Expression of a Copper-Containing Amine Oxidase by Human Ciliary Body

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Purpose: To examine the molecular structure and ultrastructural distribution of a novel amine oxidase in human ciliary body.

Methods: Human ciliary bodies were solubilized with a nonionic detergent. The solubilized material was subjected to affinity chromatography with 2B4.14.1, a monoclonal antibody which recognizes a family of ciliary body glycoproteins. Proteins eluted from the affinity column were further separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Peptides produced from a 2B4.14.1-reactive protein with an approximate molecular weight of 100 kDa were analyzed by Edman degradation. The protein thus identified was further examined by Western blotting and immunoelectron microscopy with anti-peptide antisera.

Results: Peptide sequences from the 100 kDa ciliary body protein were identical to the predicted protein sequence of an amine oxidase identified recently in a human placental cDNA library. The identity of the ciliary body protein was confirmed by Western blotting with rabbit antiserum generated against the predicted carboxy-terminal peptide of human placenta amine oxidase. Western blotting under nonreducing conditions and following glycosidase digestion indicated that the native enzyme is a disulfide-linked homodimer with multiple N-linked oligosaccharide side chains. By immunoelectron microscopy, the ciliary body amine oxidase was localized to the plasma membranes of inner epithelial cells.

Conclusions: Human placenta amine oxidase is present on the plasma membranes of ciliary body inner epithelial cells. This finding provides a potential explanation for amine oxidase enzyme activity detected in previous studies of anterior segment tissues. Though the functional role of human placenta amine oxidase in the eye is unclear, it may contribute to the production of H2O2 in aqueous humor.

Amine oxidases are a diverse group of enzymes that catalyze the conversion of biogenic amines to corresponding aldehydes by the following reaction: RCH2 NH2 + O2 + H2O -> RCHO + NH3 + H2O2. They are distributed widely in both neural and non-neural tissues [1,2], and are found in a range of organisms including prokaryotes and eukaryotes [1]. A major function of amine oxidases is the catabolism of amine neurotransmitters such as norepinephrine, serotonin, and dopamine [2]. They may also act as scavengers for potentially toxic biogenic or exogenous amines [3,4].

Two categories of amine oxidases with a minimal degree of sequence similarity have been identified. The first, designated EC 1.4.3.4, are flavoproteins. This group includes a well-characterized pair of enzymes, monoamine oxidase (MAO) A and MAO B, which are found in a variety of locations within the nervous system and visceral organs. The two enzymes catalyze similar reactions, but differ in their relative affinities for various substrates and inhibitors [2]. The cDNA sequences for human MAO A and MAO B have been published, and have approximately 70% identity [2]. At the subcellular level, both enzymes have been localized to the outer membranes of mitochondria [2].

The second class of amine oxidases, designated EC 1.4.3.6, includes copper-containing enzymes with a covalently-linked, tyrosine-derived cofactor termed “topa quinone” [1,5]. These enzymes have been described variously as “copper-containing amine oxidases,” “semicarbazide-sensitive amine oxidases,” and “benzylamine oxidases” [1, 5]. Though enzymes of the EC 1.4.3.6 class generally appear to share all of these properties, we shall hereafter refer to amine oxidases with copper-binding motifs as assessed by sequencing studies as “copper-containing” and enzymes detected by functional assays using semicarbazide as an inhibitor as “semicarbazide-sensitive.” Members of the group, whose distribution, function, and ultrastructural localization are incompletely understood, include bovine serum amine oxidase [6] and a diamine oxidase found in human kidney [7]. The cDNA for a human amine oxidase whose sequence exhibits 84% identity with that of bovine serum amine oxidase has been isolated recently from a human placental cDNA library [5].

Amine oxidases have been detected in ocular tissues, including the ciliary body, by both enzyme assays [8-13] and histochemical techniques [14,15]. Studies with selective enzyme inhibitors have suggested the presence of both MAO A and MAO B in ciliary epithelium [9,12]. “Semicarbazide-sensitive” amine oxidase activity has also been reported in anterior segment tissues [11-13]. Purification and
characterization of specific amine oxidases from ciliary body tissue has not been described, however.

We have recently isolated a group of high molecular weight glycoproteins from human ciliary body by affinity chromatography with the monoclonal antibody 2B4.14.1 [16]. By peptide sequencing and Western blotting with anti-peptide antisera, we have established the identity of one of the 2B4.14.1-reactive proteins as the copper-containing human placenta amine oxidase [5]. In addition, we have obtained information about the subunit structure, glycosylation, and ultrastructural localization of the protein.

**METHODS**

**Tissues**— Human eyes were obtained from the Durham branch of the North Carolina Eye Bank Association; donors ranged in age from 50 to 88 years. All studies were performed in accordance with the tenets of the Declaration of Helsinki and with approval of the responsible institutional human experimentation committee. Eyes were kept on ice on saline-moistened gauze until use, generally within 24 to 48 hours of the donor’s death. Ciliary bodies were isolated from the eyes by a combination of blunt and sharp dissection as previously described [16] and stored at -70 °C until use.

**Antibodies**— 2B4.14.1, a mouse IgM[kappa] monoclonal antibody, was produced by standard methods using human corneal endothelium as an immunogen [17]. 2B4.14.1 reacts with both corneal endothelium and ciliary body epithelium as assessed by immunohistochemical staining and immunoelectron microscopy [16,17].

Rabbit antiserum was generated against a 15-amino-acid synthetic peptide constituting the predicted carboxy-terminus of human placenta amine oxidase (APDLPASFHGGFSHN; amino acids 749-763) [5]. Peptide was purchased from BioSynthesis, Inc. (Lewisville, TX), and coupled to ovalbumin as a carrier using glutaraldehyde [18]. Rabbits were immunized with peptide-carrier emulsified in complete Freund’s adjuvant, followed by two boosts at four-week intervals with peptide-carrier in incomplete Freund’s adjuvant; serum was obtained two weeks after the final boost.

**Isolation and sequencing of 2B4.14.1 ligands**— Twenty human ciliary bodies were solubilized in 200 ml of 1% polyoxyethylene 9 lauryl ether (C12 E 9; Sigma Chemical Co., St. Louis, MO) in Tris-buffered saline (TBS), pH 8.0, with the aid of a mechanical tissue homogenizer. Insoluble residual material was removed by two sequential 30-min centrifugations at 10,000 g. The supernatant was applied to an affinity column of 2B4.14.1 coupled to Affi-Prep Hz (Bio-Rad Laboratories, Richmond, CA) as previously described [16]. A precolumn consisting of an isotype-matched control antibody coupled to Affi-Prep Hz was employed to eliminate proteins capable of nonspecific interaction with IgM. The 2B4.14.1 column was washed extensively with 0.1% C12 E9 in TBS, pH 8.0, and bound antigens were eluted with 0.1 M Tris, pH 11. The eluate was neutralized by addition of 1N acetic acid and precipitated by addition of 10 volumes excess of ice-cold absolute ethanol.

The ethanol precipitate was dried, redissolved in 50 mM N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES), 150 mM NaCl, pH 7.5, mixed with sample buffer containing dithiothreitol (DTT), and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 5-15% gradient gel. The upper reservoir buffer contained 2.5 mg Coomassie blue/100 ml to facilitate detection of the proteins. Following SDS-PAGE, the proteins were transferred to a polyvinylidene-fluoride-based membrane (CD-membrane, Millipore, Bedford, MA). Major bands of 80, 100, and 130 kDa were apparent. The same bands are visible in a small portion of the sample subjected to SDS-PAGE in parallel and visualized using a standard silver staining procedure [19] (Figure 1, lane A).

The portion of the membrane containing the 100 kDa band was excised and suspended in 200 μl of 2 M acetic acid. 0.4 mg of porcine trypsin (Sigma Chemical Co.) was added, and the reaction was continued for 4 hr at 37 °C [20]. The resulting peptides were separated on an Aquapore RP-300 reverse-phase column (Brownlee Labs, Perkin Elmer, Norwalk, CT) connected to a 130A high-performance liquid chromatography system (Applied Biosystems, Foster City, CA). The peptides were detected at 214 nm and collected manually. The purified...
peptides were subjected to automated Edman degradation using an Applied Biosystems 477A protein sequencer with on-line phenylthiohydantoin analysis.

**Glycosidase digestion**— Aliquots of ethanol-precipitated human ciliary body proteins were dried and resuspended in 0.1% SDS, 10 mM DTT in TBS, pH 8.0. Nonidet P-40 (Sigma Chemical Co.) was added to a final concentration of 1%. A mixture of endoglycosidase F/N-glycosidase F (Sigma Chemical Co.) was then added, followed by a 1-hr incubation at 37 °C; a control sample was treated identically with the exception that the glycosidase was omitted. Following digestion, the samples were repurified with ethanol.

**Western blotting**— Aliquots of detergent-solubilized whole human ciliary body (20 µg protein) were subjected to SDS-PAGE, with or without prior reduction and/or glycosidase digestion, and transferred to polyvinylidene-fluoride-based membranes as described above. Following incubation with a milk-powder-based blocking solution, the blots were stained with rabbit anti-peptide antiserum used at a dilution of 1:500; preimmune serum served as a negative control. Binding was assessed using horseradish-peroxidase-conjugated secondary antibody and a chemiluminescent detection system (ECL Western blotting analysis system, Amersham Corp., Arlington Heights, IL).

**Immunoelectron microscopy**— Fresh human ciliary body tissue was fixed for 2 hr in 2% paraformaldehyde in piperazine-N,N’-bis[2-ethanesulfonic acid] (PIPES) buffer, pH 7.4, cryoprotected in 2.1 M sucrose in phosphate-buffered saline (PBS), pH 7.4, snap frozen in liquid nitrogen, and stored in the vapor phase of liquid nitrogen. Ultrathin sections were produced and placed on carbon and Formvar-coated grids as previously described [16]. The grids were floated on PBS containing 5% fetal bovine serum for 10 min; washed successively in PBS, 100 mM NH4Cl in PBS, and PBS; then placed on drops containing a 1:500 dilution of rabbit anti-peptide antiserum or preimmune rabbit serum. Following a 1 hr incubation at 25 °C, the grids were washed 6 times with PBS, then incubated for 30 min at 25 °C with a 1:10 dilution of 10-nm colloidal gold-conjugated goat-anti-rabbit IgG (Janssen Biochimica; supplied by Amersham Corp.). The grids were then washed and treated with methyl cellulose containing uranyl acetate as previously described [16]. Grids were picked up in wire loops, drained on filter paper, allowed to air-dry, and examined using a Philips EM300 electron microscope (FEI-Philips, Hillsboro, OR).

**RESULTS**

**Peptide sequencing**— Unambiguous sequences were obtained for four peptides from the 100-kDa ciliary body protein isolated by affinity chromatography with 2B4.14.1 (Table 1). All four were identical to the predicted protein sequence of human placenta amine oxidase [5].

**Biochemical characterization of human placenta amine oxidase**— To confirm the identity of the 100-kDa protein as human placenta amine oxidase, rabbit antiserum raised against a synthetic peptide from the predicted carboxy-terminus of the protein was used to probe Western blots of proteins from whole, detergent-solubilized human ciliary bodies. In blots of proteins reduced prior to SDS-PAGE, the antiserum reacted strongly with a 100-kDa band (Figure 2, lane B). In samples subjected to SDS-PAGE without prior reduction, the antiserum detected a somewhat diffuse band of approximately 200 kDa (Figure 2, lane A).

Removal of N-linked oligosaccharides from reduced human ciliary body proteins by treatment with endoglycosidase F and N-glycosidase F prior to SDS-PAGE lowered the apparent molecular weight of the main band detected by the antiserum from 100 kDa to approximately 80 kDa (Figure 2, lane D). No shift was detected in a reduced, mock-digested sample (Figure 2, lane C). Glycosidase digestion of proteins isolated from human ciliary body by affinity chromatography with 2B4.14.1 led to similar downward shifts in the electrophoretic mobility of all of the major 2B4.14.1 ligands as assessed by silver staining (Figure 1, lane B).

Similar Western blotting results were obtained with a rabbit antiserum raised against a peptide near the predicted amino terminus of human placenta amine oxidase (DGEGPSQLPHC, amino acids 31-41). In all experiments, no significant staining was seen in control blots produced in parallel and stained with preimmune serum (data not shown).

**Localization of human placenta amine oxidase in ciliary body**— Immunoelectron microscopy of human ciliary body proteins with rabbit antiserum raised against the predicted carboxy-terminal sequence of human placenta amine oxidase revealed strong reactivity with the plasma membranes of inner epithelial cells (Figure 3). A small amount of staining was also seen on the surfaces of outer (pigment) epithelial cells, epithelial cell cytoplasm (Figure 3), cytoplasmic organelles, and nuclei, but this did not exceed the background staining observed when preimmune serum was substituted for the anti-peptide serum (data not shown).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Position in predicted sequence of human placenta amine oxidase</th>
</tr>
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<tbody>
<tr>
<td>YLDID</td>
<td>176-180</td>
</tr>
<tr>
<td>CVFEQNQGL</td>
<td>430-438</td>
</tr>
<tr>
<td>FLRPYNF</td>
<td>704-710</td>
</tr>
<tr>
<td>FDEDPFSF</td>
<td>711-717</td>
</tr>
</tbody>
</table>

Sequences of peptides produced from the 100-kDa protein isolated from human ciliary body by affinity chromatography with 2B4.14.1 are shown in the left-hand column. All four peptides are identical to the predicted sequence of human placenta amine oxidase; the positions of the peptides in the predicted sequence are provided in the right-hand column.
DISCUSSION

We have identified a 100-kDa glycoprotein from human ciliary body, isolated by affinity chromatography with the monoclonal antibody 2B4.14.1, as a copper-containing amine oxidase recently identified in a cDNA library from human placenta. To our knowledge, this is the first definitive identification of a specific amine oxidase protein in ciliary body, and the first demonstration of human placenta amine oxidase in any tissue at the protein level.

Digestion of the 100-kDa ciliary body glycoprotein with glycosidases specific for N-linked oligosaccharides decreases its apparent molecular weight to approximately 80 kDa; the molecular weight of the deglycosylated protein is in good agreement with the predicted molecular weight of the human placenta amine oxidase monomer (82,525 D, assuming cleavage of a putative 19-amino-acid signal peptide) [5]. The presence of N-linked oligosaccharide side chains on 2B4.14.1 ligands was not unexpected, since such oligosaccharides are known to form an integral part of the 2B4.14.1 binding site [16]. The predicted protein sequence of human placenta amine oxidase contains six potential N-glycosylation sites [5], and other copper-containing amine oxidases are known to be glycosylated [1].

Under non-reducing conditions, human placenta amine oxidase in ciliary body has an apparent molecular weight of approximately 200 kDa, suggesting that the native enzyme is a disulfide-linked dimer. This finding is consistent with previous reports that other copper-containing amine oxidases are disulfide-linked homodimers [1,21].

Human placenta amine oxidase is localized primarily to the plasma membranes of ciliary body inner epithelial cells as assessed by immunoelectron microscopy with rabbit antiserum raised against the carboxy-terminal peptide of the enzyme; similar reactivity was observed in previous experiments with 2B4.14.1 [16]. The association of a copper-containing amine oxidase with the plasma membrane was studied using an antibody raised against the carboxy-terminal peptide of human placenta amine oxidase. The antibody specifically recognizes the protein in the plasma membrane of ciliary body inner epithelial cells.

Figure 2. Western blot of human ciliary body proteins with rabbit antiserum against carboxy-terminal peptide of human placenta amine oxidase. Whole human ciliary body tissue was detergent-solubilized, subjected to SDS-PAGE, and analyzed by Western blotting with rabbit antiserum against the carboxy-terminal peptide of human placenta amine oxidase. Lane A, non-reduced sample. Lane B, reduced sample. Lane C, reduced sample mock-digested without glycosidase. Lane D, reduced sample digested with mixture of endoglycosidase F/N-glycosidase F. The faint bands at approximately 200 kDa in lanes B-D most likely represent a small amount of residual unreduced dimer (or dimers joined by linkages other than disulfide bonds). The lower molecular weight bands in lanes B-D may represent proteolytic degradation products; partial proteolytic degradation may also explain the molecular weight heterogeneity of the band in lane A. The antisera does not crossreact with the flavoprotein amine oxidases MAO A and B; no bands of appropriate molecular weight for these enzymes (approximately 60 kDa for monomers, 120 kDa for dimers) are present in the blot.

Figure 3. Immunoelectron micrograph of human ciliary body stained with rabbit antiserum against carboxy-terminal peptide of human placenta amine oxidase. Sections of human ciliary body produced by ultracytomicrotomy were stained with rabbit antiserum against the carboxy-terminal peptide of the enzyme; similar reactivity was observed in previous experiments with 2B4.14.1 [16]. The association of a copper-containing amine oxidase with the plasma membrane was studied using an antibody raised against the carboxy-terminal peptide of human placenta amine oxidase. The antibody specifically recognizes the protein in the plasma membrane of ciliary body inner epithelial cells.
oxidase with plasma membranes is not unexpected; other enzymes in this family, including bovine serum amine oxidase [6] and kidney diamine oxidase [21], are extracellular enzymes. The latter protein binds to cell surfaces via an interaction with heparin [21]; inspection of the published sequence for human placenta amine oxidase reveals no obvious heparin-binding sequences, however.

In addition to binding to plasma membrane surfaces, 2B4.14.1 also reacts strongly with the surfaces of pigment granules in ciliary body outer epithelium [16]. No significant reactivity of antiserum to human placenta amine oxidase peptide was seen in this location. Presumably, 2B4.14.1 ligands other than human placenta amine oxidase are associated with the pigment granules.

The distribution and function of amine oxidases in the eye are incompletely characterized. Monoamine oxidases A and B have been identified in ciliary body, iris, retina, choroid, and optic nerve by enzyme assay of solubilized tissue with selective substrates and inhibitors [9,12]. Topical application of selective inhibitors of both MAO A [22] and MAO B [8,9] is capable of lowering intraocular pressure, presumably by potentiating the effects of sympathetic stimulation. Patients with Norrie disease, an X-linked heritable disorder in which the genes encoding both MAO A and B are lost via deletion [23], exhibit a wide range of developmental and degenerative changes of both the anterior and posterior segments, and are generally blind at birth or become so shortly thereafter [24].

Semicarbazide-sensitive amine oxidases have also been localized to the eye by a variety of direct and indirect evidence. Semicarbazide-sensitive or benzylamine oxidase activity has been reported in bovine iris, retina, choroid, and optic nerve [12,13] and rat retina, sclera [25], and cornea [11]. The cDNA for a human retina-specific amine oxidase has recently been cloned [26]. The retinal enzyme and human placenta amine oxidase are clearly distinct, but their predicted protein sequences have a 57% degree of similarity.

The natural substrates and functions of semicarbazide-sensitive amine oxidases in general, and human placenta amine oxidase in particular, are largely unknown. Semicarbazide-sensitive amine oxidases whose enzyme activity has been studied to date include both monoamine and diamine oxidases. Based on sequence comparisons, human placenta amine oxidase is predicted to belong to the former group [5]. Patients with Norrie disease, who lack MAO A and B, have marked abnormalities in oxidatively deaminated metabolites of norepinephrine (an MAO A substrate), but normal levels of deaminated dopamine and serotonin metabolites, suggesting that the latter substances may be catabolized at least in part by semicarbazide-sensitive amine oxidases [27]. Semicarbazide-sensitive amine oxidase activity has been detected in both bovine retina and iris using the synthetic substrate benzylamine, but only the retinal enzyme has detectable activity toward dopamine [13]. The two different enzyme activities identified in this study may represent bovine versions of retina-specific amine oxidase and placenta amine oxidase.

Other functions that have been suggested for semicarbazide-sensitive amine oxidases include deamination of potentially toxic amines such as methylamine [4] and regulation of cell growth by catabolism of polyamines such as putrescine [28]. In the latter process, H$_2$O$_2$ and/or aldehydes generated as products of the amine oxidase reaction are thought to inhibit protein and DNA synthesis and ultimately elicit programmed cell death. Growth regulation by amine oxidase products has been postulated to play a role in the control of embryogenesis [28]. This possibility is especially intriguing for an amine oxidase indigenous to the placenta, but its potential relevance to the adult eye is unclear. Increased levels of benzylamine oxidase activity have also been associated with corneal angiogenesis [11].

The production of H$_2$O$_2$ by amine oxidases is of particular interest because of the documented presence of H$_2$O$_2$ in aqueous humor [29-31]. H$_2$O$_2$-mediated oxidative damage has been implicated as a possible causative factor for cataracts [30,32], decreases in aqueous outflow facility [33], and damage to the corneal endothelium [34]. Stimulation of Na-K ATPase activity in nonpigmented ciliary epithelial cells by H$_2$O$_2$ has also been reported recently [35]. Though much of the H$_2$O$_2$ in aqueous humor may be generated by non-enzymatic reactions [29,31], the possible contribution of enzymes such as amine oxidases cannot be excluded, particularly in the microenvironment immediately surrounding the ciliary body epithelium.

In summary, we have identified a novel amine oxidase, human placenta amine oxidase, on the surfaces of human ciliary body epithelial cells. This enzyme provides a potential explanation for semicarbazide-sensitive amine oxidase activity detected in previous experiments in anterior segment tissues, and may contribute to the production of H$_2$O$_2$ in aqueous humor. A related but distinct enzyme is present in human retina, suggesting that copper-containing/semicarbazide-sensitive amine oxidases may play a role in the development and/or function of both posterior and anterior segments.

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