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Hepsin, a putative cell-surface serine protease, is required for mammalian cell growth

(antisense oligonucleotide/cell morphology/developing embryo/plasma membrane/cellular process)

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ABSTRACT **Hepsin was previously identified as a putative cell-surface serine protease. When hepatoma cells were treated with anti-hepsin antibodies, their growth was substantially arrested, suggesting the requirement of hepsin molecules present at the cell surface for normal cell growth. This was further supported by a gross inhibition of cell growth with hepsin-specific antisense oligonucleotides. Upon treatment of cells with antisense oligonucleotides, rapid reduction in cellular hepsin was observed. This reduction in cellular hepsin levels was accompanied by drastic morphological changes. Various tissues in the developing mouse embryo showed greatly elevated hepsin levels in regions of active proliferation. These results indicate that hepsin plays an essential role in cell growth and maintenance of cell morphology.**

Cell-surface serine proteases have been known to play important roles in various cell functions (1). Our current understanding of this class of plasma membrane proteins, however, is significantly limited. Hepsin is a putative membrane-associated serine protease of 51 kDa (2). It is synthesized as a single polypeptide chain of 417 amino acid residues with a 27-residue-long internal hydrophobic sequence (2, 3). Hepsin is located primarily in the plasma membrane with its trypsin-type protease module (the C-terminal half) at the external surface of cells. This molecular orientation makes hepsin particularly interesting since very little is known about the biological roles of such serine proteases in spite of their predicted importance in cell growth. Hepsin is present at significant levels in many different types of mammalian cells such as human hepatoma cells (HepG2 and PLC/PRF/5 cells), mammary cancer cells (MCF784 and T470), peripheral nerve cells (PC12), and baby hamster kidney cells, but at undetectable levels in some types of cells such as human umbilical cord as well as rat capillary endothelial cells. Hepsin is produced in most tissues but at a particularly high level in the liver (2).

In the present report, we describe the importance of hepsin for mammalian cell growth. Experimental evidence indicates that the expression of hepsin is necessary for normal cell growth and morphology.

MATERIALS AND METHODS

Effects of Anti-Hepsin Antibodies and Antisense Oligonucleotides on the Growth of PLC/PRF/5 Cells. To test the importance of hepsin for cell growth, a set of antisense oligonucleotides and their thioate derivatives were prepared. Synthetic phosphodiester oligonucleotides including sense strand (SS-pd-oligo237, 5′-GGCAGTGACATGGCGCAGAAG-3′) and antisense strand (AS-pd-oligo237, 5′-CTTCTGCGCCATGTCACTGCC-3′) of hepsin, which are purified on reverse-phase high-performance liquid chromatography, corresponded to the 5′ end region of hepsin cDNA (nt 237–257) with the first in-frame ATG at the center (3). An AS oligo (AS-FIX-oligo), which corresponds to the exon IV sequence of human factor IX (5′-GCTATGTAACATTTTCGAT-3′), was also synthesized as a control (4). This has a 10-base stretch of sequence similar to that of AS-pd-oligo237 with only 2 base mismatches (underlined letters indicate identical sequences).

Phosphorothioate (pt) analogue counterparts of the pd-oligos described above (SS-pt-oligo237 and AS-pt-oligo237) and a randomized sequence of AS-pt-oligo237 (RAS-pt-oligo237, 5′-CCCGTCGTATCGATCCGTTCC-3′) were similarly synthesized, except that the oxidation reagent was replaced by tetraethylthiuram disulfide in acetonitrile (ABI P/N 401147) and was purified by thin-layer chromatography. The second set of AS and RAS pt derivatives (AS-pt-oligo952 and RAS-pt-oligo952) designed to the downstream coding sequence (nt 952–971 corresponding to amino acids 236–242) and the third set (AS-pt-oligo1720 and RAS-pt-oligo1720) designed to the sequence in the 3′ untranslated sequence (nt 1720–1739) were also made. All oligos and their analogues were free from blocking groups.

Human hepatoma cell lines, including PLC/PRF/5 cells (5) and HepG2 cells, or baby hamster kidney cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, streptomycin, and penicillin (GIBCO/BRL) under 5% CO_2/95% air at 37°C. Cells were plated in 1.5-cm plates at ≈5% confluency and grown in 0.5 ml of DMEM supplemented with 5% fetal bovine serum for 24 hr until reaching ≈10% confluency. Aliquots (5 μl) of oligos dissolved in water were then added to the medium on day 0 to a final concentration of 15 μM for pt analogues. Oligo concentrations used were optimized for effects on cell growth. Cells were incubated for another 24 hr (day 1), and the medium was changed to fresh DMEM containing 10% fetal bovine serum and the second aliquots of oligos. Cells were then incubated for subsequent days in the same medium without adding any fresh oligos. Treatment of cells with pd-oligos was carried out in a similar manner, except that an oligo concentration of 10 μM was used and fresh aliquots of the oligos were added daily after day 1. Cell growth was recorded by counting cell numbers in four separate grid areas for each culture dish. On day 0, each grid area contained 15–20 small colonies with total cell number ranging from 30 to 50. Increase in cell number on the following days was calculated by dividing the number of cells

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Abbreviations: SS, sense strand; AS, antisense strand; RAS, randomized antisense strand; pd, phosphodiester; pt, phosphorothioate; oligo, oligonucleotide; FIX, factor IX.

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in each grid by that obtained on day 0. The mean cell number increase of the four grid areas for each dish was used as representative of that time point. The experiments were repeated six times with pt and twice with pd (Fig. 1*A*) and five times with pt (Fig. 1*B*).

Effects of anti-hepsin antibody were tested by daily addition of affinity-purified rabbit anti-human hepsin antibody (HAbP5) to the culture medium (10 μg per ml of medium). This antibody was previously prepared against a synthetic peptide (20 amino acid residues) corresponding to the C-terminal unique region of hepsin, and it has a very high specificity to hepsin (2). Water, nonspecific rabbit IgG, and affinity-purified anti-angiogenin antibody were used as controls. Experiments were repeated five times, and data were processed as described for experiments with AS oligos.

Effects of AS-pt-oligo237 and RAS-pt-oligo237 on Hepsin Biosynthesis in PLC/PRF/5 Cells. To examine effects of AS-pt-oligo237 on hepsin biosynthesis, PLC/PRF/5 cells, which were grown and treated with RAS-pt-oligo237 or with AS-pt-oligo237 as described in Fig. 1, were pulse-labeled for 3 hr with [^{3}H]leucine (0.3 mCi per ml of medium; 1 Ci = 37 GBq) on day 0, 1, or 2. Immediately after pulse-labeling, cells were washed and cell protein extracts were prepared as described (6). Aliquots (50 μg) of protein extracts prepared from cells harvested on each day were incubated with 11 μg of anti-hepsin antibody (HAbP5) at 4°C for 1 hr, followed by addition of washed protein A (Sigma) in an amount sufficient to bind 33 μg of IgG and incubation for 1 additional hr. Protein A pellets obtained by centrifugation were washed five times by repeating resuspension in 500 μl of TSA buffer (10 mM Tris·HCl, pH 8.0/0.14 M NaCl/0.025% NaN_3) and centrifugation, heated at 100°C for 10 min with 1% SDS sample buffer containing 10 μl of 2-mercaptoethanol, and electrophoresed on a 12% polyacrylamide gel. Gels were soaked in isopropanol/acetic acid/water (25:10:65; vol/vol), and then in Amplify solution (Amersham), followed by drying and exposure to x-ray film (Kodak, X-Omat-AR). Hepsin bands visualized were digitized and quantitated with hardware and software from BioImage (Ann Arbor, MI).

Effects of Hepsin AS Oligos on Hepsin Level and Morphology of PLC/PRF/5 Cells. Cells were plated at ≈5% confluency on slides (eight wells per slide; Miles) and were grown in the presence of hepsin oligos for 5 days as described above. Cells were then fixed at room temperature for 10 min with 4% paraformaldehyde in phosphate-buffered saline (PBS; 171 mM NaCl/3 mM KCl/10 mM Na_2HPO_4, pH 7.2). Cells were processed for indirect immunofluorescence localization of hepsin as described (2). Anti-hepsin antibody (HAbP5) diluted 1:100 and goat anti-rabbit IgG with fluorescein isothiocyanate were used as the primary and secondary antibodies. Slides were viewed and fluorescent and phase-contrast images were photographed with a fluorescence microscope (Leitz Orthoplan).

Immunofluorescence Localization of Hepsin in Selected Tissues. Mouse embryos were obtained from matings of CD-1 strain animals (Charles River Breeding Laboratories). The morning of finding a vaginal plug was considered day 1 of pregnancy. Embryos and selected adult tissues were fixed for 1 hr at room temperature in 2% paraformaldehyde/0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). Tissue sections were cut at ≈8 μm. For immunocytochemistry, the affinity-purified anti-hepsin antibodies (HAbPM), which were previously prepared by using three synthetic peptides designed to regions unique to hepsin in the catalytic subunit (2), were used as the primary antibody (1:10 dilution) followed by fluorescein isothiocyanate-conjugated secondary antibodies. Slides were coverslipped, viewed, and photographed with a Leitz Orthoplan microscope. Controls were exposed to preimmune serum alone, to PBS in place of the primary antibody, or to antibody preabsorbed with excess hepsin peptide.

RESULTS AND DISCUSSION

Anti-Hepsin Antibodies Grossly Suppress Cell Growth. In an attempt to establish a biological role for hepsin, we first examined the effect of anti-hepsin antibodies on cells in culture. When PLC/PRF/5 cells were treated with anti-hepsin antibody (HAbP5), 89% inhibition of cell growth was observed without any significant changes in cell morphology (Fig. 1*A*). A similar strong inhibitory effect of the antibodies was also observed on HepG2 cells, whereas a significant but somewhat weaker inhibition (≈60%) was observed on baby hamster kidney cells (data not shown), agreeing well with the cross-reactivity of these antibodies with rodent hepsin (2).

AS Oligos Suppress Cell Growth. To examine possible nonspecific effects of anti-hepsin antibodies, such as steric hindrance of other cell-surface components, and to define

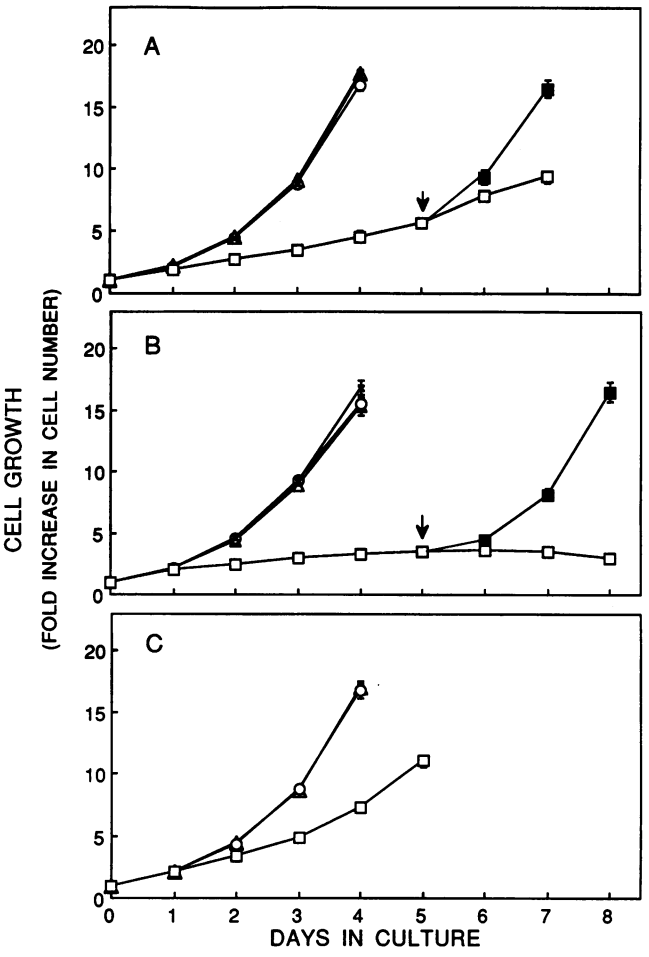

FIG. 1. Effects of anti-hepsin antibodies and AS oligos on the growth of PLC/PRF/5 cells. (*A*) Cells cultured in medium to which water (△), nonspecific rabbit IgG (○), anti-angiogenin IgG (×), or anti-hepsin (□) was added daily. Arrow indicates change from an anti-hepsin antibody-containing medium (□) (three replicates for days 6 and 7) to an anti-hepsin-free medium (■) (two replicates for days 6 and 7). Bars indicate SEM for days 1–5 and range of values for days 6 and 7. (*B*) Cells cultured in medium to which water (△), RAS-pt-oligo237 (○), SS-pt-oligo237 (×), or AS-pt-oligo237 (□) was added. Bars indicate SEM. Arrow indicates change from an oligo-containing (□) to an oligo-free (■) medium. (*C*) Cells cultured in medium to which water (△), RAS-pt-oligo952 (○), or AS-pt-oligo952 (□) was added. Mean values (five replicates) were plotted. All other conditions are the same as in *B*.

further the role of hepsin, we tested the effects of AS oligos that specifically suppress hepsin biosynthesis. AS oligos have been successfully used to modulate biosynthesis of various proteins in studying their functions (7–9).

In the first experiment, we prepared both pd oligos (SS-pd-oligo237; AS-pd-oligo237) and their degradation-resistant pt analogues (SS-pt-oligo237; AS-pt-oligo237; RAS-pt-oligo237), which were designed to the region of the translation initiation site of hepsin. These oligos were added directly to the culture medium and examined for their effects on growth and morphology of various cells. Typical effects of pt analogues on the growth of PLC/PRF/5 cells are shown in Fig. 1*B*. Cells treated with SS-pt-oligo237, RAS-pt-oligo237, or water showed normal growth with a doubling time of ≈1 day, whereas growth of cells treated with AS-pt-oligo237 was almost completely arrested by day 3. AS-pd-oligo237 caused a similar arrest of cell growth, whereas SS-pd-oligo237 had little effect on the cells (data not shown), clearly indicating that the cellular effects are not due to nonspecific effects of pt derivatives. AS-FIX-oligo, which is unrelated but has significant sequence identity with AS-pt-oligo237, did not show any obvious effects on cell growth, further supporting the specific effects of AS oligos on cells. Specific cell growth arrest was maintained as long as the AS oligos were present in the culture medium. However, upon replacing the medium containing AS-pt-oligo237 with the normal medium, even as late as day 5, the cells regained their normal growth rate and morphology in ≈2 days (Fig. 1*B*). In comparison, the cells treated with AS-pd-oligo237 regained normal cell growth within a day, agreeing well with a shorter half-life for pd oligos than for pt (data not shown). These results indicate that the arrest of cell growth by hepsin AS oligos does not impair cell viability; however, prolonged treatment of cells (longer than ≈7 days) with AS-pt-oligo237 (15 μM) gradually reduces cell viability.

Similar, but less severe, inhibitory effects were also observed with the second AS construct (AS-pt-oligo952) designed to the downstream sequences still in the coding region (Fig. 1*C*). AS-pt-oligo1720 designed to the general region in the 3′ untranslated sequence produced little inhibition of cell growth (data not shown). These results further supported the notion that the effects observed with AS-pt-oligo237 and AS-pd-oligo237 were not due to nonspecific effects.

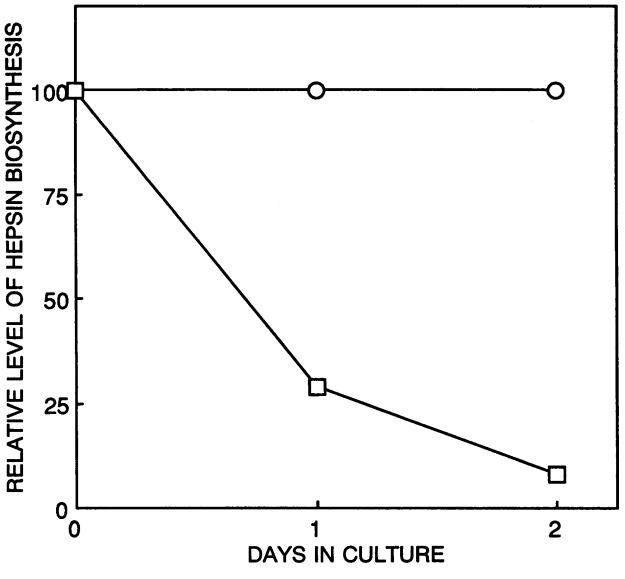

FIG. 2. Effects of AS-pt-oligo237 and RAS-pt-oligo237 on hepsin biosynthesis in PLC/PRF/5 cells. Relative levels of hepsin biosynthesis in cells treated with AS-pt-oligo237 (□) for various times are shown as a percentage of those treated with RAS-pt-oligo237 (controls) (○).

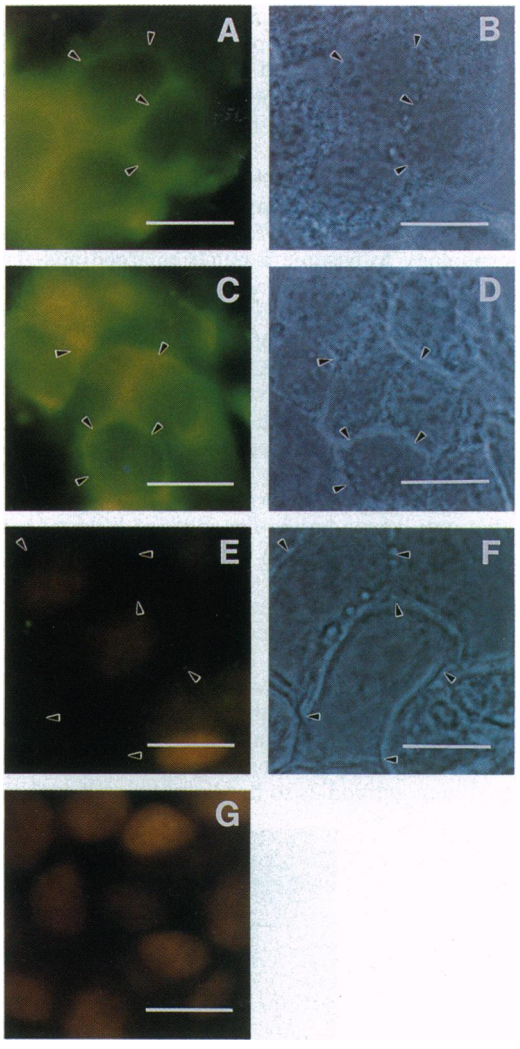

FIG. 3. Effects of hepsin AS oligos on hepsin level and morphology of PLC/PRF/5 cells. Cells on day 4 of treatment with oligo or anti-hepsin antibody (HAbP5) were subjected to indirect immunofluorescence localization of hepsin (*A*, *C*, and *E*), and their phase-contrast images were photographed (*B*, *D*, and *F*). Presence of hepsin is represented by greenish fluorescent color. (*A* and *B*) Anti-hepsin antibody. (*C* and *D*) RAS-pt-oligo. (*E* and *F*) AS-pt-oligo. (*G*) AS-pt-oligo except no anti-hepsin antibodies were added. (Bars = 24.7 μm.)

AS Oligo Inhibits Biosynthesis of Hepsin, Which Is Accompanied by a Gross Change of Cell Morphology. In the cells treated with AS-pt-oligo237, levels of hepsin biosynthesis were rapidly lowered within the first 2 days, supporting the direct inhibitory effects of AS-pt-oligo237 on hepsin biosynthesis (Fig. 2). This also agreed well with the rather short half-life of hepsin (≈3.5 hr) estimated by pulse labeling of cells with [^{35}S]methionine (data not shown). The effects of AS-pt-oligo237 on cell morphology were noticeable on day 2 of the oligo treatment and became more marked thereafter. A similar but less severe or no effect on cell morphology was observed with AS-pt-oligo952 or with AS-pt-oligo1720, respectively, in good correlation with the weaker effects of these oligos on cell growth.

Effects of AS-pt-oligo237 on hepsin level and cell morphology were then examined by indirect immunofluorescent staining with anti-hepsin antibodies and phase-contrast microscopy. Typical cell-surface staining of hepsin, previously obtained with HepG2 cells (2), was also observed for PLC/

PRF/5 cells on day 4 of treatment with anti-hepsin antibody (HAbP5) (Fig. 3*A*) or RAS-pt-oligo237 (Fig. 3*C*). Similar stainings were also observed for cells treated with SS-pt-oligo237 or water (data not shown). These cells also showed no significant differences in morphology compared to the untreated control cells (Fig. 3 *B* and *D*). However, cells treated with AS-pt-oligo237 showed a dramatically reduced level of hepsin, with little hepsin staining at the cell surface as well as boundaries (Fig. 3*E*), and a striking alteration in cell morphology to assume a much enlarged and flattened appearance (Fig. 3*F*). These results indicate that the AS-pt-oligo237 effectively suppresses hepsin biosynthesis, resulting in only a background level of hepsin. Essentially identical effects of AS-pd-oligo237 on cell morphology and hepsin biosynthesis were also observed, while SS-pd-oligo237 and AS-FIX-oligo showed no such effects (data not shown). The loss of hepsin at the cell surface following AS oligo treatment was correlated with grossly suppressed cell growth and accompanying substantial morphological changes. It is interesting that anti-hepsin antibodies induced little morphological change while substantially inhibiting cell growth, which suggests that the presence of accessible intact hepsin at the cell surface is essential for cell growth, whereas the presence of hepsin molecules associated with the plasma membrane alone or in combination with its possible presence in other subcellular organelles (2) is required to maintain normal cell morphology. The presence of the normal level of hepsin at the surface of cells treated with anti-hepsin antibodies as shown by fluorescent immunostaining (Fig. 3*A*) indicates that the binding of such antibodies to hepsin at the cell surface does not change the level of hepsin biosynthesis or its cellular concentration. Neither growth rate nor morphology of rat endothelial cells was affected by AS-pt-oligo237, further supporting the notion that the effects of hepsin AS oligos are specific and not due to any nonspecific effects of the oligos on cells (data not shown).

Expression of Hepsin at Various Developmental Stages in Mice. As a putative protease, hepsin may be involved in the degradation of extracellular proteins to create spaces for their normal growth, which requires cell shape change and migration. If hepsin does play an essential role in cell growth, it may be produced at a high level, particularly in the actively growing and rearranging tissues of the developing embryo. To test this hypothesis, we examined hepsin expression in the developing mouse embryo. At the earliest stage of development examined (day 10), there was a relatively low level of hepsin present throughout the embryo (data not shown). As shown in Fig. 4, by day 13, hepsin was sparsely deposited in the forming surface ectoderm and colonic epithelium (data not shown), increasing significantly over the next several days both in intensity and in the number of tissues stained. The observed unique pattern of developmental regulation of hepsin expression strongly supports the suggestion that this protein plays an important role in cell growth and tissue rearrangement, particularly at highly circumscribed developmental stages.

Proteases have been implicated in a number of cellular processes such as cell growth and migration (10–15), metastasis (16), and tissue rearrangements involved in morphogenesis (17). In these processes, proteases are assumed to

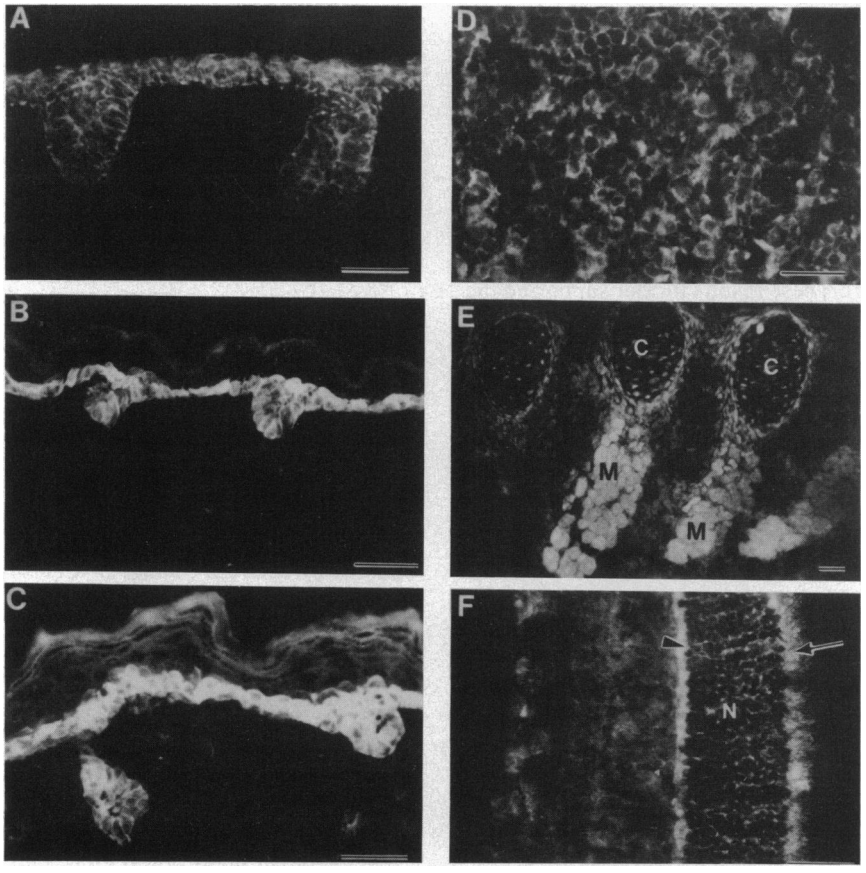

FIG. 4. Immunofluorescence localization of hepsin in selected tissues. Transverse sections through developing skin and forming hair follicles on day 14 (*A*), day 17 (*B*), and day 19 (*C*) of gestation. Presence of hepsin is shown as white area. Note progressive restriction in deposition from epithelium to basal layer of forming epidermis. (*D*) Section through fetal liver on day 17 of gestation illustrating diffuse cell-surface staining of forming hepatic cords. (*E*) Section through forming cartilage (C) and associated intercostal muscle (M) from day 19 embryo. (*F*) Transverse section through an adult retina illustrating staining of horizontal cells in outer plexiform layer (arrowhead) and diffuse staining of outer nuclear layer (N) as well as in association with rods and cones (arrow). (Bars = 50 μm.)

create space for cell migration and process extension through an extracellular matrix and cell-filled milieu. Some cell-surface proteases have also been shown to function as receptors for viruses (18–20) and to be involved in membrane protein catabolism (1). The present results demonstrate at the molecular level that hepsin, a putative trypsin-type surface protease, is indispensable for cell growth and for maintenance of normal cell morphology and suggest its possible involvement in other cellular processes. Preliminary studies show that affinity-purified hepsin can be progressively activated by a catalytic amount of trypsin, resulting in activated hepsin with substantial activity (1.2% activity relative to that of trypsin) toward a synthetic peptide substrate, *N*-benzoyl-Leu-Ser-Arg-pNA·HCl. *In vitro* activation of hepsin by trypsin is almost totally inhibited by HAbP5. HAbP5 also inhibits hepsin activity toward the synthetic peptide substrate to ≈30% that without antibodies. Whether the mechanism underlying the critical role of hepsin in cell growth *in vitro* and *in vivo* may involve solely its proteolytic activity or hitherto unrecognized properties is currently not known. Its precise substrate specificity toward both peptide and protein substrates, kinetic characteristics, and a protease(s) responsible for hepsin activation *in vivo* remain to be determined.

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