Identification and Cloning of the Membrane-associated Serine Protease, Hepsin, from Mouse Preimplantation Embryos*

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Previous studies have suggested the existence of a membrane-associated serine protease expressed by mammalian preimplantation embryos. In this study, we have identified hepsin, a type II transmembrane serine protease, in early mouse blastocysts. Mouse hepsin was highly homologous to the previously identified human and rat cDNAs. Two isoforms, differing in their cytoplasmic domains, were detected. The tissue distribution of mouse hepsin was similar to that seen in humans, with prominent expression in liver and kidney. In mouse embryos, hepsin expression was observed in the two-cell stage, reached a maximal level at the early blastocyst stage, and decreased subsequent to blastocyst hatching. Expression of a soluble form of hepsin revealed its ability to autoactivate in a concentration-dependent manner. Catalytically inactive soluble hepsin was unable to autoactivate. These results suggest that hepsin may be the first serine protease expressed during mammalian development, making its ability to autoactivate critical to its function.

Embryonic development is marked by a series of cellular divisions and morphogenetic changes (1). These processes are mediated by the complex expression and interplay of different sets of genes, some of which are derived from maternally expressed genes stored as mRNAs in the oocytes. It is generally accepted that zygotic gene expression begins at the embryonic two-cell stage (2). These newly expressed zygotic genes complement the maternally expressed genes to mediate early preimplantation development. Numerous studies have suggested the involvement of a variety of proteases during development. Members of the astacin family of metalloproteases are involved in hatching in both invertebrates and vertebrates (3–6), pat-...
EXPERIMENTAL PROCEDURES

Collection and Culture of Mouse Preimplantation Embryos—Experiments utilizing preimplantation embryos were performed with cultured two-cell stage embryos, which were obtained from B6C3F1 prepubescent female mice (Charles Rivers Lab) weighing 10–13 g. Mice were injected intraperitoneally with 5 IU of pregnant mare's serum gonadotropin (Sigma) followed 48 h later with 5 IU of human chorionic gonadotropin (Sigma). Subsequently, a single female was paired with a single male overnight, and females were checked for vaginal plugs the following day (day 1). On day 2, mice were dissected to obtain the oviducts, which were bathed in sperm washing medium (Irvine Scientific) and dissected to release the two-cell embryos. About 40–50 two-cell embryos were pooled and cultured under oil at 37 °C in a humidified atmosphere of 5% CO₂ in air in 50-μl droplets of human tubular fluid (Irvine Scientific) plus 0.5% human serum albumin (Irvine Scientific). Cultures were maintained for 4–5 days or until expanded blastocysts began to hatch.

RNA Isolation and First-strand cDNA Synthesis—Total RNA was isolated from 100–200 hatching blastocysts (embryonic day 4.5), according to the method of Chomczynski and Sacchi (27). The total amount of RNA obtained was then used in the first-strand cDNA synthesis reaction using SuperScript reverse transcriptase (Life Technologies, Inc.) and oligo(dT) as primers. The reaction was incubated at 42 °C for 1 h. Subsequently, RNase H (Life Technologies, Inc.) was added and the reaction was incubated at 37 °C for 20 min to remove the RNA template.

PCR Amplification, Cloning, and Sequencing of Mouse Hepsin—To identify the serine protease involved in mouse blastocyst hatching, degenerate oligonucleotides, 5'-TGCTCTAGATGG(A/G)TINTI(A/T)(G/C)IGCIGCICA-3' and 5'-CCGGAATTCA(A/G)IGGI(G/C)(ACT)ICCI(G/C)(A/T)(A/G)TCICC-3' (Molecular Biology Resource Facility, OUHSC), based on two conserved regions of known serine proteases, were used to amplify a 500-bp DNA fragment, encoding part of the protease catalytic domain, from hatching blastocyst RNA. Aliquots of first-strand cDNA were incubated in the presence of 0.1 μM of each 5'- and 3'-primers, 100 μM dNTP, 1·PCR buffer, and 2.5 units/100 μl of AmpliTaq DNA polymerase (Perkin-Elmer). The reactions were cycled 40 times through the following steps: 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C in a Perkin-Elmer DNA thermocycler model 2800. DNA fragments of the correct size (500 bp) were purified from agarose gels using GeneClean II (BIO 101 Inc., Vista, CA). The purified fragments were ligated into pBS-SK(+) (Stratagene) using T4 DNA ligase (New England Biolabs). Double-stranded DNA was sequenced using T3 and T7 primers and the Sequenase Version 1 kit (U. S. Biochemical Corp./Amersham Life Science). Sequences of cloned PCR fragments were compared with DNA sequences compiled in data bases.

A full-length cDNA of mouse hepsin was subsequently cloned by screening a mouse liver cDNA library (Stratagene), using the manufacturer's instruction.32P-Labeled DNA probes were generated using the Prime-It II random primer labeling kit (Stratagene) and the 500-bp cloned PCR fragment described above as a template. A 1.8-kb cDNA obtained was sequenced as described above using both pBluescript and internal primers.

Construction and Expression of Soluble Hepsin and Catalytically Inactive Hepsin—The method of site-directed mutagenesis as described previously (28, 29) was used to introduce a Stu I restriction site at the end of the coding sequence of the transmembrane (TM) domain of hepsin using the oligonucleotide, 5’-GTGACCATCCTAAGGCCTAGTGAC-CAGGAGCC-3’, which replaced nucleotides 331–336 with a Stu I site.

1 The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); RT, reverse transcriptase; PAGE, polymerase chain reaction; IVI, factor VII; IVIa, activated factor VII; pBS, pBluescript.
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This Stu1 site and the Xba1 site at the 3' end of the CDNA in PBS-SK+ were used to excise a 1.1-kb DNA fragment and cloned into the same sites in the RSV-PL4 expression vector (30). This construct included a transferrin signal peptide, followed by an amino-terminal epitope tag recognized by HPC4, a calcium-dependent monoclonal antibody (31). The soluble hepsin expressed using this vector had a new amino-terminal of Glu-Asp-Gln-Val-Asp Pro-Arg-Leu-Ile-Asp-Gly-Lys-Ile-Glu-Arg-Ser-Pro, followed by the wild-type hepsin sequence from Ser45. The terminal of Glu-Asp-Gln-Val-Asp Pro-Arg-Leu-Ile-Asp-Gly-Lys-Ile-Glu-Arg-Ser-Pro was recognized by HPC4, a calcium-dependent monoclonal antibody (31). The soluble hepsin expressed using this vector had a new functional S348A soluble hepsin mutant, which replaced the active site serine with an alanine, was constructed similarly with the addition of the oligonucleotide, 5'-TGCCAGGGCGACGCTGGGGGC-3' (31). This non-functional S348A soluble hepsin mutant, which replaced the active site serine with an alanine, was constructed similarly with the addition of the oligonucleotide, 5'-TGCCAGGGCGACGCTGGGGGC-3'. The resulting constructs were transfected into human 293 epithelial cells using LipofectAMINE (Life Technologies, Inc.) at a final concentration of 400 μg/ml G418 (Life Technologies, Inc.). High expressing clones were selected using 400 μg/ml G418 (Life Technologies, Inc.). The accuracy of the constructs were confirmed by DNA sequencing. The recombinant epitope-tagged protein was purified from conditioned medium by affinity chromatography using HPC4-linked Affi-Gel 10 and was eluted with EDTA.

**Assay of Soluble Hepsin Activity**—Soluble hepsin amidolytic activity was assayed using the chromogenic substrate Spectrozyme PCa (H-D-Pro-Arg-Naph; American Diagnostica) at a final concentration of 0.2 mM. The absorbance at 405 nm was monitored over 10 min using a microplate reader (Molecular Devices) to determine the rate of substrate hydrolysis (ΔA₄₀₅/min). Inhibitory dose-response curves were generated by preincubating the enzyme with specific inhibitors at different concentrations for 30 min at ambient temperature prior to the addition of the substrate.

**Semi-quantitative RT-PCR and Southern Blot Analysis**—RT-PCR linked Southern blot analysis to augment sensitivity of detection was utilized to investigate the temporal expression of hepsin in mouse preimplantation embryos. CDNAs from various stages of development were prepared from 40 to 50 embryos as described above. Oocytes were prepared from unmated females and treated with hyaluronidase (Sigma) to remove cumulus cells before proceeding to the total RNA isolation and cDNA synthesis as above. PCR was performed essentially as above with the mouse hepsin primers, 5'-ACCTGACAGTGGTCTC-CCTG-3' and 5'-ATCCACGCGCATGTGCTC-CC-3', but with only 15 cycles. Similar PCR reactions using, β-actin primers (a gift from Jeff Gimble, Department of Surgery, University of Oklahoma Health Sciences Center), were used as positive controls. Southern blot analysis of the PCR products was performed as described previously (30) using 32P-labeled random-primed DNA probes generated from the same amplified DNA regions as templates.

**Northern Blot Analysis**—Total RNA was isolated from cells according to published methods (27). RNA was transferred to MSI-NT nylon membranes by capillary action, then cross-linked to membranes with UV light. Membranes were incubated for 1 h at 60 °C with prehybridization buffer (500 mM NaPO₄, pH 7.4, 1% SDS, 1 ml EDTA). Membranes were then hybridized overnight in prehybridization buffer plus labeled cDNA probe at 60 °C. Probes were 32P-labeled by incorporational use of the oligonucleotide, 5'-CCCTTTGTG-3'. The resulting constructs were transfected into human 293 epithelial cells using LipofectAMINE (Life Technologies, Inc.) at a final concentration of 400 μg/ml G418 (Life Technologies, Inc.). High expressing clones were selected using 400 μg/ml G418 (Life Technologies, Inc.). The accuracy of the constructs were confirmed by DNA sequencing. The recombinant epitope-tagged protein was purified from conditioned medium by affinity chromatography using HPC4-linked Affi-Gel 10 and was eluted with EDTA.

**RESULTS**

**Strategy for the Identification and Cloning of an Embryonic Serine Protease**—A prior study using a radioiodinated active site chloromethyl ketone probe and SDS-PAGE detected a single serine protease of Mr~74,000 in mouse blastocyst lysates (26). Using RT-PCR and degenerate oligonucleotides based on conserved regions in the catalytic domain of serine proteases, we amplified and subcloned a 0.5-kb cDNA fragment encoding the putative mouse hatching enzyme from hatching blastocysts mRNAs. Ten separate clones were sequenced and found to be identical. Data base searches showed that the deduced amino acid sequence was similar to that of human hepsin, a trypsin-like serine protease previously cloned from a liver library (33). A full-length mouse hepsin cDNA (Fig. 1) was obtained after

**Fig. 2. Sequence alignment of mouse, rat, and human hepsin.** Deduced amino acid sequences of mouse, rat, and human hepsin are shown. Amino acid identity is indicated by a dash. The conserved TM domain and Asp346 are boxed.

**Fig. 3. Temporal expression of hepsin in mouse preimplantation embryos.** Total RNA from mouse embryos was isolated, then analyzed for hepsin mRNA expression by Southern blot-linked-RT-PCR analysis (n = 3). β-Actin was used as a control.

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**Mouse**

| Rat | Human |
|-----|-------|
| MAQQ----VP---- | ---- |
| R-----Q-----L-----P----- | ---- |

**Mouse**

| Rat | Human |
|-----|-------|
| DSRLAVLWRTGVARLCCSSRNSKAVAGLCCCTEMGFLRALKHSELVIRGTANAGTGSCFFC | ---- |
| R-----Q-----L-----P----- | ---- |

**Mouse**

| Rat | Human |
|-----|-------|
| VEDQERLPAGQLRLDLVIGVCCDPGPHPLTAPCQDCSRLPKLVQIRDVGQVDGQSGLQSPQVS | ---- |
| R-----Q-----L-----P----- | ---- |

**Mouse**

| Rat | Human |
|-----|-------|
| LRYDQTHLCGGSSLSDGVNLTAHCFVSRILVSRWAVFACAVARSSHPWAVGQVAYT | ---- |
| R-----Q-----L-----P----- | ---- |

**Mouse**

| Rat | Human |
|-----|-------|
| HGGYLPFFRDPITIDENSIALVHLSLPTETIQQVCPDLPAAGQALDVGKCTVTGGKNT | ---- |
| R-----Q-----L-----P----- | ---- |

**Mouse**

| Rat | Human |
|-----|-------|
| QYQQQAMVLQVBRWPILSNECNEDPFPFYQIKRFNMCAPGYPFGT | D ACQGDDCPF |
| R-----Q-----L-----P----- | ---- |

**Mouse**

| Rat | Human |
|-----|-------|
| VCESDTSIGTSRMLCGVSGMTCGCATARKFGYVTYDFRENIFKAATKEHASEO5MTQF | ---- |
| R-----Q-----L-----P----- | ---- |

**Mouse**

| Rat | Human |
|-----|-------|
| ACQGDDCPF | ---- |
| R-----Q-----L-----P----- | ---- |
screening a mouse liver library using the amplified DNA fragment as a probe. Hepsin is a type II transmembrane protein with an extracellular carboxyl-terminal catalytic domain (33, 34). Based on the predicted amino acid sequence homology with other related serine proteases, hepsin is likely to be synthesized as a single chain zymogen that requires cleavage of the Arg161-Ile162 bond to generate the mature, disulfide-linked two-chain form. In addition to the catalytic triad residues and Asp346, which is important for trypsin-like specificity, the transmembrane and short cytoplasmic domains of hepsin are all conserved among mouse, rat, and human hepsin (Fig. 2). The significance of the transmembrane domain remains to be determined.

**Temporal Expression of Hepsin in Preimplantation Embryos**—To determine if the temporal expression of hepsin was consistent with that of a hatching enzyme, we performed semi-quantitative RT-PCR-linked Southern blotting to indirectly determine the time and level of hepsin message in oocytes and in several stages of preimplantation development. Hepsin transcription was biphasic, beginning at the 2-cell stage, absent at the 8-cell stage, and peaking at the early blastocyst stage prior to hatching (Fig. 3). There was no detectable expression in oocytes, and, subsequent to embryo hatching, the level of expression clearly diminished (Fig. 3).

**Tissue Expression and Multiple Hepsin mRNAs**—Human hepsin was previously shown to be expressed primarily in liver and kidney, and mouse hepsin was similarly distributed (Fig. 4). Unlike human hepsin, mouse hepsin had two alternative forms detected by Northern blotting, migrating at 1.8 and 1.9 kb. To characterize the differences in the two mRNAs, we performed RT-PCR analysis using total RNA samples isolated from mouse liver and kidney. Several oligonucleotide primers spanning the hepsin cDNA sequence were utilized, as shown in Fig. 5. PCR analysis revealed that an insert in the 5’-end of the coding sequence distinguished the 1.9-kb message from the 1.8-kb message. DNA sequencing revealed an additional 60-bp sequence coding for 20 amino acids within the cytoplasmic domain of 1.9-kb hepsin cDNA (Fig. 6). This sequence has not been demonstrated in human hepsin.

**Expression and Autoactivation of Soluble Hepsin**—Because hepsin is a type II transmembrane serine protease, we wanted to address the possibility that a soluble form of the enzyme could be expressed and used to elucidate hepsin’s enzymatic properties. We developed an expression construct by site-directed mutagenesis that encoded for a zymogen form of hepsin lacking its transmembrane and cytoplasmic domains (soluble hepsin), and stably expressed it in human 293 epithelial cells. Soluble hepsin was expected to be expressed as a single-chain zymogen which could be activated proteolytically to a disulfide-linked two-chain form, consisting of a 12-kDa light chain and 31-kDa heavy chain. The intact precursor as well as proteolytically activated species would be expected to migrate with a Mr = 43,000 on SDS-PAGE gels. Surprisingly, upon elution, soluble hepsin was spontaneously activated from a single-chain zymogen to the active disulfide-linked two-chain form (Fig. 7, WT lanes, and data not shown); this activation was not detected in the conditioned medium not subjected to purification (Fig. 7, 5′-end of the coding sequence distinguished the 1.9-kb message from the 1.8-kb message. DNA sequencing revealed an additional 60-bp sequence coding for 20 amino acids within the cytoplasmic domain of 1.9-kb hepsin cDNA (Fig. 6). This sequence has not been demonstrated in human hepsin.

**Fig. 4. Tissue distribution of mouse hepsin expression.** Total RNA (20 μg/lane) from several adult rat tissues was analyzed for hepsin expression by Northern blots hybridized with a cDNA consisting of the entire hepsin coding region. Two hybridizing species highly detected in liver and kidney correspond to mRNAs of approximately 1.8 and 1.9 kb in size.

**Fig. 5. Localization of the region of nucleotide insertion in the 1.9-kb hepsin message.** Total RNA from both mouse kidney and liver were subjected to RT-PCR analysis using different primers sets (each primer is denoted by a letter from A-E) to localize the region of nucleotide differences between the 1.8- and 1.9-kb hepsin mRNAs. The positions of the primers (arrow) are indicated along the 5′-to-3′-nucleotide sequence as represented by a horizontal bar above the gel image. The position of the nucleotide insertion is also marked. PCR products were separated by 1% agarose electrophoresis and stained with ethidium bromide. Primer set A/B detected two different bands due to the 60-bp insertion in the coding region for the cytoplasmic domain of hepsin. Primers were as follows: A, 5′-TGGGAATCTTTTAAACAGATCCCTGAC-3′; B, 5′-AGTCGGATTCCCGTCTTACG-3′; C, 5′-AGGAAGTGCTGCGGAGCAGCAGTG-3′; D, 5′-ATCCCGCCATGATTGTGTCCCTG-3′; E, 5′-TCAGGCGTGAGTCACATGCCAC-3′.

**Fig. 6. Alternative cytoplasmic domains in the two hepsin mRNAs.** Amino acid and cDNA sequence of the hepsin cytoplasmic domain, with the inserted sequence within the 1.9-kb form shown above the 1.8-kb form of hepsin.
CM lanes). Additionally, it further processed itself from a 43- to 29-kDa form (Fig. 7, non-reduced WT lane). Upon reduction, only a 31-kDa band, which represented the heavy or catalytic chain, was seen, suggesting that only the light chain was proteolytically modified to generate the 29-kDa form seen under nonreducing conditions. The autoactivation of soluble hepsin upon elution was not seen with a catalytically inactive S352A soluble hepsin mutant, in which the active site serine was replaced by alanine (Fig. 7, S352A lanes). Of note, the initial eluate, when immediately prepared and separated by reducing SDS-PAGE, showed only a small amount of conversion to the two-chain form (data not shown). Similarly, the presence of the inhibitor benzamidine in the eluate prevented the conversion and only a small converted fraction was seen on reducing SDS-PAGE (data not shown).

**DISCUSSION**

We have identified hepsin, a membrane-bound serine protease previously shown to activate fVII (35), in preimplantation mouse embryos as early as the two-cell stage. Based on evidence that a single serine protease is present in preimplantation embryos (26), it is possible that hepsin represents the first such protease expressed during development. Prior in vitro experimentation implicated hepsin in the maintenance of cellular morphology and hepatoma cell growth (36), and in blood coagulation by human factor VII activation (35). Increased hepsin expression has also been associated with ovarian cancer (37). No developmental functions of hepsin have been described. Whether hepsin plays a critical role in early development is not clear, but it is possible that it plays a role in blastocyst hatching.

**FIG. 7. Soluble hepsin is capable of autoactivation.** Wild-type and S352A soluble hepsin was isolated from medium conditioned by transfected 293 epithelial cells, and proteins were separated by both nonreducing and reducing SDS-PAGE and blotted to nitrocellulose membrane. The primary HFP-2 and anti-goat alkaline phosphatase-conjugated antibodies were used to visualize hepsin in conditioned medium (CM), as well as purified soluble hepsin (WT) and its inactive mutant (S352A). Molecular mass markers are shown in kDa.

**FIG. 8. Model of hepsin activation.** Based on structural similarities to other serine proteases, hepsin is expressed as a single-chain zymogen and can be activated proteolytically by a single cleavage at the Arg161-Ile162 bond to generate the two-chain, membrane-bound form. Its deduced primary amino acid sequence suggests that hepsin is expressed as a type II transmembrane zymogen with an extracellular carboxyl-terminal catalytic domain. The internal signal sequence, serving as a transmembrane domain, is surprisingly conserved. The presence of this transmembrane domain is consistent with Perona and Wassarman’s (26) data suggesting that the putative mouse hatching enzyme, which would be expressed in early preimplantation embryos, is membrane-bound. The trypsin-specificity conferring Asp346 that lines the S1 subsite and composes part of the specificity pocket is present and conserved, indicating that hepsin is likely to have trypsin-like specificity. Indeed, our activity assay of the recombinant soluble hepsin using a number of chromogenic substrates have confirmed this observation. The reason for the presence of two forms of hepsin, differing in the cytoplasmic domain, is not clear. The inserted sequence in the 1.9-kb form of hepsin has no homology to any domains found in signal transducing proteins. It is unlikely that changes to the cytoplasmic domain alter hepsin’s proteolytic properties, particularly since the soluble form of the enzyme is apparently fully functional. Whether the 1.8- and 1.9-kb hepsin mRNAs are the result of two different genes or, more likely, the result of alternative splicing of a single gene transcript remains to be defined.

Since hepsin is likely to be expressed as a zymogen based on the predicted amino acid sequence, and appears to be the only serine protease present during blastocyst hatching, the question arises, what is the mechanism of its activation? Our hypothesis is that density-dependent autoactivation occurs, as suggested by data from our soluble hepsin expression study. We noted that during purification, upon elution with EDTA, soluble hepsin was spontaneously converted to the active, disulfide-linked two-chain form probably via cleavage of the Arg161-Ile162 bond. The conversion was clearly concentration dependent (activation was only seen in the eluate and not in the diluted conditioned medium) and required hepsin’s inherent enzymatic activity since it was not observed with a catalytically inactive S352A mutant soluble hepsin. These data indicate that hepsin was capable of concentration-dependent autoactivation. Since hepsin is membrane-bound via a transmembrane domain, its density and lateral diffusion on the trophoblast surface may play an important role in achieving the concentration needed for autoactivation (Fig. 8). This mode of autoactivation resembles fVII cell surface autoactivation, which utilizes distinct tissue factor molecules to localize both the fVII and fVIIa to the cell surface, forming two separate membrane-bound binary complexes. The complex with the active fVIIa then activates the adjacent tissue factor-anchoring...
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J.VII, obeying obligatory two-dimensional enzyme kinetics (38). Hepsin autoactivation is likely to follow similar kinetics, but further studies are necessary to elucidate its mechanism of cell surface autoactivation. Interestingly the recent purification of intact hepsin from rat liver microsomes also resulted in its activation (39), but it was not clear if this was the result of autoactivation or of the action of another protease. Our data with the inactive hepsin mutant suggest that membrane-bound hepsin is capable of autoactivation.

The autoactivation of soluble hepsin additionally generated a second form of the enzyme. A band of 29 kDa, which was absent in the S352A mutant, along with the intact 43 kDa, were both present when the eluate was analyzed on nonreducing SDS-PAGE and Western blot experiments. This 29-kDa form was likely to be the result of proteolytic modification of the light chain of the active two-chain form since only the intact catalytic heavy chain was seen under reducing conditions. The presence of this 29-kDa form suggests that membrane-bound hepsin can be cleaved off the trophoblast surfaces of embryos (Fig. 8). Interestingly, Sawada et al. (40) have demonstrated the presence of a soluble trypsin-like activity in blastocyst culture medium and that this activity represented that of a hatching enzyme. Whether this secreted trypsin-like activity and the 29-kDa form of hepsin are one and the same, and what roles it may play during embryogenesis, remain to be determined.

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