Identification and Characterization of Human and Mouse Ovastacin

A NOVEL METALLOPROTEINASE SIMILAR TO HATCHING ENZYMES FROM ARTHROPODS, BIRDS, AMPHIBIANS, AND FISH

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We have cloned and characterized human and mouse ovary cDNAs encoding a new protein of the astacin family of metalloproteinases, called ovastacin because of its predominant expression in ovarian tissues. Human and mouse ovastacins exhibit the same domain organization as other astacins, including signal sequence, propeptide, and metalloproteinase domain. However, ovastacins show an additional C-terminal domain of about 150 amino acids with no similarity to other ancillary domains present in the equivalent region of most astacins. No homologs of human ovastacin were revealed at the sequence level of available databases. In human and mouse unfertilized ovaries, ovastacin could be detected in the preimpla superovulatory tract. The distribution of this enzyme in human and mouse ovaries resembles the enzyme activity in oocytes and extraovarian tissues. On the basis of its expression and cellular localization, ovastacin may be involved in some physiological function. This study was supported by grants from the Comisión Interministerial de Ciencia Y Tecnologia-Spain, Gobierno del Principado de Asturias, and European Union (Cancer Degradome-FP6). The Instituto Universitario de Oncología is supported by Obra Social Cajas-Banco. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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chased from the same company. Nylon filters containing polyadenylated RNAs from human tissues were from Clontech (Palo Alto, CA). Collagenase, pregnant mare's serum glycoprotein, human chorionic gonadotropin, hyaluronidase, and M2 medium were purchased from Sigma.

Bioinformatic Screening of the Human Genome and cDNA Cloning—The BLAST program was used to search public (www.ncbi.nlm.nih.gov) and private (www.ceder.com) human and mouse genome databases, looking for regions with sequence similarity to previously described hatchin enzymes belonging to the astacin family of metalloproteinases. After identification in human chromosome 2q and mouse chromosomes 2 and 7, the amplified product was isolated and sequenced using a Superdex SE75 column pre-equilibrated with 20 mM Tris, pH 7.4, 1 M NaCl, 1 mM MnCl₂, and 0.1 M guanidinium chloride for 48 h, and then twice against 50 mM NaCl, 10 mM Na₂HPO₄, 1 mM EDTA, pH 7.4, 10 mM dithiothreitol, and 0.1 M guanidinium chloride. After cloning of the amplified PCR products in pBSII, their identities were confirmed by nucleotide sequencing.

Nucleotide Sequence Analysis—Cloned cDNAs were sequenced by the dideoxy chain termination method, using the Sequenase Version 2.0 kit (U.S. Biochemicals, Cleveland, OH) and the ABI-Prism 310 DNA sequencer (Applied Biosystems). Computer analysis of DNA and protein sequences was performed with the GCG software package of the University of Wisconsin Genetics Computer Group.

Production, Purification, and Refolding of Recombinant Proteins—A 606-bp fragment of the human ovastacin cDNA containing the entire astacin-like domain was PCR-amplified using two oligonucleotides containing BamHI and EcoRI sites, respectively: 5'-GTCGACATCCCTGAGCTGCTGAGGC-3' and 5'-TGGAGATACAACTTGTGCCCAGAGCTGAT-3' (where the BamHI and EcoRI sites are underlined). The PCR amplification was performed for 25 cycles of denaturation (95°C, 15 s), annealing (58°C, 10 s), and extension (68°C, 30 s) using the Expand™ high fidelity PCR system. The amplified product was then digested with BamHI and EcoRI and ligated in the expression vector pGEX 3X (Amersham). The resulting vector was transformed into BL21(DE3) E. coli competent cells, and expression was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside at 28°C. The cells were then washed with phosphate-buffered saline, and suspended in 100 µl of Triton X-100 overnight. The pellet was washed three times with 100 mM NaCl, 0.1% Triton X-100 by centrifugation, and then once with 50 mM NaCl, 5 mM dithiothreitol, and 0.1 M guanidinium chloride at 4°C, and then twice against 50 mM Tris, pH 7.5, with 150 mM NaCl for 24 h. The recombinant human MMP-26 used as a control of enzyme assays was produced, purified, and refolded as described previously (18).

Enzymatic Assays—Enzymatic activity of the purified recombinant human ovastacin protein was assayed using the synthetic fluorescent substrates QF-24 (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂), QF-35 (Mca-Pro-Leu-Ala-Npa-Dpa-Ala-Arg-NH₂), and QF-41 (Mca-Pro-Cha-Gly-Nva-His-Dpa-Npa-Ala). Routine assays were carried out at 37°C at substrate concentrations of 1 µM in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂, 0.05% Brij-35, pH 7.5, and 1% dimethyl sulfoxide. The fluorescence measurements were made in an LS55 Perkin Elmer Life Sciences spectrophotofluorometer (λex = 328 nm and λem = 390 nm). For inhibition experiments, the reaction mixture was preincubated for 30 min at 37°C with EDTA, batimatstat (BB-94), E-64, 4-(2-aminoethyl)-benzene-nesulfonyl fluoride, or recombinant human tissue inhibitors of metalloproteinases; its hydrolysing activity against QF-35 was determined by fluorescent measurements as above. Kinetic studies were performed using different concentrations of the fluorogenic peptides (0.5–4 µM) in 45 µl of assay buffer containing 0.2 mM recombinant metalloproteinase. Peptide hydrolysis was measured as the increase in fluorescence at 37°C over time.

Identification and Characterization of Human and Mouse Ovary cDNAs Encoding a New Astacin Metalloproteinase—To identify candidate metalloproteinases involved in the process of oocyte maturation, we used the BLAST algorithm to look for DNA contigs with similarity to previously described hatchin enzymes from other organisms. This search led to the identifi—

1 The abbreviations used are: dpc, days postcoitum; GST, glutathione S-transferase; MMP, matrix metalloproteinase; ISP, implantation serum protein.
cation of a contig in the human chromosome 2q11.1 and a syntenic contig in the mouse chromosome 2F3 containing coding information for new astacin metalloproteinases. To generate cDNA clones for these genes, we performed PCR amplifications using reverse-transcribed RNA from human and mouse ovaries and specific oligonucleotides derived from the predicted cDNA sequences. This method allowed us to amplify two fragments of the expected size (1.3 kb) containing in-frame initiator and stop codons. After cloning and sequencing the PCR-amplified products, we confirmed by conceptual translation that the generated sequences coded for two highly related proteins (78% amino acid sequence identities between them) (Fig. 1). Computer analysis of the protein sequences revealed that both contained an N-terminal signal peptide, a prodomain possibly involved in maintaining protease latency, a zinc-dependent metalloprotease domain, and a C-terminal extension with little similarity to any previously known domain. Further analysis of the predicted sequence confirmed that the putative catalytic domains of these proteins contain all the conserved features of the astacin family of metalloproteinases, including

**FIG. 1.** Nucleotide and amino acid sequences of ovastacin from human and mouse ovary. The Zn-binding site characteristic of astacins is shadowed. Other astacin-specific residues are boxed. The putative N-glycosylation sites are circled. The unique extension located C-terminal from the catalytic domain is underlined. A, human ovastacin; B, mouse ovastacin.
three zinc-ligating histidines, a general base glutamic acid, an astacin-specific glutamic acid, an RXXDRD motif, a Met-turn methionine, and a zinc-proximal tyrosine (7, 8, 12) (Figs. 1 and 2). These structural features are also perfectly conserved in the sequence deduced for the rat ortholog of these proteins after bioinformatic analysis of the recently available genome sequence of the Brown Norway rat (22) (Fig. 2A). Most astacins also present easily recognizable ancillary domains in their C-terminal region, such as MATH (meprin and TRAF homology domain), MAM (meprin, A-5 protein and receptor protein tyrosine phosphatase mμ (μ)), or CUB (complement factor C1s, urchin embryonic growth factor, bone morphogenetic protein). However, the newly identified proteins present a C-terminal extension without significant similarity to any of these ancillary domains. This extension is the most divergent fragment between the human and murine enzymes, and it is likely to be heavily O-glycosylated in both proteins, as predicted by using the NetOGlyc 2.0 program (www.cbs.dtu.dk/services/NetOGlyc/) (Fig. 1, A and B).

Detailed amino acid sequence comparisons of the catalytic domains of the identified sequences with those corresponding to other astacins revealed that the highest percentages of identities were found with hatching enzymes from different species: 49% with eel hatching enzyme-7, 48% with chorioallantoic membrane-1 protein (CAM-1); astacus embryonic astacin (AEA); medaka high choriolytic enzyme (HCE); and medaka low choriolytic enzyme (LCE). Common residues to all sequences are shaded. The alignment was performed using ClustalX (version 1.81) (59). The sequences shown in panel A were aligned with other members of the astacin family to deduce their phylogenetic relationship. The sequences shown in panel B were aligned with the protpars program of the Phylip program package (version 3.6). BP10 and SPAN correspond to sea urchin blastula proteases. h, m, and r stand for human, mouse, and rat, respectively.

**Fig. 2.** Amino acid sequence alignment and phylogenetic relationships of the catalytic domains of ovastacin and other astacins. A, the amino acid sequences deduced for the catalytic domains of human, mouse, and rat ovastacins are compared with those of *Xenopus* hatching enzyme (*XHE*), eel hatching enzyme-7 (*EHE*), and medaka high choriolytic enzyme (*HCE*), medaka low choriolytic enzyme (*LCE*), and *Astacus* embryonic astacin (*AEA*). Common residues to all sequences are shaded. The alignment was performed using ClustalX (version 1.81) (59). The sequences shown in panel A were aligned with other members of the astacin family to deduce their phylogenetic relationship. The sequences shown in panel B were aligned with the protpars program of the Phylip program package (version 3.6). BP10 and SPAN correspond to sea urchin blastula proteases. h, m, and r stand for human, mouse, and rat, respectively.

To further explore the structural and evolutionary relationships between the newly identified ovastacin and other members of the astacin family, we next performed a computational phylogenetic tree analysis (Fig. 2B). This analysis confirmed that human and rodent ovastacins are closely related to those astacins functionally characterized as hatching enzymes in arthropods, birds, amphibians, and fish but are phylogenetically separated from other astacin subgroups such as the BMP-tolloid enzymes, the meprins, and the sea urchin blastula proteases (Fig. 2B). These data suggest that ovastacin could play in mammals a physiological function similar to that performed by hatching enzymes in other species.
Expression of Ovastacin mRNA during Preimplantation Development of the Mouse Embryo—The absence of significant levels of ovastacin RNA transcripts in normal tissues suggested that the expression of this gene was likely restricted to very specific conditions. To investigate the putative presence of ovastacin during embryo development, we collected mouse oocytes and embryos at different times postcoitum and performed semiquantitative RT-PCR amplification followed by Southern blot analysis. This analysis showed that the expression of ovastacin is highest in unfertilized oocytes. Upon fertilization, the expression of this transcript drops to undetectable levels, although at 1.5 dpc a significant amount of ovastacin RNA could also be detected. No expression of ovastacin mRNA was detected from 1.5 dpc to implantation of the embryo. Interestingly, superovulation caused a dramatic increase in the expression of ovastacin, indicating that this gene is under hormonal control (Fig. 4).

Analysis of Ovastacin Distribution in Human Tissues—To investigate the presence of ovastacin in human tissues, Northern blot analysis was performed from a variety of human organs (e.g., intestine, ovary, testis, pancreas, uterus, kidney, skeletal muscle, liver, brain, and heart) were hybridized with a specific probe for human ovastacin. As can be seen in Fig. 3A, the expression of ovastacin mRNA is undetectable by this method in normal tissues. By contrast, Northern blot analysis of RNAs isolated from diverse tumor cell lines revealed the presence of a band of about 2.5 kb in Raji cells derived from Burkitt’s lymphoma and in HL-60 cells from promyelocytic leukemia (Fig. 3A). RT-PCR analysis also demonstrated the expression of ovastacin in some ovarian carcinomas (Fig. 3B).

Expression analysis of ovastacin in mouse preimplantation embryos. Total RNA from mouse embryos and oocytes was isolated and analyzed for ovastacin mRNA expression by RT-PCR followed by Southern blot analysis. β-actin was used as a control. Immature oocytes were obtained from ovaries of 12-day-old mice. Unfertilized oocytes were obtained from mature mice that were mated with vasectomized males. Superovulated oocytes were collected from mice treated with pregnant mare’s serum gonadotropin and human chorionic gonadotropin. Embryos 0.5–4.5 dpc were obtained from mature female mice mated with vasectomized males.

As previously reported for other proteases linked to GST (23, 24), we observed that the fusion protein underwent autolytic cleavage during the purification and refolding process, leading to the generation of a 30-kDa protein band that could correspond to the ovastacin catalytic domain after proteolytic release of the GST moiety. The identity of this band as ovastacin was verified by mass spectrometry analysis (data not shown). The purified catalytic domain of human ovastacin was used in enzymatic assays with fluorescent peptides commonly employed for assaying other metalloproteinases. These assays showed that the recombinant ovastacin exhibits a significant proteolytic activity. Thus, among the different substrates used, human ovastacin displayed the highest $k_{cat}/K_m$ value against QF-35 ($5.1 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$) with an optimum pH of 7.5. Similar enzyme assays performed with the recombinant catalytic domain of human MMP-26 resulted in a similar $k_{cat}/K_m$ value against QF-35 ($7.9 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$). These values were also similar to those previously described in our enzymatic analysis of the activity of the catalytic domain of MMP-19 against QF-35 ($3.1 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$) (25). We then tested the ability of different protease inhibitors to block the enzymatic activity of ovastacin (Fig. 6B). This study showed that the naturally occurring tissue inhibitors of metalloproteinases do not inhibit the activity of ovastacin. By contrast, batimatstat (BB-94), a wide spectrum inhibitor of matrix metalloproteinases (MMPs), showed a strong inhibitory activity against ovastacin. Likewise, EDTA also abolished the activity of the recombinant catalytic domain of
ovastacin. Finally, 4-(2-aminoethyl)-benzenesulfonyl fluoride and E-64, inhibitors of serine and cysteine proteases, respectively, showed no inhibitory activity against human ovastacin.

**FIG. 5.** In situ hybridization analysis of ovastacin expression in mouse ovary. 5-week-old female mice were injected intraperitoneally with 5 international units of pregnant mare’s serum glycoprotein followed by 5 international units of human chorionic gonadotropin. The following day, the mice were dissected to obtain their ovaries, which were paraffin-embedded. Sections of these ovaries (A, B) or of ovaries from immature mice (C) were hybridized with antisense and sense (D) probes for ovastacin. Bright field (left) and dark field (right) images are shown for each section. Positive signal is restricted to oocytes at different stages of maturation (arrows). Scale bars, 200 μm.

**FIG. 6.** Production and enzymatic analysis of recombinant human ovastacin. A, Coomassie Blue-stained SDS-PAGE showing the results of expression, purification, and refolding of the recombinant catalytic domain of human ovastacin. 5-μl aliquots of whole bacterial lysate (total extract), soluble fraction of the lysate (soluble fraction), insoluble fraction of the lysate (pellet), purified insoluble protein (GST-ovastacin), and refolded purified ovastacin (refolded ovast) were analyzed in each lane. The sizes of the molecular size markers are indicated in kDa on the left. The upper arrow indicates the position of the fusion protein GST-ovastacin; the lower arrow indicates the position of the autoactivated ovastacin protein. B, analysis of the effect of different protease inhibitors on the enzymatic activity of human recombinant ovastacin. In this work, we describe the finding in human and rodents of a new member of the astacin family of metalloproteinases that we have called ovastacin. This protein shows structural and evolutionary relationship with diverse hatching enzymes originally identified in other species, such as arthropods, birds, amphibians, and fish. The strategy followed to identify ovastacin was first based on a genomic search of human and mouse sequences with similarity to those encoding the catalytic domain of hatching astacins, such as Astacus embryonic astacin, chorioalantoic membrane-1, Xenopus hatching enzyme, and high and low choriolytic enzymes (13–16). After identification of candidate sequences in the human and mouse genomes and a series of PCR experiments using ovary cDNA as template, full-length cDNAs coding for human and mouse ovastacin were finally isolated and characterized. Structural analysis of the identified sequences revealed that these orthologous proteins show the same domain organization as the remaining astacins and include a signal sequence, a prodomain, and a catalytic domain with all characteristic features of astacin metalloproteinases (7, 8). In addition, and also similar to most astacins, human and mouse ovastacins contain an additional domain that is located at their C-terminal region. However, this domain shows a sequence similarity to any of the catalytic domains present in data bases. This domain was named the ancillary domain and is a distinctive feature of ovastacin that could play a role in facilitating its interaction with putative substrates or with other proteins or in mediating the formation of multimeric structures. The ancillary domain found in other proteins or in mediating the formation of multimeric structures as proposed for the ancillary domains found in other astacins (28). Kinetic analysis revealed that the catalytic efficiency of ovastacin against QF-35 (kcat/Km = 5.1 × 10⁴ M⁻¹ s⁻¹) is similar to that of other metalloproteinases with the ability to hydrolyze this peptide, such as MMP-26 (kcat/Km = 7.9 × 10⁴ M⁻¹ s⁻¹) and MMP-19 (kcat/Km = 3.1 × 10⁴ M⁻¹ s⁻¹) (25). In addition, this proteolytic activity of human ovastacin was abolished by inhibitors of metalloproteinases but not by inhibitors of other classes of enzymes. Interestingly, batimastat (BB-94), a hydroxamic acid-derived inhibitor originally designed to target MMPs overexpr.
pressed in cancer (29, 30), was also able to block the catalytic activity of human ovastacin. This finding is consistent with the recent observation that human meprins α and β are also targeted by this inhibitor (31) and reinforces the need for more selective inhibitors to block the unwanted activity of specific proteases in human diseases without interfering with the physiological functions of structurally related enzymes (29, 30).

In this work, as a step toward analyzing whether the structural similarities of ovastacin to hatching enzymes are also supported by functional relationships between them, we have analyzed the distribution of the ovastacin enzyme in human and mouse tissues. These studies have revealed that the expression pattern of ovastacin is very restricted and is only detected in oocytes from superovulated mice, indicating that it is subjected to tight hormonal control. Taken together, these findings suggest that ovastacin could be a protease implicated in some of the tissue-remodeling processes occurring during embryonic development and implantation, including the hatching process (32–40). In many animal species from echinoderms to mammals, the hatching enzyme is secreted from embryos to digest their protective extracellular coats and allow them to emerge at the time of hatching. This process has been well studied in the embryos of sea urchins, arthropods, zebrafish, water teleost medaka, and amphibians such as Xenopus, a variety of proteases from different families characterized as hatching enzymes in these organisms (13–47). Thus, the sea urchin hatching enzyme, the putative substrate of ovastacin is of the MMP family (41–44), similar to this enzyme in crayfish, birds, and mammals, the hatching enzyme is secreted from embryos to digest their protective extracellular coats and allow them to emerge at the time of hatching. This process has been well studied in the embryos of sea urchins, arthropods, zebrafish, water teleost medaka, and amphibians such as Xenopus, a variety of proteases from different families characterized as hatching enzymes in these organisms (13–47). Thus, the sea urchin hatching enzyme, the putative substrate of ovastacin is of the MMP family (41–44), similar to this enzyme in crayfish, birds, and mammals.

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