The lysosomal enzyme, β-hexosaminidase, exists as two major isoforms; HexA and HexB. HexA is an αβ-subunit heterodimer and HexB a β-subunit homodimer. Both isoforms can remove nonreducing β-N-acetyl-D-glucosamine residues, whereas HexA hydrolyzes charged substrates as G\textsubscript{M2} gangliosides as well. β-Hexosaminidase is present in both human and rabbit tear fluid and is secreted from rabbit lacrimal gland acinar cells in primary culture on stimulation with secretagogues. To further characterize the enzyme, the α- and β-subunit mRNA expression was explored in rabbit lacrimal gland tissue as well as in cultured cells. Possible correlation between mRNA expression and HexA specific enzymatic activity was also investigated. Because existing β-hexosaminidase antibodies are unable to recognize the rabbit enzyme, cloning and sequencing of the α- and β-subunits were performed. Sequencing of the these subunits indicate that both are highly conserved between human, mouse, and rabbit. In contrast to the β-subunit, showing an even mRNA expression between tissue and cultured cells, the level of α-subunit expression was higher in cultured acinar cells compared to tissue, with no alteration after cell stimulation. A minor but significant increase in total β-hexosaminidase as well as HexA activity was observed in cultured cells compared to tissue. Enzymatic activity assays also revealed that HexA is the dominating isofrom of β-hexosaminidase in lacrimal gland and cultured acinar cells.

Key words: enzyme activity/expression/β-hexosaminidase/ lacrimal gland/sequencing

Introduction

The lysosomal hydrolase β-hexosaminidase is present in human tears and rabbit lacrimal gland fluid, though there is still little knowledge about the physiological function of the enzyme at the ocular surface (Gierow et al., 1997; Van Haeringen and Glasius, 1976). β-Hexosaminidase is formed by the assembly of two noncovalently linked structurally similar subunits, α and β. Dimerization gives rise to three isoenzymes; HexA (αβ), HexB (ββ), and HexS (αα). Individual subunits are initially translated into precursor, prepro-polypeptides (later referred to as α- and β-subunits).

As in the biosynthesis of other lysosomal enzymes, signal peptide cleavage, glycosylation of specific Asn-X-Ser/Thr sites, and oligomerization takes place in the endoplasmic reticulum. In the Golgi compartments, certain mannose residues are phosphorylated, enabling recognition by mannose-6-phosphate receptors. Final maturation into Hex isozymes by proteolytic processing events occurs in the lysosome, where the internal polypeptides of the α-subunit (α\textsubscript{a}α\textsubscript{m}) and the β-subunit (β\textsubscript{b}β\textsubscript{m}) are linked together by disulfide bonds (Mahuran, 1995; Proia et al., 1984).

β-Hexosaminidases are responsible for the degradation in acidic lysosomal compartments of glycoconjugated substrates derived from plasma membrane endocytosis. Because each subunit exhibits a different substrate specificity and catalytic activity, HexA is the only isoenzyme able to hydrolyze all known β-hexosaminidase substrates—G\textsubscript{M2} ganglioside, β-N-acetylglucosaminides, β-N-acetylgalactosaminides, as well as 6-sulfated β-N-acetylgalactosaminides. The enzymatic degradation of G\textsubscript{M2} ganglioside by HexA requires the assistance of the GM2 activator protein functioning as a cofactor in the reaction (Meier et al., 1991). The B isozyme has no catalytic activity toward G\textsubscript{M2} gangliosides and sulfated compounds (Kytzia and Sandhoff, 1985). The physiological significance of HexS has been questioned because only small amounts have been detected in human tissue, but recent studies have demonstrated that HexS is active on a wide range of compounds also hydrolyzed by HexA (Hepbildikler et al., 2002).

The increased knowledge about β-hexosaminidase is to a great extent due to studies of the α- (HexA) and β-subunit (HEXB) genes in recent years. The nucleotide and deduced amino acid sequences that are of similar length in both subunits have been determined for human and mouse (Bapat et al., 1988; Beccari et al., 1992; Myerowitz et al., 1985; O’Dowd et al., 1988). The establishment of HEXA and HEXB gene organization also enabled scientists to learn more about the catalytic activity. Recent presentations of the crystal structure of HexB and of the GM2 activator protein have provided additional information about the hydrolytic mechanism for all isoenzymes (Mark et al., 2003; Wright et al., 2000). Of even greater importance were perhaps the identification of the genetic defects causing the inherited lysosomal storage disorders Tay-Sachs (Myerowitz and Hogikyan, 1986) and Sandhoff diseases (O’Dowd et al., 1986). Tay-Sachs is caused by mutations in the HEXA gene on chromosome 15; Sandhoff’s disease is due to mutations in the gene coding for HEXB. Defects in either gene, results in accumulation of the normal substrate.
for HexA, \( \text{G}_{\text{M2}} \) ganglioside, in cells. The compound is particularly abundant in nerve tissue, resulting in a progressive neurodegeneration. Rarely, \( \text{G}_{\text{M2}} \) gangliosidosis can also be caused by mutations in the \( \text{G}_{\text{M2}} \) activator protein gene, called the AB variant form (Mahuran, 1999).

Studies of \( \beta \)-hexosaminidase in the lacrimal gland were initially performed in belief that the enzyme would function as a lysosomal marker in membrane trafficking experiments. It was reported that \( \beta \)-hexosaminidase activity showed a higher steady-state content in endoplasmic reticulum and the Golgi compartments, compared with the lysosomal enzyme cathepsin B (Gierow et al., 1996; Hamm-Alvarez et al., 1997). \( \beta \)-Hexosaminidase also shows the highest catalytic activity compared with the hydrolases, \( \beta \)-glucuronidase, \( \alpha \)-sulfatase, and \( \alpha \)- and \( \beta \)-galactosidase as well as \( \alpha \)-fucosidase measured in lacrimal gland acinar cell fluid (Gierow et al., 2001; Sjögren et al., 2000), demonstrating the importance of exploring the role of \( \beta \)-hexosaminidase in tear fluid. Today, the catalytic activity of \( \beta \)-hexosaminidase commonly serves as a marker of regulated secretion in studies of rabbit lacrimal gland acinar cells in primary culture (Gierow and Mircheff, 1998; Hamm-Alvarez et al., 1997; Yang et al., 1999). Secretion from lacrimal acinar cells is regulated through several signaling pathways, including both \( \Pi \)-DAG and cAMP formation (Hodges and Dartt, 2003). Stimulation of both pathways simultaneously triggers a maximal secretory response from cultured acinar cells (Gierow et al., 1995). \( \beta \)-Hexosaminidase release parallels total protein secreted by stimulation of these pathways (Gierow et al., 1997).

The purpose of the present study was to further characterize the enzyme \( \beta \)-hexosaminidase in rabbit lacrimal gland. Currently, there are no antibodies available that recognizes rabbit \( \beta \)-hexosaminidases, which would have been a useful tool in intracellular trafficking studies. To elucidate any variations at the DNA–protein level, in different species, the nucleotide sequences for rabbit \( \beta \)-hexosaminidase \( \alpha \)- and \( \beta \)-subunits were determined, showing high identity with human and mouse sequences. Northern blot analysis demonstrates an up-regulated \( \alpha \)-subunit expression in cultured acinar cells compared with lacrimal gland tissue, which is not detected for the \( \beta \)-subunit. Enzymatic studies showed a significantly higher enzymatic \( \beta \)-hexosaminidase activity in cellular extracts, and determination of the HexA and HexB proportions revealed that HexA is the major active \( \beta \)-hexosaminidase isoform in cells and lacrimal gland tissue.

Results

Expression of \( \beta \)-hexosaminidase \( \alpha \)- and \( \beta \)-subunits

The \( \beta \)-hexosaminidase \( \alpha \)-subunit specific RNA probe detected a single transcript around 2.2 kb, which is in agreement with the reported human \( \alpha \)-subunit mRNA size (Myerowitz et al., 1985). The autoradiogram in Figure 1A shows a significantly higher mRNA expression of the \( \beta \)-hexosaminidase \( \alpha \)-subunit in cultured acinar cells compared with tissue sample. Optical density quantifications (Figure 1B), where lacrimal gland tissue expression was set to 1.00, confirm an average fourfold increased expression in

![Fig. 1. Up-regulation of \( \alpha \)-subunit expression in primary cultured acinar cells.](https://academic.oup.com/glycob/article-abstract/15/3/211/574719)
both resting (4.44 ± 0.12, n = 3) and stimulated (4.05 ± 1.66, n = 3) cultured acinar cells relative to lacrimal gland tissue. However, the increase in secretion observed after 1 h maximal cellular stimulation with carbachol and vasoactive intestinal peptide (VIP) (Figure 1C) does not induce any change at the mRNA level of the β-hexosaminidase α-subunit expression. The β-hexosaminidase β-subunit probe detected a 2.0-kb single transcript (Figure 2A) in size similar to that previously reported in mouse (Bapat et al., 1988). Neither the acinar cell culturing event nor the stimulated secretion induces any significant alterations in the expression pattern of the β-subunit, as pointed out by densitometric data in Figure 2B.

β-Hexosaminidase isoenzyme activity

HexA appears to be the predominant β-hexosaminidase isoenzyme activity in lacrimal gland tissue as well as in cultured acinar cells. Thermal inactivation of HexA (Martino et al., 2002) reduces the activity for both 4MUGlcNAc and 4MUGlcNAc6SO4 substrates by, in average, 93% in both tissue and cells (Table I). Both the total β-hexosaminidase and HexA activity had a smaller but significant higher specific activity in cellular extracts compared to tissue (Figure 3). The discrepancy in height of the bars for total β-hexosaminidase and HexA enzymatic activity, shown in Figure 3, reflects differences in specific catalytic activity of HexA for the two substrates. In correlation with mRNA expression, there is no

Table I. Percentage HexA activity in LG tissue, untreated and stimulated cultured acinar cells in relation to total β-hexosaminidase activity

<table>
<thead>
<tr>
<th>Samples</th>
<th>4MUGlcNAc (nmol/min/mg protein)</th>
<th>4MUGlcNAc6SO4 (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LG</td>
<td>94.6% ± 5.8</td>
<td>97.4% ± 1.8</td>
</tr>
<tr>
<td>Con</td>
<td>91.3% ± 4.9</td>
<td>91.9% ± 1.5</td>
</tr>
<tr>
<td>Cch + VIP</td>
<td>93.4% ± 2.5</td>
<td>91.4% ± 1.3</td>
</tr>
</tbody>
</table>

Level of HexA was determined by the percentage decrease in enzymatic activity after heating for substrates 4MUGlcNAc and 4MUGlcNAc6SO4.

Fig. 2. β-Subunit mRNA expression. (A) Northern blot analysis of the β-hexosaminidase β-subunit in lacrimal tissue and acinar cells. Equal amounts of total RNA (15 µg) extracted from rabbit lacrimal gland tissue (LG) and primary cultured cells incubated for 1 h in the absence (Con) or presence of carbachol 100 mM and VIP 0.1 mM (Cch + VIP), were loaded in each lane. The autoradiogram shown on top was exposed 6 days at −80°C with intensifying screen. Ethidium bromide fluorescence shows total RNA loaded on a denaturating formaldehyde-agarose gel. Data representative from results of three independent experiments are shown. (B) Graph representing relative mRNA expression of the β-hexosaminidase β-subunit. Accumulated densitometric data is presented in comparison to lacrimal gland tissue, set to 1.0 with error bars indicating SEM.

Fig. 3. Determination of β-hexosaminidase enzymatic activities. Total β-hexosaminidase and HexA activities in lacrimal gland tissue homogenate (LG), cellular extracts of untreated acinar cells (Con), and stimulated cells (see Figure 1 for details) (Cch + VIP), were determined using 4MUGlcNAc and 4MUGlcNAc6SO4 substrates, respectively. Enzyme activity is expressed as nmol released 4-methylumbelliferone/min/mg protein. To evaluate the proportions of HexA and HexB, the HexA isoenzyme was inactivated by heating samples for 1 h at 52°C (H). Residual enzymatic activity after heating represents that of HexB. Data represent the average of three preparations with error bars showing SEM; asterisk indicates statistically significant (p < 0.05) difference in enzymatic activity compared with LG.
significant difference in activities between untreated and stimulated cells.

**β-Hexosaminidase α-subunit sequence**

Alignment of overlapping sequences from the 3’-rapid amplification of cDNA ends (RACE), 5’-RACE as well as random hexamer amplified cDNA resulted in a 1641-bp nucleotide sequence specific for the β-hexosaminidase α-subunit. The determined rabbit α-subunit sequence, submitted to GenBank with accession number AY629243, showed 81% and 78% identity with the corresponding α-subunit sequence of human (Korneluk et al., 1986; Myerowitz et al., 1985) and mouse (Beccari et al., 1992) (data not shown). The rabbit sequence includes a suggested open reading frame coding for 490 amino acids. The translated peptide exhibited 88% and 85% identity with human and mouse α-subunit peptides, respectively (Figure 4). Unfortunately, the cDNA synthesis by the 5’-RACE method had stopped prior to the ATG start codon in the 5’ end, revealing no information about a possible signal peptide sequence. Comparing published nucleotide sequences for the mouse and human α-subunit of β-hexosaminidase indicate that an ~120-bp 5’ end fragment still needs to be determined to obtain the full-length sequence. Using the human α-subunit peptide sequence as a framework for analysis; the obtained rabbit sequence included most of the αp-chain and all of the αm-chain (Figure 4). The amino acid alignment with human and mouse α-subunit peptide sequences displayed that the three human sites for glycosylation (Weitz et al., 1992), at Asn115, Asn157, and Asn294, were completely conserved between all three species.

**β-Hexosaminidase β-subunit sequence**

Alignment of the overlapping sequences from 3’-RACE as well as random hexamer amplified cDNA resulted in a 1411-bp nucleotide sequence specific for the β-hexosaminidase β-subunit. The determined rabbit β-subunit sequence, submitted to GenBank with accession number AY629244, showed 83% and 78% identity with the corresponding length of human (O'Dowd et al., 1985) and mouse (Bapat et al., 1988) sequences, respectively (data not shown). The rabbit sequence includes a suggested open reading frame coding for 424 amino acids. The translated peptide exhibited 78% and 76% identity with human and mouse β-subunits (Figure 5). By using the human β-subunit amino acid sequence as a framework for analysis, the obtained sequence included most of the βp-chain, the whole βm-chain, and two of the four glycosylation sites. Due to absence of 5’ end sequence data for rabbit, no information about the first glycosylation site in human is available. The amino acid alignment shows that the third and fourth human site for glycosylation (Sonderfeld-Fresko et al., 1985) were conserved between the three species.

**Fig. 4.** Multiple alignment of the amino acid sequences for part of the rabbit β-hexosaminidase α-subunit and full-length coding sequences for human and mouse α-subunits. The signal peptide sequences are underlined and are in italics. Sequences representing the αp chains are surrounded by square brackets and sequences corresponding to the αm chains are enclosed by normal brackets. Human and mouse sequences are from GenBank accession number NM000520 (Korneluk et al., 1986; Myerowitz et al., 1985) and GenBank accession number NM010421 (Beccari et al., 1992), respectively. Amino acids removed during posttranslational processing are in italics. The possible N-linked glycosylation sites are underscored, and the preferred phosphorylation site is in boldface and underscored. Identical amino acids are marked with an asterisk. Strongly similar amino acids are marked with a colon (:) and weakly similar amino acids are marked with a dot (**).
and Proia, 1989) at Asn\(^{190}\) and Asn\(^{327}\) were completely conserved between rabbit, human, and mouse, where Asn\(^{327}\) also is the suggested site for phosphorylation of the \(\beta\)-subunit (Figure 5). In spite of the fact that the overall peptide sequence is highly conserved between all three species, the rabbit and mouse \(\beta\)-subunits apparently lack the site corresponding to the second human glycosylation site at Asn\(^{142}\). In addition the rabbit and mouse peptide sequences contain an extra Ile residue at the outermost 3\(^{\text{rd}}\) end, missing in the human \(\beta\)-subunit.

The lacrimal gland is believed to be the main source of these enzymes, demonstrated by a catalytic activity that can be correlated to that determined in tear fluid, at a low pH (Van Haeringen and Glasius, 1976, 1980). Despite the relatively high activity of \(\beta\)-hexosaminidase measured in tear fluid, the question about the functional role for this enzyme at the ocular surface, which has a neutral pH, remains unanswered. Not considering the acidic requirements for activity against the artificial substrates, \(\beta\)-hexosaminidase could participate in the turnover of mucins in the tearfilm. Also, the pH of the mucin microenvironment is not known. Abnormalities in the mucous layer, as a result of longer oligosaccharide chains on glycoproteins, have been observed in a canine model of keratoconjunctivitis sicca (Hicks et al., 1998). Preliminary results indicate that purified ocular mucins from rabbit are degraded by enzymes secreted by cultured lacrimal cells (Matthews et al., 2001). Cholinergic stimulation with carbachol accelerates both release of secretory vesicles and the membrane recycling by endocytosis in acinar cells (Gierow et al., 1995; Lambert et al., 1993). After stimulated exocytosis a disappearance of \(\beta\)-hexosaminidase from secretory membrane compartments has been reported at the same time as an increasing amount could be detected in trans Golgi compartments (Yang et al., 1999), suggesting that a large amount of \(\beta\)-hexosaminidase is recycled back (Hamm-Alvarez et al., 1997). The mechanism by which a significant portion of lysosomal hydrolases are discharged...
extracellularly is still not known but it could be a process by which enzymes are concentrated to lysosomal compart-
ments through reinternalization by mannose 6-phosphate receptors.

The possibility that β-hexosaminidases, escaping from the lysosomal route, are secreted in their nonmature form and then recaptured for final processing in the intracellular acidic membrane compartments has been addressed in attempts to restore Tay-Sachs defects by overexpressing the α-subunit in fibroblasts in vitro and then allowing for recapture in HexA deficient fibroblasts (Guidotti et al., 1998; Martino et al., 2002). This is also in agreement with the study showing that β-hexosaminidase in human serum only exists in its precursor form (Isaksson and Hultberg, 1995). The precursor form of HexA can degrade GM2 ganglioside in the presence of GM2 activator protein at the same rate as the mature HexA isoenzyme (Hasilik et al., 1982), suggesting that the secretory, nonmature portion of β-hexosaminidase is functionally active in the extracellular fluid. Furthermore, a large fraction of the GM2 activator protein is secreted (Rigat et al., 1997). It has been shown to bind HexA at pH 7 (Yadao et al., 1997) and function as a glycolipid transporter at physiological pH (Smiljanic-Georgijev et al., 1997), suggesting that the GM2 activator protein could induce the hydrolysis of GM2 gangliosides through interaction with HexA even at neutral pH. Whether the catalytic activity of β-hexosa-
minidase, observed in the secretory fluid from rabbit lacrimal gland acinar cells, arises from proteolytically processed enzymes or the nonmature form remains to be explored. Though only insignificant β-hexosaminidase activity at pH 7.0 has been detected in human tear fluid and serum (Van Haeringen and Glasius, 1976) as well as fluid secreted by rabbit lacrimal gland acinar cells (data not shown).

Northern blot studies of β-hexosaminidase α-subunit clearly show a higher mRNA expression in cultured lacri-
mal gland acinar cells compared with tissue. This could be the result of purification of acinar cells from other structures with small amounts of or no β-hexosaminidase, or the culturing environment, affecting the gene expression within the acinar cells. Isolated cells were cultured in serum-free medium, a condition previously shown to up-regulate both α- and β-subunit mRNA expression in microglial cells (Beccari et al., 1997). An up-regulated α-subunit transcription and protein synthesis followed by an increased enzyme secretion could be an attempt for the cells to modulate the surrounding matrix and stabilization of acinar structures. An interesting feature is that the mRNA expression studies of the β-subunit, present in the functionally active iso-
enzymes HexA and HexB, show an even expression between cultured cells and tissue. High levels of the α-subunit could be necessary to favor HexA formation instead of the more stable HexB isoform as discussed by Mahuran (1995). The fact that no difference could be observed at the mRNA level after stimulated secretion suggests that β-hexosaminidase is stored in secretory vesicles at the apical membrane ready to be released. Studying the expression for a longer period of time (hours) after stimulation, resulting in emptying of intracellular β-hexosaminidase stores, would perhaps reveal changes at the mRNA level.

Enzymatic assays revealed a slightly higher β-hexosaminidase activity in cellular extracts, which together with the northern blot data indicate that the α-subunit and consequently HexA is present to a higher degree than HexB in primary cultured acinar cells. Heat inactivation of HexA, resulting in an ~93% loss of both total β-hexosaminidase and HexA activity, suggests that HexA is the major active β-hexosaminidase isoform in the lacrimal gland.

Sequence analyses were performed to explore any differences between rabbit and the species human and mouse, at the DNA–protein level. Comparison of the translated peptide sequences with mouse and human clearly showed that the overall identity, glycosylation sites, and proposed catalytic sites are highly conserved both for α- and β-subunits between all three species. Absence of the human site of glycosylation at Asn142 in the rabbit β-subunit could be the reason why antibodies directed against the human protein do not detect the rabbit β-hexosaminidase. Despite several attempts, with different molecular biology approaches, we could not obtain the 5’ ends of either subunit. Comparative analysis of the sequence data will though be useful in selecting rabbit specific subunit peptide epitopes, likely to be surface exposed on the mature enzyme, which will be used for production of antibodies.

Materials and methods

Reagents

Carbachol, insulin-transferrin-sodium selenite mix, hydro-
cortisone, linoleic acid, and 4-methylumbelliferyl N-acetyl-
β-D-glucosaminide (4MUGlcNAc) were obtained from Sigma-Aldrich (St. Louis, MO). 4-Methylumbelliferyl-6-
sulfo-N-acetyl-b-D-glucosaminide (4MUGlCNActSO4) was obtained from Calbiochem (San Diego, CA). Matrigel was obtained from BD Bioscience (Bedford, MA). Penicillin, streptomycin, and glutamine were purchased from Invitrogen (Carlsbad, CA) and VIP was obtained from Bachem AG (Bubendorf, Switzerland). All nucleotide primers were obtained from Invitrogen.

Cell purification and culture

Lacrimal gland acinar cells were isolated from female New Zealand White rabbits weighing 1.7–2.0 kg (ESF
Products, Estuna AB, Norrtälje, Sweden) as described previously (Gierow et al., 1996). Animals were handled according to directions from the Ethical Committee for Animal Experiments (Linköping, Sweden) and the ARVO statement for use of animals in ophthalmic and vision research. Purified single cells were cultured on Matrigel (40 μg/ml) coated wells with a cell density of 6.5 × 10⁵ cells/cm² in PCM, a serum-free Ham’s F-12 (Invitrogen) and low-glucose Dulbecco’s modified Eagle’s medium (Invitrogen) in a 1:1 mixture and supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 0.1 mM sodium citrate, a mix of 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite, 2 mM sodium butyrate, 5 nM hydrocortisone, and 0.3 μM linoleic acid.
Cell and tissue treatment

Treatments were performed after 2 days in culture, allowing cells to reorganize into acinus-like structures with distinct apical and basolateral regions. To be able to study the \( \beta \)-hexosaminidase \( \alpha \)- and \( \beta \)-subunit expression both in resting cells and after maximal stimulated secretion, acinar cells were incubated in the presence or absence of 0.1 mM carbachol and 0.1 \( \mu \)M VIP in Hank’s balanced salt solution (Sigma-Aldrich), supplemented with 10 mM HEPES and were incubated in the presence or absence of 0.1 mM ing cells and after maximal stimulated secretion, acinar cells were incubated in the presence or absence of 0.1 mM CaCl\(_2\), with final pH 7.6, for 1 h at 37°C. Following stimulation, supernatants were collected from the culturing dishes, and detached cells were removed by brief centrifugation. Cells were lysed in Hank’s balanced salt solution, supplemented as stated, containing 1% Triton-X 100; scraped; and saved at \(-80^\circ\text{C}\) until use. To assay enzyme activity, \(~100\) mg tissue was homogenized using an Ultra-Turax homogenizer (Janke & Kunkel, Staufen, Germany) in Hank’s balanced salt solution, supplemented as stated, containing 1% Triton-X 100; filtered through a Nylon Net Filter, 180 mm (Millipore, Billerica, MA); and saved at \(-80^\circ\text{C}\) until use.

Enzymatic assays

Secretion from resting and stimulated lacrimal gland acinar cells was measured using total \( \beta \)-hexosaminidase activity as marker (Gierow and Mircheff, 1998). To evaluate the proportions of HexA and HexB activity in cell extracts and tissue samples, HexA was thermally inactivated by heating samples for 1 h at 52°C (Martino et al., 2002). Protein content was measured according to a modified method of Lowry et al. (1951), described earlier (Gierow et al., 1995). Total \( \beta \)-hexosaminidase enzyme activity, referring to the hydrolysis by HexA and HexB isoenzymes in samples was determined using the substrate 4MUGlcNAc (7.5 mM) and HexA-specific activity was confirmed with 4MUGlcNAc6SO\(_4\) (0.1 mM) using the method by Barrett and Heath (1977). Routinely, reactions are started by adding 50 \( \mu \)l reaction buffer (133 mM sodium citrate, 133 mM sodium chloride and substrate, pH 4.3) to the samples. After 2 h the reaction is terminated by addition of 2 ml quench solution (50 mM glycine and 5 mM ethylene-diamine tetra-acetic acid, pH 10.5). Absorbance measured at 460 nm using Flourolog 3-22 Fluorescence Spectrophotometer (Instruments S.A., Edison, NJ) was calibrated to a 4-methylumbelliferone standard (0.1 mM) concentration subjected to the same conditions. Data were analyzed by Student \( t \)-test, where \( p < 0.05\) was considered statistically significant.

RNA isolation

Total RNA was extracted from cultured resting and stimulated acinar cells and \(~0.2\) g rabbit lacrimal gland tissue using the Ultraspec II RNA kit (Biotec Laboratories, Houston, TX), if not otherwise stated. The tissue sample had been snap-frozen in liquid nitrogen on removal and kept at \(-80^\circ\text{C}\) until further processing according to the manufacturers instructions.

Table II. Sequences of oligonucleotide primers used for PCR and RACE

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>HexA Fw1</td>
<td>5’-GCTTCCCGAGGTCTGGAGACTT-3’</td>
</tr>
<tr>
<td>HexA Fw2</td>
<td>5’-CGAGGAGCTTCTCATCA-3’</td>
</tr>
<tr>
<td>HexA Fw3</td>
<td>5’-GAGGAGAAGGCGCTGTTGAC-3’</td>
</tr>
<tr>
<td>HexA Fw4</td>
<td>5’-AGGCAAATGTTGGCCAGGAG-3’</td>
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<td>HexA Rw1</td>
<td>5’-TCAAACTCTGCCAGCACAGC-3’</td>
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<td>5’-GACCCAGTGCGCATGGAGCAGT-3’</td>
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<td>5’-GGTTTTCCAGATAATACGCATCAT-3’</td>
</tr>
<tr>
<td>HexB Rw1</td>
<td>5’-CTGATAAGGGAGAAGCTGTCGTTCA-3’</td>
</tr>
</tbody>
</table>

Construction of complementary cDNA clones

Using 1 \( \mu \)g total RNA extracted from lacrimal gland tissue, single-stranded random hexamer-primed cDNA was generated with the GeneAmp RNA polymerase chain reaction (PCR) kit (Perkin Elmer, Roche Molecular Systems, Branchbury, NJ). The \( \beta \)-hexosaminidase \( \alpha \)- and \( \beta \)-subunit-specific cDNA fragments were PCR amplified with the following primer pairs; HexA Fw1/HexA Rw1 and HexB Fw1/HexB Rw2, respectively (See Table II for primer nucleotide sequence). Primers were designed from highly conserved regions within human (GenBank accession number NM000520) and mouse (GenBank accession number NM010421) sequences of \( \beta \)-hexosaminidase \( \alpha \)-subunit and human (GenBank accession number NM000521) and mouse (GenBank accession number MMXEB) sequences of \( \beta \)-hexosaminidase \( \beta \)-subunit. The cDNA fragments, corresponding to the \( \beta \)-hexosaminidase \( \alpha \)- (394 nt) and \( \beta \)- (343 nt) subunits, were separated on an ethidium bromide-stained \( 1\% \) agarose gel and purified with JetQuick Purification Spin Kit (Genomed, Bad Oeyenhausen, Germany) cloned into pGEM-T vectors (Promega, Madison, WI), and finally transformed into competent Escherichia coli JM 109 cells (Promega). Plasmid DNA was purified with Wizard Plus mini- and midiprep kits (Promega) and sequenced using the ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Foster City, CA) and the BigDye Terminator Cycle sequencing ready reaction DNA Sequencing kit (PE Applied Biosystems, Warrington, U.K.).

Transcription of \( ^{32}\text{P} \)-labeled RNA Probes

Purified vectors containing \( \alpha \)- or \( \beta \)-subunit specific DNA fragments were linearized with the Not I restriction enzyme and RNA probes transcribed in presence of \( ^{32}\text{P} \)-labeled rUTP as described previously (Gierow et al., 2002; Magnuson et al., 2001).

Northern blot analysis

RNA separation, transfer, and probe hybridization followed the procedure described in detail earlier (Gierow et al., 2002; Magnuson et al., 2001).
et al., 2002; Magnusson et al., 2001). Briefly, ~15 μg total RNA isolated from lacrimal gland tissue or cultured resting and stimulated acinar cells was separated on a denaturating formaldehyde-agarose gel by electrophoresis. Comparable amounts RNA loaded were visualized by UV illumination of ethidium bromide–induced fluorescence of 18S and 28S ribosomal RNA bands. Thereafter RNA was transferred onto an uncharged nylon transfer membrane (0.45 Micron, Micron Separations, Westboro, MA) and cross-linked by UV illumination. Blots were prehybridized for at least 2 h, followed by hybridization with 32P-labeled antisense RNA probes against the β-hexosaminidase α- or β-subunit overnight at 65°C. Blots were then rinsed, and hybridization signals were detected by autoradiography and exposures were made at ~80°C using Kodak X-OMAT AR or Kodak Biomax MS film. Densitometric quantification of mRNA expression from northern blots was performed as described earlier (Magnusson et al., 2003). Alterations in expression were calculated by comparing cultured cell data to lacrimal tissue data, set to 1.0. Data were analyzed by Student t-test, where p < 0.05 was considered statistically significant.

Sequence of the β-hexosaminidase α-subunit

Determination of the sequence of the β-hexosaminidase α-subunit was performed with the 3’-RACE and the 5’-RACE systems according to the manufacturers protocol (Invitrogen). All PCR products were analyzed on ethidium bromide–stained 1% agarose gels. In the 3’-RACE, first strand cDNA was synthesized from 2.9 μg total RNA using the supplied adapter primer (AP). PCR amplification was performed with the gene-specific HexA Fw1 primer and the supplied abridged universal amplification primer (AUAP). Nested PCR reactions were performed with the obtained PCR product as a template, using HexA Fw2 or HexA Fw4 primers in combination with the AUAP primer. Amplified fragments of expected size, 1500 bp and 500 bp were purified, cloned, and sequenced as described. In addition, sequencing was also performed directly on the purified 1500-bp 3’-RACE amplified cDNA with the forward primers HexA Fw2 and HexA Fw3. In the approach to amplify the 5’ end of the rabbit α-subunit, total RNA was isolated from 0.3 g lacrimal gland tissue, using the GenElute Mammalian Total RNA Kit (Sigma-Aldrich). First strand cDNA was synthesized from 3.4 μg total RNA with Hex Rw1 as reverse primer. PCR amplification of dC-tailed cDNA was performed using the puReTaq Ready-To-Go PCR Beads kit (Amersham Biosciences, Piscataway, NJ) with the supplied forward abridged anchor primer and HexA Rw2 reverse primer. A nested PCR was performed using AUAP and the HexA Rw3 primer. An amplified cDNA fragment of 550 bp was purified, cloned, and sequenced as described.

Sequence of the β-hexosaminidase β-subunit

To amplify the 3’ end of the β-hexosaminidase β-subunit mRNA, the 3’-RACE System, Version C kit (Invitrogen) was used. First strand cDNA was synthesized from 11 μg total RNA with the supplied AP. Superscript III Reverse Transcriptase (Invitrogen) was used instead of the enclosed Superscript II Reverse Transcriptase. Gene-specific primers were designed from highly conserved regions within human (GenBank accession number NM000521) and mouse (GenBank accession number NM010422) sequences of β-hexosaminidase β-subunit. PCR amplification of synthesized cDNA was performed with the HexB Fw1 forward primer and the supplied AUAP, as reverse primer. The PCR product was analyzed by ethidium bromide–stained 1.0% agarose gel electrophoresis, and a fragment of expected size was cut out and dissolved in 100 μl H2O at 94°C. A nested PCR was carried out, using the gel matrix fragment as a template and the AUAP primer and the HexB FwJ1 as forward primer. The amplified cDNA fragment of 1140 bp was purified, cloned, and sequenced as described. Because of the relative large size of the fragment, another forward primer, HexB Fw3, was designed to obtain sequence from the middle section of the fragment.

Sequence analysis

NCBI’s BLAST program (www.ncbi.nlm.nih.gov/BLAST) for nucleotide sequences was used for confirming sequence similarities of obtained rabbit nucleotide sequences with published human and mouse β-hexosaminidase α- and β-subunit nucleotide sequences. Nucleotide sequence alignment and analysis was performed with the Vector NTI Suite 7.0 program. The Clustal W 1.8 program (www.ebi.ac.uk/clustalw) was used for multiple alignments of amino acid sequences with a gap open penalty of 10.0 and a gap extension of 0.1.

Acknowledgments

We thank Dr. Sven Tägerud for critically reviewing the manuscript, Dr. Michael Lindberg for helpful discussions regarding sequence experiments, and Jan Ekelin for working on the sequencing as a student research project. This work was supported by grants to J.P.G. from the University of Kalmar Faculty Research Board, the Crafoord Foundation, and the Swedish Knowledge Foundation.

Abbreviations

AP, adapter primer; AUAP, abridged universal amplification primer; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; VIP, vasoactive intestinal peptide.

References


