Fibroblast Activation Protein, a Dual Specificity Serine Protease Expressed in Reactive Human Tumor Stromal Fibroblasts

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Proteolytic degradation of extracellular matrix (ECM) components during tissue remodeling plays a pivotal role in normal and pathological processes including wound healing, inflammation, tumor invasion, and metastasis. Proteolytic enzymes in tumors may activate or release growth factors from the ECM or act directly on the ECM itself, thereby facilitating angiogenesis or tumor cell migration. Fibroblast activation protein (FAP) is a cell surface antigen of reactive tumor stromal fibroblasts found in epithelial cancers and in granulation tissue during wound healing. It is absent from most normal adult human tissues. FAP is conserved throughout chordate evolution, with homologues in mouse and Xenopus laevis, whose expression correlates with tissue remodeling events. Using recombinant and purified natural FAP, we show that FAP has both dipeptidyl peptidase activity and a collagenolytic activity capable of degrading gelatin and type I collagen; by sequence, FAP belongs to the serine protease family rather than the matrix metalloproteinase family. Mutation of the putative catalytic serine residue of FAP to alanine abolishes both enzymatic activities. Consistent with its in vivo expression pattern determined by immunohistochemistry, FAP enzyme activity was detected by an immunocapture assay in human cancerous tissues but not in matched normal tissues. This study demonstrates that FAP is present as an active cell surface-bound collagenase in epithelial tumor stroma and opens up investigation into physiological substrates of its novel, tumor-associated dipeptidyl peptidase activity.

Solid tumor growth beyond a critical size of 1–2 mm is dependent upon the formation of a supporting tumor stroma, which, in epithelial cancer, comprises extracellular matrix proteins (ECM),¹ newly formed tumor blood vessels, activated tumor stromal fibroblasts, and, in some cases, leukocytic infiltrates (1, 2). Proteolysis of the ECM contributes to the processes of tumor cell invasion and metastasis and to sprouting of tumor capillaries, thus permitting malignant tumors to spread as well as acquire nutrients and discard wastes. The complex interplay between the multiple cell types involved, including the malignant cells themselves, the endothelium, and tumor stromal fibroblasts, remains poorly understood. Identification of uniformly expressed tumor stromal markers and elucidation of their functions in the tumor remain major challenges to better understand the dynamics of tumor tissue organization and remodeling.

One recently identified marker of reactive tumor stromal fibroblasts, the so-called fibroblast activation protein (FAP), is expressed in over 90% of common human epithelial cancers, and its expression in tumors is restricted to the tumor stromal fibroblast, as detected by immunohistochemistry with the prototype monoclonal antibody (mAb) F19 (3) and other FAP-specific antibodies (4). FAP is a type II membrane-bound glycoprotein whose sequence suggests its kinship to dipeptidylpeptidase-IV (DPP-IV) (5); in fact, the chromosomal location and organization of the FAP gene (6) suggest a common ancestry through gene duplication. Conservation of the FAP gene has been observed in several species. A mouse FAP homologue (87% identical predicted amino acid sequence) has been identified (7), and, like human FAP, it is expressed in tumor stroma. A Xenopus laevis FAP homologue (8) has also been described, using differential display, and its expression is up-regulated during hormone-induced tail resorption, indicating a possible role in tissue remodeling. This study was designed to link the known expression pattern of human FAP in tumor stromal fibroblasts with a biochemical function that may help to explain its role in tumorigenesis and other tissue remodeling events.

EXPERIMENTAL PROCEDURES

Materials—Geneticin, Lipofectin, DMEM, and DMEM/F12 media were obtained from Life Technologies, Inc. Fetal bovine serum was supplied by Biological Industries (Kibbutz Beit Hemeetz, Israel). All restriction enzymes were from Roche Molecular Biochemicals. vine trypsin, and DMEM lacking methionine and cysteine were from Sigma. Radiolabeled diisopropylfluorophosphate as14C-DFP or 3H-DFP was a kind gift of Dr. R. Barton (Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT). HT1080, a human fibrosarcoma cell line, and 293 cells (a human embryonal kidney epithelial cell line) were obtained from American Type Tissue Collection (Manassas, VA). Bati-mastat® was obtained from British Biotech (Oxford, UK). High Five cells, pVL1393 vector, and SP9 cells were obtained from Invitrogen (NV Leek, Netherlands). Molecular mass standards, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid, diisopropylfluorophosphate (DFP), bovine trypsin, and DMEM lacking methionine and cysteine were from Sigma. Radiolabeled diisopropylfluorophosphate as [3H]DFP or [14C]DFP and [35S]methionine/cysteine mix were from NEN Life Science Products. Primers for site-directed mutagenesis were obtained from MWG Biotech (Ebersberg, Germany). Dipeptidic 4-methoxy-β-naphthylamide and 7-amino-trifluoromethyl-coumarin (AFC) conjugates were obtained from Bachem (Heidelberg, Germany).

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¹ The abbreviations used are: ECM, extracellular matrix; FAP, fibroblast activation protein; mAb, monoclonal antibody; DMEM, Dulbecco’s modified Eagle’s medium; DFP, diisopropylfluorophosphatase; AFC, 7-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid, diisopropylfluorophosphate (DFP); bovine trypsin, and DMEM lacking methionine and cysteine were from Sigma. Radiolabeled diisopropylfluorophosphate as [3H]DFP or [14C]DFP and [35S]methionine/cysteine mix were from NEN Life Science Products. Primers for site-directed mutagenesis were obtained from MWG Biotech (Ebersberg, Germany). Dipeptidic 4-methoxy-β-naphthylamide and 7-amino-trifluoromethyl-coumarin (AFC) conjugates were obtained from Bachem (Heidelberg, Germany).

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Immunological Reagents—Protein A-Sepharose was obtained from Amersham Pharmacia Biotech. Antiserum used were: polyclonal rabbit anti-fibronectin (Sigma), rabbit anti-laminin (Sigma), and goat anti-type I collagen and goat anti-type IV collagen (Life Technologies, Inc.). Affinity-purified fluororescent isothiocyanate-labeled secondary antibodies were used as negative controls for 4 days. Rat anti-mouse mAb (clone 53–19C11) labeled (Fab')2 rabbit anti-mouse antibodies were obtained from Dianova (Hamburg, Germany). Affinity-purified anti-rabbit or anti-goat alkaline phosphatase-coupled antibodies were obtained from Dianova. Anti-FAP murine mAbs, FB68, FB52, and F19 have been described (3, 4); a chimerized version of F19 with a human IgG1 constant region (9) was produced for some experiments. Rat anti-mouse CD8 cDNA (clone 53–6.72) was from Roche Molecular Biochemicals. Biotinylated horse anti-mouse IgG and avidin-biotin complex immunoperoxidase were purchased from Vector Labs (Burlingame, CA).

Cell Culture and Transfections—293 cells were maintained in a DMEM/F12 mix 50:50 or DMEM, respectively. Cells were transfected with a FAP expression vector, pFAP.38 (5) using either calcium phosphate transfection (10) or Lipofectin, according to the manufacturer's instructions. Clones were selected with 200 μg/ml Geneticin; resistant clones were picked and examined for FAP expression by immunofluorescence with mAb F19 (3). Clones were expanded and maintained in DMEM plus 200 μg/ml Geneticin. High Five cells were cultured in serum free Insect Express medium (Boehringer Ingelheim Bioproducts, Heidelberg, Germany). Affinity-purified anti-rabbit or anti-goat alkaline phosphatase-conjugated antibodies were obtained from Dianova.

Metallic Labeling and Immunoprecipitations—Cells to be labeled were washed thrice with phosphate-buffered saline (PBS) and then incubated for 1 h in DMEM medium without methionine and cysteine. Cells were labeled overnight with 0.5 μCi of [35S]methionine/cysteine (Amersham, Buckinghamshire, UK) for 1 h in DMEM medium without methionine and cysteine. The culture supernatant of High Five cells infected with recombinant baculovirus were performed as described (11). The culture supernatant of High Five cells infected with recombinant baculovirus were performed as described (11). Extracts were centrifuged at 12,000 × g for 10 min, and supernatants were cleared by PD-10 size exclusion columns. Immunoprecipitations, SDS-polyacrylamide gel electrophoresis (PAGE), and fluorography were performed essentially as described (11).

FAP Fusion Constructs and Site-directed Mutagenesis—A CD8α cDNA clone (a kind gift of Dr. Marilyn Kehry, Boehringer Ingelheim Pharmaceuticals, Inc.) was used as a template to amplify the cDNA encoding the extracellular domain (ECD) of murine CD8α (GenBank™ accession number M12825), consisting of the N-terminal 189 amino acids of CD8α, using the polymerase chain reaction. A Sal I restriction site was included in the primer sequence on the 3' end for cloning purposes. Similarly, the FAP cDNA (including the first Xba I site) encoding the extracellular domain of FAP (amino acids 27–760) was amplified using PCR and a 5' Sal I site ligated to the FAP ECD cDNA by overlap extension PCR, generating a fusion protein construct, FAPmCD8α, similar in structure to the CD8α-CD40 ligand fusion protein, as described previously (12). The cDNAs were inserted into the pVL1393 vector. Control baculovirus containing only the CD8α ECD were also produced. Mutation of FAP cDNA (5) to create the SnaI → Ala mutant was performed by overlap extended PCR. The final construct was generated by restriction fragment exchange. All constructs were verified by sequencing both DNA strands.

Insect Cell Expression—Transfection of Sf9 cells and amplification of the resulting recombinant baculovirus were performed as described (13). The culture supernatant of High Five cells infected with recombinant FAPmCD8α baculovirus for 4 days was collected and clarified by ultracentrifugation, loaded onto a heparin-Sepharose column, and then for 10 min, followed by centrifugation (4,000 × g, 20 min at room temperature).

The detergent phase was diluted with 150 mM NaCl, 50 mM Tris-Cl buffer, pH 7.5, 5 mM CaCl2, 5 mM MgCl2, and 0.75% Empigen BB, and separated on concanavalin A-Sepharose, as described (14). Concanavalin A-bound fractions were eluted with 1 M glucose (or 0.25 M methyl-α-d-mannopyranoside), 150 mM NaCl, 50 mM Tris-Cl buffer, pH 7.4 and 0.1% Triton X-100. Samples were either analyzed immediately or stored frozen at −80 °C. Frozen samples of tumor or normal tissues (stored at −80 °C) were thawed on ice, diced with a scalpel, and snap frozen in liquid N2 and pulverized with a mortar and pestle. Extraction buffer was added, and the resulting slurry was homogenized with an Ular Soras homogenizer (Burlingame, CA). Affinity-purified protein eluting from the samples was carried out as described above for the cell lines.

Dipeptidyl Peptidase Assays and Enzyme Immunocapture Assay—Two quantitative assays for dipeptidyl peptidase activity were developed using Ala-Pro-afc as the substrate. In the first assay, membrane extracts (see above) were mixed with 5–10-fold volumes of 100 mM Hepes/100 mM Tris, pH 7.8, and added to an equal volume of 0.5 mM Ala-Pro-afc in reaction buffer followed by incubation for 1 h at 37 °C. Release of free AFC was measured in a Cytofluor fluorimeter (PerSeptive Biosystems, Inc.) using the 395 nm excitation/403 nm emission filter set. For 4-methoxy-β-naphthylamide-coupled substrates, a 355 nm excitation/404 nm emission filter set was used to measure 4-methoxy-β-naphthylamide release. Enzyme kinetic parameters (Michaelis–Menten Km values) were calculated (15) using membrane extracts in this assay.

In the second assay, FAP isolated from membrane extracts via an immunocapture step was tested for enzyme activity. Specifically, 96-well ELISA plates (Costar, Corning, NY) were coated overnight at 4 °C with 1 μg/ml F19 antibody or control antibody in PBS. In the case of F19 (see above), plates were coated with either 0.1 μg/ml F19 or 1 μg/ml rat anti-mouse CD8 overnight at 4 °C. Wells were then rinsed with wash buffer (PBS, 0.1% Tween 20), and excess binding sites were blocked with blocking buffer (5% bovine serum albumin in PBS) for 1 h at room temperature. Blocking buffer was removed; membrane extracts or supernatants were added and incubated for 1 h at room temperature. The unbound material was removed, wells were washed thrice with wash buffer, and dipeptidyl peptidase activity was assayed using 100 μl of Ala-Pro-afc (0.5 mM Ala-Pro-afc in reaction buffer) for 1 h at 37 °C. Background fluorescence (as measured using control antibody) was subtracted from each value. In control experiments, binding of antibody F19 to FAP did not detectably affect its dipeptidyl peptidase enzymatic activity.

Zymography—Zymography assays were performed essentially as described (16). For protease inhibitor analyses, samples were preincubated with the appropriate inhibitor for 15 min prior to addition of sample buffer. Gel incubation times were in the range of 50–60 h at 37 °C. Gelatinase activity was visualized as clear (unstained) areas of digested gelatin against a blue background following Coomassie Blue staining. Molecular mass standards run on the same gels, but under reduced conditions, were used to determine the apparent molecular masses of proteins with collagenase activity.

ELISA Assays—Indirect ELISA assays were performed using 96-well F19-coated plates prepared as described above. IgG-coated plates were used as negative controls. All incubations were performed at room temperature. All wash buffers and antibody dilutions were made in phosphate-buffered saline, pH 7.4 plus 0.02% Tween 20 (PBS-Tween). FAP isolated from membrane extracts was added in various dilutions, incubated for 1 h, and washed. Anti-FAP mAbs FB52 or F19 (1 μg/ml) were added, incubated for 1 h, and washed. Goat anti-mouse peroxidase-labeled IgG (0.1 μg/ml) was added for 1 h, and then plates were washed. Bound peroxidase-labeled antibody was detected using the substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid according to manufacturer’s instructions (Sigma)).

ECM Protein Degradation Assay—Native human ECM proteins (1 μg of fibronectin, 1 μg of laminin, 1 μg of type IV collagen, or 1 μg of type I collagen) were incubated with cell membrane extracts (5 μg of protein) for 16 h at 37 °C. Trypsin (5 ng/sample) was used as a positive control for ECM protein degradation. Reactions were terminated by addition of SDS sample buffer and heating to 95 °C for 1 min. Proteins were then subjected to SDS-PAGE (17) under reducing conditions and transferred to nitrocellulose membranes (18). Degradation of ECM proteins was visualized by immunoblotting with rabbit anti-fibronectin, rabbit anti-laminin, goat anti-type I collagen, or goat anti-type IV collagen antibodies, respectively (18). Bands were visualized using alkaline phosphatase-conjugated secondary antibodies and a color reaction with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Promega, Madison, WI).

Dipeptidyl Peptidase Overlay Assays—Cellulose diacetate membranes impregnated with Ala-Pro-afc were obtained from Enzyme Systems (Dublin, CA). Samples were separated by 8% SDS-PAGE, gels were washed with 2.5% Triton X-100 in PBS, pH 7.4, and proteins were allowed to renature in Tris-buffered saline (50 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl2, 5 mM MgCl2) for 15 min. The membrane was then
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RESULTS

Expression of Plasma Membrane-bound FAP and Soluble FAPmCD8 Fusion Proteins—First, transfection of the FAP-negative 293 cell line with an expression vector encoding full-length human FAP was used to generate stable clones (designated 293FAPwt) expressing cell surface, membrane-bound FAP protein. Cell surface expression was confirmed by indirect immunofluorescence staining of cytospin preparations with the anti-FAP mAb F19 (Fig. 1, A and B), as well as by cytofluorometric analysis of mAb F19-labeled, 293FAPwt unfixed cells (data not shown). Tissues were taken from the collection of the Ludwig Institute for Cancer Research. Indirect immunofluorescence on cytospin preparations and cytofluorometry of cell suspensions stained with mAb F19 or negative control mouse IgG was performed on nonpermeabilized cells following published procedures (20, 21).

are in close agreement with the predicted molecular mass of FAPmCD8 monomers (112 kDa) and dimers (224 kDa), following signal sequence cleavage. Moreover, purified FAPmCD8 is recognized in ELISA assays by anti-mCD8 mAbs as well as three different anti-FAP mAbs, directed against distinct FAP epitopes (data not shown). Finally, N-terminal amino acid sequencing of FAPmCD8 yielded the sequence QAPEL, corresponding to amino acids 30–34 of mCD8, confirming its identity.

FAP Is a Dipeptidyl Peptidase—To define the biochemical function of FAP, the membrane-bound and soluble forms of the protein were first analyzed for dipeptidyl peptidase activity, because sequence similarity suggests that human FAP is most closely related to dipeptidyl peptidase-IV (DPP-IV) (5, 22), a serine-type exopeptidase (23) specific for N-terminal Xaa-Pro sequences. Using a fluorogenic assay, we found that membrane extracts prepared from 293FAPwt cells specifically cleave a peptide substrate containing Ala-Pro-AFC (Fig. 3A), as well as Gly-Pro-AFC (Fig. 3B), Lys-Pro-AFC (Fig. 3C), and Asp-Pro-AFC (Fig. 3D), with the highest activity toward Ala-Pro-AFC (Fig. 3A). By contrast, similarly prepared membrane extracts of the parental, FAP nonexpressor cell line 293 lack this dipeptidyl peptidase activity. Among the dipeptidyl peptidase substrates included in this study (Fig. 4), FAP shows a preference (24) for Ala-Pro-AFC (Km = 0.46 ± 0.04 mM) as compared with Lys-Pro-AFC (Km = 0.9 ± 0.24 mM) or Gly-Pro-AFC (Km = 1.15 ± 0.32 mM).

Because the tests with 293FAPwt membrane extracts did not reveal whether FAP itself is a dipeptidyl peptidase or activates a latent dipeptidyl peptidase in 293 membrane extracts, further testing was carried out with the purified soluble FAPmCD8 fusion protein, which also possesses dipeptidyl peptidase activity (Fig. 3A). Dipeptidyl peptidase activity is recovered from FAPmCD8-containing culture supernatants fol-
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Fig. 3. A, detection of dipeptidyl peptidase activity in membrane extracts of human parental 293 cells and 293FAPw transfectants and of purified FAPmCD8 using Ala-Pro-AFC as substrate. B, immunoprecipitation of purified FAPmCD8 fusion protein followed by 8% SDS-polyacrylamide gel electrophoresis and dipeptidyl peptidase overlay assay. Dipeptidyl peptidase activity is evidenced by the appearance of a fluorescent signal (lane 1). No signal is observed when FAPmCD8 supernatant is immunoprecipitated with a control antibody (lane 2). Precipitation with mAb F19 of control CD8s baculovirus-infected supernatants yielded no signal in the overlay assay (data not shown).

Fig. 4. Eadie-Hofstee plot comparing FAP dipeptidyl peptidase substrates. AFC-coupled peptides (Gly-Pro-AFC, Ala-Pro-AFC, and Lys-Pro-AFC) were incubated at 37 °C with FAP enzyme (1 ng) derived from membrane extracts of the 293FAPw transfect cells. Release of free AFC from the peptides was measured in a fluorometer using the 390 nm excitation/538 nm emission filter set.

Fig. 5. Comparison of substrate specificities of FAP and DPP-IV. A, gelatinase zymography demonstrates gelatinolytic activity in 293FAPw extracts but not in 293 extracts or purified DPP-IV. B, FAPmCD8 shows gelatinolytic activity (lane no Mab) immunodepleted with an anti-FAP mAb (lane anti-FAP Mab) but not with IgG isotype control (lane hIgG). C, dipeptidyl peptidase activity control demonstrating that purified DPP-IV retains dipeptidyl peptidase activity after SDS-polyacrylamide gel electrophoresis as detected by dipeptidyl peptidase overlay assay. By contrast, FAP titrated to dilute out detectable dipeptidyl peptidase activity (C, lane 293FAP) still retains gelatinolytic activity (A, lane 293FAP). The amount of DPP-IV, although sufficient to produce a signal in the overlay, shows no detectable gelatinase activity (A, lane DPP-IV). SDS-PAGE was carried out under nonreducing conditions using 8% gels. Molecular masses are shown in kDa.

To compare dual enzymatic activities of FAP with those of its nearest known structural relative, DPP-IV, we tested purified DPP-IV protein (0.5 ng) for gelatinolytic activity. No DPP-IV gelatinolytic activity could be detected (Fig. 5A, lane DPP-IV), although the DPP-IV preparation used still retained a powerful dipeptidyl peptidase activity in the overlay assay (Fig. 5C, lane DPP-IV; 0.5 ng protein). In the same assay format, the dipeptidyl peptidase activity of FAP can be diluted out (Fig. 5C, lane 293FAP; 7 µl of membrane extract), while still retaining activity in the gelatinolytic assay (Fig. 5A, lane 293FAP; 7 µl membrane extract). Each enzyme retained either dipeptidyl peptidase activity (DPP-IV) or gelatinolytic activity (FAP) following SDS gel electrophoresis, demonstrating different substrate specificities for the two enzymes and ruling out the possibility that the two enzymes show different stabilities under the experimental conditions.

A Western blot-coupled protease assay was used to examine whether FAP cleaves only gelatin (denatured collagen) or also native ECM proteins. Membrane-enriched fractions of 293FAPw cell extracts and control 293 cell extracts were incubated with human fibronectin, laminin, and native collagens I and IV (Fig. 6). Immunoblotting with the relevant ECM protein-specific antibodies revealed specific degradation of type I collagen by FAP, visualized through disappearance of collagen bands (Fig. 6, top panel, lane 2). No FAP-mediated digestion was observed for the other ECM proteins tested (Fig. 6, bottom three panels). In control experiments, all ECM protein preparations tested were shown to be sensitive to trypsin cleavage (Fig. 6, lane 4 in each panel). Other ECM proteins tested (tenasin, vitronectin, collagens II and V) were resistant to FAP-mediated cleavage (data not shown). The results demonstrate that collagen I is a natural substrate of FAP.

Inhibitor Profiling of FAP Activity—The nature of the catalytic mechanism of FAP was probed using a series of inhibitors selective for distinct classes of proteases (Table I). Among the serine protease inhibitors tested, a distinctive pattern was observed. Only DFP, a broadly active serine protease and esterase inhibitor (24), significantly inhibited FAP activity with
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**TABLE I**

Characterization of FAP dipeptidyl peptidase activity with selective protease inhibitors

| Protease inhibitor | IC 
|-------------------|--------------------------|
| DFP, 1 mM               | + (IC$_{50}$ = 20 μM) |
| PMSF, 100 μM              | + (IC$_{50}$ ≥ 100 μM)  |
| TLCK, 100 μM             | -                       |
| EDTA, 10 mM             | -                       |
| EGTA, 10 mM             | -                       |
| o-Phenanthroline, 2 mM  | -                       |
| BB94, 100 μM            | -                       |
| β-Mercaptoethanol, 100 mM | -                   |
| Iodoacetamide, 5 mM    | -                       |
| Pepstatin, 0.1 mM       | -                       |

no inhibition of FAP protease activity even at high concentrations. FAP enzymatic activity was insensitive to the metallo-protease inhibitors tested including EGTA, EDTA, and o-phenanthroline (26). Batimastat®, a potent, broad spectrum inhibitor of matrix metalloproteases (27), also showed no effect at concentrations up to 100 μM. Neither the cystine/cysteine-directed reagents (28), β-mercaptoethanol and iodoacetamide, nor pepstatin, an inhibitor of aspartyl proteases (29), showed any effect on FAP dipeptidyl peptidase activity at the concentrations tested.

**Mutational Analysis of FAP Gelatinolytic and Dipeptidyl Peptidase Activity**—Next, we investigated whether both enzymatic activities of FAP, namely its dipeptidyl peptidase and gelatinolytic activities, are dependent upon a single active site. Therefore, we replaced the putative catalytic serine of FAP, Ser$^{624}$, with alanine to generate a catalytically inactive FAP mutant (FAP$^{mut}$). FAP-negative 293 cells were transfected with the FAP$^{mut}$ cDNA vector, three transfected 293 cell clones were selected, and the presence of the mutation in these cells was confirmed by PCR. Immunoprecipitation of [³⁵S]methionine-labeled 293FAP$^{mut}$ cell extracts with mAb F19 yielded a 93-kDa band that comigrates with the band detected in 293FAP$^{wt}$ cells and is expressed at similar levels (Fig. 7C). Immunofluorescence analysis of these stably transfected 293FAP$^{mut}$ cells with several anti-FAP antibodies directed against distinct FAP epitopes indicated that FAP$^{mut}$ protein was expressed and properly transported to the cell surface (data not shown).

Membrane extracts of both 293FAP$^{wt}$ and 293FAP$^{mut}$ cells were produced, and the relative amounts of FAP protein were adjusted based on ELISA binding data. The 293FAP$^{wt}$ and 293FAP$^{mut}$ extracts were then tested for dipeptidyl peptidase and gelatinolytic activity. Both the dipeptidyl peptidase activity and the gelatinolytic activity of FAP$^{mut}$ were greatly reduced when compared with FAP$^{wt}$ (Fig. 7). These results demonstrate the key role of Ser$^{624}$ in both the dipeptidyl peptidase and gelatinolytic activities of FAP and suggest a single active site.

**Enzyme Immunocapture of FAP Activity in Tumor Tissues**—With the identification of suitable test substrates for FAP enzyme function, such as Ala-Pro-AFC, and the availability of FAP-specific mAbs, it has become feasible to monitor FAP activity not only in extracts of cultured cells but also in tissue biopsies. Accordingly, we have developed a FAP-specific enzyme immunocapture assay to study FAP activity directly in samples of human epithelial cancers. Tumor tissues and matched normal control tissues were obtained from cancer patients. Immunohistochemical analysis of these tissues revealed the presence of FAP immunoreactivity in the stromal fibroblasts of the malignant tissues but not in the normal control tissues (Fig. 8, A and B). Equal amounts of these matched normal and tumor tissues were extracted and subjected to enzyme immunocapture on mAb F19-coated ELISA plates. Bound dipeptidyl peptidase activity measured with Ala-Pro-AFC as substrate showed a distinctive pattern. FAP activity could be detected in extracts of all six tumor samples examined, but little or no activity was detectable in the matched normal tissues from the same patients (Fig. 8C). The Triton X-114 aqueous phase was largely depleted of this activity, indicating that most, if not all, of the immunoprecipitable FAP is associated with cell membranes (data not shown).

To confirm the molecular identity of the tumor-derived FAP enzyme activity, mAb F19 immunoprecipitates derived from one of the colon carcinoma samples (patient 3), and its matched control normal colon tissue were incubated with [¹⁴C]DFP. Labeled samples were separated by SDS-PAGE under reducing conditions. Sub-
sequent autoradiography of these samples revealed the presence of a labeled 95-kDa protein (Fig. 8D, two left lanes), present in the colon cancer but not in the normal colon tissue (Fig. 8D, second lane from left). We conclude from these results that FAP is expressed as an active protease in epithelial tumor tissues.

**DISCUSSION**

The specific and consistent expression of FAP in the reactive (nontransformed) tumor stroma of malignant epithelial neoplasms implies that it could play an important role in the formation, remodeling, or maintenance of the tumor compartment. To identify possible mechanisms through which FAP might influence these processes, we chose to examine whether FAP possessed any enzymatic activity. We found that FAP is a serine protease capable of degrading a number of dipeptides as well as an ECM component, collagen I. Moreover, we have demonstrated that FAP proteolytic activity is present in primary human epithelial tumor samples.

We have also shown that FAP is a member of the serine protease family. The classification of FAP as a serine protease is based on three lines of evidence. First, analysis of the FAP protein sequence (5, 7) revealed that it is a member of an
extensive family of prolyl-specific serine oligopeptidases (30), including DPP-IV (23). Second, of all enzyme inhibitors tested, only serine protease-specific inhibitors were able to inhibit FAP proteolytic activity. Finally, replacement of the canonical serine at position 624 disrupted both FAP enzymatic activities.

DPP-IV shares 50% sequence identity at the amino acid level with FAP. This similarity is reflected in the ability of both enzymes to cleave Xaa-Pro dipeptides, as well as their protease inhibitor sensitivity profiles (for DPP-IV, see Ref. 31). The affinity (460–1150 μM) of dipeptidic substrates for FAP described here is higher than that previously described for dipeptidyl peptidase II (2 mM) (32) but is lower than that of DPP-IV (approximately 11 μM) (33), depending on the P2 amino acid and fluorescent leaving group. From a kinetic standpoint, DPP-IV (kcat/Km = 4.7 × 106 M−1 s−1; Ref. 33) is a more robust dipeptidyl peptidase than FAP (kcat/Km = 1.3 × 105 M−1 s−1; this study) with Ala-Pro as its substrate. However, DPP-IV lacks collagenolytic activity and has a characteristic expression pattern, for instance, being found in activated T-cells (34) and on kidney and intestinal epithelia (35, 36) but not in FAP expressing cell types (5). A search of DNA and protein data bases identifies an additional, more distantly related molecule, DPPX, expressed in brain (37). The characteristic catalytic serine is not present in DPPX (38), and no enzymatic activity has been observed. Based on biochemical studies with the human LOX melanoma cell line, a putative serine protease-type gelatinase has been described (39), but in this case, no associated dipeptidyl peptidase or collagen type I degradation has been found (40); because LOX cells also show no immunoreactivity with anti-FAP mAbs, their gelatinolytic activity is likely due to an unrelated protease.

In our tests with both recombinant and natural FAP, we saw no evidence that proteolytic cleavage of a proenzyme is required for FAP activity. There are two lines of evidence arguing against major processing steps involved in FAP enzyme activity. First, any cleavage C-terminal of amino acid 26 would eliminate the entire transmembrane domain of the FAP protein and would result in the generation of soluble, shed FAP rather than the membrane-bound form found in our studies. Second, the putative FAP catalytic triad is located at the extreme C terminus of the FAP protein, and removal of just 27 amino acids from that end would remove the predicted catalytic histidine and thereby be expected to eliminate enzymatic activity.

Cleavage of gelatin and native collagen I places FAP into the group of enzymes possibly involved in tumor tissue remodeling. As experience with the MMPs shows, the presence of immunoreactive enzyme at tumor sites is not necessarily an indicator of its local activity, if a discrete activation step is involved. This is exemplified by the complex regulation of the MMPs (41, 42). The MMPs are first synthesized as inactive zymogens that may react with FAP activity is detected in tumor samples, and our extraction experiments with human tumor tissue show a close correlation between the presence of immunohistochemically detectable FAP and its dipeptidyl peptidase activity. The low level of FAP dipeptidyl peptidase activity seen in occasional, matched normal control tissues may reflect a quantitative, rather than absolute, difference in expression between malignant and normal tissues. It could also be the result of inflammatory processes (known to induce FAP expression; see Ref. 3) that may be present in some of the patient samples not involved by cancer. Alternatively, because some normal control tissues were derived from apparently uninvolved portions of surgical specimens in patients with metastatic disease (patient 4, for example), minor contamination of these tissues with micrometastases cannot be fully excluded.

Recent approaches to cancer therapy include inhibition of proteases present in tumor tissues (27, 48, 49). It is tempting to speculate that such enzymes, by degrading ECM components and/or activating latent growth factors, facilitate neangiogenesis, invasion, and metastasis. FAP may play multiple roles in these processes by virtue of its dual enzyme activities. DPP-IV is known to cleave and, in some cases, alter receptor specificities of peptide hormones and chemokines, such as neuropeptide Y (50), glucagon-like peptide (51), peptide YY (52, 53), and GCP-2 and RANTES (54, 55). Similarly, FAP may be capable of cleaving and, thereby regulating, as yet unidentified peptide growth factors in the tumor stroma via its dipeptidyl peptidase activity. In addition, invasive events, such as the recruitment of a tumor stroma or metastasis of tumor cells, require tissue remodeling mediated by ECM-degrading enzymes. Although a host of potential enzyme systems have been implicated in these processes, there appears to be considerable redundancy with partial overlap built into tissue proteolysis. Our demonstration that FAP possesses collagenolytic activity places it in the context of these events. The consistent correlation between FAP protease activity and its restricted expression in tumor stromal fibroblasts provides a striking contrast to the variability seen with some other tumor-associated proteases, including some MMPs that have already been activated (44), or via furin-dependent intracellular processing (45). MMPs are themselves transcriptionally regulated by various growth factors and cytokines (46, 47). After activation, protein inhibitors known as the tissue inhibitors of metalloproteases (42) may inactivate the MMPs. Thus, although immunologically reactive MMPs may be present in many tissues, their disease-related activity is subject to additional control. By contrast, the membrane-type MMPs are constitutively active, and it has been speculated that the local restriction through membrane anchoring is sufficient to prevent uncontrolled tissue damage. In this context, it is important that FAP is a cell surface-anchored serine protease of tumor stromal fibroblasts rather than a secreted, diffusible protease. The regulation of FAP activity therefore may parallel in some ways that of the membrane-type MMPs by combining constitutive biochemical activity with regional control of action through membrane association.

**Table II**

**Characterization of tumor samples and matched normal tissues from the same patients used for FAP enzyme capture assays and immunohistochemical analysis with anti-FAP mAbs**

| Patient (symbol) | Tumor type | Histological diagnosis | Matched normal tissue |
|-----------------|------------|------------------------|-----------------------|
| 1 (▼)           | Primary breast cancer | infiltrating ductal carcinoma | breast |
| 2 (●)           | Primary breast cancer | infiltrating ductal carcinoma | breast |
| 3 (●)           | Primary colon cancer | adenocarcinoma | colon |
| 4 (●)           | Metastatic colon cancer | adenocarcinoma (hepatocellular metastasis) | liver |
| 5 (▲)           | Primary lung cancer | adenocarcinoma | lung |
| 6 (●)           | Primary lung cancer | squamous cell carcinoma | lung |

[^2]: P. Garin-Chesa, J. E. Park, and W. J. Rettig, unpublished results.
MMPs (56–58), or urokinase (59, 60). We may have to await the results of careful gene inactivation studies for multiple proteases or, more likely, studies with highly selective inhibitors of these various proteolytic pathways to understand their importance for the invasive and metastatic phenotype of specific cancers as well as other developmental or disease-related tissue remodeling programs.

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