A ubiquitin C-terminal hydrolase gene on the proximal short arm of the X chromosome: implications for X-linked retinal disorders

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We report the cloning of a novel human cDNA which encodes a 690 amino acid protein with high homology to ubiquitin C-terminal hydrolases. Northern blot analysis shows expression of a 3.3 kb transcript in all tissues examined, with 5- to 10-fold higher levels in retina than elsewhere. We mapped the structural gene to Xp21.2-p11.2. This gene’s relatively high levels of retinal expression and recent work showing that perturbations in protein turnover and processing can lead to retinal disease make it an excellent candidate for several X-linked retinal disorders mapping within this interval. Additionally, there is evidence that members of the ubiquitin hydrolase family may play a role in oncogenesis and a locus implicated in ovarian cancer is also located within this region.

INTRODUCTION

Ubiquitination of cellular proteins is involved in diverse cellular activities including DNA repair, cell-cycle control, peroxisome biogenesis, and the stress response [for reviews on the ubiquitin system and the enzymes involved see (1–4)]. One of the best understood functions of ubiquitin is its role in targeting proteins for non-lysosomal proteolysis mediated by the 26S proteasome. Additionally, reversible ubiquitination may play a role in regulating protein function. For example, IgE receptors are polyubiquitinated immediately after antigen-induced engagement and are deubiquitinated once the receptors are disengaged (5). There are several enzymatic steps in the ubiquitination pathway. The formation of protein-ubiquitin conjugates is mediated by the sequential action of ubiquitin activating enzymes, conjugating enzymes, and ligases. Ubiquitin is cleaved from its various conjugates by ubiquitin C-terminal hydrolases. Ubiquitin hydrolases are thiol proteases with several functions including release of ubiquitin from its biosynthetic precursors, recovery of ubiquitin from ubiquitinated proteins undergoing proteolysis, and reversal of non-degradative ubiquitination. Multiple ubiquitin C-terminal hydrolase genes have been described in eukaryotes, including at least five in S. cerevisiae (6,7).

Retinal photoreceptors are exquisitely specialized cells responsible for detecting photons of light and transforming that signal into a chemical one which is then transmitted to the brain. Photoreceptors have a high metabolic rate (8) and a high rate of protein turnover due to shedding and renewal of outer segment discs (9). Ubiquitin-conjugating activity is present in retinal homogenates from rat (10), and a neuron-specific ubiquitin carboxyl-terminal hydrolase, PGP 9.5 (11,12), has been localized to horizontal and ganglion cells in vertebrate retinas (13). We report the cloning of a novel cDNA from human retina that encodes an apparent new member of the family of ubiquitin hydrolases and whose structural gene maps to the X chromosome. The map location of this gene and the function of its presumed protein product make it a candidate for several inherited X-linked retinal disorders.

RESULTS

Isolation and characterization of cDNA clones

We used a differential hybridization screen of arrayed clones (14) from a human retinal cDNA library to isolate conserved, abundantly expressed cDNAs found preferentially or exclusively in retina. One clone (clone 2) was selected on the basis of its preferential hybridization to a total cDNA probe prepared from DNA isolated from 13-line ground squirrel (Citellus tridecemlineatus) retina compared to a total cDNA probe prepared from human fibroblast RNA. We opted to use the cone-predominant retina of the 13-line ground squirrel in an attempt to increase the likelihood of identifying novel cone-specific genes in our screen (Swanson, Freund, Steel, Ploder, McInnes and Valle, in preparation). Sequence analysis revealed an ORF of approximately 600 bp followed by approximately 400 bp of presumed 3′ UTR. Using this partial length cDNA as a probe, we screened a once amplified retinal cDNA library, detecting first round positives with a frequency of 1:700. Thirty of these were screened by PCR for sequence extending 5′ beyond clone 2. The three phage clones

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which gave the longest products were plaque purified. Clone 2.11 is the longest and has an insert of ~2.8 kb which overlaps about 500 bp of clone 2 at its 3' end. The composite cDNA sequence from clones 2 and 2.11 and the conceptual translation of the ORF are shown in Figure 1. There is a 2070 bp ORF preceded by 679 bp of 5' UTR and followed by a 410 bp 3' UTR which ends in a string of nine As. An atypical polyadenylation signal, TATAAA (15), is present 17 bp upstream of the As. The putative initiation codon has an acceptable Kozak consensus sequence, ATG, and there is an in-frame stop codon 396 bp upstream with no other intervening methionine codons.

We used the cDNA sequence to search for homologous sequences in the public databases. Initial searches (BLASTN 1.3.11MP) (16) with 209 bp of 3' UTR and 204 bp of C-terminal sequence from the ORF revealed high nucleotide sequence similarity (89% identity in a 68 bp stretch for the former and 92% identity across all 204 bp for the latter) to a clone identified as canine tracheal mucin (17). Subsequent searches (BLASTP 1.4.8MP) (16), with the full length sequence, identified high homology between the protein encoded by this cDNA and a variety of ubiquitin hydrolases (p values ranging between 2.4e–265 and 1.4e–13). Significant homology was also found with the fat facets (faf) gene of Drosophila (p = 4.5e–15). Based on its strong similarity to previously known ubiquitin hydrolases over the entire length of the ORF, we conclude that our cDNA encodes a new member of this family of proteins and we name the gene UHX1 for ubiquitin hydrolase on the X chromosome. A phylogenetic tree and alignments of UHX1 with some of these related proteins in two highly conserved domains, known as the Cys and His domains, are shown in Figure 2.

Tissue distribution of UHX1 expression
We used the inserts from UHX1 clones 2 and 2.11 to probe a Northern blot and detected a single mRNA of approximately 3.3 kb in all tissues examined, with highest levels of expression in retina (Fig. 3).

Mapping of UHX1
We hybridized a UHX1 probe to a somatic cell hybrid mapping panel (Oncor) and localized the UHX1 structural gene to the X chromosome (data not shown). UHX1 PCR primers were then used on an X chromosome regional mapping panel (generously provided by David Ledbetter) and UHX1 was sublocalized to Xp21.2-p11.2 (Fig. 4). Interestingly, another enzyme involved in the ubiquitin system, ubiquitin activating enzyme E1 (UBE1) also maps within this region at Xp11.23 (18, 19). UHX1 was not found on OA TAC 1 or 10, from the OA TL2 region at Xp11.22-p11.21 (20) or on OA TAC 2, 3, or 6 from the OA TL1 region at Xp11.3-p11.23 (18, 20–22) (data not shown).

DISCUSSION
We isolated a novel cDNA from a human retinal library which encodes a protein of 690 amino acids and has strong homology to the family of ubiquitin C-terminal hydrolases. The structural gene encoding this cDNA, designated UHX1, maps to Xp21.2-p11.2. We showed that it is expressed in all tissues examined, with levels 5- to 10-fold higher in retina than elsewhere.
Figure 2. Relationship of UHX1 to select significant matches from the database searches. (A) Phylogenetic tree of UHX1 and other members of the ubiquitin hydrolase family. These are human ubiquitin protease (HSU20657_1 gp:U20657); murine probable ubiquitin carboxyl-terminal hydrolase (UBP_MOUSE sp:P35123); yeast ubiquitin carboxyl-terminal hydrolase 4 (UBP4_YEAST sp:P32571); human probable ubiquitin carboxyl-terminal hydrolase (UBPX_HUMAN sp:P40818); and human ubiquitin carboxyl-terminal hydrolase TRE-2 (TRE2_HUMAN sp:P35125). The length of each pair of branches represents the distance between sequence pairs. The scale beneath the tree measures the distance between sequences. Units indicate the number of substitution events. (B) Alignment of UHX1 with members of the ubiquitin hydrolase family and the Drosophila fat facets (Faf) gene in the conserved Cys and His domains. Analyses in (A) and (B) were done by the Clustal method using MegAlign.

Figure 3. Tissue distribution of UHX1. (A) Northern blot of total RNA from various tissues and cell lines (10 μg RNA/lane) hybridized to a UHX1 probe. (B) The same filter from (A) stripped and rehybridized to a mouse β-tubulin probe to control for loading and RNA quality.

Ubiquitination plays a role in protein degradation and also provides a means of reversible protein modification. At least three lines of evidence suggest that disturbances in protein processing and turnover can lead to retinal degeneration. First, expression of human wild type rhodopsin in transgenic mice at levels approximately five times that of endogenous murine levels leads to retinal degeneration. By contrast, expression of the same transgene at levels comparable to endogenous murine levels does not (23). Second, TIMP3, the gene responsible for Sorsby’s fundus dystrophy, encodes a tissue inhibitor of metalloproteinases which is important in the processing of extracellular matrix proteins (24). Third, deficiency of enzymes involved in post-translational modifications of proteins has been shown to cause retinal disease. The choroideremia gene, CHM or REP, encodes a subunit of geranylgeranyl transferase, an enzyme which attaches isoprenoid groups to Rab proteins (25,26). The gene responsible for infantile neuronal ceroid lipofuscinosis, a neurodegenerative disorder with pigmentary retinopathy, has recently been shown to encode palmitoyl protein thioesterase, an enzyme which removes palmitate moieties from proteins (27). Interestingly, TIMP3 and CHM, like UHX1, are expressed in many tissues but mutations in these genes result solely in an ocular phenotype. These observations suggest that photoreceptors, with their high levels of protein
synthesis and trafficking, are particularly sensitive to disturbances in protein turnover and processing.

There are at least four X-linked retinal diseases which map to a region within or overlapping with the UHX1 interval: two retinitis pigmentosas (RP2 and RP3), a type of congenital stationary night blindness, and a cone dystrophy (28). Recently, two different candidate genes for RP3 have been identified, but definitive proof of either as the RP3 gene has not yet been shown (29,30). Given the importance of correct protein processing to retinal health and the role of the ubiquitin system in protein modification and targeting, UHX1 is a strong candidate gene for these disorders. There are data that suggest deubiquitination of modification and targeting, UHX1 is a strong candidate gene for retinal health and the role of the ubiquitin system in protein overexpressing transgenic mouse model and the latter has been seen in some neurodegenerative disorders (1,32,33). Additionally, the ubiquitin system is involved in the degradation of short lived regulatory proteins (3,4), suggesting it is possible that mutations in UHX1 may prolong the half life of such proteins, thus altering crucial timing of regulatory processes. Yet another possibility is that a reversible modification may no longer be possible. Interestingly, UHX1 has regions of similarity over its entire length to the fat facets gene of Drosophila, which encodes two proteins involved in Drosophila retinal development (34). For these reasons, we are investigating a possible role for UHX1 in retinal disease.

As a last note, some oncoproteins and tumor-suppressor proteins, such as c-Mos, p53 and c-Jun (4), are degraded by ubiquitin-dependent pathways and some members of the ubiquitin hydrolase family have been associated with oncogenesis. The mouse Unp gene encodes a nuclear ubiquitin protease which causes oncogenic transformation of NIH3T3 cells when over-expressed (35,36). Expression of its human ortholog, designated Unph, is elevated in certain lung tumors (37). One transcript of the human tre-2 oncogene encodes a truncated ubiquitin hydrolase which lacks enzyme activity (7) but is tumorigenic when overexpressed in nude mice (38). Papa and colleagues (7) propose that this inactive protein interferes with the function of the normal tre-2 product(s) suggesting that tre-2 may normally have some role as a tumor suppressor. Yang-Feng et al. (39) found frequent LOH on Xp in ovarian cancers and have mapped a common deletion region to Xp21.2-p11.4, a region within the UHX1 interval. Given the activity of the ubiquitin system in growth regulation and the precedence for ubiquitin hydrolases being associated with oncogenesis, the possible involvement of UHX1 in the development of cancer should also be considered.

MATERIALS AND METHODS

Probe preparation

To make total cDNA probes for the differential hybridization screen, total RNA was isolated by guanidinium thiocyanate extraction from cultured human fibroblasts and from 13-line ground squirrel retina (40). First strand cDNA was then reverse transcribed using oligo(dT) as a primer and the second strand was synthesized and labeled using random hexamer primers with incorporation of [32P]dCTP [modified from Sambrook et al. (41)]. To make UHX1 probes for use on the mapping panel and Northern blot and for library screening, inserts from UHX1 plasmid clones were band purified on 1% LMP agarose gels and DNA was extracted using a QiaGen Gel Extraction Kit. The probes were labeled using an Amersham redilPrime Kit. A mouse tubulin control probe for the Northern blot was labeled according to the method of Feinberg and Vogelstein (42).

Differential hybridization screen

Duplicate lifts of arrays of well isolated phage clones from a retinal cDNA library (14) were hybridized (one with human fibroblast probe, one with ground squirrel retina probe) with 10^6 c.p.m./ml of probe in 30% formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate at 42°C overnight. Probes were preannealed prior to hybridization as described (20). Filters were washed in 2x SSC, 1% SDS 3× 20 min at 65°C.

Mapping

A UHX1 probe was hybridized to a somatic cell hybrid panel from Oncor, localizing it to the X chromosome. Once mapped to X, UHX1 was sublocalized utilizing a PCR-based X chromosome mapping panel. (Cell lines: RJK88 (rodent parent), GM10324, GM11173, GM10501, GM11172, GM11174, GM10664 and GM10662A (43-48)). Four sets of PCR primer pairs which give products using human genomic DNA as a template were used: F2,R1; F6,R3; F6,R5; and F1,R4. All reactions were done in 100 µl using 100 ng of template, 120 ng of each primer, 0.5 µl Taq, and 0.2 mM of each dNTP using a Hybaid thermocycler and the following conditions: 1× 7 min at 95°C; 30× 1 min at 60°C, 3 min at 72°C, 1 min at 95°C; 1× 1 min at 60°C, 10 min at 72°C. (Primers: F2: 5′-GAAAGACACCAGCTGCTGTCTC-3′; R1: 5′-ACCTCTG TTTCATGTTGAACC-3′; F6: 5′-GATGTGTTA-TTGAGACCCCTGG-3′; R3: 5′-GGACCACTCAA CACGATA-AGAG-3′; R5: 5′-ACACATGAGCAGACTAAGCC-3′; F1: 5′-ACCTGGACT TCTCTGAGTTTG-3′; R4: 5′-GTGTAAGTG-TCCATACGCCAT-3′).

Northern blot analysis

Ground squirrel retina and human cerebellum fibroblast, retina and retinoblastoma cell line (weri-1, were-27) RNAs were isolated by guanidinium thiocyanate extraction (40). Human kidney and liver RNAs were purchased from Clontech. Northern blotting was performed as described (49) and the filter was probed with a UHX1 probe, stripped and reprobed with a mouse tubulin control probe.

Cloning of UHX1 cDNA

The insert from the original UHX1 (clone 2) phage clone was subcloned into pGEM4 (Promega) by standard techniques. The insert was also used to screen 5x 10^5 phage clones from a human retinal cDNA library (generously provided by Jeremy Nathans). Fifty duplicate positives were picked into 1 ml of SM buffer (41) and 30 were screened by PCR using a reverse primer from the 5′ end of clone 2 (R2: 5′-GGTGAGTAGA TGAAGAATCTCCG-3′) and a Agt10 phage arm primer. Reactions were done in 100 µl with 5 µl of phage in SM buffer as template, 120 ng of each primer, 0.5 µl Taq and 0.2 mM of each dNTP using a Hybaid
thermocycler and the following conditions: 1x 7 min at 95°C; 30x 1 min at 60°C, 5 min at 72°C, 1 min at 95°C; 1x 1 min at 60°C, 10 min at 72°C. The three phage clones yielding the longest PCR products were then plaque purified by subsequent rounds of screening. The insert from the longest of these, clone 2.11, was subcloned into pGEM4 (Promega) by standard techniques.

Sequencing

Inserts in pGEM4 were sequenced using a Sequenase (U.S. Biochemicals, Inc.) kit (50).

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