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Expression, Purification, and Kinetic Characterization of Full-Length Human Fibroblast Activation Protein

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Human fibroblast activation protein (FAP), an integral membrane serine protease, was produced in insect cells as a hexa-His-tagged protein using a recombinant baculovirus expression system. Two isoforms of FAP, glycosylated and nonglycosylated, were identified by Western blotting using an anti-His-tag antibody and separated by lectin chromatography. The glycosylated FAP was purified to near homogeneity using immobilized metal affinity chromatography and was shown to have both postprolyl dipeptidyl peptidase and postgelatinase activities. In contrast, the nonglycosylated isoform demonstrated no detectable gelatinase activity by either zymography or a fluorescence-based gelatinase activity assay. The kinetic parameters of the dipeptidyl peptidase activity for glycosylated FAP were determined using dipeptide Ala-Pro-7-amino-trifluoromethyl-coumarin as the substrate. The k_{cat} is 2.0 s^{-1} and k_{cat}/K_m is 1.0×10^4 M^{-1} s^{-1} at pH 8.5. The pH dependence of k_{cat} reveals two ionization groups with pK_{a1} of 7.0 and pK_{a2} of 11.0. The pH profile of k_{cat}/K_m yields similar results with pK_{a1} 6.2 and pK_{a2} 11.0. The neutral pK_{a1} is associated with His at the active site. The basic pK_{a2} might be contributed from an ionization group that is not involved directly in catalysis, instead associated with the stability of the active site structure.

Fibroblast activation protein (FAP)[3] is expressed specifically in reactive tumor stromal fibroblast cells, granulation tissue in healing wounds, and chronic inflammatory lesions, but not in normal human tissue (1–4). FAP acts as a matrix-degrading enzyme involved in tissue remodeling and cancer cell invasion via an unknown mechanism (5). Since it is highly expressed in tumor stroma, FAP has gained attention as tumor marker and a possible target for cancer therapy. For instance, radiolabeled antibody against FAP has been tested as a treatment for cancer patients (2).

FAP is located on the cell surface as a glycosylated 95-kDa protein. It was initially identified by a FAP-specific monoclonal antibody (4, 6). Sequence analysis reveals that FAP belongs to a family of membrane serine proteases with a catalytic triad of conserved Ser, Asp, and His residues. FAP has a type II membrane protein topology with a short N-terminal cytoplasmic tail (6 amino acids), a transmembrane domain (20 amino acids), and a large C-terminal extracellular domain (732 amino acids), which contains the putative catalytic site. FAP has been found to exhibit dipeptidyl peptidase and collagenase activities *in vitro* (7), but little is known about its natural substrate and the significance of its peptidase activity *in vivo*. The closest relative of FAP is dipeptidyl peptidase IV (DPPIV) with 54% amino acid sequence identity (8). Unlike FAP, DPPIV lacks the gelatinase activity and is expressed

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[3] Abbreviations used: FAP, fibroblast activation protein; DPPIV, dipeptidyl peptidase IV; ConA, concanavalin A; AFC, 7-amino-trifluoromethyl-coumarin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; TLCK, L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone–hydrochloride; PMSF, phenylmethylsulfonyl fluoride; FBS, fetal bovine serum; PVDF, polyvinylidene difluoride; BSA, bovine serum albumin; RFU, relative fluorescent units.

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in activated T-cells, kidney, and intestinal epithelia cells but not in FAP-expressing tissues, suggesting that FAP and DPPVI biological roles could be significantly different in spite of their sequence homology (9).

The extracellular domain of FAP (amino acids 27–760) has been produced as a CD8α fusion protein (7). In the same study, the full-length FAP was expressed in HEK293 cells and enriched by concanavalin A (ConA) affinity chromatography selective for glycoproteins, but was not purified to homogeneity, possibly due to solubility problems associated with a membrane protein (7). In this paper, we describe the production in insect cells and purification of full-length human FAP to near homogeneity for the first time. We also examine the enzymatic activity of FAP as a serine protease, which may help to understand its biochemical function.

MATERIALS AND METHODS

Materials

ConA–Sepharose 4B resin was purchased from Sigma-Aldrich Corp. Talon resin was from Clontech. Ala-Pro-AFC (7-amino-trifluoromethyl-coumarin) was supplied from Bachem. EnzCheck gelatinase/collagenase kit was from Molecular Probes. Zymogram gels (10% Tris–glycine gel with 0.1% gelatin) and zymogram developing and renaturing buffers were from Invitrogen. AFC was from Enzyme Systems Products. Triton X-100, methyl-D-mannopyranoside, imidazole, DMSO, DTT, and mercaptoethanol were from Sigma-Aldrich Corp. The protease inhibitors aprotinin TLCK (L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone–hydrochloride), PMSF (phenylmethylsulfonyl fluoride), chymostatin, phosphoramidon, and Pefabloc were from Roche Diagnostic Corporation. An anti-hexa-His monoclonal antibody was generated in our laboratories. ExpresSF+ cells were from Protein Sciences Corporation. *Trichoplusia ni* cells (BTI-Tn-5B1-4) were obtained from Dr. M. Shuler at Cornell University (Ithaca, NY) (10). These cells are also sold commercially by Invitrogen, known as High-5.

Construction of FAP Plasmid

Standard molecular biology methods were used to construct the FAP baculoviral transfer vector (11). Briefly, the 5′ portion of the FAP cDNA, containing residues −65 to +1449 (GenBank Accession No. U76833), was isolated by RT/PCR using cDNA prepared from WI38 cells. The resulting PCR fragment was ligated into the pTOP4 vector (Invitrogen). The final baculoviral transfer vector that expressed FAP with an N-terminal histidine tag was constructed using a three-way ligation. The 5′ portion of the FAP cDNA was amplified by PCR from the pTOP4/FAP vector using a 5′ oligonucleotide that introduced a *Bam*HI site immediately upstream of the initiator ATG. The resulting PCR fragment was digested with *Bam*HI and *Cla*I. The 3′ portion of the FAP cDNA was obtained by digesting IMAGE clone 965933 (GenBank AA528235) (Research Genetics) with *Cla*I and *Not*I. The 5′ and 3′ fragments of the FAP cDNA were then ligated with pFastBacHTb (Life Technologies Corp.) that was previously digested with *Bam*HI and *Not*I. DNA sequencing of the final vector confirmed that the FAP cDNA insert in pFastBacHTb was identical to the GenBank U76833 sequence.

Expression of Full-Length FAP

A recombinant baculovirus was prepared for expression of His-tagged FAP according to the Bac-to-Bac protocols from Life Technologies. The plasmid containing full-length FAP cDNA was transformed into DH10Bac *Escherichia coli*, allowing production of infectious viral DNA by transposition within the cells. Recombinant bacmid DNA was recovered and transfected into expresSF+ cells to allow production of virus. For viral amplification, a 1-ml aliquot of clarified medium containing the recombinant virus was recovered 4 days posttransfection and used subsequently to infect a T-75 flask containing 10^7 expresSF+ cells. Amplification was carried out for 3 days in 15 ml of culture medium (SF900 II SFM, Life Technologies, supplemented with 10% FBS, 2 mM glutamine, and 50 μg/ml gentamicin). The medium containing the amplified recombinant virus was clarified by centrifugation (5 min at 1500 rpm in a Sorvall H1000B rotor), and aliquots of 1 ml were further amplified in a 400-ml culture medium containing expresSF+ cells infected at an initial cell density of 2×10^6 cells/ml. Amplified virus was harvested by centrifugation 3 days postinfection at 27°C and the resulting viral stock was titered by a plaque assay and stored in the dark at 4°C before use.

Infections (m.o.i. = 5) were carried out at 27°C, typically in shake flasks containing 8×10^8 cells in 400 ml of expression medium. For expresSF+ cells, the expression medium was Sf900 II SFM supplemented with 2 mM glutamine and 50 μg/ml gentamicin. For *T. ni* cells, the expression medium was ExCell 405 (JRH BioSciences) supplemented with 2 mM glutamine and 50 μg/ml gentamicin. Cells were harvested 72 h postinfection by centrifugation (5 min at full speed in a benchtop microcentrifuge or 5 min at 2500 rpm in a Sorvall H-6000A rotor) and cell pellets containing recombinant FAP were stored at −80°C until purification.

Purification of Full-Length FAP

The cell pellet from 500 ml of cell culture was resuspended in 100 ml of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 1%

Triton X-100). Cells were lysed by sonication and cellular debris was removed by centrifugation at 16,000*g* for 30 min. The supernatant was collected and added to 20 ml of ConA–Sepharose resin. The suspension was incubated for 4 h at room temperature with gentle mixing. The ConA resin was collected by centrifugation and then washed with 10 vol of washing buffer (50 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Triton X-100) followed by incubation with elution buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 0.25 M methyl-D-mannopyranoside) for 1 h at room temperature. The ConA eluate was collected and then incubated with 1 ml Talon resin at 4°C overnight followed by 3 h at room temperature with gentle mixing. The Talon resin was washed extensively with washing buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 20 mM imidazole, and 0.1% Triton X-100) and then incubated with elution buffer (50 mM Tris, pH 7.5, 150 mM NaCl, and 500 mM imidazole) at room temperature for 1 h with gentle mixing. The supernatant containing purified FAP was collected and stored at 4°C.

Western Blotting and SDS–PAGE Analysis

The expression of FAP was analyzed by Western blot. Samples were prepared by suspending pelleted cells in 2× Laemmli sample buffer. One aliquot (10 μl) containing 10^5 lysed cells was subjected to electrophoresis on 4–20% polyacrylamide gel. Proteins in SDS–PAGE gels were transferred to a PVDF membrane (Bio-Rad) and processed with the WesternBreeze kit (Invitrogen) using an anti-His monoclonal antibody as the primary antibody.

The purification of recombinant FAP was analyzed by SDS–PAGE and Western blot. Protein samples were reduced by DTT and denatured by boiling in 1× SDS sample buffer. SDS–PAGE was carried out using Nu-PAGE 4–12% Bis-Tris gels (Invitrogen). Proteins were visualized by silver stain using the GelCode SilverSNAP stain kit (Pierce). The silver stained gels were scanned and analyzed using One-D scan analysis software (Scanalytic, Inc). Protein purity and concentration were calculated by comparing the density of the FAP protein band to the densities of all the other bands in the lane and to the density of the band of a known amount of BSA (Pierce) loaded on the same gel, respectively.

Dipeptidase and Gelatinase Activity Assays

The dipeptidyl peptidase activity of FAP was monitored by fluorometrically measuring hydrolysis of the synthetic dipeptide Ala-Pro-AFC. In a 96-well black plate (Packard), FAP was added to a final volume of 100 μl assay buffer (25 mM glycine, 25 mM acetic acid, 25 mM Mes, 75 mM Tris, and 100 mM NaCl, pH 8.0). The reaction was initiated by addition of Ala-Pro-AFC prepared in DMSO. DMSO concentration was adjusted to 3% in the final assay solution. Release of free AFC was monitored by CytofluorII (PerSeptive Biosystems, Inc.) using a 360-nm excitation/530-nm emission filter set. The initial rate was obtained by fitting the time course to a linear curve. All assays were performed at room temperature.

A serial dilution of free AFC was prepared in the same assay buffer and the corresponding fluorescence signal was measured. A standard curve of free AFC from 0 to 4 μM versus fluorescence signal was linear and independent of pH from 6.0 to 9.5.

To measure gelatinase activity, 10 μl of a 10 mg/ml solution of collagen type I or gelatin conjugated with fluorescein was mixed with FAP in 100 μl of assay buffer. Digestion of collagen or gelatin by FAP resulted in an increase in fluorescence, monitored by CytofluorII (PerSeptive Biosystems, Inc.) using a 485-nm excitation/530-nm emission filter set. The initial reaction rate was derived from a linear fit of the first 5 to 10 min time course data.

The zymogram was performed as described previously (12). Briefly, protein samples in sample buffer (80 mM Tris, pH 7.5, 1% SDS, 4% glycerol, 0.006% bromphenol blue) were loaded onto 10% polyacrylamide gel containing 0.1% gelatin and subjected to SDS–PAGE at 20 mA current for 1.5 h. The gel was incubated with 100 ml zymogram renaturing buffer for 1 h on a shaker to restore enzyme activity. The gel was then transferred to 100 ml zymogram developing buffer and incubated at 37°C for 3 days. The gel was stained in 0.5% Coomassie blue G-250 in 30% methanol and 10% acetic acid for 5 h at room temperature and subsequently destained with 30% methanol and 10% acetic acid overnight. The area of digestion by gelatinase did not bind stain and showed up as a clear band on the gel.

Inhibitor Profile

A number of protease inhibitors and reducing regents were tested for the effects on FAP dipeptidyl peptidase activity. The selected inhibitor was preincubated with FAP for 15 min at room temperature prior to addition of the substrate Ala-Pro-AFC. The reaction was followed for 15 min and the initial rate was obtained by fitting the time course to a linear curve. The inhibitory effect was measured as the activity remained relative to the enzyme activity in the absence of inhibitor (100%).

pH Profile

Peptidase activity was measured over the pH range of 6.0–9.5 using 25 mM glycine, 25 mM acetic acid, 25 mM Mes, 75 mM Tris, and 100 mM NaCl as a buffer. The buffer was titrated with NaOH or HCl to the desired pH. The change in ionic strength due to the pH adjustment was negligible with respect to total ionic strength.

RESULTS

Expression and Purification of FAP

The expression of His-tagged FAP in SF+ and *T. ni* cells was monitored by Western blot (Fig. 1). Maximal expression level of FAP occurred approximately 48 h following infection and remained constant to 72 h in both cell lines. Two major bands, adjacent to each other with slightly different intensities, were detected at molecular weight around 90 kDa with anti-His antibody, indicating the production of two FAP isoforms. SF+ and *T. ni* cells were similar in the expression of FAP, though the relative intensity of the major bands differed between the cell types.

Nonionic detergent Triton X-100 (1%) combined with high salt concentrations in lysis buffer effectively extracted full-length FAP into solution. Soluble FAP was purified by two affinity chromatographic steps, a ConA resin to bind glycoproteins followed by a Talon resin to bind His-tagged FAP. Samples from each purification step were analyzed by Western blot (Fig. 2a) and silver-stained SDS–PAGE gel (Fig. 2b). The gelatinase activity of FAP was monitored by a zymogram throughout the purification steps (Fig. 2c). The negative control was the host cell infected with FAP-negative baculovirus. As shown in Fig. 2a, lanes 2 and 4, the FAP isoform represented by the upper band was bound to ConA resin and eluted at 0.25 mM methyl-D-mannopyranoside, while the low-molecular-weight isoform remained in the flowthrough of ConA, suggesting that the two isoforms differed by glycosylation. The glycosylated His-tagged FAP was bound to Talon resin efficiently when incubated for an extended period of time (Fig. 2a, lane 5). Buffers with low imidazole (20 mM) and high salt concentration (500 mM) were employed to wash away nonspecific and weakly bound proteins from the Talon resin. This wash step proved to be critical in removing the background proteins and increasing the purity of the His-tagged protein. The final purified His-tagged FAP appeared as a dominant band at the expected molecular weight, around 95 kDa, on the silver-stained gel and was recognized by anti His-tag antibody in the Western blot. The yield was approximately 10 μg FAP from a 1-L culture of *T. ni* or SF+ cells with up to 80% purity as judged by SDS–PAGE.

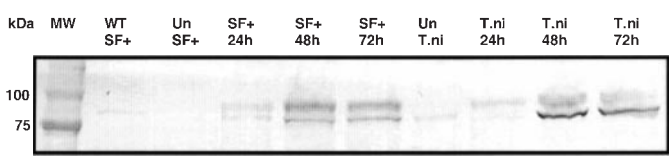

FIG. 1. Expression time course study of full-length FAP in SF+ and *T. ni* cells. The expression level was analyzed by the Western blot. His-tagged FAP was probed with monoclonal anti-His antibody. The controls were uninfected host cells (Un SF+ and Un *T.ni*) and cells infected with wild-type virus (WT SF+).

In a zymogram, protein with gelatinase activity was identified as a clear band resulting from digestion of gelatin, which corresponded to the protein band at the molecular weight of gelatinase. Multiple bands were observed at the low-molecular-weight region from the crude soluble fraction, suggesting the presence of gelatinase-like proteins, which were also observed in the control lanes (Fig. 2c, lanes c1 and c2). The non-FAP gelatinases were removed by Talon affinity chromatography (Fig. 2c, lane c3). The final purified FAP was visualized as a single band on a zymogram (Fig. 2c, lane 7). In contrast, the ConA flowthrough, which contained the unglycosylated FAP isoform, showed little gelatinase activity (Fig. 2c, lane 2). Gelatinase activity was enriched by ConA resin, which was consistent with the observation that little gelatinase activity was detected in the ConA flowthrough by the fluorescence-based gelatinase activity assay (data not shown).

Tracking FAP recovery during purification by following dipeptidyl peptidase and gelatinase activities using fluorescence-based assays was difficult because of nonspecific FAP-like activity in the crude cell lysate. For example, FAP-negative host cells exhibited significant proteolytic activity with Ala-Pro-AFC, as well as gelatinase activity with gelatin (or collagen type I). The control cells treated with the same purification procedure displayed no dipeptidyl peptidase and gelatinase activities in the final eluate, indicating the removal of other proteases. Less than 0.5% of the total activity in the crude cell lysate was observed in the final purified FAP.

Enzymatic Activity of FAP

A fast, continuous activity assay using fluorescein-conjugated gelatin as substrate was employed to measure the gelatinase activity of purified FAP quantitatively. The fluorescence is quenched in the intact gelatin due to extensive fluorescein labeling. Fluorescein was released when gelatin was degraded by FAP, resulting in an increase in fluorescence proportional to FAP proteolytic activity. FAP exhibited gelatinase activity with both gelatin and collagen type I, consistent with the previous observation based on zymography (7). The specific gelatinase activity is 5 relative fluorescent units (RFU) min^{-1} nM^{-1} at 0.1 mg/ml collagen type I. This gelatinase activity reflected in RFU is an arbitrary unit.

The dipeptidyl peptidase activity of FAP was characterized using the synthetic dipeptide Ala-Pro-AFC as substrate. Ala-Pro-AFC was dissolved in DMSO, which may effect enzyme activity, as it did with prolyl oligopeptidase (13). Thus we first investigated the DMSO effect on FAP activity. As shown in Fig. 3, DMSO had moderate inhibitory effect on the dipeptidyl peptidase activity. The inhibition of activity increased with the increased concentration of DMSO, and ~50% inhibition

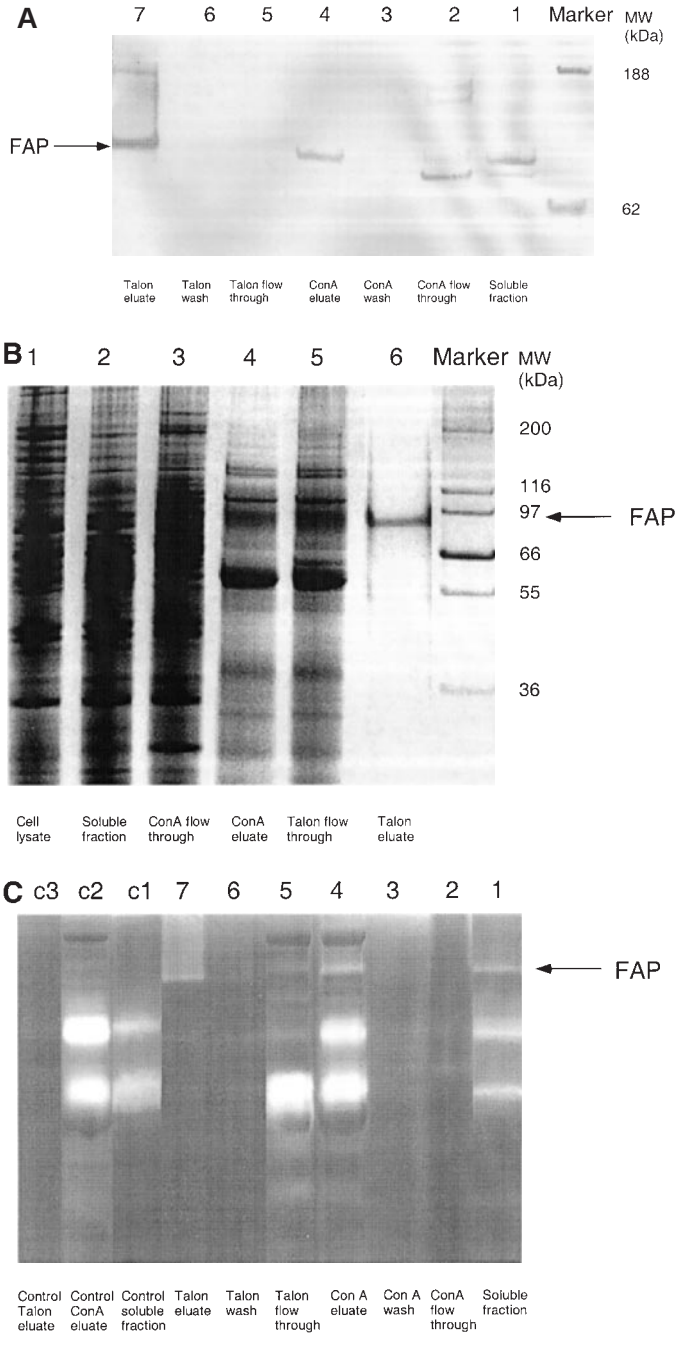

FIG. 2. Analysis of FAP purification. (a) Western blot probed by monoclonal anti-His antibody. Lane 1, soluble fraction; lane 2, ConA flowthrough; lane 3, ConA wash; lane 4, ConA eluate; lane 5, Talon flowthrough; lane 6, Talon wash; lane 7, Talon eluate. The band at high molecular weight ~180 kDa might be part of the smear artifact probably due to overheating during the electrophoretic transfer of protein from SDS–PAGE gel to PVDF membrane with the overloaded protein sample. Repeated experiments showed a single band at MW ~95 kDa for FAP when the electrophoretic transfer was carefully controlled with limited protein sample (data not shown). (b) Silver-stained SDS–PAGE gel electrophoresis. Lane 1, whole cell lysate; lane 2, soluble fraction; lane 3, ConA flowthrough; lane 4, ConA eluate; lane 5, Talon flowthrough; lane 6, Talon eluate, purified FAP. (c) Zymogram. Lane 1, soluble fraction; lane 2, ConA flowthrough; lane 3, ConA wash; lane 4, ConA eluate; lane 5, Talon flowthrough; lane 6, Talon wash; lane 7, Talon eluate; lane c1, control cell, FAP-negative host cell (*T. ni*), soluble fraction; lane c2, control cell, ConA eluate; lane c3, control cell, Talon eluate.

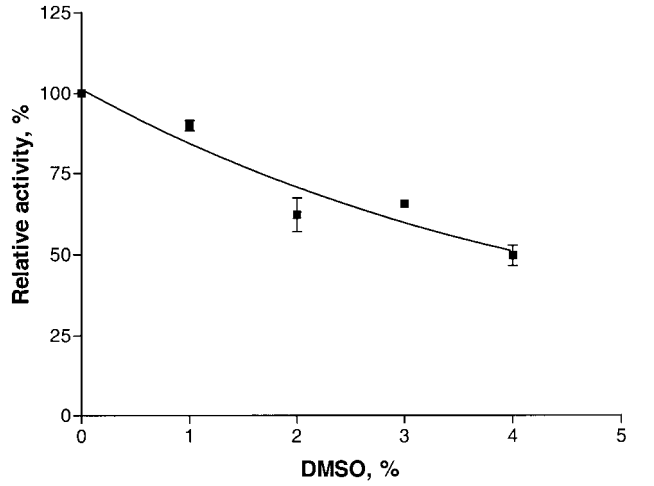

FIG. 3. Effect of DMSO on FAP dipeptidyl peptidase activity. The activity was measured as the initial rate in the presence of 0–4% DMSO and 0.5 mM Ala-Pro-AFC, pH 8.0, and expressed as relative activity compared to that in the absence of DMSO. The curve was arbitrarily drawn through the data.

was observed in the presence of 4% DMSO. To eliminate the activity variation by DMSO concentration, all assays were performed in the presence of 3% DMSO when maximal concentration of substrate was used.

FAP exhibited normal saturation kinetics with Ala-Pro-AFC as the substrate (Fig. 4). The enzyme concentration was calculated using a MW 95 kDa. The data were fitted to the Michaelis–Menten equation, yielding $K_m = 0.20 \pm 0.03$ mM and $k_{cat} = 2.0 \pm 0.1$ s^{-1} at pH 8.5. Thus k_{cat}/K_m is calculated to be 1.0×10^4 M^{-1} s^{-1}. The curve was drawn based on the nonlinear fit of the data. The kinetics of full-length FAP was analyzed for its dipeptidyl peptidase activity in the rest of the study.

Inhibitor Profile of FAP Dipeptidyl Peptidase Activity

The effects of protease inhibitors and reducing agents on FAP dipeptidyl peptidase activity were studied at a single concentration of inhibitor. As shown in Fig. 5, FAP has a specific inhibition spectrum. A serine protease inhibitor, PMSF, inhibited more than 50% of FAP activity at 1 mM ($IC_{50} < 1$ mM), consistent with previous observations (7). Chymostatin, an inhibitor of the serine protease chymotrypsin, showed 50% inhibition at 0.16 mM. FAP shares low structural homology with chymotrypsin. Other serine protease inhibitors, including aprotinin, TLCK, and Pefabloc, demonstrated no inhibitory effects at the concentration tested. FAP was insensitive to the metalloprotease inhibitor EDTA and

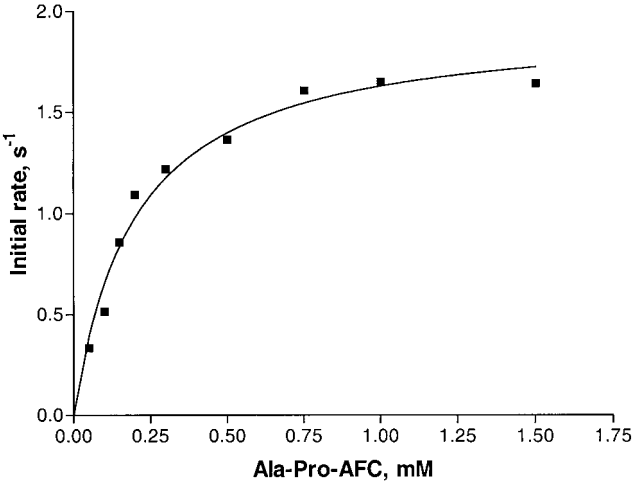

FIG. 4. Dipeptidyl peptidase activity of FAP with Ala-Pro-AFC as the substrate. The curve was the best fit of the data to Michaelis–Menten equation.

phosphoramidon, a collagenase inhibitor. The reducing reagents, β-mercaptoethanol and DTT, exhibited inhibitory effects at 140 and 10 mM, respectively, indicating reduction of disulfide linkages changed protein conformation important for catalysis.

pH Profile

The pH dependence of the kinetic parameters was constructed from k_{cat} (Fig. 6a) and k_{cat}/K_m (Fig. 6b). Both profiles are bell-shaped, indicating two significant ionization states contributing to enzyme function and stability. The data were fitted to the equation

$$k_{cat}/K_m \text{ (or } k_{cat}) = k_{HA}/(1 + 10^{pK_{a1}\text{-}pH} + 10^{pH\text{-}pK_{a2}}). \quad [1]$$

The curve fitting yielded $pK_{a1} = 7.0$ and $pK_{a2} = 11$ for k_{cat} and $pK_{a1} = 6.2$ and $pK_{a2} = 11$ for k_{cat}/K_m. Curves were drawn based on the fitted results.

DISCUSSION

FAP is an integral membrane glycoprotein, which shares homology with members of the dipeptidyl peptidase family such as DPPVI and DPP8 (14). It is present on the cell surface as a dimer or higher molecular weight multimer. Dimerization is required for its catalytic function (3). In a previous study, CD8 was fused to the N-terminal extracellular domain of FAP allowing dimerization via a CD8 interchain disulfide bond (7).

In our study, the full-length human FAP was expressed in SF+ or *T. ni* cells infected by FAP recombinant baculovirus and purified to near homogeneity. Since the putative catalytic domain is located at the C-terminus, a His-tag was added to the N-terminus of the full-length FAP to avoid potential perturbation of the catalytic activity and to facilitate the purification. A purification challenge with full-length FAP is that the His-tag is located close to the hydrophobic transmembrane domain, which could fold inward, making the His-tag less accessible to the immobilized metal ion resin. This limitation was alleviated by incubating protein with the Talon resin for an extended time.

One of the advantages of the baculoviral expression system is that it allows eukaryotic posttranslational modifications, such as glycosylation, which may be critical to the stability and activity of the recombinant protein. Recombinant FAP produced in SF+ or *T. ni* cells was found to be a mixture of two isoforms, which can be separated by ConA chromatography. Expression in baculovirus infected insect cells produces predominantly oligomannose N-glycosylated protein (15), which can be recognized by ConA. Thus the low-molecular-weight FAP isoform which does not bind to ConA is

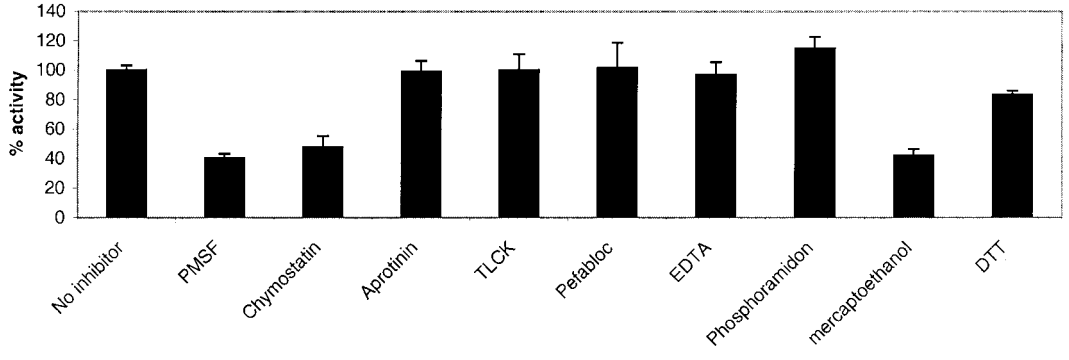

FIG. 5. Inhibitor profile of dipeptidyl peptidase activity of FAP. Inhibitor was incubated with FAP for 10–15 min before substrate was added at pH 8.0. The activity in the absence of inhibitor was taken as reference, 100%. Ala-Pro-AFC was 0.5 mM. The concentration of inhibitor in the reaction was 1 mM PMSF, 0.16 mM chymostatin, 15 μM aprotinin, 0.27 mM TLCK, 0.4 mM Pefabloc, 5 mM EDTA, 0.18 mM phosphoramidon, 140 mM mercaptoethanol, and 10 mM DTT.

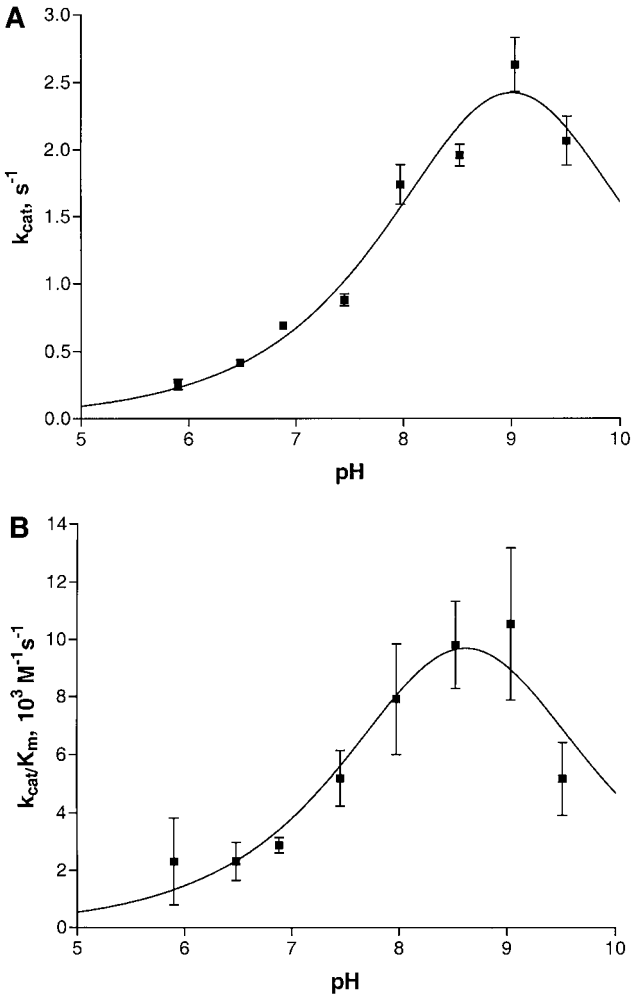

FIG. 6. pH profiles on k_{cat} (a) and k_{cat}/K_m (b). The dipeptidyl peptidase activity was measured using Ala-Pro-AFC as the substrate. The curve was the best fit to Eq. [1].

likely a nonglycosylated protein. It is interesting to notice that the relative amount of glycosylated and nonglycosylated proteins differed in SF+ and *T. ni* cells. Studies of the glycosylation pattern of recombinant secreted alkaline phosphatase from three insect cell lines revealed both qualitative and quantitative differences (16). The *T. ni* cell lines produced smaller oligosaccharides with relatively little fucose and no detectable terminally linked α-1,3-mannose. Donaldson and Shuler have been able to identify subpopulations of insect cells that differ in overall glycosylation capacity and that this difference could be identified via lectin affinity (17). In addition, environmental factors such as culture conditions, time of harvest, baculovirus type, and infection conditions have also been shown to lead to differences in overall glycosylation capacity and composition (18).

The glycosylated FAP was purified to near homogeneity and shown to have dual activities of peptidase and gelatinase. The unglycosylated FAP isoform was found to possess no gelatinase activity, suggesting that the carbohydrate content may play a role in the proteolytic activity of FAP. It is not clear whether nonglycosylated FAP has dipeptidyl peptidase activity, since the fraction containing nonglycosylated FAP (ConA flowthrough) has significant background protease activity from the host cells. The effects of glycosylation on proteolytic activity of β-secretase, an aspartic protease (19), and on glycoprotein folding (20) have been demonstrated. Glycosylation that occurs to nascent proteins in the endoplasmic reticulum prior to folding is thought to increase solubility and prevent aggregation, thereby aiding the folding process. It has less impact on the overall conformation when folded (21). FAP contains five potential N-glycosylation sites, one of which is located within the catalytic domain. Perhaps glycosylation facilitates FAP gelatinase activity by promoting proper folding and dimerization, which is required for catalytic activity. It is not clear whether glycosylation has a direct impact on substrate binding or catalysis.

Site-directed mutagenesis of Ser^{624} to Ala at the putative catalytic site abolished both peptidase and gelatinase activities suggesting the dual activities share the same activity site (7). The kinetics of FAP were characterized with respect to its dipeptidyl peptidase activity with Ala-Pro-AFC. The kinetic parameter for the full-length FAP, k_{cat} of 2.0 s^{-1}, is close to the value obtained in the previous study with FAP derived from membrane extracts (Fig. 4; Ref. 7). The K_m is comparable to the reported value (0.2 vs 0.46 mM). Compared to the structurally homologous protein DPPVI, the substrate specificity of FAP dipeptidyl peptidase activity is similar. However, their catalytic activities could be significantly different. For example, with the dipeptide substrate Ala-Pro-4-nitroanilide, the kinetic constants for DPPVI are K_m = 0.011 mM and $k_{cat}/K_m = 4.7 \times 10^6$ M^{-1} s^{-1} (22). The leaving group of the substrate may also contribute to the difference in the kinetics.

FAP has an optimal dipeptidyl peptidase activity around pH 8.5, similar to the pH 7.8 optimum of DPPVI (23, 24). The k_{cat}, a first-order rate constant, describes a series of kinetic events after substrate binding to product release. The activity requires ionization from two groups, one group with pK_{a1} 7.0 and the other one with pK_{a2} 11. The group exhibiting a neutral pK_{a1} is likely associated with the histidine of the catalytic triad, which is commonly observed with classical serine proteases including chymotrypsin and subtilisin (25). The group with high pK_{a2} may not be directly involved in catalysis but rather is associated with the stabilization of protein. The k_{cat}/K_m is a second-order rate constant, which reflects substrate binding and subsequent chemical steps. The pH dependence of k_{cat}/K_m yields a slightly lower pK_{a1} (6.2 vs 7.0) and the same pK_{a2}. The

apparent pK_{a1} value may reflect a combination of multiple ionization groups contributing to the substrate binding to the free enzyme and subsequent reaction steps.

In summary, we describe an expression and purification procedure to produce nearly homogenous, active full-length human FAP using a two-step affinity chromatography procedure. The purification protocol is simple, efficient, and suitable for further scale-up. The kinetic studies confirm that FAP belongs to the serine protease family. The availability of recombinant FAP will facilitate studies on its structure, substrate specificity, and screens for specific inhibitors.

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