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β-Hexosaminidase From *Xenopus laevis* Eggs and Oocytes: From Gene to Immunochemical Characterization

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ABSTRACT

Glycosidases are present both in sperm and eggs in vertebrates and have been associated with different fertilization steps as gamete binding, egg coat penetration, and polyspermy prevention. In this manuscript, we have analyzed the activity of different glycosidases of *Xenopus laevis* eggs. The main activity corresponded to *N*-acetyl-β-D-glucosaminidase (Hex), which was reported to participate both in gamete binding and polyspermy prevention among phylogenetically distant animals. We have raised homologous antibodies against a recombinant N-terminal fragment of a *X. laevis* Hex, and characterized egg's Hex both by Western blot and immunohistochemical assays. Noteworthy, Hex was mainly localized to the cortex of animal hemisphere of full-grown oocytes and oviposited eggs, and remained unaltered after fertilization. Hex is constituted by different pair arrangements of two subunits (α and β), giving rise to three possible Hex isoforms: A (αβ), B (ββ), and S (αα). However, no information was available regarding molecular identity of Hex in amphibians. We present for the first time the primary sequences of two isoforms of *X. laevis* Hex. Interestingly, our results suggest that α- and β-like subunits that constitute Hex isoforms could be synthesized from a same gene in *Xenopus*, by alternative exon use. This finding denotes an evolutionary divergence with mammals, where α and β Hex subunits are synthesized from different genes on different chromosomes. J. Cell. Biochem. 113: 3709–3720, 2012.

KEY WORDS: AMPHIBIANS; BLOCK TO POLYSPERMY; EGG; FERTILIZATION; GLYCOSIDASES; HEXOSAMINIDASE

Fertilization is a complex process that requires multiple coordinated events leading to sperm and egg fusion. Some of these events involve protein–carbohydrate interactions. Experimental evidence suggests that sperm surface glycosidases could act in a non-catalytic or lectin-like manner, recognizing specific sugar moieties in the egg envelope (*zona pellucida* in mammals and vitelline envelope in amphibians) involved in sperm–egg binding [Miranda et al., 1997; Martinez et al., 2000; Perotti et al., 2001; Miller et al., 2002; Intra et al., 2006, 2011; Zitta et al., 2006; Hedrick, 2008; Honegger and Koyanagi, 2008]. In addition to this role, glycosidases released from activated-eggs have been proposed to act on glycosidic residues present in the egg envelope modifying sperm binding sites, thus ensuring a block to polyspermy [Prody et al., 1985; Lambert, 1989; Matsuura et al., 1993; Miller et al., 1993a; Vo et al., 2003]. Glycosidases seem to be present in gametes of all vertebrate species studied. Among glycosidases, the lysosomal enzyme *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.52) has been long linked to gamete binding and polyspermy prevention in many animal species [Prody et al., 1985; Lambert, 1989; Matsuura et al., 1993; Miller et al., 1993ab; Godknecht and Honegger, 1995; Martinez et al., 2000; Miranda et al., 2000; Perotti et al., 2001; Zitta et al., 2006]. It is classified as β-*N*-acetylhexosaminidase or β-hexosaminidase (hereafter Hex) since it efficiently catalyzes the removal of β (1→4) linked *N*-acetylglucosamine (GlcNac) or *N*-acetylgalactosamine (GalNac) residues from non-reducing glycoconjugate ends. In mammals, three different Hex isoforms have been found, each composed by non-covalent homo or heterodimerization of two (α and β) subunits: Hex A (αβ), Hex B (ββ), and Hex S (αα). Both α and β subunits have a catalytic site and, in the case of mammals, are encoded by genes on different chromosomes, believed to evolve from a common ancestor gene [Proia, 1988]. All three isozymes can hydrolyze β-linked GlcNac or GalNac residues from neutral substrates; however, the presence of an α-chain, renders to the Hex A and Hex S isoforms capable of

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hydrolyzing substrates containing negatively charged groups like sulfate or sialic acid [Kresse et al., 1981; Hepbildikler et al., 2002]. In the extensively characterized human Hexs, the difference in substrate specificity arises from the only two variable amino acid residues (αAsn423 and αArg424 or βAsp452 and βLeu453) that distinguish α from β active site. Thus, whereas the presence of Arg424 in the α active site is critical for binding of negatively charged groups, the presence of Asp452 in the active site of β-subunit allows only the efficient cleavage of neutral substrates [Lemieux et al., 2006].

In anuran amphibian eggs, two mechanisms triggered by fertilization are known to prevent polyspermy. First, a rapid and transient depolarization of the egg plasma membrane prevents fusion of further sperms; seconds after, enzymes released during egg cortical reaction, including glycosidases, and proteases, modify the vitelline envelope of the activated egg ensuring a long-term block to polyspermy. In this regard, Hex has been found to be released during the cortical reaction of *X. laevis* eggs [Greve et al., 1985]. This enzyme hydrolyzes non-reducing terminal GlcNac residues from vitelline envelope ZPC, contributing to long-term polyspermy prevention [Hedrick, 2008]. Besides its critical role in fertilization, molecular, and biochemical characterization of Hex in amphibian eggs remains incomplete. In the present work, we show that Hex is the most important glycosidase activity in *X. laevis* eggs. In addition, we present for the first time the sequence of two putatives *X. laevis* Hex proteins. Based on human Hex subunits, our evidence suggests that different polypeptides with α- and β-like active sites are synthesized in *Xenopus* by alternative splicing from the same gene. Finally, we expressed an amino terminal fragment of *X. laevis* Hex in *E. coli* and raised specific antibodies against it. A complete immunochemical characterization of eggs and oocytes Hex is presented.

MATERIALS AND METHODS

REAGENTS

Jack bean Hex was obtained from Sigma-Chemical Co. (St. Louis, MO). 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (4-MU-NacGlc) and 4-methylumbelliferyl-6-sulfo-*N*-acetyl-β-D-glucosaminide (4-MU-NacGlc-SO_4) were obtained from Calbiochem (San Diego, CA). Complete and incomplete Freunds adjuvants were purchased from Bio-Rad (Hercules, CA). Nitrocellulose membrane, goat anti-rabbit IgG peroxidase conjugated and X-ray film (Amersham Hyperfilm™ ECL) was purchased from Amersham Biosciences (Buckinghamshire, UK). Glutathione Sepharose™ 4 Fast Flow was purchased from Amersham Biosciences (Uspalla, Sweden). Centricon YM-10 centrifugal filters device were obtained from Amicon (Bedford, MA). Anti-rabbit Cy3 conjugated antibodies were obtained from Chemicon International (Temecula, CA). All other chemicals were obtained from Sigma-Chemical Co.

ANIMAL SAMPLES

Ovarian tissue from sexually mature *X. laevis* specimens was obtained following approved protocols of the Faculty of Sciences, University of Chile, and kindly supplied by Dr. Francisco Romero. Oviposited non-fertilized and recently fertilized eggs from sexually mature *X. laevis* specimens were kindly supplied by Dr. Manuel Aybar, Department of Developmental Biology, INSIBIO (CONICET-National University of Tucumán), Argentina.

SEQUENCE ANALYSIS, DATABASE HOMOLOGY SEARCHES AND SEQUENCE ALIGNMENTS

Database homology searches were performed using tblastn and blastx programs. Multiple alignments were performed using ClustalW2 programs. Sequence analyses were carried out using tools and software packages of ExPASy Proteomics Server (http://ca.expasy.org/). Antigenic index of *X. laevis* Hex was determined using Protean 4.03 program (DNASTAR, Inc.).

OBTAINING OF *X. LAEVIS* AND *TROPICALIS* HEX SEQUENCES

Consensus sequences belonging to the N-terminal (VWGALRGLETFSQL), central (HLGGDEVDFTCW), and C-terminal (IGGEACMWGEYVD) regions of known vertebrate Hexs (human β-subunit: UniProt ID P07686; human α-subunit: UniProt ID P06865; mouse β-subunit: UniProt ID P20060; mouse α-subunit: UniProt ID P29416; rat β-subunit: UniProt ID Q6AXR4; rat α-subunit: UniProt ID Q641X3; cat β-subunit: UniProt ID P49614; pig β-subunit: UniProt ID Q29548 and bovine α-subunit: UniProt ID Q0V8R6) were obtained from multiple alignments performed with ClustalW2 programs. These sequences were used as a query to search *X. laevis* EST databases and verify that the cDNA insert corresponding to XL250c11ex clone of XDB3.2 web data catalogue of NIBB/NIG/NBRP *X. laevis* EST project (http://xenopus.nibb.ac.jp) contained the N-terminal and C-terminal ends of a putative *X. laevis* Hex. The corresponding plasmid was kindly provided to us by National Institute for Basic Biology (NIBB) of Japan. To obtain the sequence coding the complete ORF of *X. laevis* Hex the complete cDNA of XL250c11ex plasmid was sequenced using SP6 and GCTGGAGACTTTCAGTCAAC (internal to cDNA sequence) as forward primers and T7 and AATTGGTTGGTTCAACTCTG (internal to cDNA sequence) as reverse primers. The sequence, and deduced protein, were deposited in the Genbank database under accession numbers JN127371 and AEJ87970 respectively. The NIBB contig 035561 was used to find the first amino acid residue (initial Met) of Hex signal peptide that was absent in the XL250c11ex clone. *X. tropicalis* sequences and gene structure were obtained from the USCS genome browser (http://genome.ucsc.edu; *X. tropicalis* database) using the *X. laevis* sequence as a query.

PLASMID CONSTRUCTS, EXPRESSION AND PURIFICATION OF N-TERMINAL FRAGMENT OF *X. LAEVIS* HEX

A cDNA fragment (~1.6 kpb) from XL250c11ex plasmid coding for the complete AEJ87970 *X. laevis* Hex protein, without the signal peptide, was amplified by PCR using Pfu Turbo DNA Polymerase (Stratagene), with GGTACCGGAGACGCAATTGACTGTAG and GCGGCCGCTGCTCCCTTGCTGATTG as forward and reverse primers respectively. The purified PCR fragment was first cloned in a pCR-BluntII-TOPO vector (Invitrogen) and then subcloned into a pEF1/HistA vector (Invitrogen). This sequence was then subcloned into two different prokaryotic expression vectors: pQE31 (Qiagen) and pRSET-C (Invitrogen). The resulting plasmids (pEF1-Hex; pQE31-Hex and pRSET-C/Hex) were then used to transform different *E. coli* strains: M15[pREP4] (Qiagen), BL21(DE3), BL21(DE3)pLysS, and

BL21(DE3) Codon Plus (Novagen). Expression of the complete ORF was attempted under different conditions of temperature and IPTG concentrations. No induction was observed. Thus, a cDNA fragment was excised from pRSET-C/Hex between a BamHI and an EcoRI (internal to Hex ORF) restriction sites, encoding an amino terminal fragment of ~18.3 kDa of *X. laevis* AEJ87970 Hex, and subcloned as a GST-fused protein in a pGEX-3X vector (Amersham). This construct, called pGEX-Hex, was used to transform *E. coli* DH5α strain (Invitrogen). Transformants were selected on LB agar plates containing 100 μg/ml of ampicillin and used for expression. Single transformant colonies were inoculated into LB-ampicillin medium and grown at 37°C to an $OD_{600\,nm}$ of 0.5. Then, 0.1 mM IPTG was added and the cultures were further incubated at 25°C for 90 min. Finally, cells were pelleted for 15 min at 5,000*g*, resuspended in lysis buffer (50 mM Tris, 200 mM NaCl, 1 mM EDTA; 1 mM PMSF, 5 mM DTT, 0.5% Triton X-100, pH 8.0) and sonicated in a Branson Sonifier 250 ultrasonic cell disruptor (40% amplitude, 5 × 20 s on/60 s off). The lysate was centrifuged for 30 min at 20,000*g* and the N-terminal fragment of Hex was purified from the supernatant using a Glutathione Sepharose resin following manufacture instructions. Recombinant GST/Hex was maintained at −20°C until use.

ANTISERUM PRODUCTION

Recombinant GST/Hex was further purified by preparative SDS–PAGE on 10% polyacrylamide gel followed by electroelution. Approximately 100 μg of electroeluted protein were injected to a rabbit at day 0, following with 50 μg at weeks 3, 8, and 14. Blood was collected 10 days after each injection, allowed to clot at 37°C, centrifuged at 2,500 rpm, and the supernatant stored at −20°C until used. Anti-GST control antiserum was obtained by inoculation of purified GST alone. Normal sera were obtained from animals before the first injection and stored at −20°C until use. Anti-GST antibodies were affinity purified.

EGG EXTRACTS PREPARATION

Oviposited unfertilized eggs (hereafter referred as eggs) from sexually mature *X. laevis* were obtained by injection of hCG as described by Wolf and Hedrick [1971], and dejellied with 0.3% β-mercaptoethanol in extraction buffer (110 mM NaCl; 2 mM KCl; 10 mM EDTA; 50 μM DTT; 10 mM Tris pH 7.6). Eggs were homogenized in extraction buffer supplemented with 1 mM PMSF in a Potter-Elvehjem homogenizer (Thomas Scientific, Swedesboro, NJ). The homogenate was centrifuged at 10,000*g* for 30 min at 4°C and the resulting supernatant, named total egg extract, stored at −20°C until used. When removing the yolk, the total egg extract was treated with 3% polyvinylpyrrolidone for 4 h on ice. The extract was centrifuged for 10 min at 20,000*g* and the supernatant called clarified egg extract.

ENZYMATIC ACTIVITY ASSAYS

Enzymatic activities present in clarified egg extracts were assayed using 3 mM of the corresponding *p*-nitrophenyl-glycoside as substrate, in 10 mM citrate buffer at pH 3.6, over 30 min at 37°C. Enzymatic activities were linear during the reaction time. Reactions were stopped by the addition of 2 vol. of 0.2 M glycine pH 10.4 and the released *p*-nitrophenol was determined spectrophotometrically at 400 nm. For each reaction, the absorbance of the corresponding control (reaction without egg extract) was subtracted. One unit of enzymatic activity is defined as the amount of the enzyme that catalyzes the release of 1 μmol of *p*-nitrophenol per hour under given conditions. Results were analyzed by ANOVA and Scheffe tests. Hex activity was also analyzed in native gels. After electrophoresis, gels were equilibrated in 400 mM citrate buffer pH 3.6, and incubated with 5 mM 4-MU-NacGlc or 4-MU-NacGlc-SO_4 in 100 mM citrate buffer pH 3.6. Hex active bands were evidenced at 302 nm with a UVP M-20 transilluminator.

PARTIAL PURIFICATION OF *X. LAEVIS* EGG HEX FROM NATIVE GELS

Hex active bands visualized in native gels (see above) were excised and crushed with a Teflon pestle. Proteins were allowed to diffuse overnight into 2 vol. of 0.1× extraction buffer at 4°C with gentle rocking. The suspension was centrifuged at 4°C for 15 min at 20,000*g* and concentrated using a Centricon YM-10 ultrafiltration device (Millipore).

SDS–PAGE AND WESTERN BLOT ANALYSIS

SDS–PAGE was performed on 10% polyacrylamide gel [Laemmly, 1970]. Gels were electro-transferred to nitrocellulose membranes [Towbin et al., 1979]. The membranes were first blocked with 5% non-fat dry milk, 0.05% Tween 20 in PBS and incubated with the appropriate antiserum: anti-*X. laevis*-Hex (1:1,000 dilution); normal serum (1:1,000 dilution) or purified anti-GST (1:20 dilution in PBS buffer). The immunoreactive proteins were identified using 1:5,000 dilution of anti-rabbit IgG HRP conjugated antibodies and Super Signal West Pico Chemiluminescent substrate (Pierce, Rockford, IL).

IMMUNOHISTOCHEMISTRY

Dejellied unfertilized eggs, dejellied recently fertilized eggs (1 cell; referred also as zygotes) and ovaries were washed in PBS, fixed in 4% paraformaldehyde for 4 h, embedded in paraffin and cut in 5 μm sections that were laid on gelatin-covered slides. Sections were hydrated and permeabilized with 0.5% Triton X-100 in TBS for 30 min. After washes in TBS, the slides were incubated in blocking solution (5% BSA, 0.02% Tween 20% and 5% ram serum in TBS) for at least 60 min and exposed to primary antibody (1:200 dilution for anti-*X. laevis*-Hex antiserum or normal serum and 1:50 dilution for purified anti-GST antibodies in blocking solution) for 60 min. Unbound antibodies were removed with five washes in TBS and sections incubated with anti-rabbit Cy3 conjugated antibodies (1:1,000 in TBS) for 60 min. Sections were washed with TBS mounted with Dabco reagent and examined under light and fluorescence microscopy with an Olympus BH-2 microscope. Lectin staining was performed on blocked section, incubating for 60 min with FITC-conjugated *D. biflorus* lectin (20 μg/ml) in TBS supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Oocyte developmental stages were identified as described by Dumont [1972].

RESULTS

N-ACETYL-β-D-GLUCOSAMINIDASE IS THE MAJOR GLYCOSIDASE ACTIVITY IN *X. LAEVIS* EGGS

In order to have a better understanding of the glycosidases present in *X. laevis* eggs, nine different glycosidase activities were studied in clarified egg extracts. Free *p*-nitrophenol released from *p*-nitrophenyl-glycoside substrates was spectrophotometrically measured and correlated to its corresponding glycosidase. The major activity belonged to *N*-acetyl-β-D-glucosaminidase (Fig. 1). This enzyme was at least 4 times more active than other glycosidases tested, including *N*-acetyl α and β D-galactosaminidase, α and β D-glucosidase, L-fucosidase, and D-mannosidase among others.

IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF *X. LAEVIS* HEX SEQUENCES

As shown in Figure 1, *N*-acetyl-β-D-glucosaminidase (Hex) was the major glycosidase activity detected in clarified egg extract. In addition, *X. laevis* Hex from eggs has been involved in polyspermy prevention [Prody et al., 1985; Vo et al., 2003]. However, information regarding its molecular nature is unknown. Thus, a bioinformatic study was carried out as a first step to a molecular characterization of *X. laevis* Hex. The amino acid sequences from three conserved peptides of amino, central, and carboxy terminal regions of known Hex proteins were used for homology blast searches in *X. laevis* EST databases. This methodology led to the identification of an EST, which was sequenced to obtain the complete open reading frame (ORF) of a putative *X. laevis* Hex (Fig. 2, view materials and methods for details). The ORF (1,662 bp) encodes a polypeptide of 553 amino acids residues consisting of three main regions: (1) an amino terminal region of 23 residues predicted to comprise a signal peptide with its potential cleavage site between residues 23 and 24; (2) the β-*N*-acetylhexosaminidase-like domain (residues 49–194); and (3) the catalytic core (residues 195–512). The Hex-like domain and the catalytic core are common to all members of the glycosidase family 20, to which Hexs belongs. An alignment analysis revealed that 62% of the amino acid sequence is identical to human β subunit (similarity of 78%). 56% of identity was obtained when compared to human α Hex subunit (71% of similarity). Noteworthy, *X. laevis* Hex is also similar (54% of identity) to the ascidian *P. mammillata* Hex (UniProt ID: Q7YTB2; Table I). An alignment of deduced amino acid sequence of *X. laevis* Hex together with human (α and β subunit precursors) and *P. mammillata* Hex is shown in Figure 2. The amino terminal was the most variable region of all Hex polypeptides analyzed; however, a small domain of 10–14 amino acid length inside this region is highly conserved in all of them (Fig. 2, box 1), even among evolutionary distant species. All amino acid residues that form the active site in human Hexs [Lemieux et al., 2006], are conserved in *X. laevis* sequence (Fig. 2, white arrowheads). The *X. laevis* sequence displays three potential *N*-glycosylation sites (Fig. 2, open boxes). Two of these sites were also found in human (Fig. 2) and other known vertebrate Hex sequences (data not shown). The third *N*-glycosylation site was only conserved in the ascidia *P. mammillata* Hex. All cysteine residues involved in disulfide bonds in human Hexs were also conserved in *X. laevis* sequence.

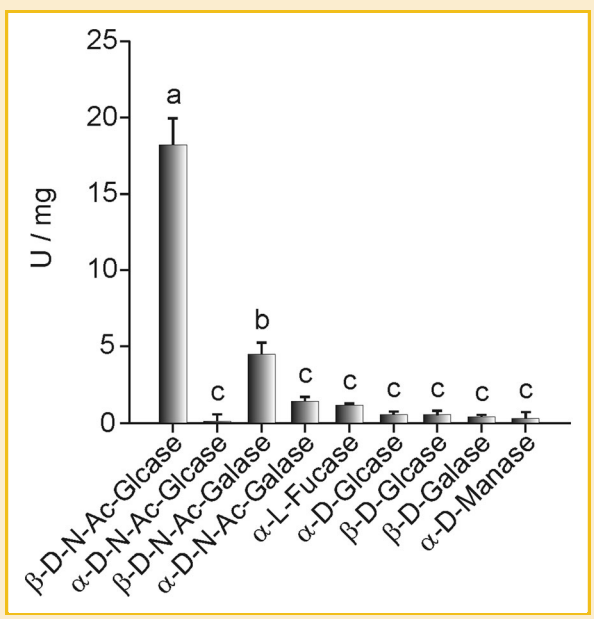

Fig. 1. Glycosidase activities in *X. laevis* egg extract. 1.5 μg of clarified egg extract was incubated with the corresponding *p*-nitrophenyl substrates. Glycosidase activities were estimated by measuring the amount of *p*-nitrophenol released from *p*-nitrophenyl conjugates as described in methods and expressed in units (U) per mg of total protein. Results are expressed as mean ± SEM of two independents experiments performed in triplicate; $P < 0.01$. *N*-acetyl α and β D-glucosaminidase (α or β D-*N*-Ac-Glcase); *N*-acetyl α and β D-galactosaminidase (α or β D-*N*-Ac-Galase); α-L-fucosidase (α-L-Fucase); α and β D-glucosidase (α or β D-Glcase); β-D-galactosidase (β-D-Galase) and α-D-mannosidase (α-D-Manase).

Two separate Hex genes are found in the mammalian genome, corresponding to α (Hex A gene) and β (Hex B gene) Hex subunits. However, nothing is known about amphibian Hex genes. Thus, a bioinformatics blast search was conducted using the JN127371 cDNA sequence (see Methods, under "Obtaining of *X. laevis* and *tropicalis* Hex sequences") as a query. Only one Hex gene was found in the *X. tropicalis* genome (JGI 4.2/xenTro3 assembly: GL172749:1,795,244–1,819,981; ~90% sequenced genome). In humans, 12 amino acid residues of the active site define the Hex alpha or beta identity, based on two variable amino acids: the presence of Asn423 and Arg424 in the human Hex active site defines the identity of α subunit, while Asp452 and Leu452 define a β subunit [Lemieux et al., 2006] (Fig. 2, box 2). These residues are codified by exon 11 of both Hex A and Hex B gene. Noteworthy, homology based studies between human and *Xenopus* Hex genes showed that these exons found on human Hex A and Hex B genes (denoted as exon 11 in Fig. 3A) are contained in the same *X. tropicalis* Hex gen (Fig. 3A α and β white exons; view Supplemental Fig. 1 for details). In order to gain insight into the functionality of these exons, the intron-flanking sequences between these exons were studied. Interestingly, invariant GT (donor splice site; GU in ARNm) and AG (acceptor splice site) dinucleotides were observed at either ends (Fig. 3B), as required for correct ARN splicing in all higher eukaryotes. The conservation of these splicing sites

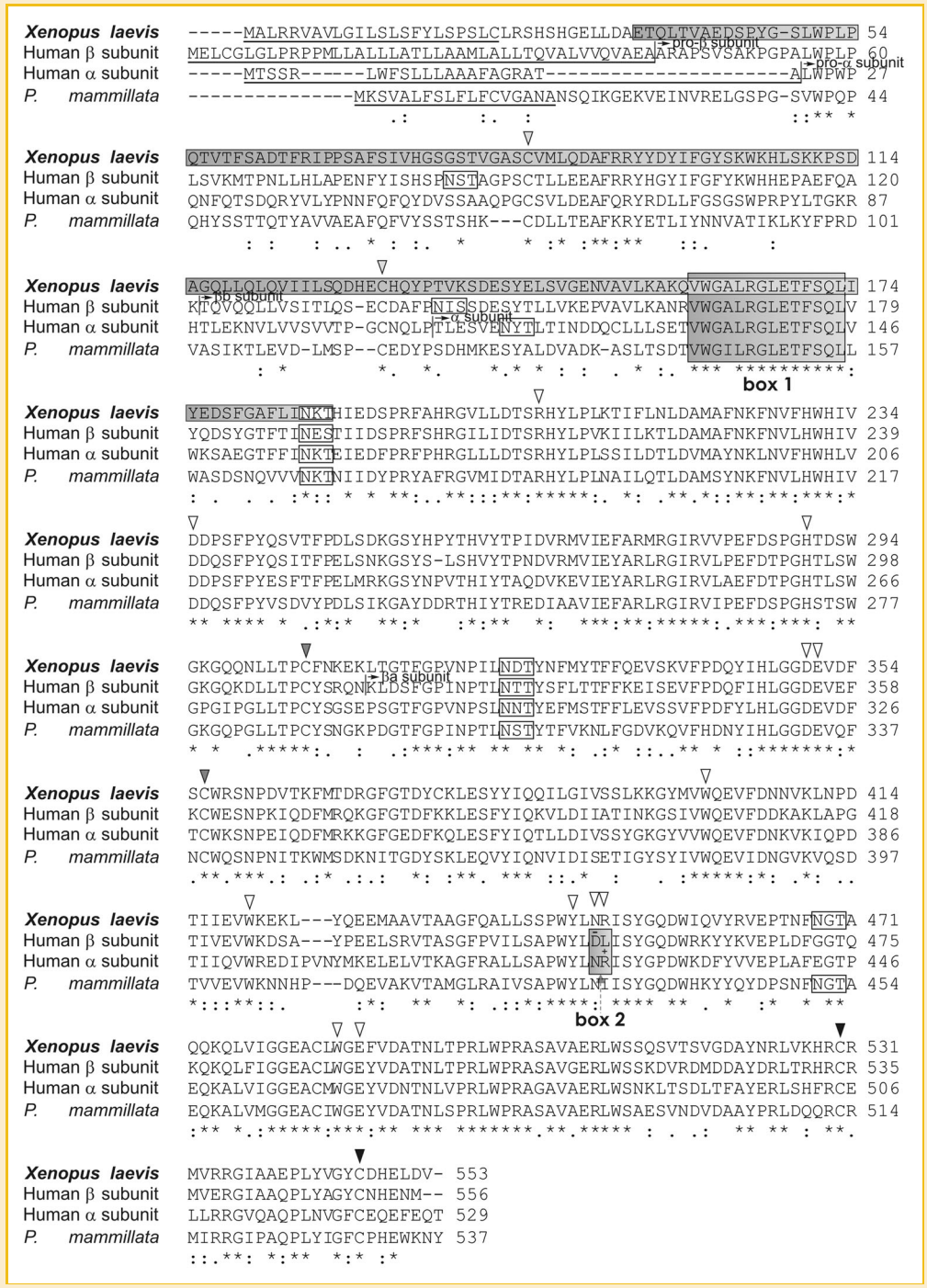

Fig. 2. Amino acid sequence of *X. laevis* Hex and comparison among other known Hex sequences. Conserved amino acids in all four sequences are indicated by asterisks. Signal peptides are underlined. Box 1 indicates a highly conserved amino acid sequence in the amino terminal region of Hexs. In human sequences, both pro and mature α and β subunits are indicated. White arrowheads indicate the amino acid residues composing active sites of α and β human Hex subunits. Box 2 indicates the only two variable amino acid residues of human Hex active site that define α (NR) or β (DL) Hex active site. Same shade arrowheads (gray or black) indicate cysteine residues involved in disulfide bonds. Open boxes indicate *N*-glycosylation sites in human or predicted ones in the rest of the sequences. Shaded *X. laevis* sequence indicates the fragment used to obtain anti-*X. laevis*-Hex antibodies. Human β-subunit: UniProt ID: P07686; human α-subunit: UniProt ID: P06865; *P. mammillata:* UniProt ID: Q7YTB2; X. laevis: GenBank ID: AEJ87970.

strongly suggests that these exons remained functional through evolution.

If *Xenopus* Hex gene was capable of giving rise to different Hex isoforms, at least two mRNA should be transcribed from it. In this regard, two different Hex mRNAs could be inferred when analyzing all existing full ORF cDNA sequences from expressed *X. tropicalis* libraries (GenBank ID: BC161740 and GenBank ID: BC161249; Fig. 3A). An alignment of both *X. tropicalis* polypeptides showed an

TABLE I. Sequence Similarity Between *X. laevis* Hex and Other Known β-Hexosaminidases

Organism	Identity (%)	Similarity (%)
Human (β subunit)	62 (318/507)	78 (397/507)
Human (α subunit)	56 (289/510)	71 (365/510)
P. mammillata	54 (281/529)	70 (369/529)

extensive identity between them (~95%) except in the region that defines the α- or β-like active site (Supplemental Fig. 2; shaded sequences). In this sense, it was found that one of the translated polypeptides (GenBank ID: AAI61249; mRNA BC161249) displays an α-like active site (conserved the Asn and Arg defining the human Hex α active site) while the other one (GenBank ID: AAI61740;

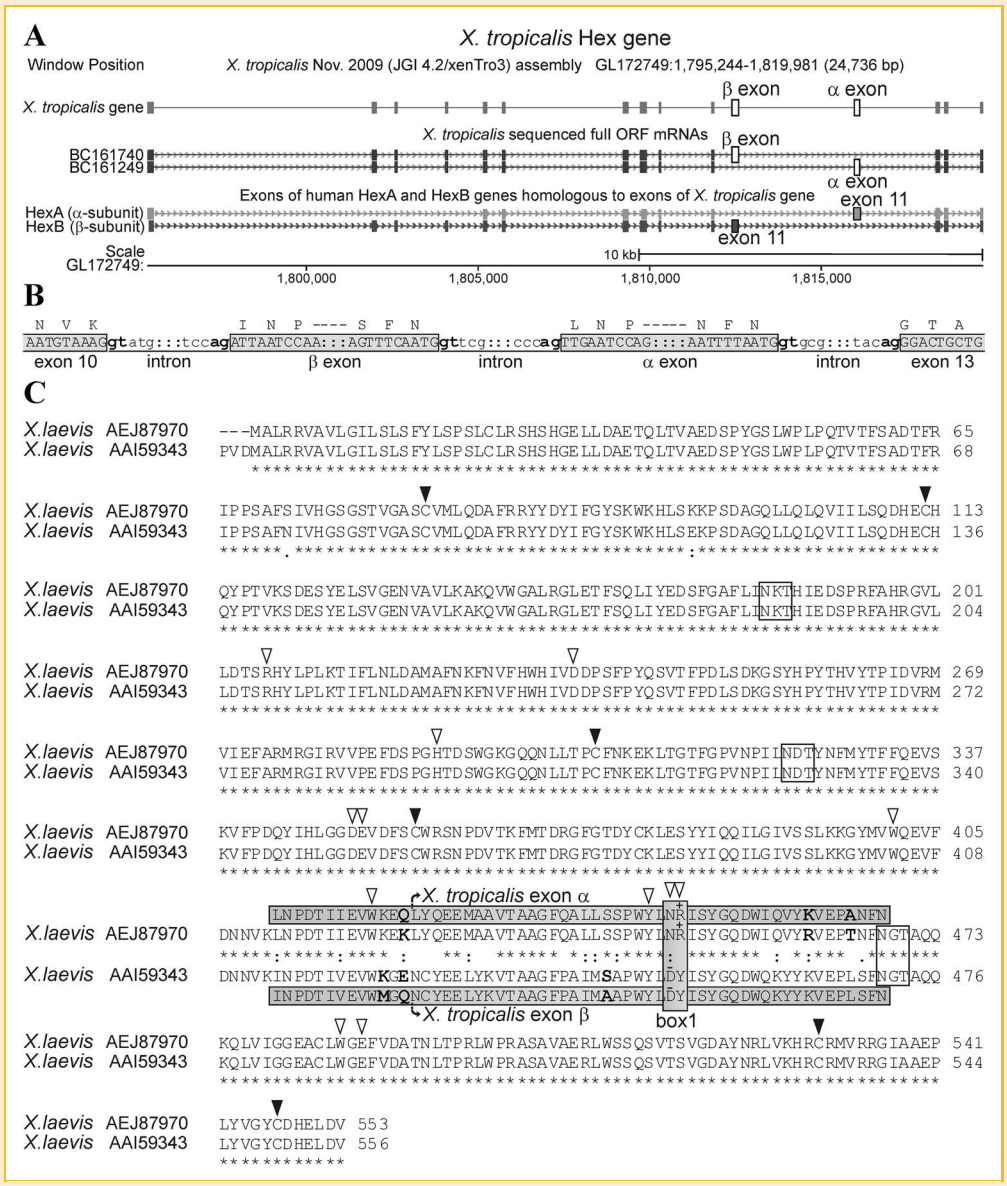

Fig. 3. Molecular characterization of *Xenopus* Hexs. A: Diagram showing the intron–exon structure of *X. tropicalis* Hex gene (JGI 4.2/xenTro3 assembly) and the two full ORF Hex transcripts (GenBank ID: BC161740 and GenBank ID: BC161249) that could be generated by alternative splicing. The figure has been taken from USCS Genome Browser and slightly modified for better understanding. Exons encoding the polypeptidic fragment that defines α or β *X. tropicalis* Hex active sites (named as α and β exon in the figure) have been white boxed. B: Diagram showing the intron-flanking sequences between α and β exons of *X. tropicalis* Hex gene. Exon sequences are boxed. Intron sequences are underlined. Bold nucleotides in both intron ends show the invariant GT (donor site; GU in primary transcript) and AG (acceptor site) splicing sites. The figure does not show internal sequences of both exons and introns for space sake. Translated sequences are shown above for comparison with sequences of panel C. C: Alignment of two *X. laevis* proteins (GenBank ID: AAI59343 and GenBank ID: AEJ87970) highly similar to *X. tropicalis* Hexs deduced from BC161740 (AAI59343) and BC161249 (AEJ87970) mRNA sequences. Asterisks indicate conserved residues in both sequences. White arrowheads indicate amino acid residues forming the Hex active site. Box 1 indicates two variable amino acid residues defining α or β *Xenopus* Hex active sites. The shaded sequences above and below *X. laevis* aligned sequences show the polypeptidic fragments codified by α and β exons of *X. tropicalis* Hex gene (α and β white exons in panel A). Bold letters indicate amino acid residues that differ between polypeptides deduced from α and β exons of *X. tropicalis* gene and their homologous polypeptidic fragments in *X. laevis* AEJ87970 or AAI59343 sequences respectively. Black arrowheads indicate putative Cysteine residues involved in disulfide bonds. Open boxes indicate putative *N*-glycosylation sites.

mRNA BC161740) displays a β-like active site (conserved the key Asp defining the human Hex β active site; Supplemental Fig. 2, box 1). Remarkably, these divergent polypeptidic fragments appear to be codified by α and β exons of *X. tropicalis* Hex gene (Fig. 3A, α and β white exons; view Supplemental Figs. 2 and 3 for details). This means that the nature of *X. tropicalis* Hex in terms of substrate specificity would result from an alternative use of two different exons of *X. tropicalis* Hex gene.

Highly similar polypeptides (89% of amino acid identity) to these *X. tropicalis* Hexs were found in *X. laevis* databases (GenBank ID: AEJ87970, presented in Fig. 2 and GenBank ID: AAI59343; view Supplemental Fig. 4 for details). As in *X. tropicalis*, an alignment of both *X. laevis* polypeptides showed an extensive identity between them (~96%) except in the region that defines the α- or β-like active site (Fig. 3C). It is remarkable that the polypeptidic sequences that define this region in *X. laevis* polypeptides are highly similar (95% of identity and 99% of similarity) to those codified in exons α and β, that define α- or β-like active site in *X. tropicalis* Hex polypeptides (Fig. 3C; shaded sequences).

EXPRESSION OF AN IMMUNOGENIC N-TERMINAL FRAGMENT OF *X. LAEVIS* HEX

We selected an amino terminal fragment of *X. laevis* Hex (GenBank ID: AEJ87970; Fig. 2, shaded sequence of *X. laevis* Hex: residues 36–187) that displayed an adequate antigenic index for raising antibodies. This fragment displayed low homology with other *X. laevis* polypeptides deposited in public databases (data not shown), predicting low possibilities of cross reaction with other proteins. It was cloned in a GST fusion vector (pGEX-3X) and expressed in prokaryote cells. The expression of a recombinant protein of ~43.5 kDa was observed upon IPTG-induction (Supplemental Fig. 5A). This molecular weight is in agreement with the predicted molecular weight for the selected Hex fragment (~18.3 kDa) fused to GST (26 kDa). Thus, cells were induced for 1.5 h, since highest expression level was observed, and the recombinant protein (GST/Hex) was purified from cytosolic fractions using a Glutathione Sepharose resin (Supplemental Fig. 5B,C), in order to raise antibodies against Hex. GST/Hex recombinant protein was inoculated in rabbits. The titters of antisera obtained at second and third bleeds were estimated by Western blots using 350 and 100 ng of purified GST/Hex. Both antisera were able to detect these amounts of GST/Hex even at 1/20,000-fold dilutions (Supplemental Fig. 6). To determine the Hex specificity of obtained antisera (thereafter anti-*X. laevis*-Hex antiserum), Western blot experiments were performed using commercial *Canavalia ensiformis* Hex (hereafter jack bean Hex), and using BSA as negative control. Anti-*X. laevis*-Hex antiserum reacted with jack bean Hex but not with BSA (Fig. 4A). Neither normal serum nor purified anti-GST antibodies reacted against these proteins (Fig. 4B,C).

ELECTROPHORETICAL CHARACTERIZATION OF HEX FROM *X. LAEVIS* EGG

We performed in-gel activity assays in order to gain biochemical insight into substrate specificity and molecular nature of Hex present in *X. laevis* eggs. In agreement with a previous report [Greve et al., 1985], two different bands with Hex activity were detected in native gels (Fig. 5A; LM and HM bands). These bands showed activity with both the non-sulfated and the sulfated 4-MU-NacGlc substrates (Fig. 5A), indicating the presence of at least one α-like subunit in these Hexs, as in the case of Hex A or S mammalian isoforms. To further investigate the polypeptidic composition of these Hexs isoforms, they were obtained from native gel and submitted to SDS–PAGE and Western blot analysis, using anti-*X. laevis*-Hex antiserum described above. Three polypeptides of ~63 kDa (a faint signal), ~59 and ~49 kDa were immunodetected under reducing conditions (Fig. 5B; HM + LM lane, +β-Me). When HM Hex band was analyzed separately, same three polypeptides (63, 59, and 49 kDa) were found (Fig. 5B; HM lane, +β-Me). However, only the 59 kDa polypeptide was found in Hex of lower electrophoretic mobility (LM band; Fig. 5B, LM lane, +β-Me). Only one single immunoreactive band at ~55 kDa could be detected when both LM and HM bands were analyzed together in non-reducing conditions (Fig. 5B, HM + LM lane, −β-Me). As a control, total egg extract was analyzed by same SDS–PAGE and Western blot procedure. Same three polypeptides (63, 59, and 49 kDa) were detected in these experiments (Fig. 5C; anti-*X. laevis*-Hex lane), demonstrating that all the egg immunoreactive Hex polypeptides were present in activity bands of native gels. Additionally, the 63 and 59 kDa polypeptides were also detected, although less efficiently, by a specific anti-jack bean-Hex antiserum, obtained from the pure commercial enzyme [Martinez et al., 2000]; demonstrating the Hex identity of these polypeptides (Fig. 5C; anti-jack bean-Hex lane). Neither normal serum nor anti-GST antibodies (Fig. 5C; normal serum lane or anti-GST lane respectively) showed immunoreactive proteins, suggesting the specificity of the polypeptides immunodetected.

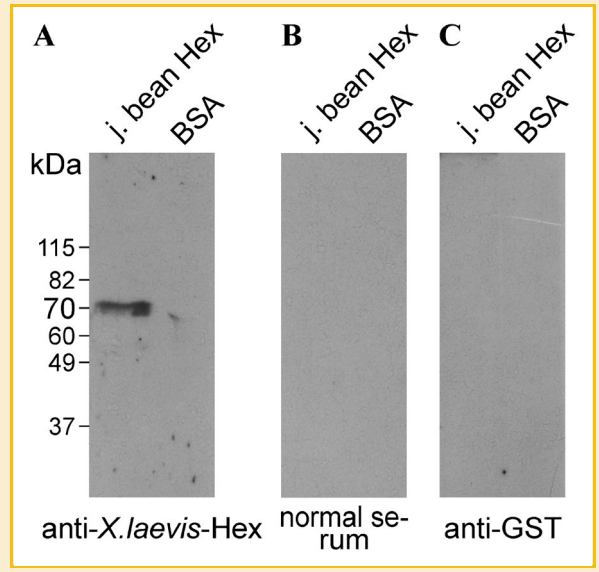

Fig. 4. Specificity of anti-*X. laevis*-Hex antiserum. 3 μg of both pure commercial jack bean Hex (jack bean Hex lanes) and BSA (BSA lanes) were run in a 10% SDS–PAGE and analyzed by Western blot using either (A) anti-*X. laevis*-Hex antiserum, (B) normal serum or (C) purified anti-GST antibodies.

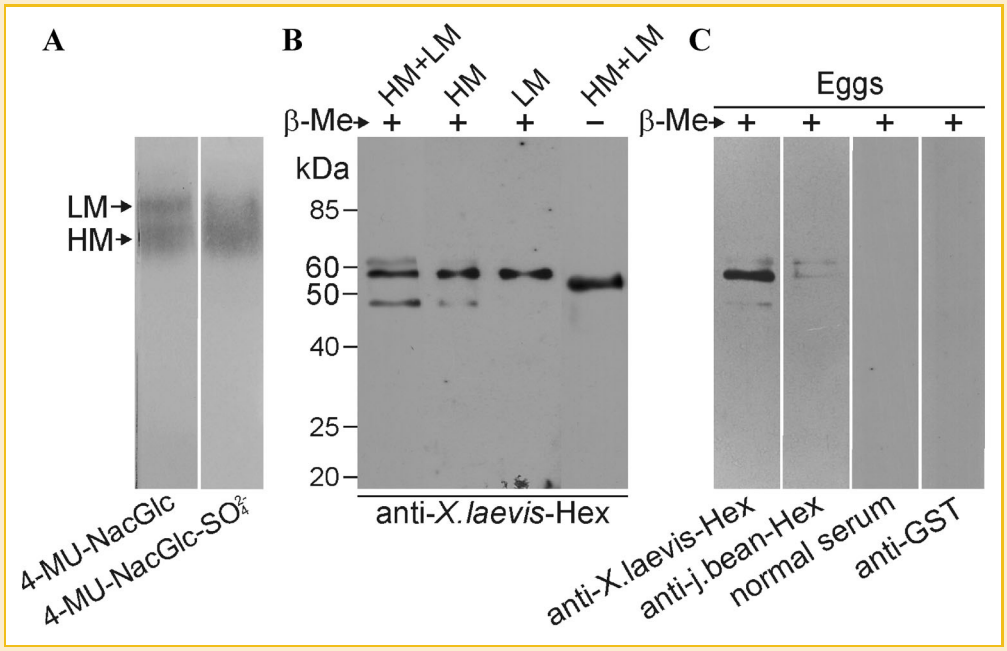

Fig. 5. Electrophoretical characterization of Hex of *X. laevis* egg. A: Clarified egg extract (160 μg of total protein) was run on an 8% native polyacrylamide gel. Hex activity was assayed using either the fluorogenic substrate 4-MU-NacGlc or 4-MU-NacGlc-SO4. Two isoforms with Hex activity were designed as LM (low electrophoretical mobility) and HM (high electrophoretical mobility). The gel is representative of five different gels. B: Bands with Hex activity were excised together (HM + LM lanes) or separately (HM or LM lane) and loaded on a 10% SDS-PAGE in the presence (+) or absence (−) of β-mercaptoethanol. Constituting Hex polypeptides were evidenced by Western blot using anti-*X. laevis*-Hex antiserum. C: Total egg extracts (eggs) were run in 10% SDS-PAGE under reducing conditions and detected with the anti-*X. laevis*-Hex antiserum (anti-*X. laevis*-Hex lane), the anti-jack bean-Hex antiserum (anti-jack bean-Hex lane), the normal serum (normal serum lane), or purified anti-GST antibodies (anti-GST lane).

IMMUNOHISTOCHEMICAL CHARACTERIZATION OF HEX IN *X. LAEVIS* OOCYTES AND EGGS

The intracellular localization of Hex in different developmental stages of *X. laevis* oocytes was determined in fixed ovary sections using the anti-*X. laevis*-Hex antiserum. Strikingly, a strong immunoreactive signal was mainly observed in the cortical zone of animal (pigmented) hemisphere of stage IV and later oocytes (Fig. 6A–F). The same animal to vegetal asymmetrical Hex distribution was also observed both in oviposited and recently fertilized eggs (1 cell; referred to as zygotes; Supplemental Fig. 7). This signal originates from small vesicle-like structures (diameter of ~1.5–2.5 μm), found to be scattered throughout the oocyte (Fig. 6C,D). In addition, a specific Hex signal was detected in the follicular layer surrounding the oocytes (indicated in Fig. 6C). In earlier stages of oocyte maturation (stages I and II) Hex was localized to the entire cortical region of oocytes (Fig. 6H). This signal was mostly coincident with the signal originated by *D. biflorus* lectin, which is used as a cortical granule marker in *X. laevis* oocytes [Sokac et al., 2003] (Fig. 6I,J). Lectin staining was not specifically detected in external integuments surrounding the oocytes. This unspecific binding was not jeopardized by haptenic sugar preincubation of the lectin (Fig. 6K,M). Control sections incubated with normal serum and/or anti-GST purified antibodies showed only background signal (see controls in Fig. 6 and Supplemental Fig. 7). This is the first time that Hex could be histochemically localized in oocytes of later developmental stages, overcoming both the non-specific staining of yolk platelets and accumulation of black pigment in the animal hemisphere.

DISCUSSION

Glycosidases have been involved in different fertilization steps, from gamete binding to polyspermy prevention in phylogenetically distant animals. Several studies have shown that *N*-acetylglucosamine acts as a complementary ligand for sperm in egg coats [Hedrick, 2008]. Treatment of unfertilized eggs with β-*N*-acetylglucosaminidase eliminates the activity of the vitelline envelope for sperm binding [Prody et al., 1985]. Thus, the importance of glycosidases in the fertilization process is beyond doubt. In our study, several glycosidases have been assayed in *X. laevis* egg extract. Activity of *N*-acetyl-β-D-glucosaminidase was 4 times higher than *N*-acetyl-β-D-galactosaminidase and at least 10 times higher than α-fucosidase, α and β-glucosidase, β-galactosidase, and α-mannosidase. Both *N*-acetyl-β-D-glucosaminidase and *N*-acetyl-β-D-galactosaminidase activities may account for same enzyme, since most *N*-acetyl-β-D-glucosaminidase also have *N*-acetyl-β-D-galactosaminidase activity [Beeley, 1985]. Our results are in accordance with previous studies showing high Hex activity in *X. laevis* oocyte and egg lysates [Decroly et al., 1979; Wall and Meleka, 1985]. Noteworthy, activities reported in this manuscript are different from those reported by Greve et al. [1985]. Even though these authors did find high Hex activity in egg cortical granules,

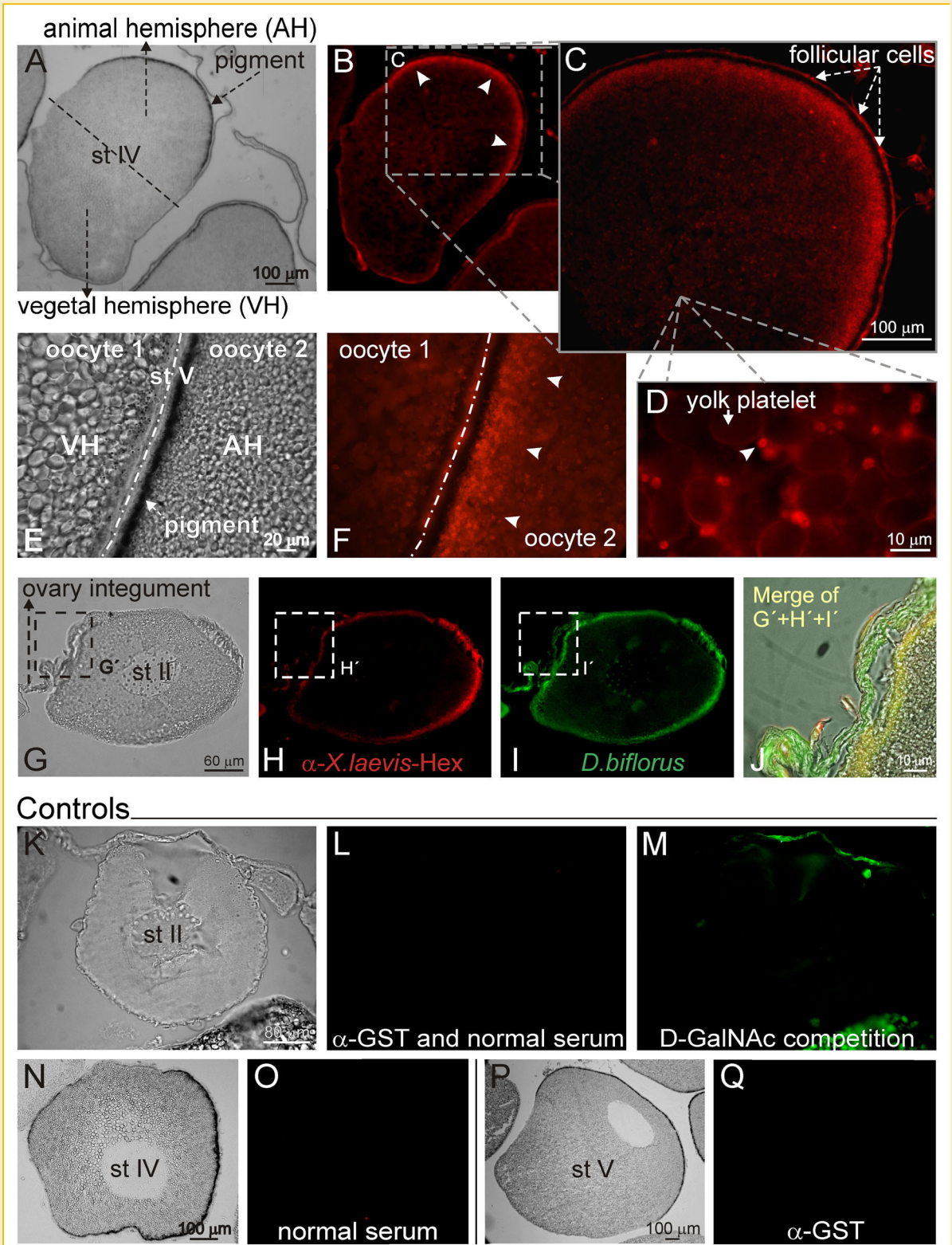

Fig. 6. Immunohistochemical localization of Hex in *X. laevis* oocytes. A–F: Micrographs of *X. laevis* ovary sections incubated with the anti-*X. laevis*-Hex antiserum. Arrowheads in micrograph B and F show the differential localization of Hex in the animal cortex of a stage IV (st IV) and V (st V) oocytes respectively (red fluorescence). Insets C (animal hemisphere) and D (non-cortical cytoplasmic compartment) are optical magnifications of stage IV oocyte show in B. Arrowhead in D indicates one of the small immunoreactive vesicles originating the immunoreactive Hex signal surrounded by yolk platelets. E and F: A vegetal hemisphere facing an animal hemisphere of stage V oocytes, showing their differential Hex localization. G–J: Micrographs of ovary sections showing a stage II (st II) oocyte, co-incubated with anti-*X. laevis*-Hex antiserum (H) and FITC-conjugated *D. biflorus* lectin (I). Insets of micrographs G, H and I (indicated as G', H' and I' respectively) were merged in figure J. K–M: Control micrographs of anti-GST and normal serum (L) and D-GalNAc preincubated lectin (M) treated stage II oocytes. N–Q: Control micrographs of normal serum (O) or anti-GST (Q) treated stage IV (N) and V (P) oocyte respectively. B, F, H, I, L, M, O, and Q correspond to fluorescence micrographs of A, E, G, K, N, and P fields respectively.

they found only minor amounts of Hex activity in egg lysates. This apparent discrepancy might be due to the technical procedure for egg extract preparation: first, they did not use polyvinylpyrrolidone during lysis, what in our hands resulted crucial for high activity yields (not shown); second, Greve et al. [1985] forced dejellied eggs through a 19G hypodermic needle followed by filtering through a 167 μm mesh and finally centrifuged the lysate to remove cell debris, egg envelopes, pigment granules, and yolk platelets. Interestingly, it has been later demonstrated that during this same procedure, the egg cortical components are lost [Gundersen et al., 2001], which could certainly accounts for low Hex activity in their extracts.

Neither cDNA nor amino acid sequences of amphibian Hex had been reported before. Our in-silico studies identified complete Hex ORFs from *X. laevis* and *X. tropicalis*. Deduced sequences showed great similarity with other Hexs characterized. All amino acid residues found in human Hex active sites as well as cysteine residues involved in disulfide bonds are conserved in *Xenopus* sequences, substantiating their identity. As in the case of human Hexs, the three *N*-glycosylation sites encountered in *Xenopus* Hexs could be playing important roles both in stabilization of the enzyme and in sub-cellular localization [Sonderfeld-Fresko and Proia, 1989; Weitz and Proia, 1992].

All *Xenopus (tropicalis* and *laevis)* Hex isoform showed high homology between them, with the exception of the region containing two variable amino acid residues of the active site that defines its α or β Hex identity, as defined for human Hex. In this sense, we found that two *Xenopus* Hexs identified (*X. tropicalis* AAI61249 and *X. laevis* AEJ87970 proteins) showed an α-like active site whereas the other two (*X. tropicalis* AAI61740 and *X. laevis* AAI59343 proteins) showed a β-like active site, and could correspond to an α- and β-like polypeptides of *Xenopus* Hex, in terms of substrate specificity. Considering the evidence from *X. tropicalis* Hex gene regarding conserved splicing sites, and the similarities between *X. tropicalis* and *X. laevis*, it is possible to assume that this Hex gene could generate either α- or β-like Hex proteins by alternative use of two exons (exons α and β). Worth noticing, this clearly denotes an evolutionary divergence with mammals, where α and β Hex subunits are synthesized from different genes on different chromosomes [Proia, 1988]. Interestingly, these exons that codify the polypeptidic fragments that define *Xenopus* Hex α or Hex β active site are conserved in human HexA or HexB genes. Alternative splicing could be an ancestral mechanism to generate Hex bearing α or β active site from same gene, before Hex gene was duplicated and diverged during the course of evolution. However, these data do not rule out the possible existence of other Hex genes in *Xenopus* genome, considering that 10% of this genome has not been sequenced so far.

One main drawback in the study of *Xenopus* Hexs was the lack of homologous antibodies. To overcome this problem, we expressed an 18 kDa N-terminal fragment of Hex codified in JN127371 cDNA and raised antibodies against it. The expressed fragment was highly conserved in all the *Xenopus* Hexs presented. It also contains the 14-amino-acid sequence highly conserved in all Hexs studied, either α or β subunits, of vertebrates (human, bovine, mouse, rat, cat, orangutan, and pig). This sequence is also conserved in *P. mammillata* Hex. It is then expected that antibodies against the expressed fragment could recognize both α and β subunits of Hex, including those of phylogenetically distant species. In this sense, our antibodies were able to specifically recognize both the recombinant *X. laevis* and jack bean Hex. These antibodies allowed us to analyze the polypeptidic composition as well as to determine the histochemical localization of Hex in *X. laevis* eggs for the first time.

In agreement with previous reports [Greve et al., 1985], our experiments demonstrated the existence of two different Hex isoforms in *X. laevis* egg. Both isoforms catalyzed negatively charged substrates (although with lower efficiency over non-sulfated substrates). This substrate specificity indicates that Hexs from *X. laevis* eggs are of A or S type isoforms. These isoforms showed different electrophoretic mobility in native gels. The higher electrophoretic mobility Hex isoform is shown to be composed by three types of polypeptides of 63, 59, and 49 kDa, while only a 59 kDa polypeptide was found in the Hex isoform of lower electrophoretic mobility. The predicted molecular weight of both *X. laevis* Hex ORFs presented in this paper is 60.5 kDa after signal peptide cleavage; this is in close agreement with the observed polypeptides of 59 and 63 kDa. Molecular weights were also similar to those predicted for full ORFs of proteins AAI61740 and AAI61249 codified in *X. tropicalis* Hex gene (~61.3 kDa, after signal peptide cleavage). In humans, both α and β Hex subunits are sequentially processed, first by signal peptide release, and by cleavage of a small N-terminal pro-peptide of ~10 kDa [Mahuran et al., 1988]. These pro-peptides are thought to remain linked to mature subunits through disulfide bonds [Quon et al., 1989; Hubbes et al., 1989], and are observed as ~56–58 kDa polypeptides when analyzed by non-reducing SDS-PAGE [Mahuran et al., 1988]. We tested this hypothesis in *X. laevis* egg Hexs and only one band at ~55 kDa was immunodetected; suggesting that all immunodetected polypeptides are chemically and structurally related and that, the 49 kDa polypeptide found in the Hex isoform of higher electrophoretic mobility could correspond to a polypeptide derived from any of the polypeptides of higher molecular weight, as in the case of human Hexs.

Histochemical localization of Hex in *X. laevis* oocytes has been previously assayed. Wall and Meleka [1985] reported that Hex is uniformly distributed on the periphery of *X. laevis* stage I and II oocytes. We confirmed these results and colocalized the Hex signal to that of *D. biflorus* lectin, a known cortical granule marker, supporting previously reported work and further demonstrating that Hex is located also to cortical granules [Greve et al., 1985; Prody et al., 1985]. However, no histochemical localization of Hex in oocytes of late developmental stages or eggs has been reported so far. We have been able to determine for the first time the localization of Hex in both types of cells. Our results demonstrate that Hex is mainly localized to the cortex of animal hemispheres of *X. laevis* stage IV and later oocytes, suggesting either relocation or preferential hemisphere-specific synthesis of Hex during oocyte growth. In support of this last hypothesis, it has been shown that the periphery of full-grown oocytes is the major site of proteins synthesis [Capco and Mecca, 1988], and that the bulk of Golgi apparati, and thus Hex transited organelles, are located to the cortex

of the animal hemisphere [Imoh et al., 1983]. This asymmetrical localization of Hex in full-grown oocytes is maintained throughout progesterone induced maturation of oocytes into fertilizable (oviposited) eggs. Hex of *X. laevis* eggs has been found in lysosome-like organelles associated to vitellogenin endocytosis [Wall and Meleka, 1985] and catabolism of recently formed zygote [Decroly et al., 1979]. Hex also participates in polyspermy prevention [Vo et al., 2003]. Noteworthy, animal hemisphere of amphibian eggs has been long known as the sperm entry site [Elinson, 1975; Cabada et al., 1989]. Whether these facts are correlated awaits further investigation.

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