HYAL2, a Human Gene Expressed in Many Cells, Encodes a Lysosomal Hyaluronidase with a Novel Type of Specificity*

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Using Expressed Sequence Tags (ESTs) deposited in the data banks, a cDNA has been assembled that encodes a protein related to the hyaluronidases from bee venom and mammalian sperm. Expression of this cDNA yielded a polypeptide termed HYAL2, which is located in lysosomes. The HYAL2 protein was shown to have hyaluronidase activity below pH 4. However, it only hydrolyzed hyaluronan of high molecular mass from umbilical cord, rooster comb, and a Streptococcus strain. The reaction product was a polysaccharide of about 20 kDa, which was further hydrolyzed to small oligosaccharides by the sperm hyaluronidase. Conversely, hyaluronan fragments from vitreous humor, which had a molecular mass of about 20 kDa, were not cleaved by the HYAL2 enzyme to any detectable extent. These results provide evidence for the existence of structural domains in hyaluronan, which are resistant to the action of this enzyme. The structural and functional implications of these findings are discussed.

Hyaluronidases have been isolated from many different sources such as mammalian testes and serum, snake and insect venoms, salivary glands of leeches, and pathogenic streptococci (1–3). These enzymes degrade hyaluronan (hyaluronic acid (HA)1), a glycosaminoglycan present in the extracellular matrix of vertebrates to oligosaccharides (4). The sequences of two related hyaluronidases from animal cells have recently been elucidated via cDNA cloning. These are the enzymes from honeybee venom (5) and from mammalian testis (6, 7). The testicular enzyme originally termed PH-20 (8) is located at the head of the sperm; upon contact with the egg, it hydrolyzes the HA present in its outermost cumulus layer (9). The PH-20 protein is normally expressed only in mammalian testis. However, it has recently been shown that this enzyme is present in some tumor cells (10). A related hyaluronidase termed HYAL1, which is present in human serum, has recently been characterized (11).

In recent years, several partial cDNA sequences have been deposited in the data banks that are derived from genes that potentially code for polypeptides related to the PH-20 hyaluronidase. Three of these genes are located close to each other on chromosome region 3p21.3 (12). This region is deleted in some cell lines from small cell carcinomas of the lung (13). The genes were thus provisionally termed LuCa-1, -2, and -3. The serum enzyme HYAL1 is the product of the LuCa-1 gene (11). Starting from commercially available ESTs we have now assembled the complete LuCa-2 cDNA. Expression of this cDNA yields a hyaluronidase with a rather unusual substrate specificity, which is located in lysosomes. It is proposed to replace the term LuCa-2 by HYAL2 for both the gene and its product.

EXPERIMENTAL PROCEDURES

Materials—HA from human umbilical cord, human vitreous humor, rooster comb, and Streptococcus zooepidemicus as well as hyaluronidase from bovine testis, i.e. PH-20 (14), were purchased from Sigma. Radioactive HA was prepared using recombinant DG42 HA synthase and ((14)C)UDP-glucuronic acid (NEN Life Science Products) as described previously (15).

Assembly of the HYAL2 cDNA—DNA and protein sequences of human PH-20 (GenBank U67798) were compared with the EST subset of the GenBank library using Smith-Waterman algorithm. Clones of interest were purchased from Research Genetics, Inc. EST 38383 (GenBank R51257) overlapped at the 5'-end with yet another EST cloned by The Genexpress cDNA program (GenBank F11962). In addition, they all matched a cDNA deposited under the name of LuCa-2 (GenBank U69577). The full-length HYAL2 cDNA could be assembled from the EST sequence R51257 by PCR amplification with 5'-specific primers. DNA was sequenced using the chain termination method with T7 DNA polymerase (Amersham Pharmacia Biotech). For multiple tissue Northern blots (CLONTECH), DNA was labeled with [a-32P]dATP and hybridized to poly(A)+ RNA on Nylon filters at 68 °C using standard procedures. Filters were washed twice with 2× saline/sodium phosphate/EDTA at 65 °C and exposed to x-ray films at ~70 °C.

Expression of the HYAL2 Protein in Escherichia coli—Using two synthetic oligonucleotides (GAG AGG ATC CAT GCC CCA AGG CTT TAG G and GAG AGA AAG CTT CAA GGT CCA GGG TAA AGG CCA GG) and the clone EST 38383, a DNA fragment was amplified by PCR that coded for the HYAL2 protein without the putative signal sequence. The amplified DNA fragment and the plasmid PMW172 were digested with BamHI and HindIII, ligated with T4 ligase, and transformed into E. coli BL21 (D3) as described (16). After a 3-h induction with isopropyl-thio-β-galactoside, bacteria were lysed with lysozyme in 10 mM Tris, pH 7.6, 10 mM NaCl. The insoluble fraction was washed twice with 4 mM NaCl, followed by washes with distilled water. The resulting pellet was enriched in insoluble inclusion bodies, which were resuspended in water and stored at ~20 °C.

Antibody Production—An antisera against HYAL2 was produced in rabbits by standard techniques as described previously (16). The serum was purified by affinity chromatography as follows. Insoluble protein from inclusion bodies was dissolved in 8 M urea, 10 mM Tris, pH 8.8, and dialyzed against buffer overnight. Soluble protein was immobilized on Sepharose Q (Amersham Pharmacia Biotech), rinsed with 1 M NaCl and equilibrated with PBS. Serum was diluted in PBS and mixed with protein beads overnight at 4 °C. The loaded beads were washed extensively with PBS. Antibodies were eluted with 100 mM glycine at pH 2.5. The neutralized antibody solution was stored at 4 °C.

Western Blots—Various tissues and cells were homogenated in PBS. Aliquots containing two micrograms of protein were separated on 10% polyacrylamide gels in presence of SDS, blotted onto nitrocellulose filters, and incubated with diluted antiserum in phosphate-buffered saline, 200 μg/ml bovine serum albumin, 0.2% Tween 20 overnight at 4 °C. Filters were washed twice with phosphate-buffered saline, 0.2% Tween 20) for 20 min and then incubated with diluted goat-anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) for 2 h and washed.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AJ000999.

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1 The abbreviations used are: HA, hyaluronan (hyaluronic acid); PCR, polymerase chain reaction; EST, expressed sequence tag; vv, vaccinia virus; EGFP, green fluorescent protein.
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again as before. Bound secondary antibodies were detected using the ECL Western blotting analysis system (Amer sham Pharmacia Biotech).

Preparation of Recombinant Vaccinia Virus (vo) Expressing HYAL2—Using synthetic oligonucleotides (Hy-1, AGG CCC AGC CCC CAC GTG TAC CTT GCC GCT GCT GGC GCT GGC ATG GGC C; Hy-2, GAG AGA GAA TTC AGC ACA TGA TCA TGA TGG GAG GCC C CCG T; Hy-3, GAG AGA GAA TTC CAA GCT CCA GGG TAA AGG CCA GG) and the clone EST R3838, a cDNA fragment was amplified by PCR that contained the complete coding sequence of the human HYAL2 gene. Hy-1 and Hy-3 primers were used in the first 10 PCR cycles, and a diluted portion of the reaction product was then amplified with Hy-2 and Hy-3. The PCR fragment and the vector pGEM-ATC-18 (17) were digested with EcoRI and ligated to yield the plasmid pGEM-ATC-H2. Recombinant vv was selected and prepared as described before (6, 16). Cellular lysates were fractionated by centrifugation. The supernatant of a 10,000 × g spin was used for the activity measurements. Fractions were extracted with Triton-X114, and the detergent phase was discarded. Triton X-100 was added to the aqueous phase to a final concentration of 1%, and samples were stored at −30 °C. Cells were pulse-labeled with [35S]methionine (American Radiochemicals), and protein products were precipitated with SPS-lysylamine gel electrophoresis, and dried gels were exposed for 24 h at −70 °C.

Assay for Hyaluronidase Activity—Initial tests for hyaluronidase activity were carried out as described (6). Hyaluronidase activity was also monitored by agarose gel electrophoresis. HA samples from different mouse tissues were electrophoresed, and stained with Stains-All (Sigma). DNA prepared from Leuconostoc mesenteroides (Quiagen) was used to stably transfect C6 cells with pHYAL2-EGFP. This yielded the plasmid pHYAL2-EGFP, which expressed the fusion protein under the control of the cytomegalovirus promotor. Superfect (Qiagen) was used to stably transfect C6 cells with pHYAL2-EGFP. This yielded the plasmid pHYAL2-EGFP, which expressed the fusion protein under the control of the cytomegalovirus promotor. Superfect (Qiagen) was used to stably transfect C6 cells with pHYAL2-EGFP. Transfected cells were selected in the presence of G418. Acidic compartments were labeled using Lysotracker Red DND-99 (Molecular Probes). Green and red fluorescence was distinguished using a Bio-Rad MRC600 confocal microscope equipped with an Argon laser and 100 × lenses. Fluorescence was measured by BHS and A1/A2 filtersets, respectively.

RESULTS

Assembly of the HYAL2 cDNA—From ESTs deposited in the data banks and a partial cDNA assigned to the human LaCa-2 gene on chromosome 3p21.3 (GenBank® U09577) the complete coding region was assembled as described under "Experimental Procedures." The polypeptide encoded by this cDNA is 35.2% identical to the human PH-20 hyaluronidase (see Fig. 1). We suggest calling this the HYAL2 gene and hyaluronidase.

Tissue Distribution of HYAL2 mRNA and Protein—Using the HYAL2 cDNA, we could show by Northern blot analysis that mRNAs hybridizing with this probe are present in all human tissues tested, the sole exception being adult brain (see Fig. 2). Using an antisense gerated against the human HYAL2 probe, extracts from different mouse tissues were tested in Western blots. A protein with the molecular mass of about 60 kDa was detected in all tissues, adult brain again being a conspicuous exception (Fig. 3). These results demonstrated both at the mRNA and the protein level that the gene encoding HYAL2 is widely expressed.

Expression of HYAL2 by Recombinant Vaccinia Virus—Recombinant vv containing the HYAL2 cDNA after the late 11,000-dalton promotor was prepared. Expression of the HYAL2 protein was then tested in HeLa and C6 glioma (see "Experimental Procedures"). In the first set of experiments, HeLa cells were infected with recombinant vv as well as with wild type virus as a control. After 18 h, cells were harvested and lysed by freezing and thawing. HYAL2 protein was detected by Western blotting, and hyaluronidase activity was assayed by turbidity measurements (21) and agarose gel electrophoresis (18).

As shown in Fig. 4A, hyaluronidase activity at pH 3.8 was about 2–3 times higher in HeLa cells infected with recombinant vv than in control cells infected with wild type vv. The enzymatic activity in the latter probably corresponds to the lysosomal hyaluronidase present in HeLa cells (22). Indeed, on a Western blot a protein with about the same molecular mass was detected in HeLa cells infected with wild type or recombinant vv (see Fig. 4D). The endogenous and the HYAL2 enzyme both have an acidic pH optimum (see also Fig. 4C). Moreover,
after centrifugation, hyaluronidase activity could be detected in the 1,000 \( \times g \) as well as in the high speed pellet. Upon extraction with Triton-X114 (23), the enzymatic activity remained in the aqueous layer. This indicates that the endogenous and the HYAL2 enzymes are soluble proteins with a similar subcellular distribution.

Since adult brain does not contain detectable amounts of the HYAL2 mRNA and protein, we next used C6 glioma cells. As shown in Fig. 4B, only C6 cells expressing the HYAL2 cDNA contained hyaluronidase activity, whereas control cells or cells infected with wild type vv were negative. This was confirmed by a Western blot (see Fig. 4D, lanes 3 and 4). The enzyme activity had a pH optimum of about 3.8 in this cell line (Fig. 4C).

Hyaluronidase activity was also tested using separation of substrates and products by gel electrophoresis and subsequent staining with Stains-All. The electrophoresis was calibrated using HA preparations of known molecular mass (see Fig. 5, A and B). Surprisingly, HA from umbilical cord was only digested to a fragment with a molecular mass of about 20,000 Da (Fig. 5C, lanes 2, 5, and 8). The same results were obtained with HA from S. zoopidemicus (see Fig. 5C, lane 9) and from rooster comb (data not shown). Upon subsequent addition of testicular PH-20 hyaluronidase, this intermediate size HA was rapidly degraded to small oligosaccharides (Fig. 5, lane 7).

Subsequently, we tested a preparation of HA from vitreous humor (Sigma) that already had a size of about 20 kDa (Fig. 5C, lane 12). This substrate was not cleaved to any detectable extent by the HYAL2 enzyme (Fig. 5C, lane 13). We also prepared radioactively labeled HA using the recombinant DG42 HA synthase. This yields an intermediate size product comparable with the HA fragments prepared from vitreous humor (15). Labeled substrate (1500 dpm) was used in a standard reaction mixture. After incubation with the HYAL2 enzyme, the product was separated by agarose gel electrophoresis. As compared with a control incubated in the absence of enzyme, 96.4% of the radioactivity was recovered in the region corresponding to the original substrate.

To confirm this result, radioactively labeled HA was also immobilized on microtiter plates. The radioactivity released after incubation with either HYAL2 or PH-20 was measured in a liquid scintillation counter. In a typical experiment, incubation with HYAL2 or the PH-20 hyaluronidase resulted in the release of 150 and 1650 cpm, respectively (the control was 137 cpm).

We also checked the extent of hydrolysis by measuring the increase of reducing sugar termini (20). After hydrolysis of high molecular weight HA with HYAL2, the increase of reactive termini was below the detection limit of this reaction. The subsequent addition of PH-20 hyaluronidase yielded significant amounts of reducing ends. The optical density at 585 nm increased from zero after incubation with HYAL2 to 0.32 after degradation with PH-20.

Several control experiments were performed in an effort to find an explanation for this unexpected observation. Thus, at the end of the incubation with HYAL2, an aliquot was removed, boiled for 5 min, cooled rapidly and then incubated further in the presence of freshly added enzyme. No further hydrolysis of...
the reaction product could be observed after this denaturation step. To test for possible product inhibition, we also added to the reaction mixture HA oligomers (up to 10 mg/ml) generated by digestion of HA with the PH-20 enzyme. This did not alter the activity of the HYAL2 enzyme.

As tested by gel electrophoresis, HYAL2 does not hydrolyze commercial preparations (Sigma) of chondroitin sulfates A, B, and C, heparan sulfate, and heparin (data not shown).

**Biosynthesis and Subcellular Localization of HYAL2—C6 glioma and other cells (RK13, AtT-20) were infected with recombinant vv. After 12 h, cells were incubated for 1 h in the presence of [35S]methionine and then chased with an excess of unlabeled methionine for 3 h. Cells and media were harvested, immunoprecipitated with anti-HYAL2 serum, and subjected to gel electrophoresis. In these experiments, neither immunoprecipitated HYAL2 protein nor hyaluronidase activity could be detected in the media (data not shown).

To learn more about the subcellular localization of this protein, an in-frame fusion of cDNAs coding for HYAL2 and the green fluorescent protein (EGFP) was constructed. C6 cells were stably transfected with this plasmid and then selected in the presence of G418. As shown in Fig. 6, the HYAL2-EGFP fusion protein gave green signals in vesicular structures. This fluorescence colocalized with the Lysotracker Red (see Fig. 6B). Control C6 cells transfected with pEGFP showed only a diffuse green fluorescence in the cytoplasm of cells (data not shown).

**DISCUSSION**

The human HYAL2 cDNA encodes a mature polypeptide of 452 amino acids that shows 36.5% identity with the PH-20 enzyme.
hyaluronidase present on the head of spermatozoa. At the nucleotide level, the identity is 43.1%. The HYAL2 gene is located on chromosome 3p21.3 in a region that is deleted in most small cell carcinomas of the lung as well as other tumor cells (12, 13). This gene was tentatively termed LuCa-2; in view of the results presented in this communication, we propose to now call it HYAL2. The hyaluronidase present in mouse (24) and human (11) serum was termed HYAL1 earlier. This enzyme is apparently secreted by a variety of cells.

The human HYAL2 gene is expressed in all cells tested, the sole exception being adult brain. It is noteworthy that several of the ESTs encoding part of the HYAL2 enzyme have been isolated from infant human brain. The expression of the HYAL2 gene may thus be developmentally regulated. Indeed, the mouse HYAL2 mRNA is present in brains from embryos but disappears for unknown reasons soon after birth.2

Using recombinant vV, the HYAL2 protein has been expressed in several cell lines; here we present the results obtained with HeLa and C6 glioma cell. The HYAL2 cDNA codes for a preprotein with an amino-terminal signal peptide, yet the protein is not secreted into the media to a measurable extent. The product of this gene is a soluble hyaluronidase that resembles the lysosomal enzyme present in HeLa cells. Both have a pH optimum below 4 and react with the same antiserum. A fusion protein of HYAL2 and EGFP could be shown to be localized in lysosomes of C6 glioma cells. We conclude from these results that HYAL2 encodes a lysosomal hyaluronidase present in many cell types. It was apparent from initial turbidity measurements that the activity of HYAL2 was rather low compared with that of the testicular PH-20 enzyme. Subsequent experiments with a gel electrophoresis assay yielded a totally unexpected result. The HYAL2 enzyme hydrolyzed only HA of high molecular mass, as is present in umbilical cord, rooster comb, and the coat of a Streptococcus strain. The reaction product was a polysaccharide of about 20 kDa, which corresponds to 50–60 disaccharide units. After heating and rapid cooling, no further hydrolysis by the HYAL2 enzyme provided the first biochemical evidence that HA chains can form a complex network containing numerous helical structures (27, 28). Antiparallel helices have indeed been observed in x-ray diffraction studies with HA films (29).

Evidence from physico-chemical studies suggests that similar structures exist in HA solutions (25, 30). Our results on the activity of the HYAL2 enzyme provide the first biochemical data in support of defined domains in HA.

Second, HA fragments generated by the HYAL2 enzyme may have distinct biological functions. One could be the stimulation of angiogenesis, which has been observed in different experimental situations (10, 31). Moreover, it is of some interest that HA fragments can induce the expression of enzymes such as nitric oxide synthase via a NF-κB-like autoregulatory loop in murine macrophages (32, 33). The participation of HYAL2 in this signaling process can now readily be tested.

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REFERENCES

1. Frost, G. I., Cso´ka, T. B., and Stern, R. (1997) Trends Glycosci. Glycotechn. 8, 419–434
2. Kreil, G. (1995) Protein Sci. 4, 1666–1669
3. Meyer, K. (1971) in Hyaluronidases (Boyer, P. D., ed) Vol. V, Academic Press, New York
4. Laurent, T. C., and Fraser, J. R. (1992) FASEB J. 6, 2397–2404
5. Gmachl, M., and Kreil, G. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 3569–3573
6. Gmachl, M., Sagan, S., Ketter, S., and Kreil, G. (1993) FEBS Lett. 336, 545–548
7. Lin, Y., Mahan, K., Lathrop, W. F., Myles, D. G., and Primakoff, P. (1994) J. Cell Biol. 125, 1157–1163
8. Lathrop, W. F., Carmichael, E. P., Myles, D. G., and Primakoff, P. (1999) J. Cell Biol. 111, 2839–2849
9. Cherr, G. N., Meyers, S. A., Yudin, A. I., VandeVoorst, C. A., Myles, D. G., Primakoff, P., and Overstreet, J. W. (1996) Dev. Biol. 175, 142–153
10. Liu, D., Pearlman, E., Diaconu, E., Guo, K., Mert, H., Hasqi, T., Markowitz, S., Wilson, J., and Sy, M. S. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 7832–7837
11. Frost, G. I., Csoka, T. B., and Stern, R. (1997) Biochem. Biophys. Res. Commun. 236, 10–15
12. Latif, F., Duh, F. M., Bader, S., Sekido, Y., Li, H., Gei, L., Zbar, B., Minna, J. D., and Lerman, M. I. (1997) Hum. Genet. 99, 334–341
13. Daly, M. C., Xiang, R. H., Buchhagen, D., Hensel, C. H., Garcia, D. K., Killary, A. M., Minna, J. D., and Naylor, S. L. (1993) Oncogene 8, 1721–1729
14. Meyer, M. F., and Kreil, G. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 4543–4547
15. Lepperding, G., Strobil, B., Jüch, A., Weber, A., Thalhamer, J., Flechner, H., and Mullay, C. (1996) Protein Sci. 5, 1250–1260
16. Stunnenberg, H. G., Lange, H., Philpion, L., van Miltenburg, R. T., and van der Vlet, P. C. (1988) Nucleic Acids Res. 16, 2431–2444
17. Lee, H. G., and Cowman, M. K. (1994) Anal. Biochem. 219, 278–287
18. Reissig, J. L., Strominger, J. L., and Leulier, L. F. (1955) J. Biochem. 216, 959–966
19. Hausmann, A. (1955) in Mucopolysaccharidases (Calmonwick, S. P., and Kaplan, N. O., eds) Vol. I, pp. 166–173 Academic Press, New York
20. Vaes, G. (1966) in Methods in Enzymology (Neufeld, E. F., and Ginsburg, V., eds) Vol. 8, pp. 509–514, Academic Press, New York
21. Bordier, C. (1981) J. Biol. Chem. 256, 1604–1607
22. Natowicz, M. R., and Wang, Y. (1996) Clin. Chim. Acta 245, 1–6
23. Fitzer Szafarz, B., and De Maeyer, E. (1989) Somatic Cell Mol. Genet. 15, 79–83
24. Sampson, P. M., Rochester, C. L., Freundlich, B., and Elias, J. A. (1992) J. Cell Biol. 119, 1324–1326
25. Laurent, T. C. (1989) CIBA Found. Symp. 143, 1–5
26. Scott, J. E. (1989) CIBA Found. Symp. 143, 6–15
27. Arnott, S., and Mitra, A. K. (1983) J. Mol. Biol. 160, 861–872
28. Scott, J. E., Cummings, C., Greiling, H., Stuhlsatz, H. W., Gregory, J. D., and Damle, S. P. (1990) Int. J. Biochem. Macromol. 12, 180–184
29. Mitra, A. K., Arnott, S., and Sheehan, J. K. (1983) J. Mol. Biol. 169, 813–827
30. Scott, J. E., Cummings, C., Brass, A., and Chen, Y. 699–705 (1991) Biochem. J. 274, 699–705
31. West, D. C., Hampson, I. N., Arnold, F., and Kumar, S. (1985) Science 228, 1324–1326
32. Noble, P. W., Mckee, C. M., Cowman, M., and Shin, H. S. (1996) J. Exp. Med. 183, 2773–2787
33. McKeel, C., Lowenstein, C. J., Horton, M. R., Wu, J., Bao, C., Chin, B. Y., Choy, A. K., and Noble, P. W. (1997) J. Biol. Chem. 272, 8013–8018