Isolation of a Novel Iris-Specific and Leucine-Rich Repeat Protein (Oculoglycan) Using Differential Selection

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PURPOSE. To identify and characterize genes expressed in the iris.

METHODS. A human adult iris cDNA library was constructed and subjected to a differential selection screen to identify genes preferentially expressed in iris or trabecular tissue versus those expressed in lymphoblasts. Selected cDNAs were partially sequenced. Novel cDNAs were chosen for further analysis. The cDNAs were localized within chromosomes using a radiation hybrid (RH) mapping panel. The tissue expression profile of each cDNA was found through computer-based searches. One novel cDNA was subjected to 5′ rapid amplification of cDNA ends and Northern blot analysis.

RESULTS. Of 24 differentially selected clones, 14 cDNAs had homology to known genes, whereas the other 10 were previously uncharacterized cDNA clones. IR185 was one novel iris cDNA identified. Northern blot analysis with IR185 indicated that it is expressed in human fetal liver as a 2.7-kb transcript and in adult iris as a 1.6-kb transcript. Computer-based searches of public databases and reverse transcription-polymerase chain reaction experiments have determined that IR185 is also expressed in retina. RH mapping experiments have localized IR185 to the chromosomal interval 1q31-q32, near the loci for age-related degeneration (1q25-q31) and retinitis pigmentosa 12 (1q31-q32), and IR185 is in the region for posterior column ataxia with retinitis pigmentosa (1q31-q32). It has a 996-bp open reading frame encoding a putative protein with homology to the small leucine-rich proteoglycan (SLRP) family. The IR185 gene has been tentatively named oculoglycan.

CONCLUSIONS. Differential selection is a technique that has been useful in identifying genes specific to a variety of tissues. This is the first time this technique has been applied to the iris. Characterizing genes highly or uniquely expressed in the iris can assist in clarifying our understanding of iris function and lead to a better understanding of the molecular pathogenesis of ocular disease. IR185 is a tentative candidate for one eye disorder genetically localized to chromosome 1q31-q32. (Invest Ophthalmol Vis Sci. 2000;41:2059–2066)

Methodologies used to search for genes involved in hereditary disease can be separated into two categories. The first, the positional cloning approach,¹ involves using linkage analysis to identify the genetic region within which lies a disease-causing gene. Once the interval is of a manageable size, genes within the minimal region are tested as candidates for the disorder of interest. The second method, the candidate gene approach,¹ entails examining individual genes that encode proteins with known functions or that have an expression profile that makes them candidates for the disorder in question. Both methods are typically used simultaneously to discover genes causing hereditary disorders.

One factor examined when considering the eligibility of any candidate gene is whether the gene is expressed in the tissue affected by the disorder. Alternatively, researchers can identify highly or specifically expressed genes in a given tissue. Genes expressed in this manner can be assumed to be important for the function of that tissue. Once isolated, genes that are highly expressed or tissue specific can become candidate genes for disorders that affect the tissue used for gene isolation. For example, genes expressed in the retina can be candidate genes for retinal disorders. There are a variety of techniques available to identify genes expressed in some tissues and not in others, including subtractive hybridization² and computer-based (in Silico) searches of public databases.³ We have used another technique, differential selection,⁴ to search for genes expressed in the iris and trabecular meshwork. In a differential selection screen, cDNA pools from different tissues are used as probes against a cDNA library. cDNAs found to be expressed in the tissue of interest and not in the control tissue are selected for further analysis. cDNAs found to be more highly expressed in the tissue of interest than in the control tissue may also be chosen for further characterization, as we did in the current work. Differential selection has been suc-

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cessfully used to isolate new genes involved in retinal function (i.e., Rom1, Chx10), but this technique has not been applied previously to the iris. Genes identified using our differential selection screen can be tested as candidates for ocular disorders, including glaucoma.

This article describes the screening of an iris cDNA library to identify cDNAs preferentially expressed in the iris or trabecular meshwork. cDNAs were identified through a differential selection screen. One novel cDNA, IR185, isolated using this method, was analyzed in detail. The expression of IR185 in fetal liver, iris, and retina together with its genetic co-localization with posterior column ataxia with retinitis pigmentosa make IR185 a candidate for ocular disease.

**MATERIALS AND METHODS**

**Preparation of Iris, Trabecular Meshwork, Retina, and Lymphoblast RNA and Construction of an Adult Iris cDNA Library**

Iris and trabecular meshwork total RNA were obtained from four and eight pairs of human donor eyes, respectively, less than 24 hours after death. The collection of human eyes was approved of by the Ethics Board of the Centre Hospitalier de l’Université Laval (CHUL). The tissues obtained were within the tenets of the Helsinki Declaration. The tissues were mixed in denaturing buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7] and 0.5% N-lauroylsarcosine) before phenol-chloroform extraction. The RNA was then precipitated using isopropanol, washed in 70% diethyl pyrocarbonate (DEPC)-treated ethanol and resuspended in DEPC-treated water. Total lymphoblast RNA was obtained from lymphoblastoid cell lines and isolated using TRIzol reagent (Canadian Life Technologies–Gibco, Burlington, Ontario, Canada). Poly(A+ RNA was isolated from iris using oligo-d(T)–coated Dynabeads (Dynal, Oslo, Norway). Five micrograms of iris poly(A+ RNA was used as the source material for the iris cDNA library. The iris cDNA library was constructed using a cDNA synthesis kit. Retinal total RNA was donated by Paul Wong, University of Alberta.

**TABLE 1. Genes Isolated in an Iris cDNA Differential Selection Screen**

<table>
<thead>
<tr>
<th>cDNA</th>
<th>cDNA Size</th>
<th>Location</th>
<th>Expression†</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR42</td>
<td>~870 bp</td>
<td>Xq25</td>
<td>Br, Co, FBr, FHe, FK, FLS, FLu, FS, FSp, Gb, He, LI, Mu, Ne, Nr, Pa, Pi, Sk, T, Th, U</td>
<td></td>
</tr>
<tr>
<td>IR99</td>
<td>~800 bp</td>
<td>7q9, 11, 13, 15*</td>
<td>Br, FBr, FHe, He, LI, Mu, O, PI, Pr, T, U</td>
<td></td>
</tr>
<tr>
<td>IR108</td>
<td>~870 bp</td>
<td>7p14~7p15</td>
<td>Lv, Sp</td>
<td></td>
</tr>
<tr>
<td>IR185</td>
<td>~300 bp</td>
<td>1q31~1q32</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>TM257</td>
<td>~500 bp</td>
<td>7q31</td>
<td>Br, Bs, Co, E, FHe, FK, FLu, Mu, Ne, Nr, Pa, PI, Sk, U</td>
<td></td>
</tr>
<tr>
<td>TM258</td>
<td>~500 bp</td>
<td>5q35~q34</td>
<td>Br, FC, FHe, LI</td>
<td></td>
</tr>
<tr>
<td>TM267</td>
<td>~1 kb</td>
<td>19q13</td>
<td>Bs, FHe, FSu, FSp, LI, Pa, PI, Sk</td>
<td></td>
</tr>
<tr>
<td>TM305</td>
<td>~700 bp</td>
<td>10p12</td>
<td>Br, Co, Li</td>
<td></td>
</tr>
<tr>
<td>TM324</td>
<td>~870 bp</td>
<td>11q21~q22</td>
<td>FLS, FHe, FSu, FSp, He, Mu, PI, Sk, U</td>
<td></td>
</tr>
<tr>
<td>IR355</td>
<td>~800 bp</td>
<td>10q23~10q24</td>
<td>Br, FHe, LI, LIV, PI, Pr, U</td>
<td></td>
</tr>
</tbody>
</table>

The cDNAs beginning with IR were seen to be more highly expressed in iris than in lymphocyte tissue. cDNAs beginning with TM were seen to be more highly expressed in trabecular meshwork than in iris or lymphocyte tissue. At, adipose tissue; Bm, bone marrow stroma; Bn, bone; Br, brain; Bs, breast; Ch, ciliary body; Co, colon; E, epididymus; F, fibroblast; FBr, fetal brain; FC, fetal cochlea; FFe, fetal heart; FK, fetal kidney; FLS, fetal liver and spleen; FLu, fetal lung; FLv, fetal liver; FR, fetal retina; FS, fetal skin; FSp, fetal spleen; Gb, gallbladder; Go, greater omentum; He, heart; K, kidney; Ke, keratinocyte; Le, lenses; Lu, lung; Lv, liver; LI, lymphocytes and lymphokines; Mu, muscle; Ne, neuroepithelium; Nr, neuron; O, ovary; Pa, pancreas; PI, placenta; Pr, prostate; R, retina; SI, small intestine; Sk, skin; Sm, skeletal muscle; Sp, spleen; T, testis; Th, thyroid; Ty, thymus; U, uterus.

* IR99 mapped to multiple chromosomes.
† The expression pattern was determined through computer (in Silico) searches of public databases.

**Differential Screen**

The iris cDNA library was grown at low density on 245-mm square bioassay dishes (Fisher Scientific, Nepean Ontario, Canada). Each dish was plated with either 6,000 or 12,500 plaque forming units (pfu). A total of 72,000 pfu, representing approximately 24,000 recombinant cDNAs, was plated. Triplicate filter lifts were made of each plate using Hybond-N (Amersham Pharmacia Biotech, Baie d’Urfé, Québec, Canada). Each filter lift was treated as follows: 5 minutes’ denaturing in 0.5 M NaOH and 1.5 M NaCl, followed by 5 minutes' neutralizing in 1.5 M NaCl and 0.5 M Tris-HCl, and 5 minutes' rinsing in 2× SSC. The filters were then air dried and baked overnight at 80°C in a vacuum oven. Before hybridization, each nylon blot was prewashed with 2× SSC before it was prehybridized for 1 hour in Church and Gilbert hybridization solution* at 65°C.

Iris, trabecular meshwork, or lymphoblast cDNA (50 ng) were radiolabeled with 32P dCTP (Random Primed Labeling Kit; Roche Diagnostics, Laval, Québec). The radiolabeled probe was denatured at 95°C, cooled rapidly on ice, added to the prehybridized filter lifts, and hybridized at 65°C overnight. The filters were washed under low-stringency conditions with 2× SSC and 1% sodium dodecyl sulfate (SDS) for 30 minutes at room temperature, were washed again under high-stringency conditions with 0.2× SSC and 0.1% SDS for 45 minutes at 65°C, and were exposed to film (Biomax; Eastman Kodak, Rochester, NY) for 1 week at −70°C. Primary cDNA plaques detected with iris cDNA but not as highly with lymphoblast cDNA or those detected with trabecular meshwork cDNA but not as highly with iris or lymphoblast cDNA were selected for further analysis.

Plaques of interest were selected and the recombinant cDNAs isolated using the in vivo excision protocol supplied by the manufacturer (Stratagene). Individual cDNAs were manually sequenced using a 32P-radiolabeled terminator sequencing kit (ThermoSequenase Cycle Sequencing Kit; Pharmacia Biotech).
PCR Primers

cDNA-specific polymerase chain reaction (PCR) primers were designed using the Primer 3 program at the Whitehead Institute for Biomedical Research (Cambridge, MA; web page available at http://www.genome.wi.mit.edu).

Northern Blot Hybridization

IR185 cDNA (50 ng) was randomly primed and radiolabeled as described. Radiolabeled IR185 cDNA was hybridized to a commercially available multiple tissue Northern blots (Human Multiple Tissue Northern Blot I, Human Multiple Tissue Northern Blot II, and Human Fetal Multiple Tissue Northern Blot II; Clontech, Palo Alto, CA). Each Northern blot contained approximately 2 μg polyA+ RNA per lane. Hybridization was performed at 68°C for 1 hour using Express Hyb solution (Clontech). The Northern blots were washed under low-stringency conditions (2× SSC and 0.05% SDS for 30 minutes at room temperature), washed again under high-stringency conditions (0.1× SSC and 0.1% SDS for 40 minutes at 50°C), and exposed to film (Biomax; Kodak). Northern blots were probed with actin cDNA to control for RNA loading. The actin control hybridization experiment was performed in an identical manner.

A Northern blot containing 3 μg human iris, human retina, and human lymphocyte total RNA was made using standard methods. The Northern blot was probed with actin cDNA to control for RNA loading. The actin control hybridization experiment was performed in an identical manner.

Figure 1. The IR185 locus relative to the loci for age-related macular degeneration (1q25-q31), retinitis pigmentosa 12 (1q31-1q32), and posterior column ataxia with retinitis pigmentosa (1q31-q32). Centimorgan (cM) distances listed were obtained from the Marshfield Genetic Database, Marshfield, WI (http://www.marshmed.org/genetics).
and 0.1% SDS for 5 minutes at room temperature) and then under high-stringency conditions (0.1× SSC and 0.1% SDS for 30 minutes at 42°C) and exposed to film.

**Radiation Hybrid Mapping**

IR185 primers were used to screen the Genebridge 4 radiation hybrid (RH) panel (Research Genetics, Huntsville, AL). Primers IR185–1F (cccaggtcatcatctttggacc) and IR185–1R (atggagacctttgcctagc) amplified a 142-bp fragment. PCRs were performed under the following conditions: 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds; followed by a final extension step at 72°C for 7 minutes. The PCR products from all 93 RH cell lines, HFL121 (human positive control), and A23 (hamster negative control) were separated on agarose gels and scored as positive, negative, or ambiguous. The results were electronically submitted to the Whitehead Institute for Biomedical Research Web site (http://www.genome.wi.mit.edu) and to the Sanger Center (Hinxton, UK) Web site for analysis (http://www.sanger.ac.uk/RHserver).

cDNA-specific primers were also used for RH mapping to chromosomal regions of any unlocalized novel or known cDNAs.

**Computer-Based Searches**

The in Silico expression profile of each cDNA was determined by an electronic search of the cDNA sequence on the TIGR database (http://www.tigr.org/tdb/hgi/hgi.html). The IR185 putative amino acid sequence alignment was performed at the Baylor College of Medicine search launcher Web site (http://
We decided to focus on IR185, a 300-bp cDNA clone, because RH mapping placed it near the locus for retinitis pigmentosa 12 at 1q31-q32\(^\text{9}\) and an age-related macular degeneration locus (1q25-q31).\(^{10}\) Recently, a posterior column ataxia with retinitis pigmentosa locus (1q31-q32)\(^{10}\) was found to be in the same chromosomal region as IR185. The location of IR185 relative to these three eye disorders is shown in Figure 1. RT analysis of the novel cDNAs demonstrated that almost all the cDNAs were expressed in both choroidal ring and lymphoblasts. IR185 was the only novel cDNA listed in Table 1 that was amplified from choroidal ring cDNA and not amplified from lymphoblast cDNA using RT-PCR (Fig. 2). Further RT-PCR experiments demonstrated that IR185 was present in retina cDNA but not in trabecular meshwork cDNA (data not shown). Northern blot analysis of IR185 (using Human Multiple Tissue Northern Blot I, Human Multiple Tissue Northern Blot II, and Human Fetal Multiple Tissue Northern Blot II, each containing approximately 2 \(\mu\)g polyA\(^+\) RNA in each lane; Clontech), identified a 2.7-kb transcript in fetal liver only (Fig. 3). A Northern blot containing 3 \(\mu\)g human iris, human retina, and human lymphocyte total RNA, probed with IR185, showed that IR185 was much more highly expressed in iris than in retina (Fig. 4). The IR185 transcript size in human iris is approximately 1.6 kb. The 1.6-kb size estimate in iris was based on internal controls used (\(\beta\)-actin, s26 cDNA, 28s RNA, and 18s RNA). In Silico searches of public databases revealed that IR185 has sequence homology to eight ESTs also obtained from retina (EST 112502, EST 112486, EST 112481, EST 112488, aa86a04.s1, aa86a04.r1, EST 19878, and EST 20234). IR185 is
therefore expressed in three tissues: iris, retina, and fetal liver. We used 5’ RACE on choroidal ring cDNA to obtain the entire open reading frame of IR185. We sequenced 1359 bp of the estimated 1.6-kb iris transcript of IR185 (Fig. 5), which appears to represent the entire protein encoding region of IR185 in the eye, because there are two in-frame stop codons upstream of the predicted start site.

To determine the putative function of IR185, we compared its predicted amino acid sequence to other sequences through in Silico searches of public databases. IR185 has homologies at the amino acid level with two proteoglycans, epiphycan and osteoglycin (47% identity-60% similarity and 40% identity-58% similarity, respectively). The amino acid alignment of all three proteins is shown in Figure 6. There are six leucine-rich motifs bounded by conserved cysteines in all three proteins. A predicted N-glycosylation site is in the same location in the putative amino acid sequence of IR185 and epiphycan. IR185 is also predicted to have casein II kinase and protein kinase C phosphorylation sites, as well as myristilation sites (data not shown).

**DISCUSSION**

Differential selection is one method that can be used to isolate tissue-specific genes. One strength of differential selection is that, unlike a subtracted cDNA library, the differential screen may be repeated with cDNA from different tissues without having to reconstruct the cDNA library. One limitation of a differential screen is that genes important in ocular function but also expressed in lymphoblasts are missed by the screen. In our differential screen, we opted to choose cDNAs that appeared to be more highly expressed in iris tissue than in lymphoblasts or more highly expressed in trabecular meshwork.
work tissue than in iris tissue or lymphoblasts. Although the described limitation still exists for our screen, this strategy allows the identification of cDNAs with some lymphoblast expression that are highly expressed in iris tissue or trabecular meshwork tissue that would otherwise have been excluded from the screen. In addition, because the iris cDNA library was made using adult human iris mRNA, any developmentally related ocular disease-causing genes not expressed in the adult iris would also be missed. These two limitations would also be present, however, in a subtractive cDNA library screen, depending on the tissues used.

We have isolated and partially characterized a novel gene, IR185, from a differential selection screen. IR185 is homologous to osteoglycin and epiphycan, two members of the SLRP family of proteins, which are secreted and associated with the extracellular matrix. SLRP proteins are characterized by several leucine-rich motifs bounded by conserved cysteine residues that are hypothesized to form internal disulfide bonds. The 24-amino acid leucine-rich repeat consensus sequence is: x-x-I/V/L-x-x-x-x-F/P/L-x-x-L-x-x-L/I-x-L-x-x-N-x-I/L, where x represents any amino acid. Within the consensus sequence, the multiple amino acids listed in positions 3, 8, 11, 17, and 24 are in order of decreasing frequency. Leucine-rich repeat consensus amino acids 3 to 11 and 14 to 24 are predicted to form a β-sheet and an α-helix, respectively.

The SLRP family is divided into three main classes based on the cysteine spacings on the amino flank of the leucine repeats. Osteoglycin and epiphycan are the sole members of the class 3 SLRPs. IR185's predicted homology to these proteins at the amino acid level and conservation of cysteine residues among the three proteins strongly suggest that IR185 is also a class 3 SLRP.

Expression of SLRP genes appears to vary depending on their class. Class 1 SLRP genes, decorin and biglycan, are expressed in virtually every tissue tested, whereas class 2 genes, fibromodulin, keratocan, lumican, and proline-arginine–rich and leucine-rich repeat protein (PRELP) are expressed in seven or fewer tissues. Class 3 genes appear to be expressed in the fewest tissues. Mouse osteoglycin is expressed in skeletal muscle, lung, kidney, and testis. Osteoglycin was originally cloned from bovine bone. Epiphycan was only seen in placenta in Northern blot experiments. IR185 also has a limited tissue expression profile, suggesting that the class 3 SLRP proteins may have a more tissue-specific function than the other SLRP proteins. Because other SLRPs are known to bind to collagen, IR185 may be a novel collagen-associated glycoprotein with a role in eye function.

The size differences between the iris and fetal liver IR185 transcripts may be the result of alternative splicing of IR185. Alternatively, the increased transcript size may be additional untranslated 3' or 5' sequence or could encode for a larger IR185 protein product in fetal liver. Further studies are needed.

**Figure 6.** (A) Amino acid alignment of the putative IR185 (oculoglycan) proteins, epiphycan and osteoglycin. Identical residues among all three proteins are shaded in gray. Filled circles: Conserved cysteine residues. A putative N-glycosylation site is underlined. Each leucine-rich repeat between the conserved cysteine residues is in a box. (B) Schematic depiction of the predicted IR185 protein. Filled circles: Conserved cysteine residues. Filled rectangle: leucine repeat motif. Tree: Putative N-glycosylation site.
to determine the nature of the longer IR185 transcript in fetal liver.

IR185 is located near the same chromosomal region as two eye-related disorders, age-related macular degeneration (1q25-q31) and retinitis pigmentosa 12 (1q31-1q32). Of interest, IR185 is located within the same chromosomal region as posterior column ataxia with retinitis pigmentosa (1q31). Further studies are needed to determine whether IR185 is a candidate for posterior column ataxia with retinitis pigmentosa. Because IR185 is not very highly expressed in retina, the candidacy of IR185 for posterior column ataxia with retinitis pigmentosa should be considered tentative.

Based on IR185's putative glycosylation site and expression in iris and retinal tissues we have named IR185 oculoglycan. Oculoglycan and the other genes identified in our screen will be subjected to further analysis to examine their roles in eye function and disease.

Authors' Note
While this paper was being published, Reardon et al. (J Biol Chem. 2000;275:2123–2129) published the isolation of a gene with the same sequence as Oculoglycan which they called Opticin.

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References