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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. Northern blot analysis of human tissues and cell lines revealed that ovastacin is only detected at significant levels in leukemia and lymphoma cells of different origin. In addition, RT-PCR analysis demonstrated that ovastacin is expressed in human and mouse ovary, in unfertilized mouse oocytes, and in 1.5-day-postcoitum preimplantation embryos. Further studies showed that superovulation caused a dramatic increase in the expression of mouse ovastacin, indicating that the production of this enzyme is under hormonal regulation. Human ovastacin was expressed in *Escherichia coli* and the purified recombinant protein hydrolyzed synthetic substrates used for assaying metalloproteinases. These activities were abolished by inhibitors of metalloproteinases, but not by inhibitors of other classes of proteases. On the basis of these results, we suggest that ovastacin could play in mammals a physiological function similar to that performed by hatching proteases in evolutionary distant species from arthropods to fish.

Proteolytic enzymes play essential roles in all living organisms. These proteins were originally characterized as nonspecific hydrolytic enzymes involved in the catabolism of dietary proteins. However, over recent years the study of proteases has gained additional relevance after multiple findings demonstrating their influence in the regulation of many events on which cell life and death depends. Thus, proteases through their ability to perform highly selective reactions of proteolytic processing of specific substrates participate in the control of cell cycle progression, tissue morphogenesis, and remodeling, cell proliferation and migration, ovulation and fertilization, angiogenesis, hemostasis, and apoptosis (1, 2).

This growing diversity and complexity of protease functions derive from the evolutionary invention in all organisms of multiple enzymes with the common ability to hydrolyze peptide bonds. To date, more than 550 genes encoding proteases and protease homologs have been annotated in the human, mouse, and rat genomes (3, 4) (web.uniovi.es/degradome). It is also remarkable that the model organism *Drosophila melanogaster,* despite having a gene content much lower than humans, also contains more than 500 protease genes because of the large expansion of a family of insect genes encoding trypsin-like serine proteases (5). The complexity of protease systems is also appreciated in other model organisms such as *Caenorhabditis elegans* and *Arabidopsis thaliana*, whose genomes encode more than 400 and 600 proteases, respectively (www.merops.ac.uk). Recently, and as part of our efforts aimed at characterizing mammalian degradomes (the complete set of proteases present in these organisms), we have evaluated the possibility that mammalian tissues could produce proteases previously described in other species but whose occurrence in mammals had not been yet reported. Among different proteases satisfying these criteria, we focused our attention on hatching enzymes belonging to the astacin family of metalloproteinases. The astacins represent a widespread family of proteolytic enzymes described in a variety of organisms and include several subfamilies of closely related members, such as the bone morphogenetic protein/tolloid-like enzymes, the meprins, and the hatching proteases (6–8). These enzymes may target a wide range of substrates and participate in multiple biological processes such as early embryo patterning and morphogenesis in *Drosophila* (9), cartilage and bone formation and remodeling in vertebrates (10, 11), digestion of diet proteins in crayfish (12), and extracellular coat degradation during the course of embryo hatching in arthropods, birds, amphibians, and fish (13–17).

In this work, we report the identification and characterization of a novel mammalian astacin metalloproteinase that has been tentatively called ovastacin because of its predominant expression in ovarian tissues. We also analyze the tissue distribution of this enzyme in human and mouse tissues and perform an analysis of its enzymatic activity. Finally, we discuss the potential roles of ovastacin in reproductive processes, including hatching.

EXPERIMENTAL PROCEDURES

Materials—Human and mouse ovary cDNAs were obtained by reverse transcription of total mRNA using the ThermoScript RT-PCR kit from Invitrogen. Restriction endonucleases and other reagents used for molecular cloning were from Roche Applied Science. Double-stranded DNA probes were radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol) from Amersham Biosciences, using a commercial random-priming kit pur-

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AJ537599 and AJ537600.

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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.celera.com) human and mouse genome databases, looking for regions with sequence similarity to previously described hatching enzymes belonging to the astacin family of metalloproteinases. After identification in human chromosome 2q and mouse chromosome 2F of DNA contigs encoding regions similar to the catalytic domain of eel hatching enzymes, we designed specific oligonucleotides to PCR-amplify cDNAs for these proteins, using cDNA from human and mouse ovaries and the Expand™ high fidelity PCR system (Roche Applied Science). All PCR amplifications were performed in a GeneAmp 2400 PCR system from PerkinElmer Life Sciences. 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′-CGTGGGATCCCCTTCCGACTGCTGTCAG-3′ and 5′-TCGCGAATTCAACTTGGGCTGCAGCCGTAG-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, 50 s) using the Expand™ high fidelity PCR system. The amplified product was then digested with BamHI and EcoRI and ligated in the corresponding sites of the pGEX·3X expression vector (Amersham Biosciences). The resulting vector was transformed into BL21(DE3)pLysE *Escherichia coli* competent cells, and expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside, followed by 5 h of incubation at 28 °C. The cells were then harvested by centrifugation, washed with phosphate-buffered saline, and lysed by incubation in phosphate-buffered saline with 100 μg/ml lysozyme, 10 μg/ml Dnase, and 0.1% Triton X-100 overnight at 4 °C. The extract was centrifuged, and the pellet was washed three times with phosphate-buffered saline and dissolved in a buffer containing 20 mM Tris, pH 7.4, and 6 M guanidinium chloride. The recombinant protein was then purified by size-exclusion chromatography using a Superdex SE75 column pre-equilibrated with 20 mM Tris, pH 7.4, and 3 M guanidinium chloride. The purified protein was refolded at 4 °C in two dialysis steps, first against a buffer containing 50 mM Tris, pH 8.5, 150 mM NaCl, 5 mM dithiothreitol, and 0.1 M guanidinium chloride for 48 h, 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-NH_2), QF-35 (Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2), and QF-41 (Mca-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH_2). 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 $CaCl_2$, 0.05% Brij-35, pH 7.5, and 1% dimethyl sulfoxide. The fluorometric measurements were made in an LS55 PerkinElmer Life Sciences spectrofluorometer (λ_{ex} = 328 nm and λ_{em} = 390 nm). For inhibition experiments, the reaction mixture was preincubated for 30 min at 37 °C with EDTA, batimastat (BB-94), E-64, 4-(2-aminoethyl)-benzenesulfonyl fluoride, or recombinant human tissue inhibitors of metalloproteinases; its hydrolyzing activity against QF-35 was determined by fluorometric 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 nM recombinant enzyme. Peptide hydrolysis was measured as the increase in fluorescence at 37 °C over time. Initial velocities were calculated using the analysis package FL WinLab 2.01 (PerkinElmer Life Sciences), and the k_{cat}/K_m ratio was calculated as described previously (19).

Northern Blot Analysis—Nylon filters containing 2 μg of poly(A)+ RNA of a wide variety of human tissues and cell lines were prehybridized at 42 °C for 3 h in 50% formamide, 5× SSPE (1× SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 10× Denhardt's solution, 2% SDS, and 100 μg/ml of denatured herring sperm DNA and then hybridized with a radiolabeled human ovastacin-specific probe 858 bp long containing nucleotides 490–1348 of the isolated cDNA. Hybridization was performed for 20 h under the same conditions used for prehybridization. The filters were washed with 0.1× SSC, 0.1% SDS for 2 h at 50 °C and exposed to autoradiography. RNA integrity and equal loading was assessed by hybridization with an actin probe.

Detection of Ovastacin RNA in Ovarian Carcinomas—0.5 μg of total RNA from human ovarian carcinomas (a generous gift from Dr. E. Campo, Hospital Clinic, Barcelona, Spain) and uterus were reverse-transcribed using the GeneAmp kit from Perkin-Elmer. Ovastacin was then PCR-amplified with the human ovastacin-specific primers 5′-GTCTCGGAGTAGCAACATGCTGACG-3′ and 5′-CCAAAAGCCGCTGCAGAGATAGGG-3′ for 25 cycles of denaturation (94 °C, 20 s), annealing (60 °C, 20 s), and extension (72 °C, 30 s). The resulting PCR products were analyzed by Southern blot using DNA probes from the same amplified regions. To ensure that a similar amount of template was used in all the reactions, β2-microglobulin was PCR-amplified from the same samples and detected by electrophoresis in agarose gel with ethidium bromide.

Collection of Mouse Oocytes and Preimplantation Embryos—For the collection of immature oocytes, 12-day-old Swiss female mice were sacrificed and their ovaries were collected and treated with collagenase (20). Oocytes devoid of granulosa cells were collected in GIT (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% *N*-lauroylsarcosine, and 0.1 M 2-mercaptoethanol). For the collection of unfertilized oocytes, Swiss albino male mice were vasectomized and mated with mature females, which were inspected for the presence of a mating plug. When a plug was found, the mice were dissected to obtain the oviducts, whose ampullae were torn to collect the oocytes in M2 medium. To obtain superovulated, unfertilized oocytes, 3–5-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 48 h later. The following day, the oocytes were collected in M2 medium. Preimplantation embryos were collected at different postcoitum times from mated Swiss albino mice by flushing the oviducts (0.5–2.5 dpc)[1] or the uterus (3.5 and 4.5 dpc) with M2 medium. Cumulus cells were removed from unfertilized oocytes and 0.5-dpc embryos by incubation in M2 medium supplemented with 300 μg/ml of hyaluronidase. Oocytes and embryos were washed six times in M2 medium and collected in GIT.

Semiquantitative RT-PCR and Southern Blot Analysis—Total RNA was isolated from mouse unfertilized oocytes and embryos from 0.5–4.5 dpc according to the method of Chomczynski and Sacchi (21). About half of the obtained product was reverse-transcribed using the GeneAmp kit from Perkin-Elmer. A PCR reaction was then performed with the mouse ovastacin primers 5′-GCCCACCATCATACCACTCTGGAC-3′ and 5′-CCAAGGTCACACTGAATGTGC-3′ for 25 cycles of denaturation (94 °C, 20 s), annealing (62 °C, 20 s), and extension (72 °C, 30 s). As a control, β-actin was PCR-amplified from all samples under the same conditions. The resulting PCR products were analyzed by Southern blot using DNA probes from the same amplified regions.

In Situ Hybridization—Ovaries from immature (14–16-day-old) or superovulated mice were fixed overnight in 4% paraformaldehyde at 4 °C and then dehydrated in ethanol and embedded in paraffin. Sections (5 μm) were hybridized to ^{35}S-labeled antisense ovastacin riboprobe. Slides were exposed to photographic emulsion at 4 °C for 6 days and then developed, fixed, and cleared. Sections were counterstained with 0.02% toluidine blue. Sections hybridized with a labeled sense ovastacin riboprobe were used as negative controls. No positive hybridization signal was found in negative controls. Bright field and dark field images were captured with a SPOT digital camera. Either sense or antisense ^{35}S-uridine triphosphate-labeled RNA probes were synthesized from the corresponding linearized DNA using the appropriate RNA polymerases.

RESULTS

Identification and Characterization of Human and Mouse Ovary cDNAs Encoding a New Astacin Metalloproteinase—To identify candidate metalloproteinases involved in the process of embryo hatching, we used the BLAST algorithm to look for DNA contigs with similarity to previously described hatching 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 serine protease.

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ATGGAGGGTGTAGGGGGTCTCTGGCCTTGGGTGCTGGGTCTGCTCTCCTTGCCAGGTGTGATCCTAGGAGCGCCCCTGGCCTCCAGCTGCGCAGGAGCCTGTGGTACCAGCTTCCCAGAT
M  E  G  V  G  G  L  W  P  W  V  L  G  L  L  S  L  P  G  V  I  L  G  A  P  L  A  S  S  C  A  G  A  C  G  T  S  F  P  D

GGCCTCACCCCTGAGGGAACCCAGGCCTCCGGGGACAAGGACATTCCTGCAATTAACCAAGGGCTCATCCTGGAAGAAACCCCAGAGAGCAGCTTCCTCATCGAGGGGGACATCATCCGG
G  L  T  P  E  G  T  Q  A  S  G  D  K  D  I  P  A  I  N  Q  G  L  I  L  E  E  T  P  E  S  S  F  L  I  E  G  D  I  I  R

CCGAGTCCCTTCCGACTGCTGTCAGCAGCCAGCAACAAATGGCCCATGGGTGGTAGTGGTGTCGTGGAGGTCCCCTTCCTGCTCTCCAGCAAGTACGATGAGCCCAGCCGCCAGGTCATC
P  S  P  F  R  L  L  S  A  A  S  N  K  W  P  M  G  G  S  G  V  V  E  V  P  F  L  L  S  S  K  Y  D  E  P  S  R  Q  V  I

CTGGAGGCTCTTGCGGAGTTTGAACGTTCCACGTGCATCAGGTTTGTCACCTATCAGGACCAGAGAGACTTCATTTCCATCATCCCCATGTATGGGTGCTTCTCGAGTGTGGGGCGCAGT
L  E  A  L  A  E  F  E  R  S  T  C  I  R  F  V  T  Y  Q  D  Q  R  D  F  I  S  I  I  P  M  Y  G  C  F  S  S  V  G  R  S

GGAGGGATGCAGGTGGTCTCCCTGGCGCCCACGTGTCTCCAGAAGGGCCGGGGCATTGTCCTTCATGAGCTCATGCATGTGCTGGGCTTCTGGCACGAGCACACGCGGGCCGACCGGGAC
G  G  M  Q  V  V  S  L  A  P  T  C  L  Q  K  G  R  G  I  V  L  H  E  L  M  H  V  L  G  F  W  H  E  H  T  R  A  D  R  D

CGCTATATCCGTGTCAACTGGAACGAGATCCTGCCAGGCTTTGAAATCAACTTCATCAAGTCTCGGAGTAGCAACATGCTGACGCCCTATGACTACTCCTCTGTGATGCACTATGGGAGG
R  Y  I  R  V  N  W  N  E  I  L  P  G  F  E  I  N  F  I  K  S  R  S  S  N  M  L  T  P  Y  D  Y  S  S  V  M  H  Y  G  R

CTCGCCTTCAGCCGGCGTGGGCTGCCCACCATCACACCACTTTGGGCCCCCAGTGTCCACATCGGCCAGCGATGGAACCTGAGTGCCTCGGACATCACCCGGGTCCTCAAACTCTACGGC
L  A  F  S  R  R  G  L  P  T  I  T  P  L  W  A  P  S  V  H  I  G  Q  R  W  N  L  S  A  S  D  I  T  R  V  L  K  L  Y  G

TGCAGCCCAAGTGGCCCCAGGCCCCGTGGGAGAGGGTCCCATGCCCACAGCACTGGTAAGAGCCCCGCTCCGGCCTCCCTATCTCTGCAGCGGCTTTTGGAGGCACTGTCGGCGGAGTCC
C  S  P  S  G  P  R  P  R  G  R  G  S  H  A  H  S  T  G  K  S  P  A  P  A  S  L  S  L  Q  R  L  L  E  A  L  S  A  E  S

AGGAGCCCCGACCCCAGTGGTTCCAGTGCGGGAGGCCAGCCCGTTCCTGCAGGGCCTGGGGAGAGCCCACATGGGTGGGAGTCCCCTGCCCTGAAAAAGCTCAGTGCAGAGGCCTCGGCA
R  S  P  D  P  S  G  S  S  A  G  G  Q  P  V  P  A  G  P  G  E  S  P  H  G  W  E  S  P  A  L  K  K  L  S  A  E  A  S  A

AGGCAGCCTCAGACCCTAGCTTCCTCCCCAAGATCAAGGCCTGGAGCAGGTGCCCCCGGTGTTGCTCAGGAGCAGTCCTGGCTGGCCGGAGTGTCCACCAAGCCCACAGTCCCATCTTCA
R  Q  P  Q  T  L  A  S  S  P  R  S  R  P  G  A  G  A  P  G  V  A  Q  E  Q  S  W  L  A  G  V  S  T  K  P  T  V  P  S  S

GAAGCAGGAATCCAGCCAGTCCCTGTCCAGGGAAGCCCAGCTCTGCCAGGGGGCTGTGTACCTAGAAATCATTTCAAGGGGATGTCCGAGGATTAA
E  A  G  I  Q  P  V  P  V  Q  G  S  P  A  L  P  G  G  C  V  P  R  N  H  F  K  G  M  S  E  D  *
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B

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ATGGGTATCATGGGAAGCCTGTGGCCTTGGATATTAACTATGCTCTCCTTGCTAGGTTTGAGCATGGGAGCACCCTCAGCATCCAGATGTTCTGGAGTCTGCAGTACCAGTGTTCCAGAA
M  G  I  M  G  S  L  W  P  W  I  L  T  M  L  S  L  L  G  L  S  M  G  A  P  S  A  S  R  C  S  G  V  C  S  T  S  V  P  E

GGCTTCACTCCTGAGGGAAGCCCGGTATTTCAGGACAAGGACATCCCCGCAATTAACCAAGGGCTCATCTCAGAGGAGACCCCAGAAAGCAGCTTCCTGGTAGAAGGGGACATTATCCGG
G  F  T  P  E  G  S  P  V  F  Q  D  K  D  I  P  A  I  N  Q  G  L  I  S  E  E  T  P  E  S  S  F  L  V  E  G  D  I  I  R

CCAAGCCCTTTCCGATTGTTGTCAGTGACCAATAATAAATGGCCCAAGGGCGTTGGTGGCTTTGTGGAGATCCCCTTCCTGCTTTCCAGAAAGTATGATGAACTCAGCCGCCGGGTCATT
P  S  P  F  R  L  L  S  V  T  N  N  K  W  P  K  G  V  G  G  F  V  E  I  P  F  L  L  S  R  K  Y  D  E  L  S  R  R  V  I

ATGGATGCCTTTGCTGAGTTTGAACGTTTCACATGCATCCGGTTTGTTGCCTACCATGGTCAGAGAGACTTTGTTTCCATTCTTCCTATGGCGGGGTGTTTCTCTGGTGTGGGACGCAGT
M  D  A  F  A  E  F  E  R  F  T  C  I  R  F  V  A  Y  H  G  Q  R  D  F  V  S  I  L  P  M  A  G  C  F  S  G  V  G  R  S

GGAGGGATGCAGGTGGTGTCCTTGGCACCCACTTGTCTCCGGAAGGGCCGAGGCATTGTCCTACATGAGCTCATGCACGTACTTGGCTTCTGGCATGAGCATTCACGGGCAGATCGGGAC
G  G  M  Q  V  V  S  L  A  P  T  C  L  R  K  G  R  G  I  V  L  H  E  L  M  H  V  L  G  F  W  H  E  H  S  R  A  D  R  D

CGCTACATCCAAGTCAACTGGAACGAGATCCTCCCGGGCTTTGAAATCAACTTCATCAAGTCACGGAGTACCAATATGTTAGTTCCCTATGACTACTCATCTGTGATGCATTATGGGAGA
R  Y  I  Q  V  N  W  N  E  I  L  P  G  F  E  I  N  F  I  K  S  R  S  T  N  M  L  V  P  Y  D  Y  S  S  V  M  H  Y  G  R

TTTGCCTTCAGCTGGCGTGGGCAGCCCACCATCATACCACTCTGGACCTCCAGTGTTCACATTGGCCAGCGATGGAACCTGAGTACCTCAGATATCACCCGGGTCTGCAGGCTGTATAAC
F  A  F  S  W  R  G  Q  P  T  I  I  P  L  W  T  S  S  V  H  I  G  Q  R  W  N  L  S  T  S  D  I  T  R  V  C  R  L  Y  N

TGCAGCCGGAGTGTCCCTGACTCCCACGGGAGAGGGTTTGAGGCCCAGAGTGATGGAAGCAGCCTCACCCCTGCCTCTATATCACGTCTACAAAGACTTCTCGAGGCACTGTCAGAGGAA
C  S  R  S  V  P  D  S  H  G  R  G  F  E  A  Q  S  D  G  S  S  L  T  P  A  S  I  S  R  L  Q  R  L  L  E  A  L  S  E  E

TCTGGAAGCTCTGCCCCTAGTGGCTCCAGGACTGGAGGCCAGAGTATTGCCGGGCTTGGTAACAGCCAGCAAGGATGGGAGCATCCTCCTCAGAGCACATTCAGTGTGGGAGCCTTGGCA
S  G  S  S  A  P  S  G  S  R  T  G  G  Q  S  I  A  G  L  G  N  S  Q  Q  G  W  E  H  P  P  Q  S  T  F  S  V  G  A  L  A

AGACCACCTCAGATGCTAGCCGATGCTTCAAAATCGGGGCCTGGAGCAGGTGCAGACAGCTTGTCTCTAGAGCAGTTCCAGCTAGCCCAGGCCCCCACTGTACCTCTTGCTCTATTTCCA
R  P  P  Q  M  L  A  D  A  S  K  S  G  P  G  A  G  A  D  S  L  S  L  E  Q  F  Q  L  A  Q  A  P  T  V  P  L  A  L  F  P

GAAGCCAGAGACAAGCCAGCACCTATCCAAGATGCCTTTGAGAGGCTAGCTCCACTTCCAGGAGGCTGTGCACCTGGAAGTCACATTAGAGAGGTGCCCAGAGACTGA
E  A  R  D  K  P  A  P  I  Q  D  A  F  E  R  L  A  P  L  P  G  G  C  A  P  G  S  H  I  R  E  V  P  R  D  *
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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 *O*-glycosylation sites are *circled*. The unique extension located C-terminal from the catalytic domain is *underlined*. *A,* human ovastacin. *B,* mouse ovastacin.

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

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hOvast : WPMGGSGVVEVPFLLSSKYDEPSRQVILEALAEFERSTCIRFVTYQDQRDFISIIPMYGCFSSVGRSGGMQVVSLAPTCLQKGRGIVLHELMHVL
mOvast : WPKGVGGFVEIPFLLSRKYDELSRRVIMDAFAEFERFTCIRFVAYHGQRDFVSILPMAGCFSGVGRSGGMQVVSLAPTCLRKGRGIVLHELMHVL
rOvast : WPKGVDGIVEIPFLLSSKYDEPSRQVIMEAFAEFERFTCIRFVAYRGQRDFVSILPMAGCFSGVGRSGGMQVVSLAPTCLQKGRGIVLHELMHVL
EHE7   : WKKSSNGLVEVPYTVSRQFSYYQRKRIVKAMKTFNTQTCIRFVPRSRQRDYISIKSRGGCYSYLGRTGGKQVVSLAKYGC-VYHGIIQHELSHAL
CAM    : WPQSMDGIVRIPYVLDPTYEENHVRGILEAMAEFETLTCINFVKRKTERDYLIIRSADGCWSNYGKVGGGQTVSVMKGGC-MWKGIIQHELDHAL
XHE    : WPKSADGIVPVPYNLSYSYNADQLALFKKAIQEFEALTCVRFVPWTTEVNFLNIMSNGGCGSLIGKNGGAQRLELDANGC-MNMGIIQHELNHAL
HCE    : WKKASNGLVVIPYVISSEYSGGEVATIEGAMRAFNGKTCIRFVRRTNEYDFISVVSKTGCYSELGRKGGQQELSINRGGC-MYSGIIQHELNHAL
LCE    : WPKSSNGIVKVPYVVSDNYESDEKETIRNAMKEFAEKTCIHFVPRNNERAYLSLEPRFGCKSMMGYVGDKQVVVLQRFGC-IKHAVIQHELLHAL
AEA    : WPKASEGFVVIPYVVTDQYDKENIDIAIDAMAEFDEITCVRFVPRRTETDFLLIDSRSGCKSIIGKLGELQKISLEKMGC-MNTGIIQHELEHAL

hOvast : GFWHEHTRADRDRYIRVNWNEILPGFEINFIKS-QSSNMLTPYDYSSVMHYGRLAFSRR-GLPTITPLWAPSVHIGQRWNLSASDITRVLKLYGC
mOvast : GFWHEHSRADRDRYIQVNWNEILPGFEINFIKS-RSTNMLVPYDYSSVMHYGRFAFSWR-GQPTIIPLWTSSVHIGQRWNLSTSDITRVCRLYNC
rOvast : GFWHEHSRADRDRYIRVNWNEILPGFEINFIKS-RNSNMLAPYDYSSVMHYGRFAFSWR-GQPTIIPLWTSSVHIGQRWNLSTSDITRVCRLYSC
EHE7   : GFYHEHTRSDRNKYVKINWENVAPRSIYNFQKQ-NTNNLNTPYDYTSIMHYGKAAFSTN-GKDTITPIPNPKQSIGQRRSMSKGDILRIKKLYNC
CAM    : GFLHEHSRSDRDKYVKIMWEYISPACRPDFRKFENSNNLGLPYDYSSVMHYGPHTFTNTTGKATIVPVPDGSVHIGQRLGLSNLDVAKINKLYNC
XHE    : GFYHEQNRSDRDDYVIIHTENIIPDFLKMFEKY-NTNNLGIEYDYASVMHYSRYHYSIN-GDITIEPKPDPNVPIGQRDGLSILDISKINKLYEC
HCE    : GFQHEQTRSDRDSYVRINWENIIPASAYNFNKH-DTNNLNTPYDYSSIMHYGRDAFSIAYGRDSITPIPNPNVPIGQRNGMSRWDITRINVLYNC
LCE    : GFYHEHTRSDRDQHVKINWENIIKDFTHNFDKN-DTDNLGTPYDYGSIMHYGRTAFGKD-RKETITPIPNPKAAIGQTERMSDIDILRVNKLYKC
AEA    : GFYHEHSRSDRDTYVKIMWENISPDNVRMFDKE-MNMQLGLPYEYTSIMHYARYVYSIE-GDESIDPAPNGNVPIGQRDGISQYDIAKINKLYNC
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B

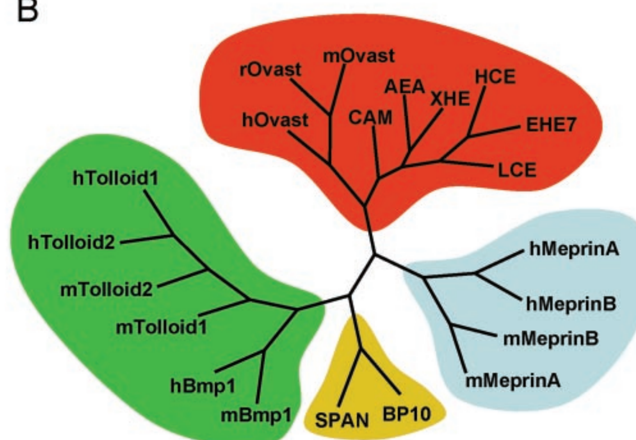

FIG. 2. **Amino acid sequence alignment and phylogenetic relationships of the catalytic domains of ovastacin and other astacins.** *A*, the amino acid sequences deduced for the catalytic domains of human, mouse, and rat ovastacins are compared with those of *Xenopus* hatching enzyme (*XHE*), eel hatching enzyme-7 (*EHE*), Japanese quail 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) *B*, the sequences shown in *panel A* were aligned with other members of the astacin family to deduce their phylogenetic relationship. The tree was calculated 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.

three zinc-ligating histidines, a general base glutamic acid, an astacin-specific glutamic acid, an RXDRD 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. 2*A*). 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 mu (μ), or CUB (complement factor C1s/C1r, 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 chorioalantoic membrane-1 protein from Japanese quail, 47 and 44% with high and low choriolytic enzymes from the teleost *Oryzias latipes*, 43% with *Xenopus* hatching enzyme, and 40% with *Astacus* embryonic astacin from crayfish. According to the structural features of the identified proteins, we have concluded that the cloned human and mouse ovary cDNAs encode a new member of the astacin family of metalloproteinases that we propose to call ovastacin.

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. 2*B*). 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. 2*B*). These data suggest that ovastacin could play in mammals a physiological function similar to that performed by hatching enzymes in other species.

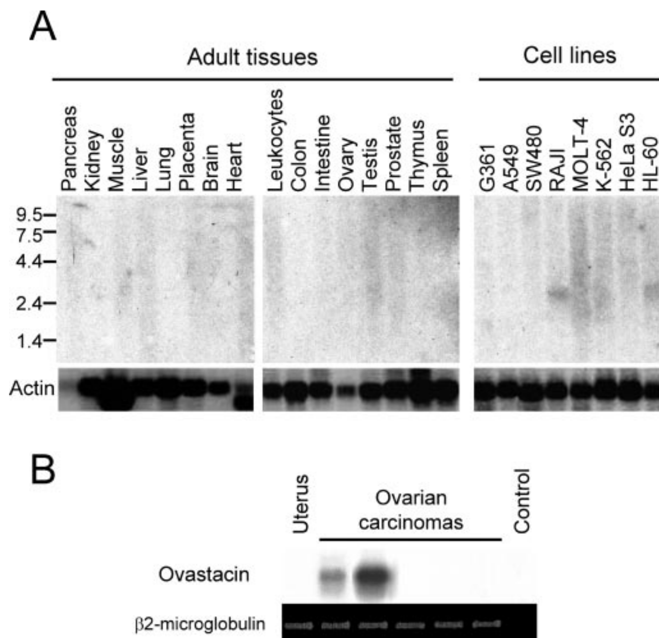

FIG. 3. **Analysis of ovastacin expression in human tissues and cell lines.** *A*, filters containing ~2 μg of polyadenylated RNAs from the indicated normal tissues and cancer cell lines were hybridized with a human ovastacin-specific probe. RNA sizes are indicated in kbp. The origins of the cell lines are: G361, melanoma; *A549*, lung carcinoma; *SW480*, colorectal adenocarcinoma; *Raji*, Burkitt's lymphoma; *MOLT-4*, lymphoblastic leukemia; *K-562*, chronic myelogenous leukemia; *HeLa S3*, cervix epithelioid carcinoma; and *HL-60*, promyelocytic leukemia. *B*, total RNA from human ovarian carcinomas and uterus was reverse-transcribed and used to PCR-amplify a 254-bp ovastacin band, which was detected by Southern blot. The reverse-transcribed samples were also used to PCR-amplify a 261-bp β2-microglobulin band as a control.

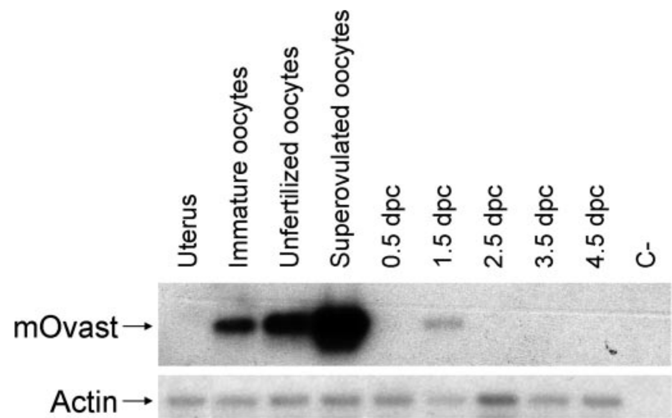

FIG. 4. **Expression analysis of ovastacin in mouse preimplantation embryos.** Total RNA from mouse embryos and oocytes was isolated and analyzed for ovastacin mRNA expression by RT-PCR followed by Southern blot analysis. β-actin was used as a control. Immature oocytes were obtained from ovaries of 12-day-old 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 glycoprotein and human chorionic gonadotropin. Embryos 0.5–4.5 days postcoitum were obtained from mature female mice mated with fertile male mice.

Analysis of Ovastacin Distribution in Human Tissues—To investigate the presence of ovastacin mRNA transcripts in human tissues, Northern blots containing poly(A)+ RNAs prepared from a variety of human tissues (leukocytes, colon, small intestine, ovary, testis, prostate, thymus, spleen, pancreas, kidney, skeletal muscle, liver, lung, placenta, brain, and heart) were hybridized with a specific probe for human ovastacin. As can be seen in Fig. 3*A*, 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. 3*A*). RT-PCR analysis also demonstrated the expression of ovastacin in some ovarian carcinomas (Fig. 3*B*).

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). *In situ* hybridization experiments in ovaries from immature or superovulated mice confirmed that ovastacin is specifically expressed in oocytes (Fig. 5).

Enzymatic Activity of the Recombinant Human Ovastacin—To investigate the enzymatic activity of human ovastacin, we expressed in *E. coli* a fusion protein containing the putative catalytic domain of the enzyme linked to an N-terminal GST tag. After isopropyl-1-thio-β-D-galactopyranoside induction of bacterial cells transformed with the plasmid encoding the fusion protein, a band of the expected size (45 kDa) was detected by SDS-PAGE analysis of bacterial protein extracts (Fig. 6*A*). This recombinant GST-ovastacin protein was contained in insoluble inclusion bodies that were solubilized, purified by size-exclusion chromatography, and refolded as described under "Experimental Procedures" (Fig. 6*A*). 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 then 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 (5.1×10^4 M^{-1} 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 (7.9×10^4 M^{-1} 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 (k_{cat}/K_m 3.1×10^4 M^{-1} s^{-1}) (25). We then tested the ability of different protease inhibitors to block the enzymatic activity of ovastacin (Fig. 6*B*). This study showed that the naturally occurring tissue inhibitors of metalloproteinases do not inhibit the activity of ovastacin. By contrast, batimastat (BB-94), a wide spectrum inhibitor of matrix metalloproteases (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.

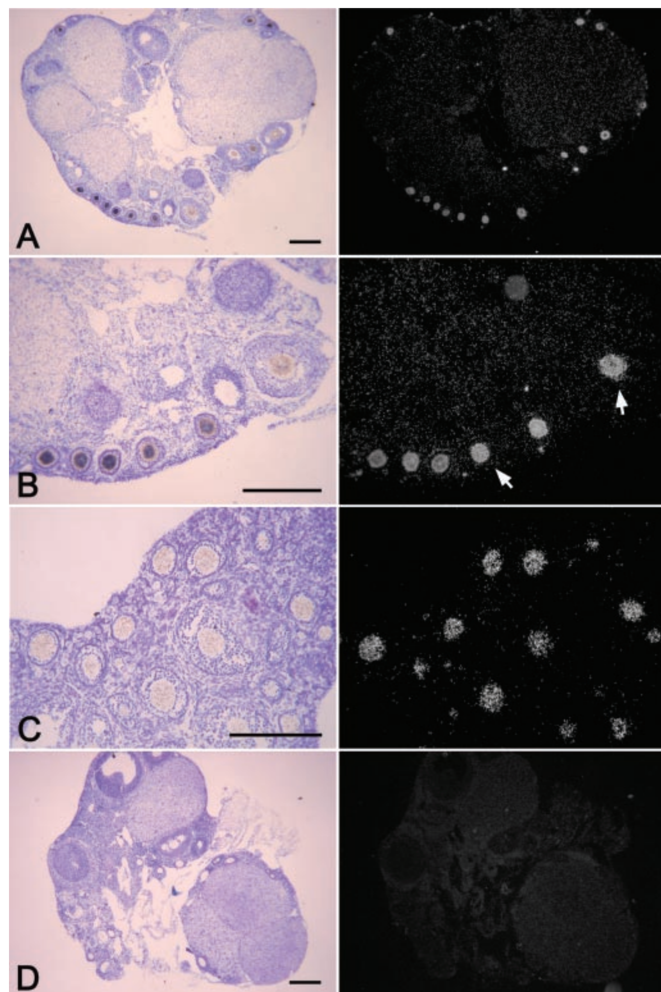

FIG. 5. ***In situ* hybridization analysis of ovastacin expression in mouse ovary.** 5-week-old female mice were injected intraperitoneally with 5 international units of pregnant mare's serum glycoprotein followed 48 h later by 5 international units of human chorionic gonadotropin. The following day, 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.

DISCUSSION

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, 12). In addition, and also similar to most astacins, human and mouse ovastacins contain an additional domain located at their C-terminal region. However, this domain does not exhibit overall sequence similarity to any of the ancillary domains present in the equivalent region of astacins or to any other protein domain present in databases. Accordingly, this ovastacin-specific domain is a distinctive feature of this enzyme and could play a role in facilitating its interaction with putative substrates or with other proteins or in mediating the formation of multimeric structures as proposed for the ancillary domains found in other astacins (26, 27).

Consistent with the structural characteristics of these novel proteins, functional analysis of a recombinant form of human ovastacin produced in *E. coli* revealed that it is a catalytically active metalloproteinase. Thus, the purified recombinant protein is able to hydrolyze several synthetic peptides used for analysis of the enzymatic activity of diverse metalloproteinases, including astacins (28). Kinetic analysis revealed that the catalytic efficiency of ovastacin against QF-35 (k_{cat}/K_m = 5.1 × 10^4 M^{-1} s^{-1}) is similar to that of other metalloproteinases with the ability to hydrolyze this peptide, such as MMP-26 (k_{cat}/K_m = 7.9 × 10^4 M^{-1} s^{-1}) and MMP-19 (k_{cat}/K_m = 3.1 × 10^4 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 overex-

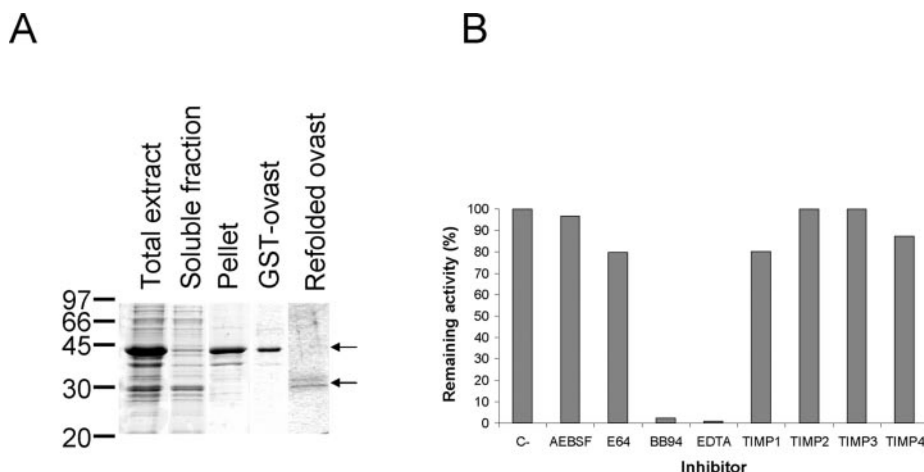

FIG. 6. **Production and enzymatic analysis of recombinant human ovastacin.** *A*, Coomassie Blue-stained SDS-PAGE showing the results of expression, purification, and refolding of the recombinant catalytic domain of human ovastacin. 5-μl aliquots of whole bacterial lysate (*total extract*), soluble fraction of the lysate (*soluble fraction*), insoluble fraction of the lysate (*pellet*), purified insoluble protein (*GST-ovast*), 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.

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 after RT-PCR amplification from RNA prepared from ovarian normal and tumoral tissues. A more detailed analysis of the presence of ovastacin in cells from mouse reproductive tissues revealed that it could be detected at significant levels in unfertilized oocytes and in 1.5-dpc preimplantation embryos. Interestingly, the expression of ovastacin in oocytes was strongly induced in 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 embryo 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 widely studied in the embryos of sea urchins, arthropods, the fresh water teleost medaka, and amphibians such as *Xenopus laevis*; a variety of proteases from different families have been characterized as hatching enzymes in these organisms (13–16, 41–47). Thus, the sea urchin hatching enzyme is a metalloproteinase of the MMP family (41, 42), whereas the corresponding enzymes in crayfish, birds, amphibians, and different fish species belong to the astacin family of metalloproteinases (13–17). In medaka fish, the hatching enzyme consists of two similar but distinct astacins (high and low choriolytic enzymes) that colocalize in the same hatching gland cells and perform a cooperative choriolytic action (43, 44). Likewise, the *Xenopus* hatching enzyme is secreted from hatching gland cells as a mixture of two distinct molecular forms that cooperate to achieve the complete solubilization of the embryo envelope (28). There is also detailed information about the mechanisms controlling the expression and activity of these hatching proteases (14, 44–47). By contrast, little information is available on the characteristics and functional properties of mammalian hatching enzymes, even though embryo hatching and outgrowth are critical steps for a successful pregnancy. In mouse, several reports have suggested that different serine proteases, ISP-1 (implantation serine protease-1), ISP-2, and hepsin, could be responsible for this process (48–50). The putative relevance of ISP-1 and -2 for embryo hatching was originally based on *in vitro* experiments (48, 49). However, further studies have suggested that ISP-mediated hatching occurs as an artifact of embryo culture and that the main role of these enzymes should be initiation of blastocyst invasion from the abembryonic pole (51, 52). Interestingly, we have recently reported the absence of functional genes for ISP-1 and -2 in the human genome (3); hence it is unlikely that these murine enzymes could be involved in an evolutionary conserved process such as hatching. Likewise, the membrane-bound hepsin has also been proposed to function as a hatching enzyme, but gene disruption studies have failed to support this possibility (53). Accordingly, our finding that human, mouse, and rat genomes encode a new astacin with significant sequence similarity to hatching enzymes from evolutionary distant species, together with its expression in early stages of embryogenesis, suggests that ovastacin could be functionally involved in the hatching process. Nevertheless, the observed expression of ovastacin in unfertilized oocytes should also be consistent with the possibility that ovastacin could play a role in some of the protease-mediated processes occurring in the ovary during the reproductive cycle. These processes include follicular development, ovulation, corpus luteum formation and regression, and follicular atresia (32–36). Further studies aimed at identifying the *in vivo* substrates of this protease will be necessary to define the precise role of this enzyme in the context of other proteolytic enzymes associated with reproductive processes.

The finding of a restricted expression of ovastacin is a common feature of several astacins and agrees well with the proposal that this enzyme plays a very specific role under physiological conditions. However, and in contrast to its restricted expression pattern in normal tissues, ovastacin mRNA was detected in some ovarian carcinomas and in leukemia and lymphoma cells of diverse origin. These findings, together with the observed hormonal regulation of ovastacin in oocytes, provide additional evidence that this enzyme is subjected to tight control in its spatio-temporal pattern of expression, which is likely lost in tumor processes. This is a common feature of many proteases from different catalytic classes and has been extensively studied (54–59). Additionally, recent studies have shown that expression and secretion of other human astacins is dysregulated in cancer (60–62), generating an increase in the proteolytic potential that could facilitate the degradation of major protein components of the extracellular matrix and the migration of tumor cells across the basement membrane (31). Further work will be required to evaluate the clinical significance of the expression of ovastacin in ovarian carcinomas as well as to extend the preliminary observations indicating that this protease can also be overproduced by other malignant cells, including leukemia cells. In this regard, it is of interest that the gene encoding ovastacin is located at 2q11, a recurrent site of translocations in hematological malignancies (63–65).

In conclusion, we have cloned and characterized ovastacin, a new astacin metalloproteinase that, according to our exhaustive screening of human and rodent genomes, represents the only member of this protease family that remained to be identified in these organisms. Ovastacin is a proteolytically active enzyme with a profile of activity and sensitivity to inhibitors characteristic of metalloproteinases, although it exhibits some structural peculiarities such as a unique C-terminal extension that may act as an ancillary domain in the recognition of its putative substrates. The expression of ovastacin is highly restricted in both human and rodent tissues. It is mainly detected in unfertilized oocytes, where it is subjected to hormonal control as assessed by the dramatic increase in ovastacin mRNA levels in oocytes from superovulated mice. Further experimental work, including the generation of mutant mice deficient in this protease, will be necessary to clarify the role of ovastacin in reproductive processes.

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