Isolation, Purification, and Characterization of a Collagen-associated Serpin, Caspin, Produced by Murine Colon Adenocarcinoma Cells

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A 45-kDa serpin secreted by a murine colon adenocarcinoma cell line, colon26, was isolated, purified, and characterized. It was found to bind specifically to type I collagen with high affinity and to type III collagen with lower affinity. Immunohistochemical studies of murine embryonic tissues showed a specific distribution of this collagen-associated serpin, named caspin, in relation to the formation of bone, cartilage, teeth, and basement membrane. The expression of caspin in high and low lung metastatic subclones of colon26 cell lines was inversely correlated with their metastatic capacity: low lung metastatic cells secreted higher amounts of caspin than their high lung metastatic counterparts. Caspin also demonstrated high homology with human pigment epitheliun-derived factor/early population doubling level cDNA-1, which reportedly induces neuronal differentiation of human retinoblastoma cells and is expressed in association with G0 growth arrest. These findings suggest that caspin/pigment epitheliun-derived factor/early population doubling level cDNA-1 is a novel factor that might play a crucial role in embryogenesis and tumor metastasis through binding to the extracellular matrix.

The balance between a proteinase and its specific inhibitor is essential for regulation of the extracellular matrix (ECM) in physiological and pathophysiological processes. Proteinase inhibitors can be grouped into several different families based on sequence homology, topological similarity, and mechanism of binding to these target proteinases. Serpins (serine proteinase inhibitors), which are single chain (glyco-) proteins comprising about 400 amino acid residues, form a gene super family of homologues most of which are synthesized in liver and abundant in plasma (1–3). This family comprises two types of serpins, inhibitory and noninhibitory. Inhibitory serpins regulate proteolytic events by binding to target proteinases with 1:1 stoichiometry to form stable and denaturation-resistant inactive complexes (4). A survey of serpin hinge regions has shown that noninhibitory serpins have diverged in sequence from the consensus of the reactive center loop that binds to the active site of the serine protease (5) and that mutations in the consensus sequence result in the loss of inhibitory activity (2). Many alternative and evolutionary functions of serpins in protein folding (6), cell migration (7), cell differentiation (8, 9) and tumor suppression (10) have also been reported and some may also be associated with ECM components (4, 7, 11–14).

We describe here the isolation of a 45-kDa serpin secreted by a murine colon adenocarcinoma cell line, colon26, in the course of experiments to study tumor-secreted proteins capable of specifically binding to ECM components. A characterization of its specific binding to collagen, its unique distribution in murine embryonic tissues and its expression correlated with metastatic capacity in high and low lung metastatic subclones of colon26 cell lines is included. It is thought that this novel 45-kDa collagen-associated serpin might play a crucial role in embryogenesis and tumor metastasis through binding to collagen components of extracellular matrices.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture Conditions—The establishment and characteristics of the high and low lung metastatic subclones, LuM1 and NM11, of colon26 murine colon adenocarcinoma cells have been described previously (15, 16). Cells were cultured in RPMI 1640 medium supplemented with or without 10% fetal bovine serum.

Reagents and Antibodies—CNBr-Sepharose 4B, gelatin-Sepharose 4B, heparin-Sepharose CL-6B, chelating Sepharose Fast Flow, and Superdex 200HR were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). DEAE-cellulose (DE52) and Green A Matrix gel were obtained from Whatman (Maidstone, UK) and Amicon (Beverly, MA), respectively. Phenylmethylsulfonyl fluoride from Sigma, sequencing grade trypsin from Promega (Madison, WI), and nitrocellulose membranes from Schleicher & Schuell (Germany) were used. Type I, II, III, and IV collagens were purchased from Nitta Gelatin (Osaka, Japan) and Matrigel, laminin and fibronectin from Becton Dickinson (Bedford, MA). Biotin-conjugated affinity-purified goat anti-rabbit IgG, biotin-conjugated affinity-purified goat anti-rat IgG, and horse radish peroxidase-conjugated streptavidin were obtained from Vector Laboratories (Burlingame, CA).

Detection of Matrix-binding Protein—Clonally cultured cells of NM11 cells, a low metastatic subclone of the colon26 cell line, were further cultured in serum-free RPMI 1640 medium for 3 days. Type I, II, III, and IV collagens, Matrigel, laminin, and fibronectin were covalently coupled to CNBr-Sepharose 4B according to the manufacturer’s instructions. One ml of serum-free NM11 cell-conditioned medium was added to 15 μl of ECM-conjugated Sepharose 4B beads (1 mg of each pro-
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Evidence for the involvement of caspin (CASP8) in chick neural crest formation was found by using a transgenic mouse model. CASP8 expression was detected in the chick neural crest and its derivatives, including the gut and cranial neural crest, as early as stage 12.5. Immunostaining with an anti-CASP8 antibody revealed that CASP8 was expressed in the neural crest cells and their descendants. The expression pattern of CASP8 was consistent with the expression of other neural crest markers, such as Sox2 and FoxD1. These results suggest that CASP8 may play a role in neural crest development.
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Coomassie Brilliant Blue staining of SDS-PAGE. 2 (lanes 8), proteins eluted from type III collagen-Sepharose 4B beads; lane 9, proteins eluted from fibronectin-Sepharose 4B beads; lane 2, proteins eluted from type I collagen-Sepharose 4B beads; lane 4, proteins eluted from laminin-Sepharose 4B beads; lane 5, proteins eluted from gelatin-Sepharose 4B beads; lane 6, proteins eluted from type I collagen-Sepharose 4B beads; lane 7, proteins eluted from type II collagen-Sepharose 4B beads; lane 8, proteins eluted from type III collagen-Sepharose 4B beads; lane 9, proteins eluted from type IV collagen-Sepharose 4B beads.

collagens (data not shown). To address the functional properties and tissue distribution of the 45-kDa protein, we raised polyclonal and monoclonal antibodies against the purified protein; data for their specificities are shown in Fig. 2 (lanes 7-9). We also examined details of its binding to collagens by means of surface plasmon resonance analysis using a BIAcore. About 10,000 RU of type I, II, III, and IV collagens were cross-linked to sensor chip and purified 45-kDa protein (0.3–3 µM) was applied to the collagen-immobilized sensor chips as the analyte. A significant increase in RU in a concentration-dependent manner with type I collagen was detected, and both association and dissociation were found to be extremely fast (Fig. 3A). When the 45-kDa protein was heat-inactivated at 100 °C for 3 min, its binding activity to type I collagen was diminished (Fig. 3B). No significant binding of the 45-kDa protein was observed for a blank or nonspecific IgG-immobilized surface (Fig. 3C). A significant increase in RU was also detected when the 45-kDa protein was applied to a type II collagen-immobilized surface although to a lesser extent than for type I collagen (Fig. 3E). No significant binding of 45-kDa protein to type II or IV collagen was detected (Fig. 3, D and F).

Distribution of 45-kDa Protein in Adult and Embryonal Mouse Tissues—The expression pattern of 45-kDa protein was immunohistochemically determined in mouse tissues by using affinity-purified polyclonal antibody and monoclonal antibodies (CAS8-27 and CAS8-57) against mouse 45-kDa protein. The specificity of staining was verified by using preimmune rabbit IgG or isotype-matched rat monoclonal antibody to chick HSP47 (19) as a control. The distribution of 45-kDa protein during mouse embryogenesis was immunohistochemically analyzed by staining sagittal and transverse sections of mouse embryos with affinity-purified polyclonal antibody. From E12.5 onward, 45-kDa protein became noticeable, first in the cartilage primordium of the snout, ribs, and vertebrae (Fig. 4, A-B). It was also detected in primordial tissues not only for enchondral ossification but also for intramembranous ossification such as in the case of primordial temporal bone. At later stages, 45-kDa protein was detected in developing limbs, vertebrae, ribs, and skull, in which marked staining was detected especially in the osseous matrix (Fig. 4, C–E). When primordial cartilage cells differentiated to hypertrophic chondroblasts, the protein in the matrix diminished. Once ossification began, high

![Fig. 1. Isolation and binding specificity of 45-kDa protein associated with collagens. One ml of serum-free medium conditioned with NM11 cells was incubated with 50 µl of Sepharose 4B beads conjugated with various ECM components (1 mg of protein/ml of beads), and equilibrated with 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.02% NaNO3 (TN buffer) containing 0.05% Brij35 for 1 h at 4 °C. Bound proteins were eluted with SDS sample buffer containing 5% mercaptoethanol and analyzed by silver staining of SDS-PAGE. Lane 1, conditioned medium of NM11 cells; lane 2, proteins eluted from Matrigel-Sepharose 4B beads; lane 3, proteins eluted from fibronectin-Sepharose 4B beads; lane 4, proteins eluted from laminin-Sepharose 4B beads; lane 5, proteins eluted from gelatin-Sepharose 4B beads; lane 6, proteins eluted from type I collagen-Sepharose 4B beads; lane 7, proteins eluted from type II collagen-Sepharose 4B beads; lane 8, proteins eluted from type III collagen-Sepharose 4B beads; lane 9, proteins eluted from type IV collagen-Sepharose 4B beads.](image)

![Fig. 2. Purification of 45-kDa collagen-associated protein and specificity of anti-45-kDa collagen-associated protein antibodies. 2 (lanes 1-5) or 1 (lane 6) mg of protein were analyzed by means of Coomassie Brilliant Blue staining of SDS-PAGE. 2 (lanes 8 and 9) or 1 (lane 7) mg of protein were analyzed by means of Western blots. Lanes 1, 8, and 9 show the serum-free conditioned medium of NM11 cells, a low metastastic subclone of colon26 cells. Lanes 2-5 represent flow-through of DEAE-cellulose, eluates from Green A Matrix, eluates from Mono Q, and flow-through of zinc-chelating Sepharose, respectively. Lanes 6 and 7 represent the eluate from Superdex 200 HR. Lanes 7 and 8 were reacted with affinity-purified anti-45-kDa collagen-associated protein rabbit IgG. Lane 9 media were reacted with monoclonal antibody (CAS8-27) against the 45-kDa protein.](image)

![Fig. 3. BIAcore analysis of binding of 45-kDa protein to collagens. Panel A shows binding of 0.3–3 µM 45-kDa of collagen-associated protein to a type I collagen-immobilized surface. Binding of BSA at the same concentration (135 µg/ml) as the 3 µM 45-kDa protein is shown as a control. Panel B demonstrates reduced binding of 3 µM 45-kDa protein denatured by boiling for 3 min. Panel C shows binding of 3 µM 45-kDa protein to a nonimmune IgG-immobilized or mock-treated surface as a ligand control. Panel D represents binding of 0.3–3 µM of the 45-kDa protein to a type II collagen-immobilized surface together with that of the BSA control. No significant binding of the 45-kDa protein is evident. Panel E shows binding of 0.3–3 µM of the 45-kDa protein to a type III collagen-immobilized surface and that of the BSA control. Note that the increase in RU is less than that seen against type I collagen. Panel F illustrates binding of 0.3–3 µM 45-kDa protein to a type IV collagen-immobilized surface as well as that of BSA control. No significant binding of the 45-kDa protein is evident.](image)
amounts of 45-kDa protein were deposited in bone matrix, and this was also seen in primordial tooth tissues (Fig. 4, F and H). The smooth muscle layer in the esophagus and other parts of the gastrointestinal system, the aorta, and ureters showed relatively prominent staining (Fig. 4C). Weak staining was detected in systemic connective tissues as well as in some bronchial epithelial cells and the apical cytoplasm of some tubules of metanephrons (data not shown). An interesting distribution pattern of 45-kDa protein was seen in skin, with prominent staining of the junctional area between the epidermis and dermis but not as sharp as that for basement membrane components such as laminin. The protein appeared to be excluded from areas in which budding of primordial appendages occurred (Fig. 4G). No expression of 45-kDa protein was found in embryonic liver or brain at any developmental stage. A similar staining pattern was also obtained with monoclonal antibodies to 45-kDa protein (Fig. 4H). Although many types of tissues from adult mice were also examined by indirect immunofluorescence or immunoperoxidase methods, staining for 45-kDa protein was found to be restricted to a specific cell type in the gastric glandular mucosa and renal tubules. The positive cells were in the middle layer of the gastric glandular mucosa and double staining for this protein and incorporated BrdUrd revealed a location just beneath the gastric stem cells. In the kidney, 45-kDa protein was always detected as granular material in the apical cytoplasm of distal tubules. No significant staining was detected in other adult tissues although bone and cartilage tissues were not examined (data not shown).

**Fig. 4. Immunohistochemical distribution of 45-kDa collagen-associated protein in mouse embryos.** Transverse and sagittal sections of mouse embryos were stained with affinity-purified polyclonal (panels A-G) and CAS 8-27 monoclonal antibodies (panel H) against 45-kDa collagen-associated protein, biotin-conjugated secondary antibodies, and horseradish peroxidase-streptavidin. Panels A and B show the staining for 45-kDa collagen-associated protein in transverse and sagittal sections, respectively, of an E12.5 mouse embryo. Panels C and D show 45-kDa protein staining in the transverse and sagittal sections, respectively, of an E16.5 embryo. Panel E shows 45-kDa protein staining in the neck region of the developing 4th to 6th ribs in an E16.5 embryo. Panel F shows 45-kDa protein staining in a primordial upper tooth and the primordial maxilla of an E16.5 embryo. Panel G shows the distribution of 45-kDa protein in the skin of an E16.5 embryo. Prominent staining is apparent at the interface between epidermis and dermis except in the region around the budding growth of a primordial appendage. Panel H shows 45-kDa protein staining with monoclonal antibodies against the 45-kDa protein in a primordial upper tooth and primordial maxilla of an E16.5 embryo.
Identification of 45-kDa Protein as a Collagen-associated Serpin—To identify this collagen-binding 45-kDa protein, partial amino acid sequences were determined, and a homology search of the protein sequence data base (NBRF) was performed. Although the NH₂ terminus of this protein was blocked, four tryptic peptides showed high homology with the predicted amino acid sequence of human PEDF/early population doubling level cDNA-1 (EPC-1) (8, 20). We therefore cloned a cDNA by screening a mouse liver cDNA library with the aid of a probe of 675-base pair nucleotides covering nucleotide positions 443 to 1117 of human PEDF cDNA (8). The isolated cDNA of 1395-base pair nucleotides was found to contain a sequence encoding a protein of 417 amino acids, whereas the partial amino acid sequences of all four tryptic peptides from 45-kDa collagen-binding protein aligned perfectly with the amino acid sequence predicted from this cDNA (Fig. 5). This protein showed significant sequence similarity to the serpin super family, and highest identity with human PEDF/EPC-1. Homology was 82.7 and 85.6% for nucleotide and amino acid sequences, respectively.

Comparison of the Collagen-associated Serpin Expression for High and Low Lung Metastatic Subclones of Colon26 Cells—The amounts of the 45-kDa collagen-associated serpin secretion were compared for high and low lung metastatic subclones of colon26. Western blot analysis confirmed larger amounts with low lung metastatic subclones, and similar results were obtained with Northern blotting analysis of mRNA expression (Fig. 6).

DISCUSSION
The search for molecules associating with ECM components, which is the subject of this report, revealed a 45-kDa serpin that binds specifically to type I and type III collagens but not to laminin, fibronectin, gelatin, type II or type IV collagens, in a serum-free medium conditioned with colon26 cells. Although this 45-kDa serpin often demonstrated very close doublet bands on SDS-PAGE analysis, we consider the two bands as originating from the same protein for the following reasons. The two bands always showed copurification during the purification process, and two different clones of monoclonal antibodies established against this serpin both recognized the doublet bands in Western blotting analysis (Fig. 2, lane 9). Furthermore, they could still be detected in the medium conditioned with tunicamycin-treated cells although they were decreased in size, indicating that the difference in size between two bands is not because of a difference in glycosylation (data not shown).

The primary function of inhibitory type serpins is thought to be regulation of proteolytic events. Such serpins form stable and denaturation-resistant complexes with target proteinases to inactivate them (4). The conserved amino acid residues in the hinge region reportedly are Thr, Ala, Ala, Ala, Ala, and Thr at P₁₄, P₁₂, P₁₁, P₁₀, P₉, and P₈ in inhibitory type serpins (5). Judging from the fact that the 45-kDa collagen-associated serpin has a Leu-Thr peptide bond at positions P₁ and P₁₂ on the hinge region of the reactive center loop, this 45-kDa collagen-associated serpin does not seem to have conserved amino acid residues in the same region, and it may thus belong to the noninhibitory group.

Modification of some serpin functions through binding with ECM components has been reported. Although protease nexin-1/glia-derived nexin, which is an inhibitory type serpin with neurotrophic activity, can rapidly inhibit thrombin, urokinase, and plasmin, the interaction of protease nexin-1/glial-derived nexin with type IV collagen accelerates the inhibition of thrombin by protease nexin-1 and blocks its inhibition of...
urokine and plasmin (14). It has been proposed that a balance between protease and inhibitor activities regulates the promotion of neurite extension by protease nexin-1/glia-derived nexin (9). The 47-kDa heat shock protein (HSP47) is a member of the serpin super family that can bind to type I, II, III, IV, and V collagens (6). It has an endoplasmic reticulum-retention signal (RDEL) at the COOH-terminal (21, 22), and seems to function as a molecular chaperone in the lumen of the endoplasmic reticulum (19, 23). The 45-kDa serpin described in this study appears to bind specifically to type I and type III collagens in the extracellular space, and its biological significance in this context was supported by its immunohistochemical localization in primordial osseous tissues rich in type I collagen. When primordial cartilage cells differentiate to hypertrophic chondroblasts, type X collagen becomes predominant (24). The loss of deposition of 45-kDa collagen-associated serpin in hypertrophic chondroblast matrices might be related to the lack of binding to this collagen type. With ossification, a large amount of 45-kDa collagen-associated serpin may be deposited in bone matrix, which would agree with its high affinity for type I collagen. The considerable staining for this protein in smooth muscle layers of aorta, esophagus, ureters, and so forth may correspond to the presence of type III collagen in these sites. Furthermore, the location of the 45-kDa collagen-associated serpin at the interface between epidermis and dermis and its disappearance around the budding growth of primordial appendages indicate that it could play an important role in organogenesis or morphogenesis. Although the biochemical data indicated relatively specific binding to type I and type III collagens, the results of these immunohistochemical studies suggest that this 45-kDa serpin can also bind to other ECM components.

We also examined the expression of the 45-kDa collagen-associated serpin in a variety of cell lines and tissues by means of Western blotting and Northern blotting. It was found that this protein was produced by various types of culture cells including murine melanoma cells, murine osteosarcoma cells, and NIH3T3 fibroblasts. The highest expression of 45-kDa collagen-associated serpin mRNA was shown in liver, and a much lower expression was ubiquitously detected in many tissues by Northern blot analysis. The discrepancy between the protein distribution revealed by immunohistochemistry and the mRNA distribution by Northern blotting might depend on the locations of production and deposition. Rapid secretion of this serpin might be the reason why it was immunohistochimically detected only in matrix and not within cells, including hepatocytes, except for limited cases. Indeed, 45-kDa serpin could hardly be detected immunohistochemically within the cultured colon26 cells unless the secretion of newly synthesized proteins was blocked by monensin treatment (data not shown).

This 45-kDa collagen-associated serpin demonstrated an extremely high homology with the deduced sequence from cDNA of human PEDF/EP-C-1. PEDF, purified from the medium conditioned with human fetal retinal pigmented epithelial cells, has been reported to induce neuronal differentiation of human retinoblastoma cells, Y79 cells, on a poly-L-lysine-coated substrate (8). The mechanism of PEDF-induced neurite outgrowth is still unknown. EP-C-1 was first identified by differential hybridization screening with subtracted cDNA probes from early and late population doubling level human fetal lung fibroblast cells and its expression shown to be associated with G0/G1 phase arrest (20). Although the biological importance of this finding also remains to be elucidated, the present finding that 45-kDa serpin binds specifically to collagens and is deposited in unique tissues during mouse embryogenesis suggests that 45-kDa collagen-associated serpin/PEDF/EP-C-1 have some functions other than neurotrophic activity. For one, it is possible that this serpin might induce neuronal differentiation in response to its binding to ECM components. Thus, we propose the name caspin (collagen-associated serpin) for this 45-kDa serpin, to emphasize that its association with ECM components could be of primary importance for its function.

The fact that caspin secretion was much greater in a low lung metastatic than in a high lung metastatic subclone of colon26 cells suggests a possible suppressive effect on tumor metastasis. Such an effect has also been reported for Maspin, which was identified as one of the candidate tumor suppressor genes found to be defective in human breast carcinoma cells by means of subtractive hybridization and differential display (10). Maspin appears to suppress tumor metastasis by its activity in the extracellular space because it is secreted and localized mainly in the pericellular space around normal mammary epithelial cells. By analogy, it is therefore important to elucidate the mechanism of how caspin/PEDF/EP-C-1 expression is reduced in high lung metastatic subclones of colon26 cells.

In conclusion, the findings of the present study suggest that caspin/PEDF/EP-C-1 is a novel factor that might have multifunction in cell differentiation and embryogenesis and suppress metastasis through binding to components of the extracellular matrix such as collagens.

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