Xenopus kidney hyaluronidase-1 (XKH1), a novel type of membrane-bound hyaluronidase solely degrades hyaluronan at neutral pH[1]

Stephan Reitinger[2], Johannes Müllegger[2], Günter Lepperdinger*

Institute of Molecular Biology, Austrian Academy of Sciences, Billrothstr. 11, A-5020 Salzburg, Austria

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Abstract In search for *Xenopus laevis* hyaluronidase genes, a cDNA encoding a putative PH-20-like enzyme was isolated. In the adult frog, this mRNA was only found to be expressed in the kidney and therefore named XKH1. When expressed by means of cRNA injection into frog oocytes, XKH1 solely exhibited at physiologic ionic strength hyaluronidase activity at neutral pH and in weakly acidic solutions. The enzyme was inactive below pH 5.4. In addition to hyaluronic acid hydrolysis, chondroitin sulfate also was degraded at low yield as assessed by fluorophore-assisted carbohydrate electrophoresis analysis of the degradation products. The enzyme is sorted to the outer surface of the cell membrane of XKH1 expressing oocytes. From there, it could not be removed by phospholipase C nor was secreted hyaluronidase activity detectable. We conclude that XKH1 represents a membrane-bound hyaluronan-degrading enzyme exclusively expressed in cells of the adult frog kidney where it either may be involved in the reorganization of the extracellular architecture or in supporting physiological demands for proper renal functions.

Key words: Hyaluronan; Hyaluronidase; PH-20; Kidney

1. Introduction

Hyaluronan (hyaluronic acid, HA), a linear polysaccharide, is a component of the extracellular matrix of higher animals with sugar moieties all connected by β-linkages. Sugar polymers with β-glycosidic bonds are rare in vertebrate tissues and require specialized enzymes for turnover [1]. HA catabolism depends primarily on hyaluronidases, endoglycolytic enzymes with, in most cases, an exclusive specificity for the β-1,4 glycosidic bond between glucuronic acid and *N*-acetyl glucosamine [2].

The first eukaryotic hyaluronidase analyzed at the molecular level was the one from bee venom. This enzyme shares significant homology with a sperm head protein termed PH-20 [3]. The human genome is known to contain at least seven hyaluronidase genes. Six of those are arranged in two clusters, one on chromosome 3p21 and another on chromosome 7q31. This arrangement suggests that two tandem gene duplication events from an original ancient sequence were followed by a more recent duplication and translocation, thus yielding the final chromosomal localizations [4].

In the course of our studies on hyaluronidase genes in the African clawed frog, *Xenopus laevis*, we isolated a cDNA sequence with striking similarities to the PH-20-type enzymes. Recently, the embryonic hyaluronidase expression pattern of a *Xenopus* cDNA sequence, XEH1, has been reported [5]. In spite of different biochemical properties, it is very likely that the sequence presented here is the twin homolog to XEH1 within the pseudotetraploid arrangement of this frogs genome [6]. In analogy and the fact, that this gene is only expressed in the adult kidney we named it *Xenopus* kidney hyaluronidase-1 (XKH1).

2. Materials and methods

2.1. Isolation and Northern analysis of XKH1

A *X. laevis* stage 24 cDNA library (#725) spotted on nylon filters and the corresponding cDNA clone (DKFZp725K1913Q2) were purchased from RZPD, Berlin, Germany. The library was screened under low stringency conditions with radiolabeled cDNA insert encoding human Hyal2 (GenBank accession AJ000099). Polymerase chain reaction (PCR) amplification of cDNA fragments encoding the open reading frame of XKH1 were performed using the following primer pair: up: GAC TTA TGC GCT GCA CAA TGG CAA AAC; down: TTA AGA GTA TGA TCT ACT TGT TAT TTA TGT TC. cDNA inserts were sequenced on both strands with the aid of an automated LICOR system (MWG, Germany). The GenBank accession number AF394961 was assigned to XKH1.

Total RNA was extracted from tissues of adult *X. laevis* and Northern blot analysis was performed as described earlier [7].

2.2. Frog surgery, oocyte preparation

A small lobe of the ovary was surgically removed from an adult *X. laevis* female. The incision in the body was then sutured and the animal was left to recover in shallow water. The isolated ovary was rinsed in OR2 buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES, pH 7.2) and individual oocytes were manually defolliculated using fine watchmaker's forceps. Oocytes were cultured in OR2 containing 100 U/ml penicillin and 100 μg/ml streptomycin at 16°C.

2.3. RNA injection

The encoding regions of XKH1 were cloned into pT7TS (gift from P.A. Krieg, Univ. of Arizona, Tucson, AZ, USA), suitable for generating in vitro-transcribed capped mRNA after linearization by *Bam*HI. Capped cRNAs were transcribed with the aid of T7 mMESSAGEmMACHINE Kit (Ambion). 50 ng of the resulting RNA was injected with the aid of a Nanoliter Injector (World Precision Instruments) into oocytes, which were subsequently cultured in OR2 at 16°C for up to 3 days. This construct was also used for in vitro

*Corresponding author. Present address: NIH/NICHD/LMG/SVD, Bethesda, MD 20892, USA. Fax: (1)-301-496 0243.
E-mail address: lepperdg@mail.nih.gov (G. Lepperdinger).

[1] GenBank accession: AF394961.
[2] Both authors contributed equally.

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translation in the presence of [^{35}S]-labeled methionine and cysteine (Translabel, American Radiochemicals) with the aid of the TNT-coupled transcription translation kit (Promega).

2.4. Characterization of XKH1 protein

In vitro translated protein products were analyzed on SDS–PAGE. GPI-linked proteins were removed from the plasma membranes using 0.1 U of phospholipase C (Sigma) in 50 μl of OR2 buffer per oocyte for 5 h at room temperature.

Hyaluronidase activity was measured as described recently [8]. Fluorophore-assisted carbohydrate electrophoresis (FACE) analysis was performed as recently published by Calabro and colleagues [9] using the MONO gel system (Glyko). Standards were generated by digesting HA with HA Lyase (Sigma) and chondroitin sulfate A and C digested with chondroitinase ABC (Sigma). Using this method, saccharide structures found after hyaluronidase or chondroitinase digestions can be tagged at the free reducing group with the fluorescent label, 2-aminoacridone. After separation by electrophoresis, the relative fluorescence in each band was quantitated by simple imaging techniques.

3. Results and discussion

Hyal2 cDNA was used as a probe to screen a *X. laevis* cDNA library under low stringency conditions. A cDNA sequence nearly identical to the previously published XEH1 could be isolated. However, several differences with respect to the published XEH1 cDNA sequence were observed. Besides several point mutations, a conspicuous single nucleotide insertion in the protein-encoding region was found. This insertion furthermore also was found in three cDNA clones generated by reverse transcriptase (RT)-PCR from embryonic stage 17 and 24 mRNAs. This insertion gives rise to a shift in the reading frame, 27 amino acids upstream from the stop codon that yields XEH1. In consequence, the translated gene product contains 512 amino acid residues, with a C-terminal tail distinctly different and 54 residues longer than that of XEH1. This novel sequence is similar to the PH-20-type hyaluronidases on the primary structural level (Fig. 1). The expression pattern of this gene was analyzed using mRNA prepared from tissues of adult *X. laevis* with the aid of Northern blots. Since this gene was solely and abundantly expressed in the kidney, we refer to this gene as XKH1 (Fig. 2) with the GenBank accession AF394961.

Most of the known hyaluronidases differ in length but exhibit high homology within the N-terminal part of their sequences. The C-terminal parts on the other hand, differ greatly within this gene family [1]. The shortest hyaluronidase is found in bee venom. In this case, the enzyme is secreted as a soluble polypeptide [10]. Longer forms, such as the sperm head hyaluronidase, PH-20, are attached to the cell membrane by a GPI anchor [11]. This particular enzyme is active under mild acidic conditions and at neutral pH. PH-20-type hyaluronidases can however also be proteolytically cleaved to yield a soluble product with a lower pH optimum in the extracellular space [12]. Furthermore, PH-20 also exhibits chondroitinase activity. Since XKH1 is similar to both with respect to the protein sequence as well as the length of PH-20 (Fig. 1), we reasoned that it might be an extracellular membrane-bound enzyme that exhibits biochemical properties similar to PH-20.

Hence, XKH1 was first translated in vitro using pT7TsXKH1 as a template. The resulting protein product exhibited a molecular weight of 60 kDa on SDS–PAGE (data not shown). We then expressed XKH1 and PH-20 in

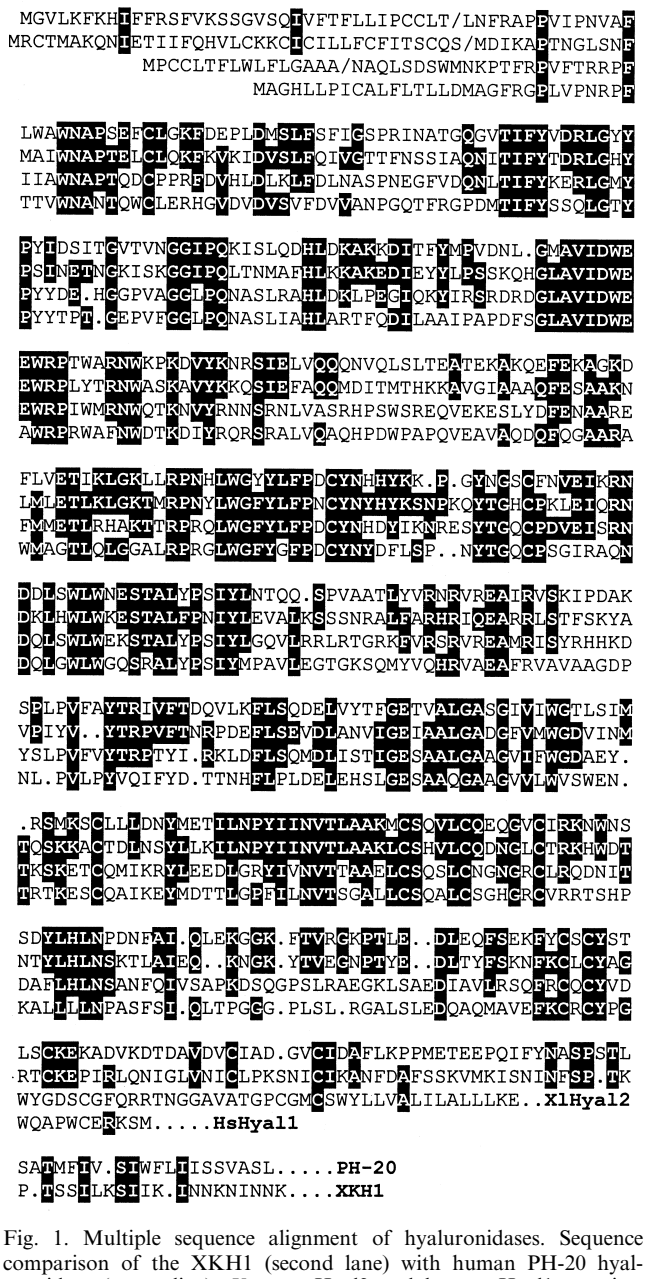

Fig. 1. Multiple sequence alignment of hyaluronidases. Sequence comparison of the XKH1 (second lane) with human PH-20 hyaluronidase (upper line), *Xenopus* Hyal2 and human Hyal1 proteins. The ends of the putative signal peptides are marked (/). Some gaps, marked by dots, have been introduced to maximize identity between the sequences. Sequences have been arranged manually. Amino acid residues identical to XKH1 are highlighted.

Xenopus oocytes with the aid of cRNA injection. The enzymatic specificity of XKH1 was monitored by FACE analysis. We first checked if both HA and chondroitin sulfate A and C were used as substrates at neutral pH. XKH1-degraded HA yielding primarily hexasaccharides as products. In *Xenopus* oocytes, some but little chondroitinase activity is present endogenously. However, when XKH1 or PH-20 is expressed, more chondroitin sulfate oligo saccharides were detectable compared to the background (Fig. 3). This indicates that XKH1 exhibits a spectrum of enzymatic activity similar to PH-20. In order to characterize the cellular localization of the expression product, injected oocytes were manually dissected and processed to yield two cellular compartments:

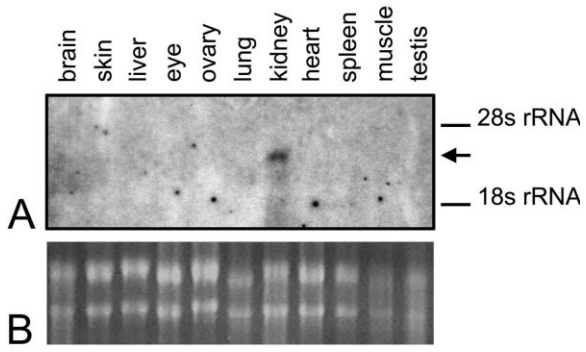

Fig. 2. Northern blot analysis. A: Radioactively labeled XKH1 cDNA was hybridized to a Northern blot containing 10 μg/lane total RNA of various tissues from adult *Xenopus*. The blot was exposed to X-ray films with intensifying screens for 5 days at −70°C. B: As a loading control, the ethidium bromide-stained agarose gel was photographed before nucleic acids were transferred to the nylon membrane.

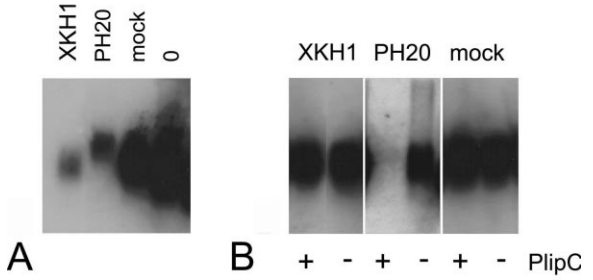

Fig. 4. Hyaluronidase activity of XKH1 and PH-20 expressing *Xenopus* oocytes. A: Oocytes injected with cRNA encoding XKH1 or PH-20 and uninjected controls (mock) were cultured in 50 μl OR2 containing 5aF-HA for 3 days. A 10 μl aliquot of the medium was analyzed by agarose gel electrophoresis. In (0), a 3 day incubation of 5aF-HA in OR2 without an oocyte is shown as a control. B: After 3 days of culture, the oocytes were thoroughly washed and the medium was changed. Some oocytes were cultured in the presence of phospholipase C. In the resulting supernatant, hyaluronidase activity was monitored as described. Hyaluronidase enzyme activity was rendered soluble by this treatment only from the surface of PH-20 mRNA-injected oocytes. The membrane-bound XKH1 was still active after the phospholipase C treatment.

the plasma membrane with the adherent cortical region and cytoplasm. XKH1-corresponding enzymatic activity was found in the membranous fraction and could be solubilized by Triton X-100 (data not shown). When HA was then added to the supernatant of injected oocytes during the 3 days of culture, the polymer was nearly completely hydrolyzed, just as when oocytes were injected with PH-20 (Fig. 4A). However, PH-20-expressing oocytes also shed soluble activity into the culture medium, whereas XKH1 cRNA-injected oocytes produced no soluble HA-degrading activity. Unexpectedly, the mature sequence reveals no canonical sequence motif for post-translational modifications that would sort XKH1 to membranes. In particular, no GPI linkage and processing consensus sequence were found at the C-terminus [13]. Nevertheless, since a GPI anchor has been described for some forms of PH-20 [11], we also treated XKH1-injected oocytes with phospholipase C. This yet did not release the enzyme from the membrane (Fig. 4B). For Hyal1 actually, a similar biochemical behavior has been reported. Triton X-114 phase partitioning showed that this serum protein exhibits an unpredictable lipophilic property, which is due to either lipid modifications or conformational features. The localization of these hyaluronidase proteins thus cannot solely and easily be inferred by simple computational methods [14].

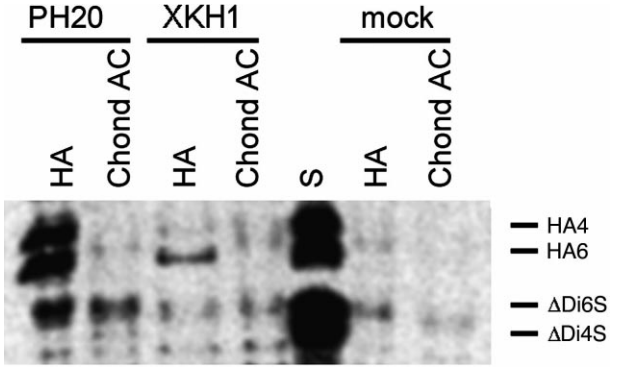

Fig. 3. FACE of 2-aminoacridone-derivatized hyaluronidase/chondroitinase digestion products. HA and chondroitin sulfate A and C were reacted with PH-20 and XKH1 which were expressed in *Xenopus* oocytes before. The enzymatic degradation products were labeled with 2-aminoacridone and subjected to FACE analysis. HA hydrolysis by PH-20 yielded HA-tetra- and hexasaccharides, whereas hexasaccharides were observed as the predominant product of XKH1 degradation. Though *Xenopus* oocytes exhibit endogenous chondroitinase activity, hydrolysis of chondroitin sulfate A and C was enhanced when PH-20 or XKH1 were overexpressed. HA: hyaluronan; ChondAC: mixture of chondroitin sulfate A and C. As standards (lane S), HA4: hyaluronan tetrasaccharide; HA6: hyaluronan hexasaccharide; ΔDi6S: Δ glcA-β1, 3-, 6S-galNAc; ΔDi4S: ΔglcA-β1, 3-, 4S-galNAc were used.

Furthermore, the pH and ionic strength requirements of the XKH1 enzyme were determined by incubating homogenated as well as intact XKH1-injected oocytes with high molecular weight HA. In both cases, XKH1 optimally hydrolyzed HA at neutral pH and physiologic salt concentration (Fig. 5). The enzyme was inactive below pH 5 (Fig. 5A–E) and low ionic strength (Fig. 5F). This is in contrast to XEH1, which optimally reacts below pH 5 in 20 mM sodium acetate buffers [5]. This reminds earlier reported data [12], that PH-20, when sorted to the sperm head or into the acrosome, differs in molecular weight and that the processed form exhibits a lower pH optimum. Taken together, this indicates that different C-termini of otherwise identical hyaluronidases are capable of changing the respective enzymatic property. Plasma membrane-attachment of XKH1 and delicate conformational changes in the substrate-binding domain and/or at the active thus also may cause the biochemical differences between XKH1 and XEH1. Experimentally, this assumption can only be tested by analyzing of the three-dimensional structure of the respective hyaluronidase polypeptides.

Though XKH1 is very similar to the PH-20-type hyaluronidases, there are differences. First, XKH1 is not expressed in the testis. We have characterized hyaluronidase activity in sperm released from frog testicles. HA degradation was only detectable in dilute salt buffers, by all means a prerequisite for fertilization in which however XKH1 is inactive (unpublished experiments). At this point, it furthermore is not clear if a sperm-bound hyaluronidase is necessary for the fertilization of frog oocytes. Therefore, we consider it unlikely that XKH1 is involved in this process.

In contrast to other hyaluronidases and in particular PH-

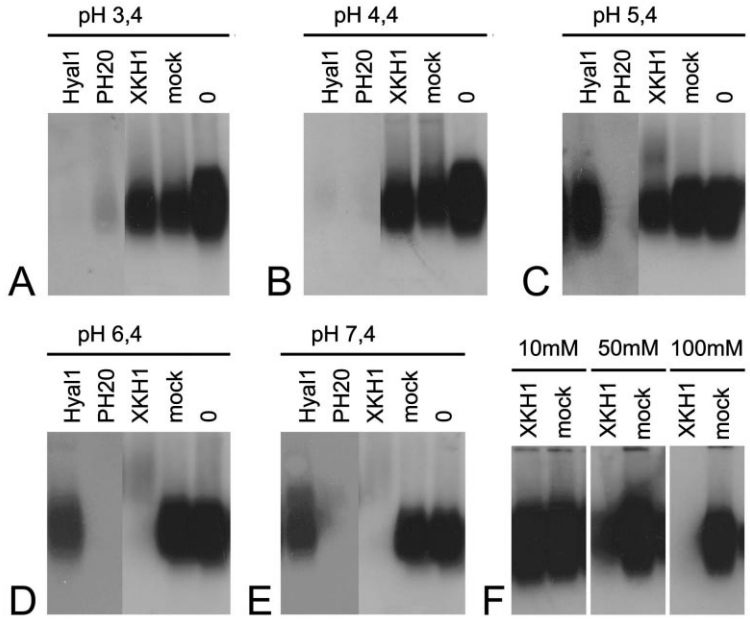

Fig. 5. Hydrolysis of fluorescein-labeled HA by *Xenopus* oocyte lysates. Oocytes were injected with cRNAs encoding human Hyal1, PH-20 or XKH1 and cultured for 3 days. 50 pg of 5aF-HA were incubated with total lysates over night at indicated pH values (A–E) or with increasing salt (F). The reaction mixture was then size-fractionated by 0.5% agarose gel electrophoresis and blotted to polyamide filters. 5aF-HA was visualized with the aid of alkaline-phosphatase-tagged anti-fluorescein-Fab and ECL.

20, which is highly active within pH 4–8, XKH1 appeared to be active only around neutral pH. Though we used a highly sensitive method in order to monitor degradation of HA [8], no hydrolysis could be detected below pH 5. All other hyaluronidases characterized so far exhibit activity at or below pH 5 except, to our knowledge, XKH1.

Taken together, XKH1 is a novel type of HA-degrading enzyme that clearly differs from the sperm hyaluronidase PH-20 by several characteristic aspects. Though it is not the functional homolog of mammalian PH-20 with respect to fertilization, its expression in the kidney is reminiscent of that of PH-20 in this organ [15]. Furthermore, at the genomic level, we cannot rule out that XKH1 and PH-20 originate from a common ancestor and thus, have to be considered structural homologs. The high level of expression of XKH1 in the adult frog kidney however implies that beside the well-known structural relevance of HA and its binding proteins for the structure of this organ [16], extracellular degradation of this polysaccharide may as well play an important role in renal physiology.

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References

[1] Kreil, G. (1995) Protein Sci. 4, 1666–1669.
[2] Meyer, K. (1971) (Boyer, P.D., Ed.), Vol. V, pp. 307–320, Academic Press, New York.
[3] Gmachl, M. and Kreil, G. (1993) Proc. Natl. Acad. Sci. USA 90, 3569–3573.
[4] Csoka, A.B., Scherer, S.W. and Stern, R. (1999) Genomics 60, 356–361.
[5] Hyde, C.E. and Old, R.W. (1999) Mech. Dev. 82, 213–217.
[6] Graf, J.D. and Kobel, H.R. (1991) Methods Cell. Biol. 36, 19–34.
[7] Lepperdinger, G., Engel, E. and Richter, K. (1997) Dev. Genes Evol. 207, 177–185.
[8] Müllegger, J., Reitinger, S. and Lepperdinger, G. (2001) Anal. Biochem. 293, 291–293.
[9] Calabro, A., Hascall, V.C. and Midura, R.J. (2000) Glycobiology 10, 283–293.
[10] Kemeny, D.M., Dalton, N., Lawrence, A.J., Pearce, F.L. and Vernon, C.A. (1984) Eur. J. Biochem. 139, 217–223.
[11] Phelps, B.M., Primakoff, P., Koppel, D.E., Low, M.G. and Myles, D.G. (1988) Science 240, 1780–1782.
[12] Li, M.W., Cherr, G.N., Yudin, A.I. and Overstreet, J.W. (1997) Mol. Reprod. Dev. 48, 356–366.
[13] Eisenhaber, B., Bork, P. and Eisenhaber, F. (1999) J. Mol. Biol. 292, 741–758.
[14] Frost, G.I., Csóka, T.B., Wong, T. and Stern, R. (1997) Biochem. Biophys. Res. Commun. 236, 10–15.
[15] Sun, L., Feusi, E., Sibalic, A., Beck-Schimmer, B. and Wuthrich, R.P. (1998) Kidney Blood Press. Res. 21, 413–418.
[16] Gupta, S., Batchu, R.B. and Datta, K. (1991) Eur. J. Cell. Biol. 56, 58–67.