and optimedin (lane 7, upper and lower panels).

GST–pull down results were collaborated by far-western experiments. The N- and C-terminal domains of myocilin and optimedin fused to GST were separated by SDS–PAGE and transferred to nylon membranes. The membranes were incubated with 35S-labeled optimedin or myocilin. Under these conditions, full-length optimedin interacted with its own N-terminal but not C-terminal domain (Fig. 6C, lanes 1 and 3, left panel). Optimedin interacted with the C-terminal domains of mouse and human myocilin (lanes 2 and 4, left panel) and showed weaker interaction with the N-terminal domain of human but not mouse myocilin (lanes 1 and 3, left panel). Full-length myocilin interacted only with its own N-terminal domain under the conditions used (Fig. 6C, lanes 1 and 3, right panel). Taken together, the results of GST–pull down and far-western experiments indicate that the C-terminal domains of optimedin and myocilin may play an essential role in the formation of heterodimers, while the N-terminal domains may be critical for the formation of homodimers.

DISCUSSION

In the present study, we describe a novel olfactomedin-related protein named optimedin that interacts with myocilin. Olfactomedin was originally identified as the structural component of the mucus layer that surrounds the chemosensory dendrites of olfactory neurons in Rana catesbeiana (13). Five proteins related to olfactomedin have been found in humans (14), and optimedin is the sixth member of this gene family. Optimedin is more closely related to noelin than to other olfactomedin-related proteins: different forms of optimedin and noelin are 65–68% identical at the protein level and their genes have very similar exon–intron structures (Fig. 1). Both optimedin and noelin are alternatively spliced. Four splice variants of olfactomedin differing at the N and C termini have been identified. We have identified three variants of optimedin, two of which differ at their N termini. While this paper was in preparation, six variants of human optimedin were deposited in GenBank as NOE3-1–NOE3-6, with accession numbers AF397392–AF397397. Rodent optimedin-A and optimedin-B correspond to human NOE3-3 and NOE3-1, respectively. Two different 5′ exons and two different promoters of the optimedin gene appear to be used in different tissues. The biological significance of this is not clear yet. In tissues of the eye angle, exon 3 encodes the...
Figure 6. Assays to reveal interactions between optedin and myocilin. (A) Co-immunoprecipitation of optedin and myocilin. $^{35}$S-labeled luciferase (lanes 1 and 4), optedin–Myc (lanes 2, 5 and 7) or myocilin–Flag (lanes 3, 6 and 8) were mixed with unlabeled optedin–Myc (lanes 4 and 8) or myocilin (lanes 4 and 7) and immunoprecipitated with Myc (lanes 4, 5 and 8) or Flag (lanes 4, 6 and 7) antibodies. Lanes 1–3 are 10% input lanes. (B) GST–pull down interaction assays. The N- and C-terminal domains of mouse (lanes 2 and 3) and human (lanes 4 and 5) myocilins and the B form of optedin (lanes 6 and 7) fused to GST were tested for their ability to pull down $^{35}$S-labeled optedin (upper panel) or $^{35}$S-labeled myocilin (lower panel). The efficiency of interactions can be assessed by comparing the amount of protein pulled down in test lanes 2–7 and in the 10% input lanes (lane 1). GST alone did not produce detectable bands under the conditions used (not shown). (C) Far-western interaction assays. Purified GST fusion proteins were separated by SDS–PAGE and transferred to nylon membranes, and membranes were incubated with $^{35}$S-labeled full-length optedin or myocilin as described in Materials and Methods. The N- and C-terminal domains of mouse (lanes 1 and 2) and human (lanes 3 and 4) myocilin and the B form of optedin (lanes 5 and 6) fused to GST were used in these experiments. Faster migrating bands in lanes 1 and 3 (right panel) correspond to the degradation products of the N-terminal myocilin domain.
N-terminal part of optimedin, while in the retina and brain, the N terminus is encoded by exon 1. It is interesting to note that different spliced forms of noelin are also used in different tissues. In brain, all four identified forms of noelin were detected, while in kidney, eye and lung, the BMY and BMZ forms were preferentially expressed (32). Optimedin-A is secreted more efficiently than optimedin-B, and optimedin-B may be more heavily post-translationally modified than optimedin-A (Fig. 5A).

Expression of optimedin and myocilin genes partially overlaps in the rat eye angle. In the adult human eye, optimedin is expressed in the retina and the trabecular meshwork among tissues tested, as judged by semiquantitative RT-PCR. Although preliminary estimates indicate that optimedin is more highly expressed in the human retina than in the trabecular meshwork, we have not done thorough quantitative estimates, since the trabecular meshwork RNA used in these experiments might be partially degraded. In the tissues of the human eye angle, MYOC is expressed in the trabecular meshwork and in the endothelial lining of Schlemm’s canal, as well as in ciliary muscle, iris stroma, beneath the anterior border of the iris and in the contractile cells of the scleral spur (9,10). Therefore, optimedin and myocilin may be coexpressed in some cells of the eye angle, and since both proteins are secreted, they should be present in the extracellular space in these tissues.

Data presented in this paper indicate that optimedin and myocilin might interact with each other and that the C-terminal olfactomedin domain is essential for these interactions. It has recently been demonstrated by a yeast two-hybrid assay that the N-terminal part of myocilin is critical for homodimer formation (31), and our data, obtained by other techniques, support these published results. Most mutations leading to glaucoma are located close to the known translocation breakpoint associated with a malignant ependymoma (33). Elucidation of mechanisms of optimedin action will help to better understand its possible contribution to glaucoma pathology as well as its role in normal development and function.

MATERIALS AND METHODS

Isolation of optimedin gene

Total RNA was isolated from dissected combined tissues of the rat eye angle (iris, ciliary body and trabecular meshwork) using RNazol B (Tel-Test). Purification of poly(A)⁺RNA, CDNA synthesis, size fractionation, and directional 5’-Sall–Nott-3’ cloning into the pSPORT1 vector (Gibco BRL) were done as a service by BioServe (Laurel, MD). Two cDNA libraries, containing $1.3 \times 10^6$ and $1.8 \times 10^6$ independent clones, respectively, were constructed. The average sizes of the inserts in these libraries were 1.7 and 1.4 kb, respectively. Two thousand clones from each library were randomly selected and sequenced from the 5’ end with fluorescent dideoxynucleotides. Sequencing was done as a service by National Intramural Sequencing Center, NIH. Clone tmwg11g07 was identified as a new olfactomedin-related cDNA, sequenced and named optimedin. The missing 5’ end of exon 2 in rat optimedin was obtained by 5’-RACE with adult rat brain PCR-ready cDNA and rat GenomeWalker kit (Clontech). A partial mouse optimedin sequence was obtained by sequencing of a PCR product synthesized using mouse brain cDNA as a template and oligonucleotides 5’-TACAGTCCATTCGAGACTTGG-3’ and 5’-TGTCATCTTCCAGCTTTAATGATGT-3’. The partial mouse optimedin sequence was used to design primers 5’-TACAGTCCATTCGAGACTTGG-3’ and 5’-CATTGGAACACCCAGATGACT-3’ for screening the mouse 129/SvJ BAC ES genomic library. This screening was performed as a service by IncyteGenomics. Three BAC clones (26567–9) were identified. DNA of BAC clones was used to sequence the coding sequence of mouse optimedin, with the exception of exon 1 and the exon–intron junctions. The sequence of the first exon, a partial sequence of the first intron and proximal promoter sequences were obtained by 5’-RACE using 5’-RACE-ready mouse brain cDNA and the mouse GenomeWalker kit (Clontech). The sizes of the introns in the mouse optimedin gene were calculated using the Celera Discovery System and Celera’s associated database. The first exon of the rat optimedin gene was amplified by PCR using rat brain cDNA as a template and oligonucleotides 5’-GGAGAGTTGTCCTCCGCACTGAGTGAAGTGC-3’ and 5’-CTTTCTCACTCAGATGTGGCGGAG-3’. The former oligonucleotide was designed on the basis of the mouse exon 1 sequence, while the latter was located in exon 4 of the rat optimedin gene. Predictions of the signal peptides were performed using the SignalP (www.cbs.dtu.dk) and PSORT (psort.nibb.ac.jp) servers.

Northern blot analysis, semiquantitative RT–PCR, and in situ hybridization

Northern blot analysis, cDNA synthesis, and semiquantitative RT–PCR were performed as previously described (34). PCR-amplified full-length coding sequences of rat optimedin (the A form) and MYOC were cloned into the pBluescript II KS vector using the HindIII (5’) and EcoRI (3’) restriction sites. For in situ hybridization, rat eyes were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) overnight and processed for paraffin embedding. Serial sections (6 μm) were
hybridized with specific $^{32}$P-labeled riboprobes transcribed from HindIII-linearized plasmids from the T7 promoter. In situ hybridization, washes and autoradiography were done as previously described (35).

Intracellular and extracellular localization of optimedin and myocilin

Optimedin-A and optimedin-B were cloned into the pDSRed1-N1 vector (Clontech) and pcDNA 3.1/Myc-His(+) B vector (Invitrogen). Human and mouse full-length myocilin proteins were cloned into the pCS2-Flag and p3XFLAG-CMV-14 (Sigma) vectors, respectively. Point mutations were introduced into human MYOC cDNA using the QuikChange site-directed mutagenesis kit (Stratagene). Identity of the constructs was confirmed by sequencing. Full-size human and mouse myocilin–GFp constructs were described in (36). Transfections into COS-7 cells was done as described in (36). Cells were stained with monoclonal β-COP antibody (Sigma) at 1:200 dilution. FITC-labeled anti-mouse antibody (Jackson Immuno Research, West Grove, PA) was used as a secondary antibody at 1:100 dilution. Images were collected using a Leica confocal microscope. To study secretion of optimedin and myocilin, cells were transfected with indicated constructs and incubated for 42 hours. Cells were kept in medium without serum for the last 12-15 hours. Incubation medium was concentrated 10 times using Centricon M-10 concentration units (Millipore). Monoclonal antibodies against FLAG and Myc epitopes (Sigma) were used for the detection of myocilin and optimedin, respectively.

GST-pull down, far-western blotting and co-immunoprecipitation

The N- and C-terminal domains of the B form of optimedin and human and mouse myocilin were cloned into the bacterial expression vector pGEX-2T (Amersham Pharmacia) as GST fusion proteins. Recombinant proteins were purified from induced cultures of Escherichia coli cells using GST purification modules (Amersham Pharmacia). $^{35}$S-labeled full-length optimedin and myocilin were synthesized using the TntT Rabbit Reticulocyte Lysate System (Promega). Labeled proteins were purified using G-25 spin columns (Amersham Pharmacia). GST-pull down interaction assays were performed essentially as described in (37). $^{35}$S-labeled myocilin or optimedin were incubated overnight at 4°C with different GST fusion proteins or with GST alone immobilized on glutathione agarose in the binding buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% NaDOC, 10 mM DTT and a protease inhibitor cocktail from Roche). The agarose beads were washed three times in the binding buffer, and proteins were eluted by boiling in the SDS-PAGE loading buffer and loaded onto SDS-PAGE gels. Gels were dried after electrophoresis and radioactive proteins were visualized by autoradiography. Efficiency of GST-pull down was estimated by comparison with a 10% input lane. Far-western experiments were performed essentially as described in (38). Purified GST fusion proteins were separated by PAGE and transferred to PVDF membranes (Millipore). The membranes were incubated with $^{35}$S-labeled myocilin or optimedin in a buffer containing 10% glycerol, 100 mM NaCl, 20 mM Tris pH 7.4, 0.5 mM EDTA, 0.1% Tween-20 and 2% dry milk at 4°C. After incubation, filters were washed in the incubation buffer at 4°C and dried, and radioactive test proteins were revealed by autoradiography. For immunoprecipitation, optimedin–Myc, myocilin–Flag and control luciferase were synthesized in the Rabbit Reticulocyte System, mixed and incubated with anti-Myc or anti-Flag antibodies overnight. The antigen-antibody complexes were captured using protein A–agarose under the conditions recommended by the manufacturer (Roche Molecular Biochemicals). The captured proteins were separated by SDS-PAGE. Gels were dried and exposed to X-ray films.

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