Glycobiology vol. 16 no. 9 pp. 786–800, 2006
doi:10.1093/glycob/cwl007
Advance Access publication on May 29, 2006

Identification and expression analysis of *Drosophila melanogaster* genes encoding β-hexosaminidases of the sperm plasma membrane

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Received on November 9, 2005; revised on May 22, 2006; accepted on May 25, 2006

Sperm surface β-*N*-acetylhexosaminidases are among the molecules mediating early gamete interactions in invertebrates and vertebrates, including man. The plasma membrane of *Drosophila* spermatozoa contains two β-*N*-acetylhexosaminidases, DmHEXA and DmHEXB, which are required for egg fertilization. Here, we demonstrate that three putative *Drosophila melanogaster* genes predicted to code for β-*N*-acetylhexosaminidases, *Hexo1*, *Hexo2*, and *fdl*, are all expressed in the male germ line. *fdl* codes for a homolog of the α-subunit of the mammalian lysosomal β-*N*-acetylhexosaminidase Hex A. *Hexo1* and *Hexo2* encode two homologs of the β-subunit of all known β-*N*-acetylhexosaminidases, which we have named β_1 and β_2, respectively. Immunoblot analysis of sperm proteins indicated that the gene products associate in different heterodimeric combinations forming DmHEXA, with an $\alpha\beta_2$ structure, and DmHEXB, with a $\beta_1\beta_2$ structure. Immunofluorescence demonstrated that all the gene products localized to the sperm plasma membrane. Although none of the genes was testis-specific, *fdl* was highly and preferentially expressed in the testis, whereas *Hexo1* and *Hexo2* showed broader tissue expression. Enzyme assays carried out on testis and on a variety of somatic tissues corroborated the results of gene expression analysis. These findings for the first time show the in vivo expression in insects of genes encoding β-*N*-acetylhexosaminidases, the only molecules so far identified as involved in sperm/egg recognition in this class, whereas in mammals, the organisms where these enzymes have been best studied, only two types of polypeptide chains forming dimeric functional β-*N*-acetylhexosaminidases are present in *Drosophila* three different gene products are available that might generate numerous dimeric isoforms.

Key words: fertilization/gamete interactions/*fdl*/*Hexo1*/*Hexo2*

Introduction

It has been demonstrated in mammals, in lower vertebrates, and in invertebrates as well that a successful interaction between sperm and egg requires as the primary event the recognition of oligosaccharide moieties of the glycoproteins of the extracellular envelope(s) of the egg by complementary molecules of the sperm plasma membrane (Dell *et al*., 1999; Koyanagi and Honegger, 2003; Talbot *et al*., 2003; Vo *et al*., 2003). In several animal models, the sperm proteins that mediate the binding to the egg zona pellucida (ZP) or vitelline envelope (VE) are glycosyltransferases or glycosidases of the plasma membrane that form complexes with oligosaccharide substrates present on ZP or VE in noncatalytic conditions. In mollusks (Focarelli *et al*., 2001), in ascidians (Koyanagi and Honegger, 2003), and in amphibians (Martinez *et al*., 2000) it has been shown that gamete recognition is mediated by glycosidases of the sperm plasma membrane. In mammals, a β1,4 galactosyltransferase (GalT I) of the sperm plasma membrane and a variety of sperm surface proteins have been demonstrated to function as ZP receptors in initial gamete recognition, and it is thought that sperm adhesion to the egg surface may involve the interaction of multiple complementary molecules on the sperm and on the oocyte (Talbot *et al*., 2003). Interestingly, sperm plasma membrane glycosidases have been indicated among molecules involved in the early interactions with the egg also in mammals, including man (Miranda *et al*., 2000; Tulsiani and Abou-Haila, 2001; Khunsook *et al*., 2003).

We have previously showed the presence in the plasma membrane of *Drosophila melanogaster* spermatozoa of two β-*N*-acetylhexosaminidases, originally named HEX1 and HEX2, which have been purified and fully characterized (Cattaneo *et al*., 2002). The evidence for their essential role in fertilization was the finding that males lacking β-*N*-acetylhexosaminidase activity from the sperm plasma membrane overlying the acrosome were sterile due to the inability of their spermatozoa to enter the egg (Perotti *et al*., 2001). In this article, HEX1 and HEX2 have been renamed DmHEXA and DmHEXB, respectively.

A search of the *Drosophila* genome indicated the presence of three genes coding for β-*N*-acetylhexosaminidase-like enzymes based on sequence homologies: *Hexo1*, *Hexo2*, and *fdl*. In this work, by combining gene expression analysis, amino acid sequence analysis, and immunochemistry and immunofluorescence, we show that all the three genes are actually expressed in the male reproductive apparatus and that they code for different subunits, which associate to form the dimeric β-*N*-acetylhexosaminidases present in the sperm plasma membrane.

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Results

Hexo1, Hexo2, and fdl encode Drosophila homologs of vertebrate and invertebrate β-N-acetylhexosaminidases

The genes of *D. melanogaster* coding for β-*N*-acetylhexosaminidases were identified using the Ensembl database for *Drosophila* and querying BLASTP with human β-hexosaminidase Hex A (GenBank accession number AAH18927) as an input. The genes *Hexo1* (CG1318), *Hexo2* (CG1787), and *fdl* (CG8824) are mapped to chromosomes 3, X, and 2, respectively (Table I). The genes have been annotated as the *Drosophila* putative homologs of genes encoding β-*N*-acetylhexosaminidases in man, in the mouse, in the ascidian *Phallusia*, and in a few insects (Table II).

Analysis of transcripts and of gene products

Four alternative transcripts of *Hexo1* are indicated in the *D. melanogaster* genome release 4.0 (Drysdale *et al.*, 2005): CG1318-RA, 2148 nt long; CG1318-RB, 2006 nt long; CG1318-RC, 1197 nt long; and CG1318-RD, 2210 nt long. CG1318-RA and CG1318-RB are predicted to code for the identical proteins PA and PB, 622 amino acid long, with a calculated relative molecular mass (M_r) of 70.5 kDa and five potential *N*-glycosylation sites (Figure 1A). The transcript CG1318-RC is predicted to code for the protein PC, 383 amino acid long, with a M_r of 43.8 kDa and three potential *N*-glycosylation sites. CG1318-RD is predicted to code for the protein PD of 606 amino acids, with a M_r of 69

Table I. Oligonucleotide primers used for PCR

Gene	Chromosome position	Oligonucleotide primers[a]		Sequence
Hexo1 (CG1318)	64B1 (3L)	sH1-A	293425–293444	5′-GGAGAACTACGTAAGGCCA-3′
		asH1-A	293724–293744	5′-CCGTATCCAGGGTGAGTCTG-3′
		sH1-B	294626–294646	5′-ATGGTACGCACACGCCCATC-3′
		asH1-B	294891–294912	5′-CGCCGGCAATGGATTTCAAAC-3′
Hexo2 (CG1787)	8A2 (X)	sH2	296805–296825	5′-GGCATGCCCGTTGGTCGCAG-3′
		asH2	297113–297133	5′-CACTGTCCTTCGTGGAGGAC-3′
fdl (CG8824)	49A9 (2R)	Sfdl	199883–199903	5′-GCACATGGCAGAACGTCTAC-3′
		Asfdl	200240–200260	5′-CCGGGATTCTGGGCACAGTA-3′
Act5C (CG4027)	5C7 (X)	sAct5C	273772–273792	5′-CCCAGATCATGTTCGAGACC-3′
		asAct5C	277065–277085	5′-AGGGCAACATAGCACAGCTT-3′

[a]H1-A and fdl probes directed to the 3′ region of the gene. H1-B and H2 probes directed to the 5′ region of the genes.

Table II. Sequence identity between catalytic domains of *Drosophila melanogaster* β-hexosaminidases and those of other known β-hexosaminidases

Organism	Gene	Peptide	Identity (%)		
			Hexo1	*Hexo2*	*fdl*
Drosophila melanogaster	*Hexo1*	CG1318-PA/PB/PD	—	42	40
	Hexo2	CG1787-PA	42	—	40
	fdl	CG8824-PC	41	40	—
Drosophila pseudobscura	FBgm0072147	GA12099-PA	88	43	42
	FBgm0074732	GA14705-PA	42	89	40
	FBgm0081336	GA21348-PA	41	40	92
Anopheles gambiae	ENSANGG00000018592	ENSANGP00000021081	63	42	41
	ENSANGG00000013720	ENSANGP00000016209	42	52	42
	ENSANGG00000018968	ENSANGP00000021457	41	40	70
Bombyx mori	HEXC	P49010	62	38	42
Trichoplusia ni	AY078172	Q8T4N1	60	43	42
Manduca sexta	AY368703	Q6UJX7	59	41	41
Phallusia mammillata	AJ518021	CAD57204	33	33	31
Mus musculus	HEXA	α-Subunit	32	35	33
	HEXB	β-Subunit	35	33	35
Homo sapiens	HEXA	α-Chain	34	37	33
	HEXB	β-Chain	34	35	34

Sequences were analyzed with BLASTP at NCBI.

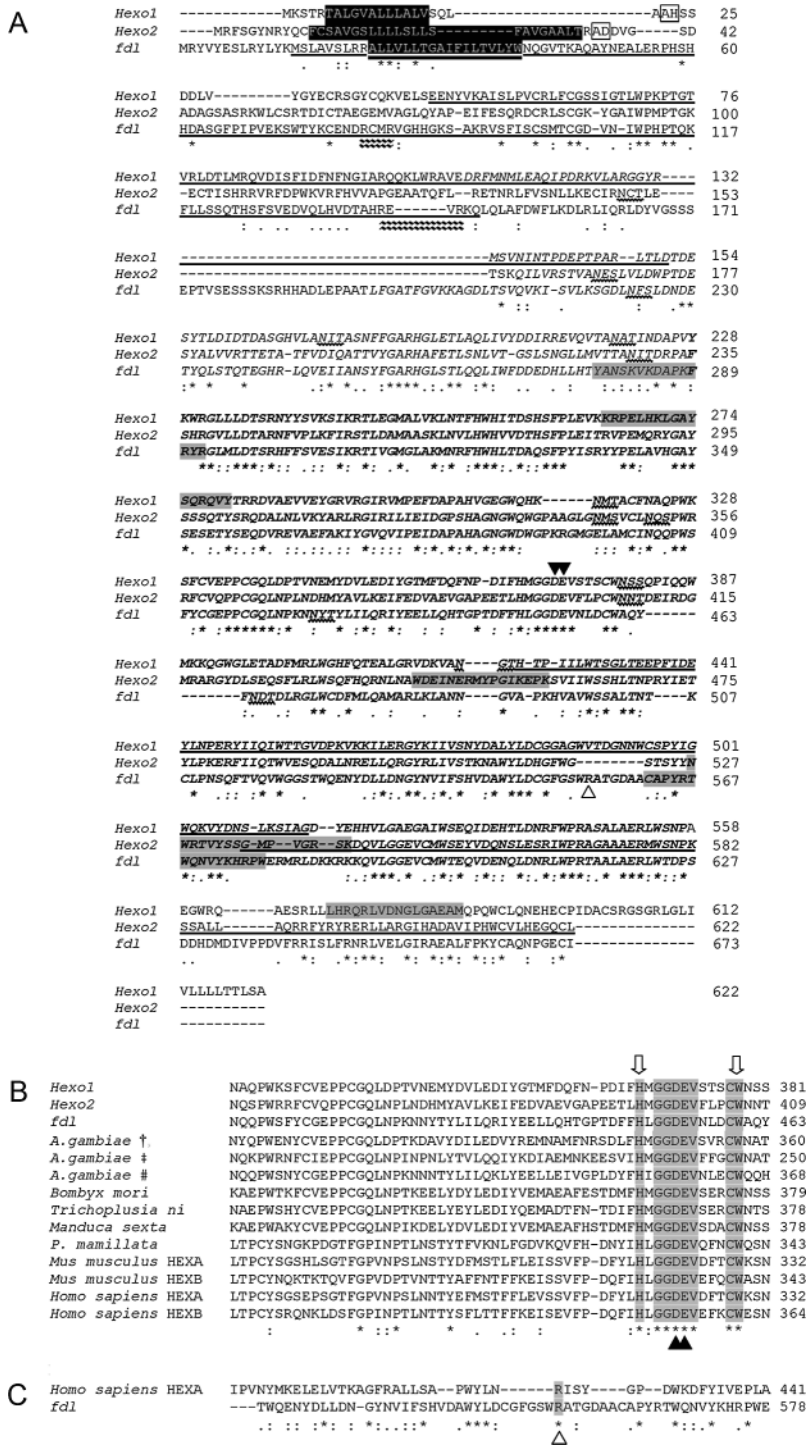

Fig. 1. Analysis of the predicted amino acid sequences encoded by *Drosophila melanogaster Hexo1* (CG1318-PA, PB), *Hexo2* (CG1787-PA), and *fdl* (CG8824-PB). (**A**) Multiple alignment of the sequences. Identical amino acids are marked with asterisks (*), strongly similar amino acids are marked with two dots (:), and weakly similar amino acids are marked with one dot (.). The putative transmembrane domains are shaded in black. The putative cleavage sites for signal peptidase are boxed. *N*-glycosylation sites are underscored with a wavy line. Putative furin consensus cleavage sites are underscored by a double wavy line. Glycohydrolase family 20 domain 2 and catalytic domain are in italics and in boldfaced italics, respectively. The active sites are indicated by solid arrowheads and the *fdl*-binding site for β-GlcNAc-6SO_4^- substrate by an open arrowhead. The sequences identified by the probes used for northern blotting and RT–PCR are underscored by a straight line. The sequences used for production of antisera are shaded in gray. Alignment was made using T-Coffee. (**B**) Extracts from T-Coffee alignment of the catalytic domains of β-*N*-acetylhexosaminidases from *D. melanogaster* and from other organisms. The motif containing the active site (open arrows) is highly conserved from *Drosophila* to man. The solid arrowheads indicate the active sites. (**C**) Alignment of human HEXA product with *fdl* product. The open arrowhead indicates the alignment of human R424 that binds negatively charged substrates with R555 of the *fdl* polypeptide CG8824-PB. Human R424 aligns also with R542 of the second *fdl* product, CG8824-PD.

kDa and five potential *N*-glycosylation sites (Figure 1A). The gene products CG1318 PA and PB differ from PD only for the presence of 12 additional amino acids at the *C*-terminus. Bioinformatic analysis of *Hexo1* theoretical products with SignalP indicated that the first 22 amino acids near to the *N*-terminus of CG1318-PA, PB, and PD have characteristics of a signal peptide (p = 1.000), with a probable cleavage site at residues 22–23 (mean D score 0.825, cutoff value 0.43) (Figure 1A). Instead, the putative translation product of the CG1318-RC transcript has no signal peptide. The analysis of CG1318-PC with Secretome 1.0b, a method for prediction of secretory proteins lacking a *N*-terminal signal peptide (Bendtsen *et al*., 2004), indicated that the protein is not secreted. This information, together with the absence of any other sorting signal, defines this conceptual translation product as a cytosolic resident protein.

Hexo2 is predicted to have a single transcript of 2142 nt, CG1787-RA, which encodes a predicted 622 amino acid long protein, with a calculated M_r of 70.7 kDa and six potential *N*-glycosylation sites (Figure 1A). The first 36 amino acids are predicted to contain a signal peptide (p = 0.959), with a probable cleavage site between residues 36 and 37 (mean D score 0.731, cutoff value 0.43).

The *D. melanogaster* database indicated the presence of two putative alternative transcripts of *fdl*: CG8824-RB of 2747 nt and CG8824-RC of 3220 nt. The transcripts yield two proteins differing for 13 amino acids at the *N*-terminal, that is, the PB protein of 673 amino acids, with a M_r of 77.3 kDa, and the PC protein of 660 amino acids, with a M_r of 75.5 kDa. Both the conceptual translation products have three potential *N*-glycosylation sites (Figure 1A). The segment spanning amino acid residues 23–40 in PB and amino acid residues 5–27 in PC constitutes a signal-anchor domain (p = 0.995). Two putative minimal furin consensus sequences RXXR, where R is arginine and X any amino acid (Thomas, 2002), are present downstream from the hydrophobic domain: one is located at amino acid residues 68–72/81–85, the second one is located at amino acid residues 127–131/140–144 (Figure 1A).

Alignment of the deduced products of *Hexo1* products CG1318-PA, PB, PD, of *Hexo2* product CG1787-PA and of the *fdl* products CG8824-PC, PB with the primary sequences of known β-*N*-acetylhexosaminidases from different organisms performed with T-Coffee and BLASTP (Figure 1A) revealed regions with relevant percentages of identity to the domain 2 and to the catalytic domain of the glycosyl hydrolase family 20 to which these types of enzymes belong (Henrissat and Bairoch, 1993) (Table II). Sequence alignment also demonstrated the presence in all *Drosophila* enzymes of the motif HXGGDEVXXXCW containing the residues Asp–Glu (DE), which are thought to form the active site in all members of the glycosyl hydrolase family 20 (Prag *et al*., 2000; Mark *et al*., 2003). Within this motif, the majority of the amino acid residues present in the enzymes from other organisms were conserved (Figure 1B). In addition, sequence alignment of the theoretical *fdl* products with the α-chain of human Hex A by T-Coffee showed that Arg424 of the human α-chain, considered to be responsible for the binding of negatively charged substrates such as β-GlcNAc-6SO_4^-, is aligned with Arg555 of *fdl* PB and with Arg542 of *fdl* PC (Figure 1C).

Topology analysis of the putative *fdl* products indicated that they belong to the type II membrane protein group, with a short *N*-terminal cytosolic domain of 9–22 residues, a single transmembrane domain of 18 amino acid residues and a large extracellular domain containing the *N*-glycosylation sites and the catalytic site.

A bioinformatic search in *Drosophila pseudobscura* and in *Anopheles gambiae* for homologs of the putative products of *Hexo1*, *Hexo2*, and *fdl* using BLASTP showed the presence in both species of three β-*N*-acetylhexosaminidase-like enzymes encoded by three distinct genes (Table II). In other insects, the presence of only one β-hexosaminidase-like polypeptide has been reported (Table II). The proteins encoded by the *D. melanogaster* genes showed 88–92% identity based on catalytic domain sequences to *D. pseudobscura* proteins, 41–70% identity to β-hexosaminidases from other insects, and 31–37% identity with those from the ascidian *Phallusia* and from mammals (Table II). Percent identities over the whole sequences were very similar (data not shown). SignalP analysis indicated that, like *D. melanogaster fdl* products, the *N*-terminus of *D. pseudobscura* GA21348-PA and of *A. gambiae* ENSANGP00000021457 contains a signal-anchor domain (p = 0.80–0.77), whereas in all the remaining β-hexosaminidases from all the other organisms, the *N*-terminus represents a cleavable signal peptide (p = 1.00–0.88). Interestingly, these polypeptides from *D. pseudobscura* and from *A. gambiae* are those with the highest identity to *fdl* product (Table II).

Expression profiles of Hexo1, Hexo2, and fdl

When gene expression was assayed by reverse transcriptase–polymerase chain reaction (RT–PCR) using *Hexo1*, *Hexo2*, and *fdl* probes that recognize all the transcripts identified in the *D. melanogaster* genome database, that is, H1-B, H2, and fdl (Table I, Figure 1A), *Hexo1* and *Hexo2* appeared to be expressed at similar levels in all of the male organs tested, that is, midgut, brain, testis, seminal vesicles, ejaculatory duct and bulb, and in the ovary (Figure 2A). Instead, *fdl* appeared to be strongly expressed in the brain and in the testis, weakly expressed in other organs of the male reproductive apparatus, and hardly detectable in the midgut and in the ovary (Figure 2A).

Northern blotting analysis on RNA from testis, whole adult males, and whole adult females with the same probes indicated above showed that all of the genes had a transcript of 1.9–2.8 kb in all samples and that *Hexo1* and *Hexo2* had also fainter bands corresponding to shorter transcripts of ~1 kb (Figure 2B). The *Hexo1* long transcript was present in whole males and females at similar levels, whereas it was not detectable in the testis. The *Hexo2* long transcript was detected in all samples, with the highest level of expression in whole males. It is of interest to notice that this transcript is not reported in the *Drosophila* testis expressed sequence tag (EST) collections available at the Berkeley *Drosophila* Genome Project (BDGP) (http://www.fruitfly.org/DGC) and at The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/projects/dbEST/). The *fdl* probe hybridized with a single transcript that appeared expressed only in testis. The *Hexo1* short transcript was present in all samples, whereas

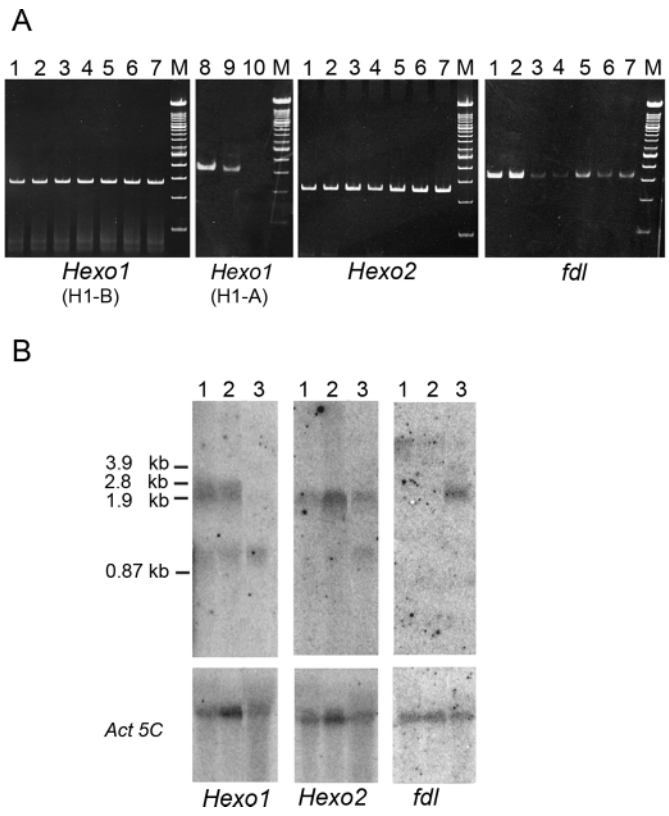

Fig. 2. Analysis of *Hexo1*, *Hexo2*, and *fdl* expression by RT–PCR and northern blotting. (**A**) RT–PCR analysis was performed using total RNA extracted from testes (1, 9), brains (2), ovaries (3), midgut (4), seminal vesicles (5), ejaculatory ducts and bulbs (6), male accessory glands (7), and whole males (8). For *Hexo1*, two sets of primers were used, H1-B and H1-A, which recognize all the gene transcripts and only the long transcripts, respectively. In all experiments, 10 ng of cDNA was used for PCR amplification, except for *Hexo1* expression in testis with the H1-A primers, which was negative when using 10 ng of template from the testis (data not shown) and positive only when 100 ng of testis cDNA was used (lane 9). M, molecular weight markers. The lane 10 shows the RT-minus control. The same control was carried out for all RT–PCR experiments. (**B**) Northern blot analysis was performed using total RNA extracted from whole females (1), whole males (2), and testes (3). The same membranes were stripped and reprobed with *Act5C* as a loading control (lower panels). Details on probes are shown in Table I and in Figure 1A.

Hexo2 short transcript was detected only in the testis. Analysis of the *Drosophila* EST libraries (http://www.ncbi.nlm.nih.gov/projects/dbEST/; http://www.flybase.org/) indicated that no alternative splice form of *Hexo2* is predicted, whereas genome analysis carried out with the UCSC genome browser (http://www.genome.ucsc. edu/) did not show the presence of a second transcription initiation site. We can therefore assume that the *Hexo2* short transcript is, most likely, a degradation product.

The lack from the testis of *Hexo1* long transcripts detectable by northern blotting prompted us to further investigate the expression of this gene in the testis and in whole males by RT–PCR using the probe H1-A directed to the 3′ region of the gene which recognizes only the gene long transcripts, that is, CG1318-RA, RB, and RC (Table I, Figure 1A). When the probe was tested on the same amount of RNA template from the testis as the one used for the probe H1-B directed to the 5′ region of the gene, no signal was detected (data not shown). However, a signal was visible in the testis after increasing 10-fold the amount of testis template (Figure 2A).

Reverse transcriptase-minus controls for all the RT–PCR experiments were negative (Figure 2A).

In situ hybridization (ISH) performed on the adult male reproductive apparatus with antisense riboprobes corroborated the results of northern blot and RT–PCR analysis. *fdl*, *Hexo2*, and *Hexo1* assayed with the fdl, H2, and H1-B probes that recognize all transcripts showed a similar pattern of expression during spermatogenesis (Figures 3A–D and 4A): mRNAs were detectable in spermatogonia, they were more abundant from premeiotic spermatocytes up to round spermatids and were found at low levels in early elongating spermatid cysts. No transcripts could be evidenced in later stages of spermatogenesis or in the stem cells. When the same probes were used on the larval gonad, the three genes appeared to be weakly expressed in spermatogonia and more intensely expressed in growing spermatocytes, whereas apical cells and the somatic terminal cells (Cooper, 1950) were negative (Figure 4D and E). In the larval testis, the levels of *Hexo1* and *Hexo2* expression were similar (Figure 4D), whereas *fdl* expression appeared slightly weaker (Figure 4E). When *Hexo1* expression was assayed in the adult testis with the probe H1-A that recognizes only the gene long transcripts, low levels of expression were detected in spermatogonia and premeiotic spermatocytes, whereas transcripts were hardly detectable in later stages (Figure 4B).

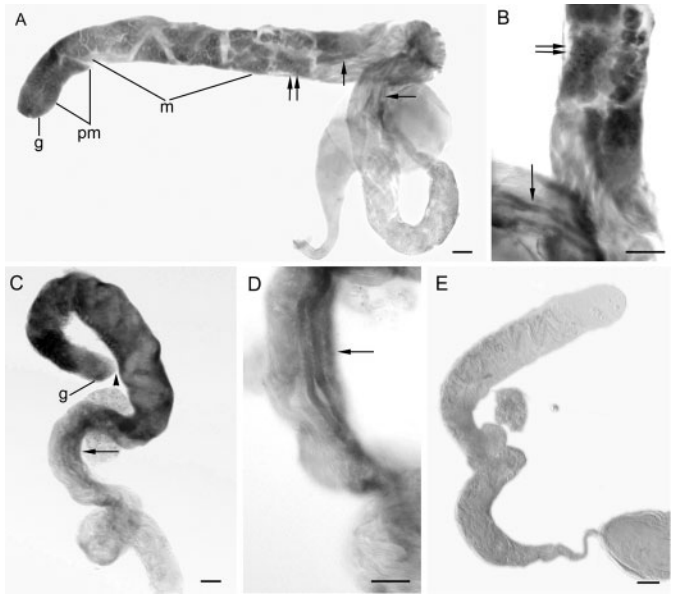

Fig. 3. ISH on testis. Whole-mount adult testes assayed with antisense riboprobes to the *fdl* (**A**, **B**) and *Hexo2* (**C**, **D**) transcripts. Gene expression is evident in spermatogonia (g), in premeiotic (pm) and meiotic (m) spermatocytes. Transcripts are still present in round spermatids (double arrows) and in early elongating spermatids (arrows). No expression is detected in stem cells (arrowhead). (**E**) Control with sense riboprobe to *fdl* transcripts. Similar results were obtained with sense riboprobe to *Hexo2* transcripts. Scale bars represent 50 μm.

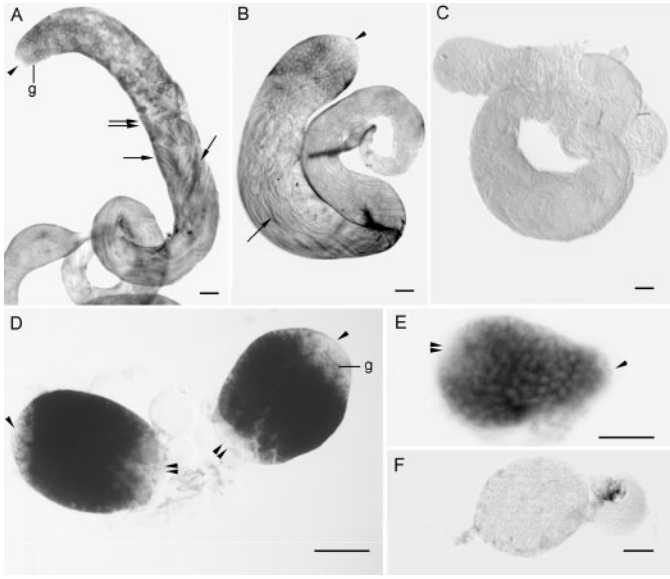

Fig. 4. ISH on whole-mount adult testis (**A**–**C**) and larval testis (**D**–**F**). (**A**, **B**) Whole-mount adult testes assayed with the antisense riboprobes to *Hexo1*, using the H1-B probe to the 5′ region (**A**) and the H1-A probe to the 3′ region (**B**) of the gene. The H1-A probe yielded a very low hybridization signal. Gene expression is absent from stem cells (arrowheads). (**C**) Control with the sense riboprobe to the 5′ region of *Hexo1*. Similar results were obtained with the sense riboprobe to the 3′ region of the gene. Details on probes are shown in Table I. For the spatial distribution of the different spermatogenic cells within the adult testis, see Figure 3. Double arrows and single arrows point to round spermatids and to early elongating spermatids, respectively. (**D**, **E**) Larval testis assayed with an antisense riboprobe to *Hexo2* (**D**) and to *fdl* (**E**) mRNAs. Similar results were obtained on the larval testis with the antisense probe to the 5′ region of *Hexo1*. Gene expression is absent from stem cells (arrowheads) and from the somatic terminal cells of the larval testis (double arrowheads). (**F**) Control on larval testis with a sense probe to *Hexo2* transcripts. Similar results were obtained with sense probes to *Hexo1* and *fdl* transcripts. g, spermatogonia. Scale bars represent 50 μm.

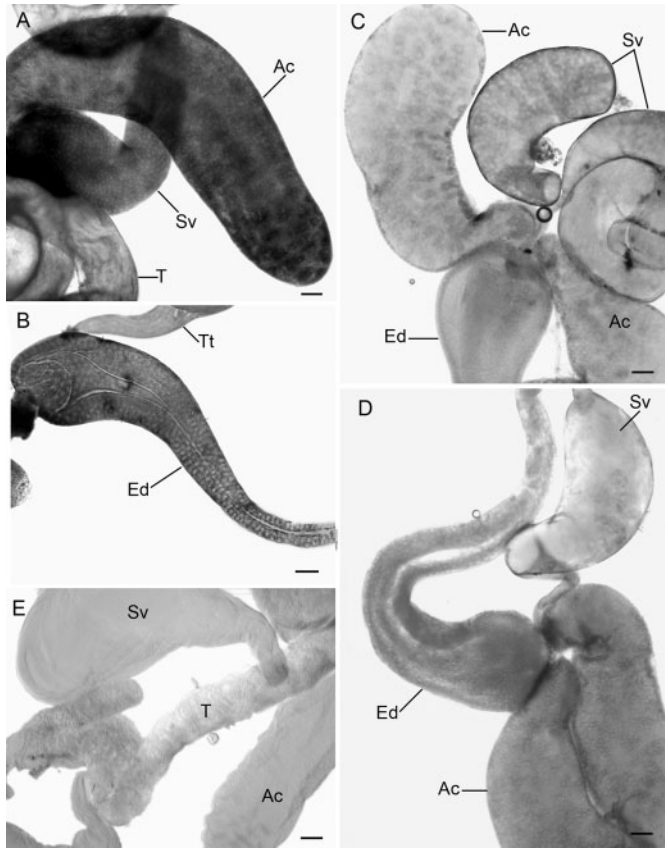

Fig. 5. ISH on whole-mount organs of the male reproductive apparatus other than the testis. (**A**, **B**) *Hexo1* expression examined with the antisense H1-B probe to the 5′ region (**A**) and with the antisense H1-A probe to the 3′ region (**C**) of the gene. (**D**) *fdl* expression assayed with an antisense riboprobe to the gene mRNAs. Analysis of *Hexo2* expression with an antisense probe gave results similar to those shown for *Hexo1* in (**A**) and (**B**). (**E**) Control with sense riboprobe to 5′ region of *Hexo1*. Similar results were obtained with the sense riboprobe to the 3′ region of the gene as well as with sense riboprobes to *Hexo2* and *fdl* transcripts. Ac, accessory glands; Ed, ejaculatory duct; Sv, seminal vesicles; T, testis; Tt, testis terminal part. Scale bars represent 50 μm.

Hexo1 and *Hexo2* expression was detected also in the terminal epithelium of the testis (data not shown). All the genes were expressed in the epithelium of seminal vesicles (Figure 5A, C, and D), of the male accessory glands (Figure 5A, C, and D), and of the ejaculatory duct and bulb (Figure 5B, C, and D). Expression of *Hexo1* assayed with the H1-B probe that recognize all the gene transcripts and *Hexo2* expression were intense in all of these organs (Figure 5A and B), whereas expression of the long transcripts of *Hexo1* appeared much weaker (Figure 5C). Also *fdl* transcripts were present at very low levels in the accessory glands and the ejaculatory duct and barely detectable in the seminal vesicles (Figure 5D).

Controls carried out with sense riboprobes on the testis and on the other components of the male reproductive apparatus were consistently negative (Figures 3E, 4C, 4F, and 5E).

Determination of β-N-acetylhexosaminidase activities

Table III shows DmHEXA and DmHEXB activities in sperm plasma membrane extracts, in adult testis, and in other tissues. DmHEXA-specific activity in the testis was not significantly different from the one determined in spermatozoa ($p > 0.05$), whereas protein concentration was 5.8 times higher ($p < 0.01$). This result indicates that noticeable levels of enzyme activity are present also in sperm precursors. In the whole male apparatus, enzyme specific activity and protein concentration were approximately two times higher than those measured in the testis ($p < 0.01$). Comparison of specific activities indicates that, all together, the other organs of the reproductive apparatus account for 45% of total activity and suggests that the testis is the major source of DmHEXA activity recovered from the entire reproductive apparatus. In whole males, DmHEXA-specific activity was significantly lower than in the reproductive apparatus ($p < 0.01$). This finding suggests that DmHEXA activity in organs other than those of the reproductive apparatus is negligible.

The specific activity of DmHEXB in the testis was significantly higher than the one measured in spermatozoa ($p < 0.01$). Comparison of the activities in the testis versus those

Table III. β-*N*-acetylhexosaminidase activities in *Drosophila melanogaster* males

Samples	Protein (mg/mL)	Specific activity	
		DmHEXA (α-chain)	DmHEXB (β-chain)
Spermatozoa	0.40 ± 0.04	3.42 ± 0.08	106.35 ± 6.39
Adult testis	2.35 ± 0.07	3.23 ± 0.28	224.18 ± 9.31
Male reproductive apparatus	4.46 ± 0.39	5.86 ± 0.26	468.29 ± 35.71
Whole male	32.67 ± 0.49	3.68 ± 0.24	487.00 ± 35.00

Specific activity expressed as nmol 4-MU/mg total protein/min. See Experimental Procedures, section *Enzyme assays* for further details. All data expressed as mean ± SEM ($n = 5$). All assays performed in triplicate.

present in the whole male reproductive apparatus and in the entire male body suggests that significant levels of DmHEXB are present also in organs other than those of the reproductive system.

β-Hexosaminidase activities were detected also in the larval testis. DmHEXA- and DmHEXB-specific activities were 1.94 ± 0.28 and 203.82 ± 6.19, respectively. DmHEXB activity of the larval testis was not significantly different from the one measured in the adults testis ($p > 0.05$), whereas the difference in DmHEXA activity between the larval and the adult testis was significant ($p < 0.01$).

Immunoblotting

Figure 6 shows the results of immunodot blot analysis of DmHEXA and DmHEXB extracted from sperm plasma membrane and partially purified by ion-exchange chromatography. DmHEXA fraction was recognized by the antisera raised against the peptides encoded by *Hexo2* and *fdl*, whereas DmHEXB fraction was reactive with the antisera raised against the peptides encoded by *Hexo1* and *Hexo2*. Controls using pre-absorbed primary antisera (Figure 6) as well as the other negative controls (data not shown) were consistently negative, indicating that neither the antisera nor the ABC method used for detection of bound primary antibodies gave nonspecific signals. When each primary antiserum was pre-incubated with the peptides encoded by the other two genes, no appreciable difference was observed from the results obtained with the unadsorbed antiserum (Figure 6), indicating the absence of cross-reactivity.

After western blotting, no protein band was detected in any of the experimental conditions used in this study, indicating that, even when electrophoresis was carried out in nonreducing and minimal denaturing conditions, the proteins were not recognized by primary antibodies.

Immunofluorescence staining of spermatozoa

The procedures employed in this study for immunofluorescence analysis of spermatozoa would allow labeling only of the cell surface. All antisera labeled the whole length of the tail surface with a punctate pattern (Figure 7A–I), whereas none of them stained the plasma membrane over the nucleus (Figure 7A, E, and G). Labeling of the tail by the antiserum against *Hexo1* product was slightly weaker than labeling by the other antibodies. The antisera against synthetic peptides encoded by *Hexo2* and *fdl* labeled also the plasma membrane overlying the acrosome (Figure 6E, F, G, and H). It is worth noticing that in *Drosophila* the acrosome is very small and is not detectable with conventional light microscopy. A strong signal over the terminal portion of the tail was evident after incubation in antisera against *Hexo1* and *fdl* peptides (Figure 7C, D, and I).

All the controls were negative.

Discussion

We have previously proposed two β-*N*-acetylhexosaminidases of the sperm plasma membrane, DmHEXA and DmHEXB, as candidates for a role as recognition proteins for the egg surface in *D. melanogaster* (Perotti *et al.*, 2001). The isoenzymes are single-pass transmembrane proteins terminally glycosylated, with the catalytic site extracellularly exposed and have a dimeric structure (Cattaneo *et al.*,

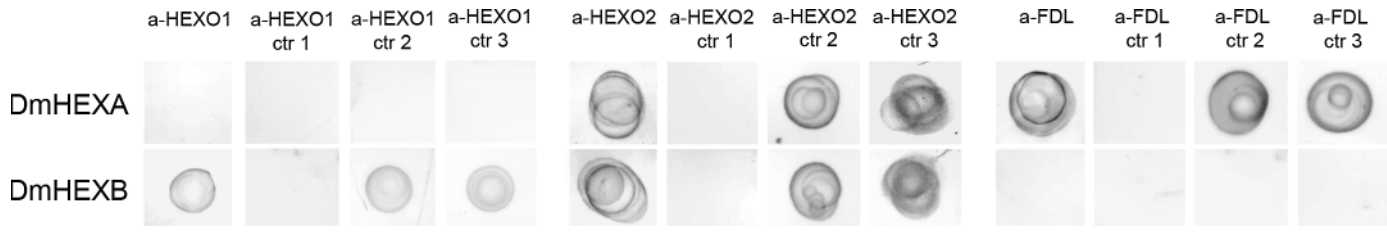

Fig. 6. Immunodot blotting analysis of DmHEXA and DmHEXB of the sperm plasma membrane. The enzymes from the sperm plasma membrane extracts have been assayed with antisera against *Hexo1* (a-HEXO1), *Hexo2* (a-HEXO2), and *fdl* (a-FDL) synthetic peptides. The corresponding negative controls carried out using the primary antisera pre-adsorbed with the synthetic peptides are indicated as ctr 1. For each primary antiserum, the absence of cross-reactivity is shown in controls 2 and 3 (ctr 2, ctr 3), carried out pre-incubating a-HEXO1 with *Hexo2* and *fdl* peptides, a-HEXO2 with *Hexo1* and *fdl* peptides, and a-FDL with *Hexo1* and *Hexo2*, respectively.

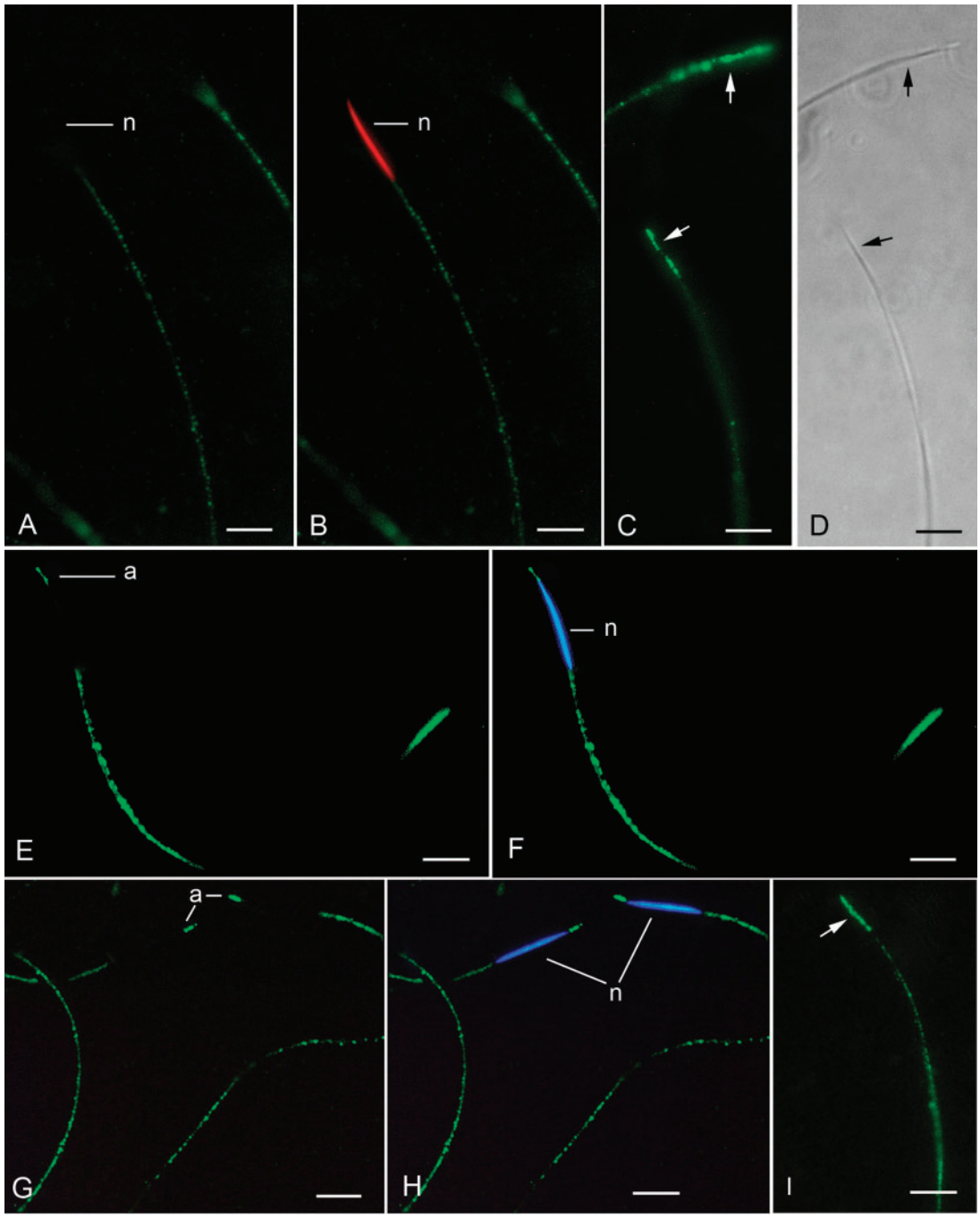

Fig. 7. Immunolocalization of *Hexo1*, *Hexo2*, and *fdl* products on the sperm plasma membrane. Primary antiserum binding was evidenced with Alexa Fluor 488-conjugated secondary antiserum (green), nuclei were counterstained with Hoechst 33342 (blue) or with propidium iodide (red) and the images were merged. (**A**–**D**) Immunolabeling with antiserum against *Hexo1* peptides. The tail is labeled over its whole length, whereas the nuclear tract of the plasma membrane is negative. (**C**, **D**) Paired micrographs with fluorescence and phase-contrast optics, showing intense labeling of the tail end piece (arrows). (**E**, **F**) Immunolabeling with antiserum against *Hexo2* peptides. The plasma membrane overlying the acrosome and the tail are labeled; the nuclear area is negative. (**G**–**I**) Immunolabeling with antiserum against *fdl* peptides. The plasma membrane over the acrosome and the tail is fluorescent, whereas it is negative over the nucleus. (**I**) The micrograph shows intense fluorescence over the tail end piece (arrow). a, acrosome; n, nucleus. Scale bars represent 5 μm.

2002). Their subunits are noncovalently associated and, as for all the other known β-acetylhexosaminidases (Mark *et al.*, 2003), they have been identified on the basis of their catalytic activities: the β-subunit hydrolyzes nonreducing terminal β-*N*-acetylglucosamine (β-GlcNAc) or β-*N*-acetylgalactosamine from neutral substrates, whereas the α-subunit hydrolyzes neutral substrates as well as negatively charged substrates like those bearing a terminal β-GlcNAc-6-sulfate (Cattaneo *et al.*, 2002). On this basis, we originally proposed that DmHEXA is a heterodimer of subunits α and β, whereas

DmHEXB is a homodimer of β-subunits (Cattaneo *et al.*, 2002). Because of their dimeric structure and catalytic activity, DmHEXA and DmHEXB are similar to the mammalian β-*N*-acetylhexosaminidases Hex A and Hex B, respectively (Proia *et al.*, 1984). The M_r of *Drosophila*-native isoenzymes is ~160 kDa, the apparent M_r of DmHEXA subunits is ~60 kDa, whereas the one of DmHEXB subunits is ~70 kDa. Isoenzymes similar to those of the sperm plasma membrane are present also in the seminal vesicles' secretion (Cattaneo *et al.*, 2002). Although β-hexosaminidases are generally present as soluble proteins in lysosomes, DmHEXA and DmHEXB are not an isolated case of nonlysosomal β-hexosaminidases. In fact, these enzymes, as well as other lysosomal glycosidases, exist also as integral proteins in the plasma membrane of invertebrate (Koyanagi and Honegger, 2003) as well as of vertebrate spermatozoa (Tulsiani and Abou-Haila, 2001; Khunsook *et al.*, 2003) and in the plasma membrane of somatic cells (Cordero *et al.*, 2001). Furthermore, they exist also as secreted species in body fluids of vertebrates (Beccari *et al.*, 2000; Tulsiani, 2003; Andersson *et al.*, 2005), of invertebrates (Marchini *et al.*, 1989; Del Pino *et al.*, 1999) and are secreted into the medium by the K_c cell line of *Drosophila* (Sommer and Spindel, 1991a,b).

In the mouse and in man, the organisms where β-*N*-acetylhexosaminidases have been best characterized, only two genes are present encoding these enzymes, HEXA and HEXB, which code for the α-subunit and the β-subunit, respectively (Mark *et al.*, 2003). In the present work, we have shown that in *Drosophila* all of the three putative genes coding for β-hexosaminidases are actually expressed in vivo. The results of the gene expression studies together with the results of immunoblot analysis demonstrating that, at least in the male germ line, all the genes are translated indicate that in the fruitfly a scenario exists more complex than the one present in mammals. In a very recent article, published after this study had been completed, *Hexo1*, *Hexo2*, and *fdl* have been recombinantly expressed in *Pichia pastoris*. The biochemical analysis performed on the fusion proteins confirmed that all the genes encode functional β-*N*-acetylhexosaminidases (Leonard *et al.*, 2006). Furthermore, determination of the specificity toward natural substrates suggested that the enzyme encoded by *fdl* might have a role in the biosynthesis of *N*-glycans (Leonard *et al.*, 2006), a function that has been previously ascribed also to a microsomal β-*N*-acetylhexosaminidase identified in lepidopteran cell lines (Altmann *et al.*, 1995).

The *D. melanogaster* genome database (http://www.flybase.org/) predicts multiple transcripts for *Hexo1*, that is, four long transcripts with a similar size and one short transcript, two very similar transcripts for *fdl* and a single transcript for *Hexo2*. All the transcripts originate from alternative splicing of pre-mRNAs. All the predicted polypeptides encoded by the transcripts contain the two typical domains of the enzymes of the glycosyl hydrolase family 20, to which β-hexosaminidases belong. We detected the transcripts in the testis as well as in other tissues and their size was generally in good agreement with the predictions. Although no quantitative evaluation of the gene expression has been carried out, the combined use of northern blots and RT–PCR suggested that the most prominent sites of *fdl* expression were the testis and the brain, whereas expression levels in other organs of the male and the female were either very low or almost undetectable. The finding that *fdl* was highly expressed in the brain was hardly surprising, since this gene is involved in the postembryonic development of this organ (Boquet *et al.*, 2000). However, the possible role of β-hexosaminidases in neuronal development remains to be determined. *Hexo1* and *Hexo2* transcripts have a broader tissue distribution. Northern blotting and RT–PCR indicated that *Hexo1* long transcripts are present at overall similar levels in whole males and in whole females, whereas their level of expression in the testis is extremely low. The testis instead preferentially expresses high levels of *Hexo1* short transcript. As to *Hexo2*, northern blotting suggests that the overall gene expression is higher in the male than in the female. Furthermore, the finding that the gene is expressed in the testis is new, since *Hexo2* cDNA is not represented in the testis EST library generated by the Berkeley *Drosophila* Genome Project (Stapleton *et al.*, 2002; http://www.fruitfly.org/DGC) or in the testis EST library available at NCBI (http://www.ncbi.nlm.nih.gov/projects/dbEST/).

Hexo1 short transcript is not a good candidate to code for *Drosophila* β-hexosaminidases because its size is too small to be compatible with any of the isoenzyme subunits. Furthermore, analysis of the predicted polypeptide encoded by this transcript suggests that the actual existence of this conceptual gene product is questionable. In fact, the absence of any sorting signal identifies it as a cytosolic protein, whereas, to the best of our knowledge, no β-*N*-acetylhexosaminidase with a cytosolic location has ever been reported. In addition, the predicted *N*-glycosylation sites and lack of a signal peptide are mutually exclusive. On the other hand, it has been demonstrated that not all the transcripts present in the *Drosophila* testis are actually translated (Andrews *et al.*, 2000). Instead, predictions related to M_r and glycosylation of the longer translation products of *Hexo1*, *Hexo2*, and of the *fdl* products are in good agreement with the results of the biochemical characterization of DmHEXA and DmHEXB and of their subunits (Cattaneo *et al.*, 2002).

D. melanogaster seems to be unique as far as the number of transcripts encoding β-hexosaminidases is concerned. In fact, the *D. pseudobscura* genome database, release 2.0, (http://www.flybase.org/) predicts the presence of only one transcript for all the genes encoding β-hexosaminidase-like polypeptides. When we analyzed the other organisms (Table II) with UCSC genome browser (http://www.genome.ucsc.edu/), we found that also in all of them only one transcript was predicted for each gene.

The catalytic domains of *Hexo1*, *Hexo2*, and *fdl* products show high conservation of the sequence containing the active site. The α- and β-subunits of the known β-hexosaminidases have identical structures of the active site (Mark *et al.*, 2003), but recent crystallographic studies of the α-subunit of the human β-hexosaminidases have demonstrated as its distinct feature the presence of Arg424 that confers the subunit the ability to accommodate negatively charged substrates at the active site (Mark *et al.*, 2003). The Arg555/542 residues of the *fdl* products might have the same role. We therefore suggest that the *fdl* products are homologs of the precursor of the mammalian α-subunit,

whereas *Hexo1* and *Hexo2* encode two distinct β-chains, β_1 and β_2, both homologs of the precursors of the β-subunit of all known β-hexosaminidases. However, bioinformatic analysis of *Drosophila* sequences raised some questions as to the possibility of reconciling the computational predictions with the experimental evidence that (1) a transmembrane and a secreted form of DmHEXA and DmHEXB exist and (2) the two forms have similar molecular masses (Cattaneo *et al*., 2002). Topology analysis of the alternative products of *fdl* identifies both as type II transmembrane proteins. This orientation places the bulk of the protein, with the potential glycosylation sites and the catalytic domain, on the extracellular face of the plasma membrane. Whereas topology prediction for *fdl* products is consistent with the experimentally determined characteristics of sperm β-hexosaminidases (Cattaneo *et al*., 2002), the long polypeptides encoded by *Hexo1* and by *Hexo2* are predicted to contain a cleavable signal peptide. Since no additional region of significant hydrophobicity that may function as a membrane-anchoring domain is predicted, the proteins should enter the secretory pathway as soluble forms. On the other hand, immunoblot analysis of DmHEXA and DmHEXB extracted from the sperm plasma membrane with procedures designed to solubilize transmembrane proteins and immunocytochemistry of whole spermatozoa clearly indicated that not only *fdl* product but also the polypeptides encoded by *Hexo1* and *Hexo2*, contrary to algorithm predictions, are present, at least in spermatozoa, as intrinsic membrane proteins. How the soluble forms secreted by the seminal vesicles (Cattaneo *et al*., 2002) and possibly in other organs might originate is, at present, a matter of speculation. In *fdl*, the presence of a potential consensus furin cleavage site between the transmembrane domain of *fdl* products and the bulk of extracellular part of the protein suggests the possibility that, along the secretory pathway, the protein might be proteolytically processed at this site by some of the furin-like proteins described in *Drosophila* (De Bie *et al*., 1995). The cleavage would yield soluble polypeptides with the same functional properties as the corresponding integral membrane forms and with a M_r ~7 to 8 kDa smaller, a difference that would have gone undetected by the experimental procedures used to define the M_r of the membrane-associated and secreted forms of the isoenzymes (Cattaneo *et al*., 2002). The current EST libraries (http://www.flybase.org/; http://www.fruitfly.org/DGC; http://www.ncbi.nlm.nih.gov/projects/dbEST/) do not report the expression in the male reproductive apparatus of any of the five *D. melanogaster* genes encoding furin or furin-like enzymes, that is, CG8481, CG10772, CG18734, CG6438, and CG7169. This issue needs therefore to be revisited, as the expression of these genes in the organs of the male reproductive apparatus might have been overlooked. As to *Hexo1* and *Hexo2*, if the signal peptide were not removed during biosynthesis, it would function as a membrane-anchoring domain, and the enzymes would be inserted in the plasma membrane with a type II protein topology similar to that of *fdl* products. Deficient cleavage of the *N*-terminal signal peptide that, despite algorithm predictions, confers a transmembrane topology has been described for other membrane-intrinsic proteins, which are found also as soluble forms (Gibson *et al*., 1993; Fleischer, 1994; Olivari *et al*., 2005). We therefore propose that the predicted long polypeptides encoded by *Hexo1*, *Hexo2* and the ones encoded by *fdl* might be post-translationally and differentially processed in different tissues, generating either integral proteins, as those of the sperm plasma membrane, or soluble secreted proteins as those present in the seminal fluid. Interestingly, when the genes were recombinantly expressed in yeast, *Hexo1* and *Hexo2* products were secreted into the medium (Leonard *et al*., 2006). Instead, the *fdl* fusion protein was predominantly localized in the plasma membrane as well as extracellularly, and only to a minor extent in the Golgi apparatus, an observation suggesting that the localization of protein in this organelle reflects its transit through this compartment while *en route* to the plasma membrane (Leonard *et al*., 2006). The findings of this study would also suggest that the *Drosophila* β-*N*-acetylhexosaminidases, at least when expressed in yeast, are not lysosomal enzymes.

The study of gene expression in the male reproductive apparatus by ISH corroborated the results by northern blotting and RT–PCR. In particular, the results of ISH for *Hexo1* using different probes for the gene 5′ and the 3′ regions confirmed that transcription of long mRNAs coding for β-hexosaminidases compatible with those evidenced on spermatozoa is very low. *Hexo2*, *Hexo1*, and *fdl* shared a similar temporal expression pattern during spermatogenesis: mRNAs were already detectable at low levels in spermatogonia; they reached the highest levels during meiosis and were present in small amounts in early elongating spermatids. The early expression of *fdl*, *Hexo1*, and *Hexo2* during spermatogenesis is in line with the thought that the late-expressed genes are more likely to be those specific for the male germ line (Eddy, 2002). Also the decrease in the levels of transcripts as the cells progressed from meiosis to early spermatid differentiation is in agreement with previous observations showing that a number of transcripts expressed in early stages of spermatogenesis do not persist into the spermatid advanced elongation stage (Fuller, 1998). ISH also confirmed that, although all of the genes are expressed both in the testis and in other organs of the male apparatus, *fdl* has a highly preferential expression in the male germ line, and its expression in other organs is extremely low. The finding that the genes coding for β-hexosaminidases are expressed also in the epithelia of the male reproductive apparatus is not unexpected, since we previously reported the presence of soluble secreted forms of DmHEXA and DmHEXB in the seminal vesicles' fluid (Cattaneo *et al*., 2002). Therefore, it is likely that the enzymes are secreted also by the accessory glands and the ejaculatory duct and bulb.

The observation that genes coding for sperm molecules that participate in gamete interactions are expressed also in somatic cells is not new. In the mouse, the best known mammalian model for the study of fertilization, a GalT I and type 1 hexokinase (HK1), two sperm plasma membrane molecules that bind to ZP, are encoded by the same genes that code for the GalT I and HK1 ubiquitously expressed in somatic cells (Travis *et al*., 1999; Rodeheffer and Shur, 2002). However, in both cases it has been demonstrated that the sperm plasma membrane forms represent germ cell-specific isoforms derived from differential

transcriptional and post-transcriptional controls. The study of the expression of *Hexo1*, *Hexo2*, and *fdl* leaves a number of questions to be answered: are all the alternative transcripts expressed at the protein levels? Is any of the predicted protein isoforms specific for the male germ line? Since the gene transcripts predicted by the genomic database are very close in size, we were not able to ascertain which transcript was expressed in the germ line as opposed to the somatic cells. Further investigations are therefore necessary to determine which is/are the transcript(s) present in spermatogenic cells and whether all the mRNA isoforms are translated.

DmHEXA and DmHEXB enzyme activities were present not only in the adult testis but also in the larval testis. This finding would suggest that the genes coding for isoenzyme subunits are translated shortly after transcription in premeiotic cells and also in meiotic cells and round spermatids. Since the α- and β-subunits are enzymatically inactive as monomers (Mahuran, 1995; Mark *et al*., 2003) and since it has been shown in other organisms that dimer formation occurs shortly after translation (Proia *et al*., 1984), it is conceivable that also in the *Drosophila* spermatogenic cells subunit translation is rapidly followed by their assembly into functional dimers. Therefore, transcription and translation of the genes coding for the subunits must be coordinated to allow the monomers to associate with each other or with the appropriate complementary monomer. However, the lower levels of *fdl* expression in the larval testis as compared to *Hexo2* expression, as shown by ISH, would indicate that the α-subunit transcription is in some degree delayed in comparison with the transcription of the β_2-subunit and might explain the significant difference in DmHEXA activity between the larval and adult gonad.

Immunodot blot analysis and immunofluorescence showed unambiguously that *Hexo1*, *Hexo2*, and *fdl* products are localized in the sperm plasma membrane. Immunodot blotting demonstrated that *fdl* and *Hexo2* code for the subunits of sperm DmHEXA, whereas DmHEXB subunits are encoded by *Hexo1* and *Hexo2*. On the basis of sequence and immunoblot analysis, we can draw the conclusion that in spermatozoa DmHEXA has a $\alpha\beta_2$ structure and DmHEXB has a $\beta_1\beta_2$ structure. However, we cannot rule out the possibility that in cells other than those of the male germ line, the subunits might assemble in a different way. In fact, assuming that also in the yeast model system used to recombinantly express the *Drosophila* genes (Leonard *et al*., 2006) β-hexosaminidases are active only when in a dimeric form (Mahuran, 1995; Mark *et al*., 2003), the expression of *Hexo1*, *Hexo2*, and *fdl* should have yielded homodimeric enzymes, with the structure $\beta_1\beta_1$, $\beta_2\beta_2$, and $\alpha\alpha$, respectively. In this case, the $\alpha\alpha$ isoform would be the homolog of the mammalian β-hexosaminidase Hex S (Proia *et al*., 1984; Mark *et al*., 2003).

Immunofluorescence showed that the enzyme subunits encoded by *Hexo2* and *fdl* are present on the plasma membrane over the acrosome. Although the series of events leading to egg fertilization in insects are totally unknown, it is conceivable that, like in all the other organisms so far studied, molecules that are localized over the acrosome might be involved in sperm–egg interactions. These findings therefore suggest a major role for *Hexo2* and *fdl* and for their products, DmHEXA, in fertilization. The immunostaining pattern of the end piece of the tail after incubation in anti-*Hexo1* and anti-*fdl* peptides is similar to the one that was previously described for lectin-binding sites of the *Drosophila* sperm surface (Perotti and Pasini, 1995), but its functional significance is obscure.

The results of enzyme assays indicating that most of DmHEXA activity in the male originates from the testis are in a good agreement with the data of gene expression showing that *fdl* expression is highest in the testis, and with the results of immunodot blotting and immunocytochemistry proving that *fdl* encodes a subunit present on sperm surface. DmHEXB activity levels in the testis are significantly higher than those measured on mature sperm. We hypothesize that some of active enzyme present in spermatogenic cells is eliminated at the end of spermatid differentiation, when considerable amounts of cytoplasm, membranes, and organelles are caudally shed within the so-called waste bag (Fuller, 1998). An additional contribution to the total DmHEXB activity measured in the testis probably originates from the epithelium of the terminal tract of the testis, where *Hexo1* and *Hexo2* are expressed. Significant levels of DmHEXB activity are present also in other components of the male reproductive apparatus and in whole males. The brain and the midgut, where the genes coding for the β-subunits of DmHEXB are expressed, are obviously good candidates as sources of the enzyme. In particular, it is known that extracellular β-hexosaminidases are present in the insect midgut with a role as digestive enzymes (Lehane, 1997) or as a component of the chitinolytic system that is involved in the turnover of the peritrophic membranes (Filho *et al*., 2002). However, other organs that are at present indeterminate might contribute to the total DmHEXB activity. In fact, the biochemical assays carried out in this study on homogenates of testes and of other organs might have detected activities related to other membrane-associated forms and soluble forms present in the supernatants. In this context, it is useful to notice that, when *fdl* was expressed in vitro, only a minor amount of the gene product localized in the Golgi apparatus (Leonard *et al*., 2006) and that a soluble and a membrane-bound protein with β-*N*-acetylhexosaminidase activity have been previously evidenced within microsomes from lepidopteran cell lines where they are supposedly involved in the metabolism of *N*-linked oligosaccharides (Altmann *et al*., 1995). Additionally, the possibility cannot be ruled out that, like in other organisms, β-hexosaminidases exist also as lysosomal species, although, to the best of our knowledge, there is no report of their presence in insect lysosomes. However, it is unlikely that the homogenization and solubilization procedures here employed would have released enzymes from microsomes/Golgi vesicles and from lysosomes massively. In addition, the activities of the potential β-hexosaminidases from lysosomes or *en route* to lysosomes through the rough endoplasmic reticulum and the Golgi apparatus would not have been efficiently detected at pH 6.5, the optimal pH for the enzymes assayed in this study (Cattaneo *et al*., 2002).

Collectively, we have identified *Hexo1*, *Hexo2*, and *fdl* as the genes coding for β-*N*-acetylhexosaminidases of the plasma membrane of *D. melanogaster* spermatozoa for

which a role as egg receptors at fertilization have been previously suggested, and we have defined the dimeric structure of these enzymes. Although none of the genes are testis-specific, *fdl* is highly and preferentially expressed in the male germ line. Expression and translation of the genes occur as early as in the larval stage.

The mechanisms of egg fertilization in insects are still enigmatic because their gametes are not amenable to in vitro manipulation, and the well-established sperm–egg binding assays with isolated cells that are used to study gamete interactions in vertebrate and in a variety of other invertebrates cannot be applied. This study represents a firm basis for future and more mechanistic investigations aimed at unraveling the role of glycosidases in gamete interactions in this animal class using *Drosophila* as a model organism.

Materials and Methods

Flies

Wild-type flies of the Oregon R strain were maintained on standard cornmeal-sugar yeast-agar medium at 25 ± 1°C. Late third instar larvae were sexed and used immediately. Adult males and females were separated at eclosion and used 6 days later.

Chemicals

All reagents were obtained from Sigma (St. Louis, MO), when not otherwise indicated.

Source of genome annotation, databases, and software

The open reading frames (ORFs) of *Hexo1* (CG1318), *Hexo2* (CG1787), *fdl* (CG8824), and *Act5C* (CG4027) were retrieved from *Drosophila* genome release 4.2 (Drysdale *et al*., 2005; http://www.flybase.org/) and analyzed using the Ensembl database for *Drosophila* (http://www.ensembl.org/Drosophila_melanogaster) and BLASTP at NCBI (http://www.ncbi.nlm.nih.gov/). Sequence alignment was performed with the program T-Coffee accessible at EMBnet.ch (http://www.ch.embnet.org/software/TCoffee.html) and the program Align accessible at the Genestream network server (http://xylian.igh.cnrs.fr/bin/align-guess.cgi). The ORFs putative products were analyzed for hydrophobicity profile, detection of signal-peptide cleavage sites and analysis of nonclassically secreted proteins with the programs TMHMM V.2.0, SignalP V3.0, and Secretome 1.0b, all accessible at the Center for Biological Sequence Analysis, Technical University, Denmark (http://www.cbs.dtu.dk/services/). The sequences were further evaluated with the combined transmembrane topology and signal-peptide predictor Phobius (http://phobius.binf.ku.dk/).

RT–PCR

Total RNA was extracted from *Drosophila* tissues using Tri-Reagent and then treated with RQ1 RNase-free DNase (Promega, Madison, WI), according to the manufacturer's instructions. PCR primers for each gene were designed on the basis of the genomic sequence information (Drysdale *et al*., 2005) (Table I). Two micrograms of total RNA was reverse transcribed with 200 U/μL of Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega) and 1 μg random hexamers (Promega) in a 50 μL reaction mixture. The cDNA was amplified by PCR in a 30 μL reaction mixture with 0.6 U of *Taq* DNA polymerase (Promega), 200 mM deoxynucleoside triphosphate (dNTP) mix, 2.5 mM $MgCl_2$, and 1 μM of each primer. The amount of each template in PCR was standardized to 10 ng of cDNA, with the exception of the testis template to be primed with the H1-A oligonucleotides that was used also at 100 ng. Ten microliters of each PCR product was electrophoresed on 1% agarose gel and stained with ethidium bromide. The PCR products were cloned into pGEM-T vector (Promega) and sequenced with M13 forward and reverse primer using SequiTherm DNA Polymerase (Epicentre Technology, Madison, WI). Sequences were analyzed using the ABI PRISM 3100 Genetic Analyzer (Hitachi Ltd, Tokyo, Japan).

Controls were performed by omitting reverse transcriptase.

RNA probes

Table I shows the oligonucleotide constructs used as probes. Digoxigenin (DIG)-labeled and ^{32}P-labeled probes were obtained using the labeling kit from Roche Diagnostics GmbH, Mannheim, Germany, and the labeling kit from Promega, respectively. *Hexo1*-DIG-labeled and radiolabeled RNA probes were obtained by in vitro transcription with SP6 RNA polymerase (Roche) of plasmids pH1-A and pH1-B linearized with the restriction enzyme NcoI (Promega). Plasmids pH1-A and pH1-B are pGEM-T derivatives carrying the *Drosophila* cDNA fragments 293425–293744 (pH1-A) and 294626–294912 (pH1-B). The fragments were obtained by PCR amplification of cDNA with the oligonucleotides sH1-A and asH1-A and sH1-B and asH1-B, respectively, and cloned with the higher coordinates close to the SP6 promoter. *Hexo2*-DIG- and radiolabeled probes were obtained by in vitro transcription with SP6 RNA polymerase of the NcoI-digested pGEM-T derivative pH2, containing the DNA fragment 296805–297133 obtained by PCR amplification of cDNA with the oligonucleotides sH2 and asH2. The *fdl*-DIG- and radiolabeled probes were obtained by in vitro transcription with T7 RNA polymerase (Roche) of the fragment 199883–200260 obtained by PCR amplification of the cDNA with the oligonucleotides s*fdl* and as*fdl*. To obtain radiolabeled probes for *Act5C*, which encodes a ubiquitous actin isoform, the fragment 273772–277085 obtained by PCR amplification of cDNA with the oligonucleotides sAct5C and asAct5C was cloned in pGEM-T. The plasmid was then digested with NcoI and used as template for in vitro transcription with SP6 RNA polymerase.

Northern blot analysis

Expression of β-*N*-hexosaminidase mRNA in whole males and females and in the testis was analyzed by northern blot with radiolabeled RNA probes. Total RNA (15 μg per lane) was fractionated by electrophoresis on a 1.5% agarose–formaldehyde gel, transferred onto a Hybond-N^+ membrane (Amersham Pharmacia Biotech, Little Chalfont, UK) by vacuum blotting and hybridized with different

^{32}P-labeled RNA probes. Hybridization to specific riboprobes was performed as previously described (Dehò *et al.*, 1992). After hybridization, the membranes were analyzed using Typhoon 8600 PhosphorImager (Molecular Dynamics/Amersham Biosciences/GE Healthcare, Little Chalfont, UK). As a loading control, all the filters were hybridized also with the *Act5C* riboprobe specific for actin mRNA.

In situ hybridization

Whole-mount ISH was carried out on testis from third instar larvae and on the reproductive apparatus of adult males following a standard procedure (White-Cooper *et al.*, 1998), using DIG-labeled antisense riboprobes and alkaline phosphatase (AP)-labeled anti-DIG antibody (Roche). Controls were performed using DIG-labeled sense riboprobes. Microscope analysis was performed using a Leica DMRB microscope (Leica AG, Heerbrug, Switzerland) equipped with the cooled CCD video camera Leica DCF480. Photographic plates were prepared using Adobe Photoshop CS2.9.

Enzyme assays

The preparative procedures originally designed to assay the sperm enzymes without disrupting the sperm plasma membrane (Cattaneo *et al.*, 2002) have been used also for the other tissues and organs studied here. Fourteen males were homogenized on ice in 240 μL extraction medium—20 mM 3-((3-cholamidopropyl)dimethylammonio(-1-propanesulfonate (CHAPS) in phosphate buffer saline (PBS) with protease inhibitors (PBS-In), pH 7.2 (Cattaneo *et al.*, 2002)—with an Ultra-Turrax T25 homogenizer (IKA-Werke GMBH, Staufen, Germany) operating at 9,500 rpm for 3 min. Testes and whole reproductive apparatuses were dissected from 14 males and collected on ice, washed thrice in 100 μL PBS-In and manually homogenized in Eppendorf tubes containing 240 μL of ice-cold extraction medium using a tight fitting pestle. The homogenized samples were kept in extraction medium for 1 h at 4°C to allow solubilization of membrane proteins and were subsequently centrifuged at 14,000 × *g* for 2 min at 4°C. The supernatants were used for protein determination and enzyme assays. Larval testes were dissected out and processed following the same procedures. Sperm were removed from the seminal vesicles of 14 males and collected on ice. Extraction of sperm plasma membrane proteins was carried out in 240 μL extraction medium (20 mM CHAPS in PBS-In, pH 7.2) as described in detail previously, and the samples were centrifuged at 14,000 × *g* for 2 min at 4°C (Cattaneo *et al.*, 2002). Fluorimetric enzyme assays were performed on supernatants using 4-methylumbelliferone (4-MU) conjugates as substrates as previously described (Cattaneo *et al.*, 2002). DmHEXA activity was measured by determining the catalytic activity of the α-subunit toward 4-MU-7-(6-sulfo-2-acetamido-2-deoxy-β-D-glucopyranoside), whereas DmHEXB activity was determined assaying the activity of the β-subunit using 4-MU-2-acetamido-2-deoxy-β-D-glucopyranoside. Protein concentration was determined in sperm extracts by measuring absorbance at 280 nm and in extracts from other samples by Bradford's method (Bradford, 1976), using bovine serum albumin (BSA) as standard. Data were expressed as specific activities and enzyme activities. All the experiments were carried out in triplicate. The results were presented as mean ± SEM, with $n = 5$. Statistical analysis was performed by one-way ANOVA test, and the data were considered statistically significant when $p < 0.01$.

Immunoblotting analysis

Antibodies were elicited against synthetic peptides encompassing two different regions for each of the polypeptides encoded by *Hexo1*, *Hexo2*, and *fdl* (Figure 1A). Synthesis of immunogens, antisera production and purification were done by Primm srl (Milan, Italy). Briefly, the immunogenic peptides were assembled using the multiple-antigen peptide (MAP) system on a 4-branched polylysine core. Structure of MAP peptides was verified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, and the corrected MAPs were purified by reverse high-performance liquid chromatography (HPLC). The two peptides encoded by each gene were then used as immunogens in a 1:1 mixture. Antisera to *Hexo1* and *Hexo2* peptides were raised in rabbits, whereas antisera to *fdl* peptides were raised in mice following standard protocols. Rabbit antisera were affinity-purified on synthetic peptide–Sepharose columns, whereas mouse antiserum was used crude. Antisera titers were monitored with enzyme-linked immunoadsorbent assay (ELISA) before and after purification.

For immunodot blotting analysis, DmHEXA and DmHEXB were extracted from the plasma membrane of sperm removed from the seminal vesicles of 1600 males and separated by ion-exchange chromatography on a diethylaminomethyl Sepharose (DEAE) column, as described previously (Cattaneo *et al.*, 2002). DmHEXA and DmHEXB fractions were concentrated in a Vivaspin concentrator (Sigma) to a final protein concentration of 0.15 and 0.12 mg/mL, respectively. Three microliters of DmHEXA and DmHEXB fractions was spotted on Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech) and air-dried. Membranes were then blocked with 50 mM Tris buffer saline (TBS) supplemented with 0.1% Tween 20 and 2% BSA, and after TBS washing, they were probed with 100 μL of serial dilutions of primary antisera in TBS/Tween 20 for 1 h at room temperature. Following extensive washing with TBS/Tween 20, binding of primary antibodies was revealed with biotinylated antirabbit/antimouse antisera and the ABC-peroxidase method using the Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA) and 3,3,5,5′-tetramethylbenzidine (TMB) as a chromogenic substrate according to the manufacturer's instructions. Antisera optimal concentration was 40 μg/mL. The following negative controls were carried out: (1) substitution of the primary antisera with pre-immune sera, (2) omission of the primary antisera, and (3) pre-absorption of the primary antisera with the corresponding 5 mM synthetic peptides for 12 h at 4°C before use. As additional controls for antisera specificity, each primary antiserum was pre-incubated with the peptides encoded by the other two genes for 12 h at 4°C before probing membranes.

For western blot analysis, following DEAE separation and concentration, DmHEXA and DmHEXB fractions were resuspended in 2% sample buffer without 2-mercaptoethanol and electrophoresed on 10% polyacrylamide gel containing 0.1% (w/v) sodium dodecyl sulfate (SDS). Staining

of proteins with silver staining performed as described previously (Cattaneo *et al*., 2002) revealed the presence of bands with the molecular masses expected for DmHEXA and DmHEXB subunits (Cattaneo *et al*., 2002). Unstained samples of the separated proteins were electrophoretically transferred to nitrocellulose membranes in 25 mM TB/192 mM glycine (pH 7.8) and 20% (v/v) methanol at 0.25 A overnight at 4°C. Reversible Ponceau S staining was used to verify transfer efficiency. The membranes were blocked as described above and probed with primary antisera undiluted and at a 40 μg/mL concentration in TBS/Tween 20. Incubation in primary antisera was carried out for 1 h at room temperature and overnight at 4°C. Bound primary antibodies were detected with biotinylated secondary antibodies and the ABC/TMB method as described above or with the ABC method followed by chemiluminescence detection of peroxidase using the SuperSignal kit (Pierce, Rockford, IL), according to the manufacturer's instructions. For chemiluminescence detection, immunoblot membranes were exposed to ECL films/X-Omat XAR (5) (Kodak, Rochester, NY) for 60 s up to 2 h. DmHEXA and DmHEXB fractions resuspended in SDS-free sample buffer were also processed for immunoblotting according to the procedures described above.

Immunofluorescence

Mature spermatozoa from the seminal vesicles were thoroughly washed in PBS (pH 7.2), fixed for 10 min with 2% paraformaldehyde in PBS at room temperature, blocked with 0.2 M NH_4Cl for 30 min, washed in PBS, and blocked again with 10% normal goat serum in PBS supplemented with 1% BSA for 30 min. They were then incubated in 40 μg/mL primary antiserum in 1% BSA–PBS for 1 h, and, following PBS washing, for 1 h in 5 μg/mL Alexa Fluor 488-goat antirabbit/antimouse antiserum (Molecular Probes, Eugene, OR) supplemented with 3 μg/mL Hoechst 33342 or with 1 μg/mL propidium iodide (PI). Negative controls were carried out as indicated for immunodot blotting analysis. Microscopic analysis was carried out with a Leica DMRB microscope equipped with a 100× oil immersion objective, the CCD-camera indicated above and manufacturer's filters for the fluorescent dyes (for Hoechst 33342, the filter set BP340–380, RKP 400, and LP 430; for Alexa Fluor 488, the fluorescein filter set BP488, BP 450–490, RKP 510, and BP 525/20; for PI, the tetramethylrhodamine filter set BP546/14, RKP 580, and LP 580). Images were recorded and processed as indicated above.

Acknowledgments

We thank G. Dehò for helpful discussions, C. Gissi and G. Pavesi for help with bioinformatics, and F. Cenni for technical help. This work was supported by PRIN grants from Italian Ministry of Research and grants from the University of Milano awarded to M.E. Perotti, and by joint JSPS-CNR fellowships to F. Cattaneo and M.E. Pasini.

Conflict of interest statement

None declared.

Abbreviations

BSA, bovine serum albumin; DEAE, diethylaminomethyl Sepharose; DIG, digoxigenin; EST, expressed sequence tag; ISH, in situ hybridization; MAP, multiple-antigen peptide; M_r, relative molecular mass; 4-MU, 4-methylumbelliferone; PBS, phosphate buffer saline; PBS-In, PBS with protease inhibitors; PCR, polymerase chain reaction; RT, reverse transcriptase; TBS, Tris buffer saline; ZP, zona pellucida.

References

Altmann, F., Schwihla, H., Staudacher, E., Glossl, J., and Marz, L. (1995) Insect cells contain an unusual, membrane-bound β-N-acetylglucosaminidase probably involved in the processing of protein N-glycans. *J. Biol. Chem*., **2270**, 17344–17349.

Andersson, S.V., Sjogren, E.C., Magnusson, C., and Gierow, J.P. (2005) Sequencing, expression and enzymatic characterization of β-hexosaminidase in rabbit lacrimal gland and primary cultured acinar cells. *Glycobiology*, **15**, 211–220.

Andrews, J., Bouffard, G.G., Jining Lü, C.C., Becker, K.G., and Oliver, B. (2000) Gene discovery using computational and microarray analysis of transcription in the *Drosophila melanogaster* testis. *Genome Res*., **10**, 2030–2043.

Beccari, T., Mancuso, F., Costanzi, E., Tassi, C., Barone, R., Fiumara, A., Orlacchio, A., Aisa, M.C., and Orlacchio, A. (2000) β-Hexosaminidases, α-D-mannosidase and β-mannosidase expression in serum from patients with carbohydrate-deficient glycoprotein syndrome type I. *Clin. Chim. Acta*, **302**, 125–132.

Bendtsen, J.D., Jensen, L.J., Blom, N., von Heijne, G., and Brunak, S. (2004) Feature-based prediction of non-classical and leaderless protein secretion. *Protein Eng*., **17**, 349–356.

Boquet, I., Hitier, R., Dumas, M., Chaminade, M., and Preat, T. (2000) Central brain postembryonic development in *Drosophila*: implication of genes expressed at the interhemispheric junction. *J. Neurobiol*., **42**, 33–48.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*., **72**, 248–254.

Cattaneo, F., Ogiso, M., Hoshi, M., Perotti, M.E., and Pasini, M.E. (2002) Purification and characterization of the plasma membrane glycosidases of *Drosophila melanogaster* spermatozoa. *Insect Biochem. Mol. Biol*., **32**, 929–941.

Cooper, K.W. (1950) Normal spermatogenesis in Drosophila. In Demerec, M. (ed.), *Biology of Drosophila*. John Wiley & Sons Inc., New York, pp. 1–61.

Cordero, O.J., Merino, A., de la Cadena, M.P., Bugía, B., Nogueira, M., Viñuela, J.E., Martínez-Zorzano, V.S., de Carlos, A., and Rodríguez-Berrocal, F.J. (2001) Cell surface human α-L-fucosidase. *Eur. J. Biochem*. **268**, 3321–3331.

De Bie, I., Savaria, D., Roebroek, A.J.M., Lazure, C., Van de Ven, W.J.M., and Seudah, N.G. (1995) Processing specificity and biosynthesis of the *Drosophila melanogaster* convertase dfurin1, dfurin1-CCR, dfurin1-x and dfurin2. *J. Biol. Chem*., **270**, 1020–1028.

Dehò, G., Zangrossi, S., Sabbatini, P., Sironi, G., and Ghisotti, D. (1992) Bacteriophage P4 immunity controlled by small RNAs via transcription termination. *Mol. Microbiol*., **6**, 3415–3425.

Del Pino, F.A.B., Brandelli, A., Termignoni, C., Gonzales, J.C., Henriques, J.A.P., and Dewes, H. (1999) Purification and characterization of β-N-acetylhexosaminidase from tick *Boophilus microplus* (Ixodide) larvae. *Comp. Biochem. Physiol. B*, **123**, 193–200.

Dell, A., Morris, H.R., Easton, R.L., Patankar, M., and Clark, G.F. (1999) The glycobiology of gametes and fertilisation. *Biochem. Biophys. Acta*, **1473**, 196–205.

Drysdale, R.A., Crosby, M.A., and The Flybase Consortium (2005) FlyBase: genes and gene models. *Nucleic Acids Res*., **33**, D390–D395.

Eddy, E.M. (2002) Male germ cell gene expression. *Recent Prog. Horm. Res*., **57**, 103–128.

Filho, B.P.D., Lermos, F.J.A., Secundino, N.F.C., Pascoa, V., Pereira, S.T., and Pimenta, P.F.P. (2002) Presence of chitinase and beta-N-acetylglucosaminidase in the *Aedes aegypti* A chitinolitic system

involving peritrophic matrix formation and degradation. *Insect Biochem. Mol. Biol.*, **32**, 1723–1729.

Fleischer, B. (1994) CD26: a surface protease involved in T-cell activation. *Immunol. Today*, **15**, 180–183.

Focarelli, R., La Sala, G.B., Balasini, M., and Rosati, F. (2001) Carbohydrate-mediated sperm-egg interaction and species specificity: a clue from the *Unio elongatus* model. *Cells Tissues Organs*, **168**, 76–81.

Fuller, M.T. (1998) Genetic control of cell proliferation and differentiation in *Drosophila* spermatogenesis. *Semin. Cell. Dev. Biol.*, **9**, 433–444.

Gibson, K.R., Vanek, P.G., Kaloss, W.D., Collier, G.B., Connaughton, J.F., Angelichio, M., Livi, G.P., and Fleming, P.J. (1993) Expression of dopamine β-hydroxylase in Drosophila Schneider 2 cells. *J. Biol. Chem.*, **268**, 9490–9495.

Henrissat, B. and Bairoch, A. (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.*, **293**, 781–788.

Khunsook, S., Bean, B.S., McGowan, S.R., and Alhadeff, J.A. (2003) Purification and characterization of plasma membrane-associated human sperm α-L-fucosidase. *Biol. Reprod.*, **68**, 709–716.

Koyanagi, R. and Honegger, T.G. (2003) Molecular cloning and sequence analysis of an ascidian egg β-N-acetylhexosaminidase with a potential role in fertilization. *Dev. Growth Differ.*, **45**, 209–218.

Lehane, M.J. (1997) Peritrophic matrix structure and function. *Annu. Rev. Entomol.*, **42**, 525–550.

Leonard, R., Rendic, D., Rabouille, C., Wilson, I.B.H., Preat, T., and Altmann, F. (2006) The *Drosophila fused lobes* gene encodes an N-acetylglucosaminidase involved in N-glycan processing. *J. Biol. Chem.*, **281**, 4867–4875.

Mahuran, D.J. (1995) β-Hesosaminidase: biosynthesis and processing of the normal enzyme, and identification of mutations causing Jewish Tay-Sachs disease. *Clin. Biochem.*, **28**, 101–106.

Marchini, D., Bertini, L.F., and Dallai, R. (1989) β-*N*-acetylhexosaminidases in the secretion of the female reproductive accessory glands of *Ceratitis capitata* (Diptera). *Insect Biochem.*, **19**, 549–555.

Mark, B.L., Mahuran, D.J., Cherney, M.M., Zhao, D., Knapp, S., and James, M.N.G. (2003) Crystal structure of human β-hexosaminidase B: understanding the molecular basis of Sandhoff and Tay-Sachs disease. *J. Mol. Biol.*, **327**, 1093–1109.

Martinez, M.L., Martelotto, L., and Cabda, M.O. (2000) Purification and biological characterization of N-acetyl-β-D-glucosaminidase from *Bufo arenarum* spermatozoa. *Mol. Reprod. Dev.*, **57**, 194–203.

Miranda, P.V., Gonzalez-Echeverria, V., Blaquier, J.A., Mahuran, D.J., and Tezon, J.G. (2000) Evidence for the participation of β-hexosaminidase in human sperm-zona pellucida interaction in vitro. *Mol. Hum. Reprod.*, **6**, 699–706.

Olivari, S., Galli, C., Alanen, H., Ruddock, L., and Molinari, M. (2005) A novel stress-induced EDEM variant regulating endoplasmic reticulum-associated glycoprotein degradation. *J. Biol. Chem.*, **280**, 2424–2428.

Perotti, M.E., Cattaneo, F., Pasini, M.E., Verni, F., and Hackstein, J.H.P. (2001) Male sterile mutant *casanova* gives clues to mechanisms of sperm-egg interactions in *Drosophila melanogaster*. *Mol. Reprod. Dev.*, **60**, 248–259.

Perotti, M.E. and Pasini, M.E. (1995) Glycoconjugates of the surface of the spermatozoa of *Drosophila melanogaster*: a qualitative and quantitative study. *J. Exp. Zool.*, **271**, 311–318.

Prag, G., Papanikolau, Y., Tavlas, G., Vorgias, C.E., Petratos, K., and Oppenheim, A.B. (2000) Structures of chitobiase mutants complexed with the substrate di-N-acetyl-D-glucosamine: the catalytic role of the conserved acidic pair, Aspartate 539 and Glutamate 540. *J. Mol. Biol.*, **300**, 611–617.

Proia, R.L., d'Azzo, A., and Neufeld, E.F. (1984) Association of α and β-subunits during the biosynthesis of β-hexosaminidase in cultured human fibroblasts. *J. Biol. Chem.*, **259**, 3350–3354.

Rodeheffer, C. and Shur, B.D. (2002) Targeted mutations in β1,4-galactosyltransferase I reveals its multiple cellular functions. *Biochim. Biophys. Acta*, **1573**, 258–270.

Sommer, U. and Spindler, K.D. (1991a) Demonstration of β-N-acetyl-D-glucosaminidase and β-N-acetyl-D-hexosaminidase in *Drosophila* K_c cells. *Arch. Insect Biochem. Physiol.*, **17**, 3–13.

Sommer, U. and Spindler, K.D. (1991b) Physical properties of β-N-acetyl-D-glucosaminidase and β-N-acetyl-D-hexosaminidase from *Drosophila* K_c cells. *Arch. Insect Biochem. Physiol.*, **18**, 45–53.

Stapleton, M., Carlson, J., Borkstein, P., Yu, C., Champe, M., George, R., Guarin, H., Kronmiller, B., Pacleb, J., Park, S., and others (2002) A Drosophila full-length cDNA resource. *Genome Biol.*, **3**, 0080.1–0080.8.

Talbot, P., Shur, B.D., and Myles, D.G. (2003) Cell adhesion and fertilization: steps in oocyte transport, sperm-zona pellucida interactions, and sperm-egg fusion. *Biol. Reprod.*, **68**, 1–9.

Thomas, G. (2002) Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat. Rev. Mol. Cell Biol.*, **3**, 753–766.

Travis, A.J., Sui, D., Riedel, K.D., Hofmann, N.R., Moss, S.B., Wilson, J.E., and Kopf, G.S. (1999) A novel NH_2-terminal, non-hydrophobic motif targets a male germ cell-specific hexokinase to the endoplasmic reticulum and plasma membrane. *J. Biol. Chem.*, **274**, 34467–34475.

Tulsiani, D.R.P. (2003) Glycan modifying enzymes in the luminal fluid of rat epididymis: are they involved in altering sperm surface glycoproteins during maturation? *Microsc. Res. Tech.*, **61**, 1827.

Tulsiani, D.R.P. and Abou-Haila, A. (2001) Mammalian sperm molecules that are potentially important in interaction with female genital tract and egg vestments. *Zygote*, **9**, 51–69.

Vo, L.H., Yen, T.Y., Macher, B.A., and Hedrick, J.L. (2003) Identification of the ZPC oligosaccharide ligand involved in sperm binding and the glycan structures of *Xenopus laevis* vitelline envelope glycoproteins. *Biol. Reprod.*, **69**, 1822–1830.

White-Cooper, H., Schaefer, M.A., Alphey, L.S., and Fuller, M.T. (1998) Transcriptional and post-transcriptional control mechanisms coordinate the onset of spermatid differentiation with meiosis I in *Drosophila*. *Development*, **125**, 125–134.