

Cleavage-site specificity of prolyl endopeptidase FAP investigated with a full-length protein substrate

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Fibroblast activation protein (FAP) is a prolyl-cleaving endopeptidase proposed as an anti-cancer drug target. It is necessary to define its cleavage-site specificity to facilitate the identification of its *in vivo* substrates and to understand its biological functions. We found that the previously identified substrate of FAP, α_2 -anti-plasmin, is not a robust substrate *in vitro*. Instead, an intracellular protein, SPRY2, is cleavable by FAP and more suitable for investigation of its substrate specificity in the context of the full-length globular protein. FAP prefers uncharged residues, including small or bulky hydrophobic amino acids, but not charged amino acids, especially acidic residue at P1', P3 and P4 sites. Molecular modelling analysis shows that the substrate-binding site of FAP is surrounded by multiple tyrosine residues and some negatively charged residues, which may exert least preference for substrates with acidic residues. This provides an explanation why FAP cannot cleave interleukins, which have a glutamate at either P4 or P2', despite their P3–P2–P1 sites being identical to SPRY2 or α -AP. Our study provided new information on FAP cleavage-site specificity, which differs from the data obtained by profiling with a peptide library or with the denatured protein, gelatin, as the substrate. Furthermore, our study suggests that negatively charged residues should be avoided when designing FAP inhibitors.

Keywords: α_2 -anti-plasmin/cleavage-site specificity/dipeptidyl peptidase/FAP/fibroblast activation protein.

Abbreviations: FAP, fibroblast activation protein; DPP, dipeptidyl peptidase; α_2 -AP, α_2 -anti-plasmin.

Fibroblast activation protein (FAP) belongs to the prolyl-cleaving peptidase family, which includes

dipeptidyl peptidase 4 (DPP4), DPP8, DPP9 and DPP2. These enzymes preferentially cleave the peptide bond following a proline residue (1). Because this prolyl-cleaving activity is distinct from that of other cellular enzymes, this family of proteases is important in various cellular functions. In contrast to other proteases in the family, which are expressed ubiquitously (1), FAP is expressed exclusively in fetal cells, in wounded tissues and in the stromal fibroblasts of >90% of malignant epithelial tumors, but it is not expressed in benign tumours or normal adult tissues (2–4). The overexpression of FAP in xenographic animal models promotes cancer formation, supporting the direct involvement of FAP in cancer formation (5). Because of its unique expression pattern and cancer-promoting capacity in animal models, FAP has long been proposed as an anti-cancer target. Recently, an FAP inhibitor has been shown to be effective to inhibit tumour growth in syngeneic animal models (6). Moreover, FAP is found to have immunosuppressive function in the tumour microenvironment (7). Elimination of FAP-containing stromal cells allows the immune system to suppress the growth of solid malignant tumours (7). How FAP exerts such function is not clear so far (7).

FAP is a type II membrane serine protease and shares 50% of its sequence identity at the amino acid level with DPP4 (8, 9). In contrast to the robust aminodipeptidase activity of DPP4, FAP is an endopeptidase with weak dipeptidase activity equivalent to around 1% of the activity of DPP4 (8). Although FAP does not cleave type I or III collagen by itself (10, 11), it cleaves the peptides into smaller fragments after matrix metalloproteinase 1 has digested these collagens (11). FAP is capable of degrading recombinant gelatin (a denatured form of collagen) *in vitro* (9). In the past few years, several studies have been performed to identify the cleavage-site specificity of FAP using a peptide library or the denatured protein gelatin. Consistent with its endopeptidase activity, FAP cleaves the Ac–P2–Pro–AFC substrate library with strict cleavage-site specificity, tolerating only Pro and Gly at the P1 and P2 sites, respectively (12). A mass spectrometry study with gelatin as the substrate showed that FAP could cleave at this site when amino acids other than Pro occur at the P1 position, including Ala, Arg, Gly, Lys and Ser (10). The same study also reported that FAP prefers to cleave gelatin after either PPGP or the following sequence: (D/E/E/P)–(R/K)–G–(D/E/P)–(A/G/S/T/P)–G–P (10).

So far, only α_2 -anti-plasmin (α_2 -AP) has been identified as being cleavable by FAP (13, 14). α_2 -AP is a 51-kD protein involved in fibrin clot formation (14). The binding of α_2 -AP to fibrin inhibits fibrinolysis (13).

FAP cleaves α_2 -AP after Pro₁₂ in the T₉S₁₀G₁₁P₁₂-N₁₃Q₁₄E₁₅Q₁₆E₁₇ sequence (Table I). Based on the α_2 -AP cleavage sequence, a fluorescence resonance energy transfer (FRET) substrate library was generated (12). Substrate profiling with this library indicated that FAP strictly requires Pro at P1, Gly at P2 and small uncharged residues such as Ser or Thr at P3 (12). However, it lacks selectivity at P4, P1' and P2' in terms of the residues introduced in that study (12, 15). Thus, the cleavage-site preferences reported in studies based on gelatin and α_2 -AP are not completely consistent (12, 13, 15).

Because of the potential of FAP as an anticancer drug target, it is imperative to define its specificity to facilitate the identification of *in vivo* substrates and to understand its biological functions. In this study, we used a full-length protein, SPRY2, to study the cleavage-site specificity of FAP. This analysis overcomes the limitations associated with the use of a peptide library or denatured gelatin, as reported previously. Our data demonstrates the cleavage-site specificities of FAP at the P3, P4 and P1' sites.

Materials and Methods

Plasmid construction

The plasmid pIRES2-EGFP-C-FLAG was constructed as described below. The FLAG tag was obtained by annealing, and PCR amplification was performed with the primers 5'-CCTAGTCGATCGACTGACCAAGGACGATGACGACAAGTAGGGATCATGC-3' and 5'-GCACTGGATCCCTACTTGTCTCGTCATCGTCC TTGTAGTCCATGTCTGACTAGG-3'. The fragment was digested with *Sal*I and *Bam*HI, and then ligated into pIRES2-EGFP (Invitrogen) to produce pIRES2-EGFP-C-FLAG.

The full-length SPRY2 cDNA was amplified by PCR from a human Jurkat cell cDNA library with the primers 5'-CAGCTAGCATGGAGGCCAGAGCTCAG-3' and 5'-CACTCGAGTGTGG TTTTCAAAGTT-3', and cloned into the pCR[®]-Blunt II-TOPO plasmid (Invitrogen). The SPRY2 DNA fragments were released by *Nhe*I and *Xho*I digestion, and ligated into pIRES2-EGFP-C-FLAG to produce pIRES2-SPRY2-C-FLAG for immunoprecipitation. Mutant SPRY2 was generated by site-directed mutagenesis, as described previously, with the primers summarized in Supplementary Table S1 (16). The FAP expression plasmid for insect cells was constructed as described previously (8). The plasmid for α_2 -AP expression in insect cells was constructed as follows. The cDNA fragment encoding α_2 -AP, without the signal sequence, was amplified with the primers 5'-CTCGAGTATGGAGCCCTGGGC TGG-3' and 5'-CTCGAGTCACTGGGGCTGCCAAAC-3' and ligated into the pCR[®]-Blunt II-TOPO vector. The DNA fragment was released from PCR-Blunt II-TOPO by *Xho*I digestion and ligated into pBac8-CD5 (16) to produce pBac8-CD5-His- α_2 -AP. CD5 is a cleavable signal sequence required for the extracellular secretion of the α_2 -AP protein (16).

The plasmid for α_2 -AP expression in mammalian cells was constructed as follows. The fragment FLAG- α_2 -AP was amplified with the primers 5'-CGTCGACATGGACTACAAGGACGATGACGA

CAAGCTCGAGATGGAGCCCTTGGGC-3' and 5'-CAGGATCCTCACTTGGGGCTGCCAAACTG-3'. The fragment was digested with *Sal*I and *Bam*HI, and ligated into pIRES2-EGFP-CD5 to produce pIRES2-CD5-FLAG- α_2 -AP.

Purification of recombinant FAP and α_2 -AP proteins from insect cells

The growth and infection of Hi5 cells have been described previously (16, 17). To obtain pure human FAP protein, a two-step purification process that coupled affinity chromatography with ion exchange chromatography was used. The purification process of affinity chromatography was performed as described previously (16, 17). More specifically, the culture medium of Hi5 cells expressing FAP was collected and precipitated by adding 3.8 M ammonium sulphate, up to 70% saturation. The pellet was resuspended in the binding buffer, which contains 20 mM Tris-HCl buffer, pH 8.0, with 500 mM NaCl and then passed through the Ni-sepharose column. Three to five times the bed volume of the binding buffer containing 10 mM or 20 mM imidazole was used to wash the column. FAP protein was eluted with 250 mM imidazole in the binding buffer. The fractions containing FAP proteins were then applied to a HiTrap Q Sepharose FF (GE Healthcare) column preequilibrated with buffer A (20 mM Tris-HCl, pH 8.0). Pure FAP proteins were eluted with a 0–500 mM NaCl gradient in buffer A.

The purification of the α_2 -AP protein from Hi5 insect cells is described below. The culture medium of the Hi5 cells expressing α_2 -AP was collected by centrifugation and precipitated by saturation with 50% ammonium sulphate. The pellet was resuspended in binding buffer [20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10 mM imidazole] and cleared by centrifugation at 9,000 rpm for 20 min. The supernatant was incubated with Ni-Sepharose resin (Novagen), which was then washed extensively with binding buffer supplemented with 20 mM imidazole. The α_2 -AP protein was eluted with the same buffer supplemented with 250 mM imidazole and concentrated with an Amicon YM-10 ultrafiltration device (Millipore) in 50 mM Tris-HCl (pH 8.0).

Cell culture, transfection and immunoprecipitation

HEK293A cells were cultured in complete Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen), 1% NEAA (GIBCO) and 1% penicillin-streptomycin (GIBCO). HEK293A cells were transfected with the indicated plasmids using Lipofectamine[™] 2000 (Invitrogen). The HEK293A cells were seeded at a density of 1×10^6 in a 6-cm culture plate. For each plate of cells, 4 μ g of DNA was diluted into 50 μ l OPTI-MEM1 medium (GIBCO) and 4 μ l of Lipofectamine[™] 2000 was also diluted into 50 μ l OPTI-MEM1 medium. After incubation at room temperature for 5 min, the diluted Lipofectamine[™] 2000 was combined with the diluted DNA and incubated for 20 min at room temperature. The mixture was added directly to each plate, and the medium were changed after 4 h incubation at 37°C. After transfection for 48 h, the cells and the culture medium were collected for the *in vitro* cleavage assay.

To isolate the FLAG-tagged α_2 -AP protein from the medium, the culture medium of the transfected 293A cells was collected and concentrated with an Amicon YM-10 ultrafiltration device. To isolate the SPRY2-Flag protein from the cell lysate, the 293A cells were collected after transfection for 48 h and lysed in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100] supplemented with EDTA-free Protease Inhibitor Cocktail (Roche). The FLAG-tagged α_2 -AP from the concentrated medium or SPRY2-Flag from the cell lysate was immunoprecipitated with

Table I. Proteins with potential or confirmed FAP cleavage sites.

Sequences ^a	P8	P7	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'
α_2 -AP (21–35)	G	R	Q	L	T	S	G	P	N	Q	E	Q	V	S
IL-1 β (16–30)	K	S	L	V	M	S	G	P	Y	E	L	K	A	L
IL-8 (30–44)	L	R	V	I	E	S	G	P	H	C	A	N	T	E
SPRY2 (137–151)	G	S	S	F	S	S	G	P	V	A	D	G	I	I

^aThe potential or confirmed cleavage sequences on α_2 -AP, IL-1 β , IL-8 and SPRY2 are shown. The sequences corresponding to P4 to P1 sites are in bold. The accession numbers are CAG46948 (for IL-8), CAG28607 (for IL-1 β), P08697 (for α_2 -AP) and NP_005833 (for Spry2), respectively.

anti-FLAG M2 agarose (Sigma-Aldrich), according to the manufacturer's instructions. The cell lysate or the concentrated medium was incubated with 30 μ l of anti-Flag M2 Agarose Affinity Gel (Sigma-Aldrich) at 4°C for 6 h. The immunoprecipitates were washed three times with washing buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl]. The protein was eluted with 75 μ l of elution buffer containing the FLAG peptide [150 ng/ μ l peptide, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl] for the *in vitro* digestion assay.

In vitro cleavage by FAP

Out of 75 μ l immunoprecipitated elutes containing α_2 -AP or SPRY2 protein, 16 μ l was incubated with purified FAP protein (100 nM) at 37°C for various times in the presence or absence of the FAP inhibitor 2F09 (10 μ M). After incubation, the samples were mixed with 5 \times loading buffer [250 mM Tris-HCl (pH 6.8), 10% SDS, 50% glycerol, 10% 2-mercaptoethanol, 0.02% bromophenol blue], boiled and analysed by western blotting with either an anti-His (Serotech, Inc.) or an anti-FLAG M2 monoclonal antibody (Sigma-Aldrich).

Results

α_2 -AP is not a robust substrate of FAP

To set up the *in vitro* cleavage reaction, we first purified FAP from insect cells using a two-step chromatographic procedure to ensure its high purity and to minimize non-specific cleavage (Fig. 1A). Subsequent gel filtration neither improved the purity greatly nor changed the results reported in this study (data not shown). The measured kinetic constant indicated that the

purified enzyme was active ($k_{\text{cat}} = 12.3 \pm 5.1 \text{ S}^{-1}$, $K_m = 413 \pm 27 \mu\text{M}$) (8). Using a method similar to that used to purify FAP, we also obtained α_2 -AP from insect cells (Fig. 1B). The molecular mass of α_2 -AP (around 65 kD) was slightly smaller than that reported in mammalian cells (around 70 kD) (Fig. 1C), indicating that the α_2 -AP from insect cells might be less glycosylated. This is not unusual because many proteins are less glycosylated in insect cells than in mammalian cells (18).

Next, α_2 -AP was incubated with FAP for various times at 37°C and the cleavage of α_2 -AP by FAP was detected either in a Coomassie-blue-stained SDS-PAGE gel or by western blot analysis using an anti-His antibody. 2F09 is a potent and selective chemical inhibitor of FAP, with an IC_{50} for FAP of 22 nM (named compound 19 in the article) (19). It effectively inhibits the enzymatic activity of FAP at 10 μ M and this compound was included as the control (Fig. 1B and 1C). As shown in Fig. 1B, the cleavage of α_2 -AP by FAP was readily detectable in western blot analysis. Not knowing whether the glycosylation of α_2 -AP affects its cleavage, we then expressed FLAG-tagged α_2 -AP in mammalian 293A cells and captured the protein by immunoprecipitation (Fig. 1C). As shown in Fig. 1C, very weak cleavage of FLAG- α_2 -AP was

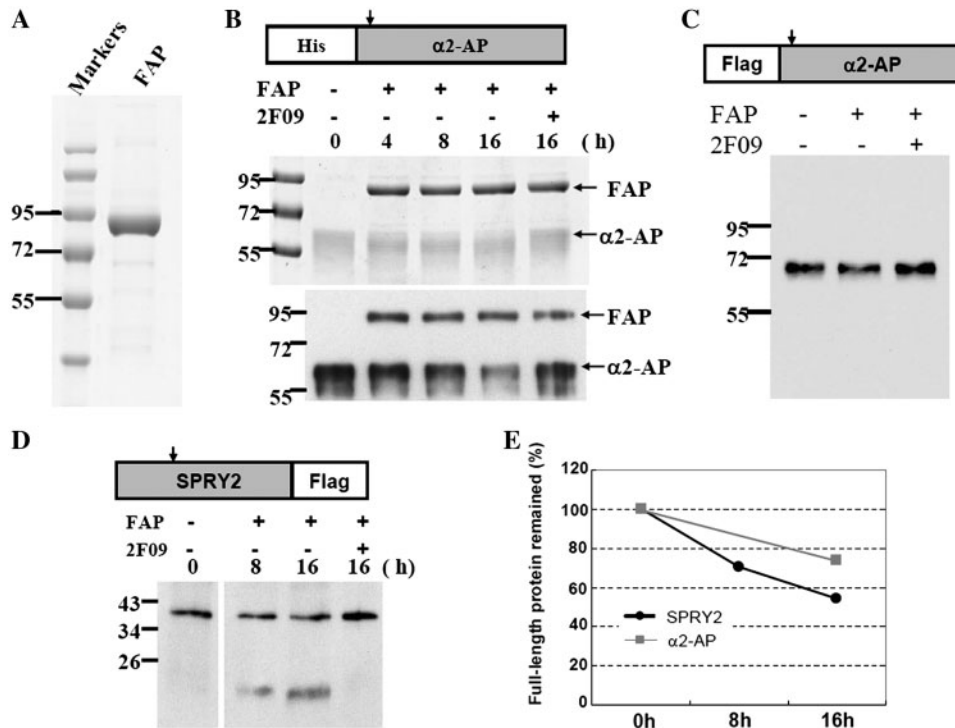


Fig. 1 α_2 -AP purified from insect cells was readily cleaved by FAP, whereas α_2 -AP expressed in mammalian cells was not a robust substrate of FAP. (A) Human recombinant FAP protein from insect cells after its two-step chromatographic purification. (B) The upper panel is a time-course study of the cleavage of purified α_2 -AP by recombinant FAP analysed by Coomassie-Blue-stained SDS-PAGE. The FAP inhibitor, 2F09, was included to inhibit the enzymatic activity of FAP. The lower panel is a time-course study of the cleavage of α_2 -AP by recombinant FAP, as analysed by western blot analysis with anti-His antibody. (C) α_2 -AP was tagged with FLAG at the N-terminus. The arrow indicates the site of cleavage by FAP. FLAG-tagged α_2 -AP was expressed in 293A cells, immunoprecipitated and incubated with purified FAP. Western blot analysis of FLAG- α_2 -AP after incubation with FAP for 16 h. (D) Time-course study of the cleavage of SPRY2 by recombinant FAP, as analysed by immunoblotting with anti-FLAG antibody. SPRY2 was tagged with FLAG at the C-terminus. The arrow indicates the site of cleavage by FAP. FLAG-tagged SPRY2 was expressed in 293A cells, immunoprecipitated and incubated with purified FAP. (E) Quantification of the rate of cleavage showed that SPRY2 was a much more robust substrate of FAP compared with α_2 -AP. The ratio of full-length protein and cleaved product was quantified by Image J software.

detected after incubation with FAP for 16 h. These results indicate that α_2 -AP from mammalian cells is not a robust substrate of FAP and is thus not a suitable protein for the further investigation of the cleavage-site specificity of FAP.

SPRY2 as an *in vitro* substrate of FAP

Using the cleavage-site sequence of α_2 -AP, we searched for and tested several proteins as potential substrates of FAP. Although interleukin 1 β (IL1 β) and IL8 contain sequences at the P3 to P1 sites identical to that of α_2 -AP (Table I), they were not cleaved by FAP, as determined by mass spectrometric analysis (data not shown). Incidentally and interestingly, we found that SPRY2 protein was cleaved by FAP (Fig. 1D). SPRY2 (also called SPROUTY2) is a known receptor tyrosine kinase inhibitor located inside cells (20). Therefore, it is not an *in vivo* substrate of FAP. Nevertheless, we wished to delineate the cleavage-site specificity of FAP in the context of a full-length globular protein. A time course study of cleavage by FAP was carried out (Fig. 1D). Quantification of the rate of cleavage clearly was done with Image J software (<http://rsbweb.nih.gov/ij/>). The data clearly showed that SPRY2 was a much more robust substrate of FAP than α_2 -AP (Fig. 1E), and was more suitable as a protein substrate to study the cleavage preferences of FAP. There is only one potential FAP cleavage site in SPRY2 with the sequence SSGPVA, with P at position 144 (Fig. 2A and Table I). Consistent with the importance of Pro144, mutation of P144 to Leu effectively abolished the cleavage of SPRY2 by FAP (Fig. 2B).

Cleavage-site specificity of FAP for SPRY2

Site-directed mutagenesis was carried out to mutate P3, P4, P1' and P2' sites of SPRY2 targeting SSGP-VA. The mutant proteins were immunoprecipitated after their expression in mammalian cells and then incubated with purified FAP for 8 or 16 h.

Quantification of the cleavage with SPRY2 remained was done with Image J software and shown below each panel. For P3, P4 and P1' sites, we changed each site individually to acidic (D), basic (R and H), hydrophobic (P and L) or small hydrophilic (G, S and A) residues. FAP showed strict selectivity at the P1' site (Fig. 3A and B).

With P at P1' site, SPRY2 was not cleaved even after incubation for 16 h. SPRY2 with either R or D at P1' site was not cleaved significantly after incubation for 8 h, but more R mutant proteins were cleaved than D after incubation for 16 h. In comparison, both small uncharged and bulky hydrophobic residues were tolerated at P1' site. With P or R at P2' site, SPRY2 was cleaved after incubation for 16 h, indicating greater selectivity for P1' site than for P2' site (Fig. 2C).

SPRY2 with R, D or P at P3 site was not cleaved significantly by FAP after incubation for 16 h (Fig. 3C and D), suggesting that charged residues are less tolerated at P3. In contrast, SPRY2 proteins with small uncharged amino acids and large hydrophobic amino acids at P3 site were cleaved after incubation for 16 h (Fig. 3C and D). Compared with P3 site, no significant selectivity was found at the P4 site, and all mutant SPRY2 proteins were cleaved to various extents after incubation for both 8 and 16 h (Fig. 3E and F). Among them, D at P4 site is the least favoured substrate.

Molecular docking of the SPRY2 cleavage site to the FAP active site

The SSGPVA peptide, corresponding to the cleavage site of SPRY2 (amino acids 141–146), was manually docked to the active site of FAP (Fig. 4). Like that of DPP4, the S1 subsite of FAP contains a number of hydrophobic residues, including Y625 (equivalent to DPP4 Y631), V650 (V656), W653 (W659), Y656 (Y662), Y660 (Y666) and V705 (V711), which are ideal for anchoring a substrate with proline at the P1 position. Furthermore, the hydroxyl oxygens of these

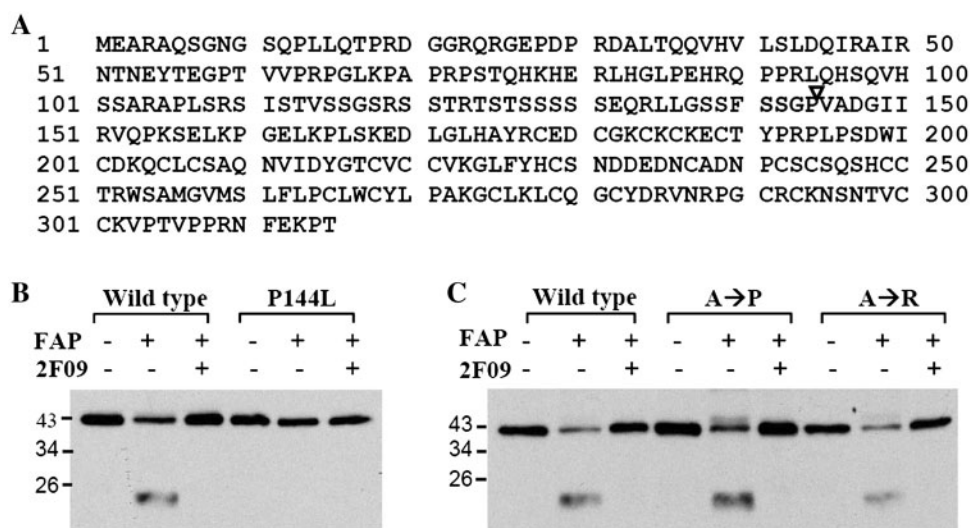


Fig. 2 SPRY2 was cleaved by FAP. (A) The amino acid sequence of human SPRY2, with the potential cleavage site marked (GenBank accession number: NM_005842.2). (B) SPRY2 was cleaved by FAP after P144. The SPRY2 protein was expressed, immunoprecipitated and incubated with purified FAP before fractionation by SDS–PAGE and western blotting analysis. (C) Mutations of the P2' site to Pro or Arg did not affect cleavage by FAP.

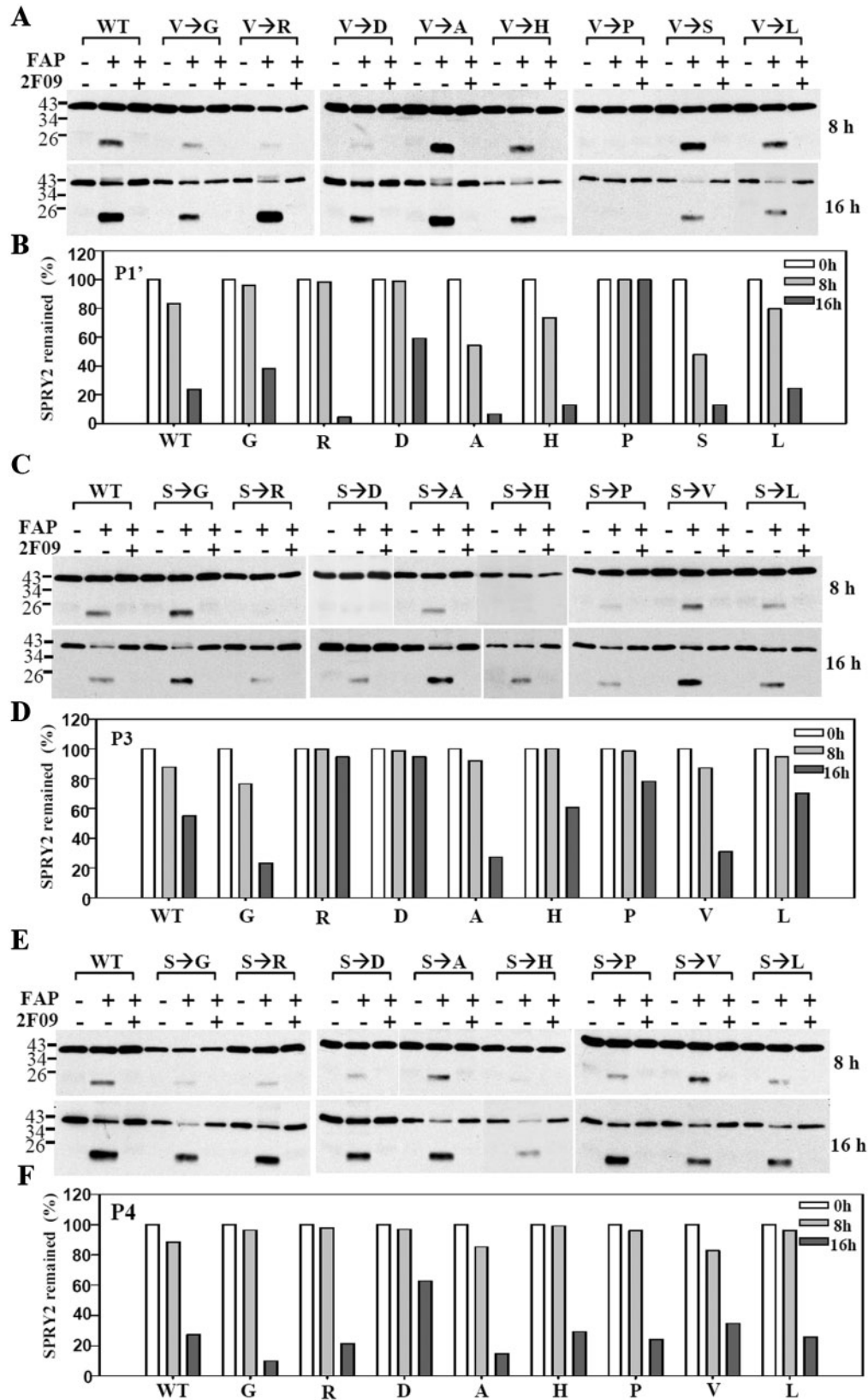


Fig. 3 Cleavage-site specificity of FAP using SPRY2 as the substrate. The residue at the P1', P3 or P4 site was replaced individually with eight different amino acids, shown at the top of the gels. The SPRY2 mutant proteins were expressed in 293A cells, immunoprecipitated, incubated with purified FAP protease for 8 or 16 h and analysed by western blotting with anti-FLAG antibody. Quantification of the cleavage with SPRY2 remained was done with Image J software. (A) Mutations at the P1' site of SPRY2. (B) Quantification of the cleavage with P1' site mutated SPRY2 remained was done with Image J software. (C) Mutations at the P3 site of SPRY2. (D) Quantification of the cleavage with P3 site-mutated SPRY2 remained was done with Image J software. (E) Mutations at the P4 site of SPRY2. (F) Quantification of the cleavage with P4 site mutated SPRY2 remained was done with Image J software. All the experiments were repeated at least once. Less than 15% difference was observed between repeats.

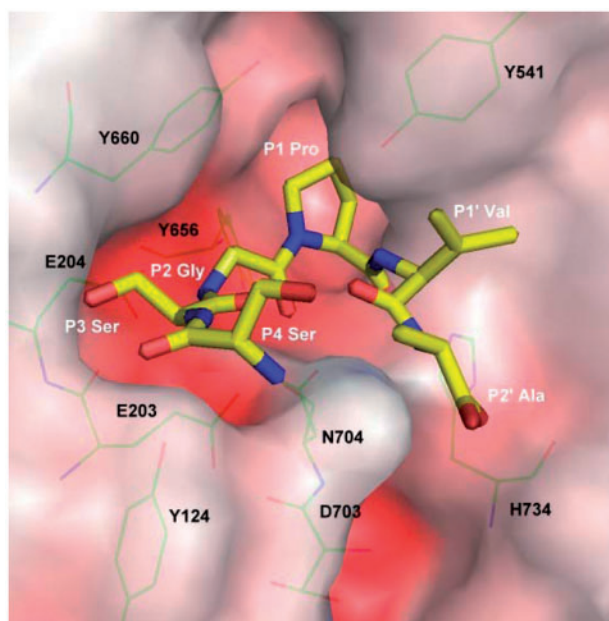


Fig. 4 A docking model of the SPRY2 fragment in the active site of FAP. Using InsightII (Accelrys Inc., San Diego, CA, USA), a rod model of SPRY2 containing the polypeptide SSGPVA (amino acid 141–146 of SPRY2) was manually docked to the FAP active site (PDB code 1Z68) based on the complex structure (PDB code 1NU8) (31) of DPP4–diprotin A (a three-amino-acid (IPI) peptide). Using GRASP program (27), the molecular surface of the FAP active site is coloured such that red indicates negative, and blue indicates positive, electrostatic molecular surface. The model is viewed from side opening, at about 20 Å depth to which the peptide binds; tunnel opening is ~30 Å to the left of the peptide binding site. The apparent acidic binding milieu (negative charged surface; in red) is mainly contributed by the hydroxyl oxygens of five tyrosines (Y124, Y541, Y625, Y656 and Y660), two glutamates (E203 and E204) and one aspartate (D703).

tyrosines, along with two glutamates (E203 and E204) and one aspartate (D703), contribute to form an acidic environment for substrate binding (Fig. 4). This is consistent with the substrate profiling of the SPRY2 protein, in that we found that substitution of D to P1', P3 and P4 sites render the proteins least cleavable by FAP (Fig. 3B, D and F).

By overlaying on the SPRY2 peptide and conducting energy minimization that followed, docking models of the α_2 -AP peptide and the two interleukin peptides were also produced (data not shown). However, probably owing to the primitive energy minimization and overlaying technique used, no notable differences were observed among the different docking models. Nevertheless, an acidic binding site is consistent with the observed low cleavage activities of the interleukins, which have negatively charged residue (glutamate) at either P4 or P2' site.

Discussion

FAP has long been considered a promising anti-cancer target molecule. In human clinical samples, a greater amount of FAP in stromal fibroblast cells is associated with worse patient survival and more aggressive disease progression and metastasis (21). Cancer treatment

using either FAP inhibitors or immunotherapy directed against the FAP antigen has been effective in different animal models (5, 6, 22–24). However, a human clinical trial with a weak and non-selective FAP inhibitor was terminated because of renal toxicity (24, 25). Treatment with anti-FAP antibody was also ineffective in human patients (26). At present, the biological functions of FAP must be better understood to confirm its potential as an anticancer target. Investigation of its cleavage-site specificity should help us identify the *in vivo* substrate of FAP and its function.

To gain more insight into the substrate specificity of FAP, we investigated its cleavage-site preference using a native and full-length protein. All studies reported so far have used either a short peptide library or the denatured protein gelatin. Our first attempt to use full-length α_2 -AP protein to delineate the specificity of FAP yielded little information. The cleavage of FLAG-tagged α_2 -AP expressed in mammalian cells was very slow (Fig. 1C). Although SPRY2 is not a real substrate of FAP, it is a much more robust substrate of FAP than is α_2 -AP. Unlike the results obtained in a peptide library study (15), FAP cleaved both small uncharged amino acids and bulky hydrophobic amino acids at P1', P3 and P4 sites. Charged amino acids were not preferred by FAP at these positions, especially the acidic residue. This is a new finding and has not been observed before in studies based on a peptide substrate library or gelatin. Consistent with this, interleukins cannot be cleaved by FAP possibly due to the fact that they have glutamate on one side or the other of the proline. As shown in Fig. 4, the substrate binding site of FAP has an acidic electrostatic surface, contributed in part by two glutamate (E203 and E204) and one aspartate (D703) residues. This could explain why glutamate at either end of the interleukin peptide would not be tolerated. In addition to the information provided from the study of (27), the present work suggests that negatively charged residues should be avoided in designing peptide inhibitors of FAP. Moreover, some aromatic moieties may interact favourably with the multiple tyrosine residues at certain sites. Similarly, a hydrophobic residue such as alanine could be favoured compared to glutamine at P2' site, making SPRY2 a more robust substrate than α_2 -AP. Consistent with our findings, mutational analysis (27) showed that alanine is better than glutamine at P2' and serine is better than threonine at P4, both favouring SPRY2 over α_2 -AP.

Interestingly, we found that FAP could not cleave SPRY2 when a proline occurred at the P1' site (Fig. 3A). However, proline was tolerated at the P3, P4 or P2' site (Figs 2 and 3). Other prolyl-cleaving enzymes, including DPP4, prolyl oligopeptidase (EC 3.4.21.26), membrane prolyl carboxypeptidase (EC 3.4.17.16) and lysosomal prolyl carboxypeptidase (EC 3.4.16.2), are unable to cleave the peptide bond between two prolines at P1–P1' (28). It is also well known that other serine proteases, including trypsin, chymotrypsin and elastase, cannot cleave at the P1 site if the P1' site is occupied by proline (29, 30). This may be because the lack of hydrogen on the imino group of

the imidazole ring of proline at P1' affects the formation of an oxyanion hole, which prevents cleavage by these serine proteases.

The active site of FAP is accessible through two openings (8). One is the tunnel formed by the eight blades of the β -propeller domain, and the other, called the 'side opening' is a cavity between the propeller and the hydrolase domains (8). From the docking results, it seems that the side opening is the more likely route for peptide entry, judging from the orientation of the peptide in the active site pocket, although this requires confirmation with further biochemical and structural studies. Our data also demonstrates that proline is essential at the P1 site (Fig. 2B). It is not clear to us whether the cleavage after Ala, Arg, Gly, Lys or Ser at the P1 site, which was observed and reported using gelatin as the substrate, can be attributed to the impurity of the FAP enzyme used in that assay (10). In the past, we observed non-specific cleavage by DPP4 when only one-step affinity purification was used (Han, Y.S. and Chen, X. unpublished observation). We believe that the purity of FAP in this kind of *in vitro* study is critical. FAP must be as pure as possible to evaluate its cleavage-site specificity. Because information is unavailable on the real *in vivo* substrates of FAP, other than α_2 -AP, the discovery of the *in vivo* substrate of FAP remains a future challenge. Nevertheless, the results of our study with SPRY2 have provided a clear answer about the preferences of FAP at the P1', P3 and P4 sites in the context of a full-length protein, which will be helpful to understand substrate specificity of FAP and its inhibitor design.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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