DOI 10.1002/prca.201300095 *Proteomics Clin. Appl.* 2014, *8*, 454–463

REVIEW

Understanding fibroblast activation protein (FAP): Substrates, activities, expression and targeting for cancer therapy

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Fibroblast activation protein (FAP) is best known for its heightened expression in tumour stroma. This atypical serine protease has both dipeptidyl peptidase and endopeptidase activities, cleaving substrates at a post-proline bond. FAP expression is difficult to detect in non-diseased adult organs, but is greatly upregulated in sites of tissue remodelling, which include liver fibrosis, lung fibrosis, atherosclerosis, arthritis, tumours and embryonic tissues. Due to its restricted expression pattern and dual enzymatic activities, FAP is emerging as a unique therapeutic target. However, methods to exploit and target this protease are advancing more rapidly than knowledge of the fundamental biology of FAP. This review highlights this imbalance, emphasising the need to better define the substrate repertoire and expression patterns of FAP to elucidate its role in biological and pathological processes.

Received: September 27, 2013
Accepted: October 16, 2013

Keywords:
Collagen / Degradomics / Dipeptidyl peptidase / Endopeptidase / Exosite / Protease

1 Introduction

Proteolysis is an irreversible and essential post-translational modification that controls the composition and activity of proteins in a biological system. Bioactive peptides influence a range of physiological processes, including glucose homeostasis, the immune response and cellular signalling, and modification of these peptides can have significant impacts on such processes. Identifying proteases and how they modify their substrates is imperative in understanding cell biology, physiology and pathogenesis and in determining the potential of these proteases as drug targets.

The dipeptidyl peptidase (DPP) 4 protein family has four enzymatic members; DPP4, fibroblast activation protein (FAP), DPP8 and DPP9 (Fig. 1), which are able to hydrolyse a prolyl bond that is two amino acids from the N-terminus of a protein. Due to the cyclic nature of the proline (Pro) residue, this is a rare catalytic ability that makes this group of proteases interesting in many aspects of biology and as therapeutic targets [1, 2].

DPP4 is the most profoundly characterised member and prototype of this family; it is expressed ubiquitously and has a role in a wide range of physiological and pathological processes via its cleavage of bioactive peptides [1]. DPP4 was identified as a drug target for type 2 diabetes after it was observed to cleave and inactivate glucagon-like peptide-1 in vivo, thereby muting insulin secretion by the pancreas. This is just one example of how an understanding of the substrates of a protease can provide crucial information on its physiological role and thus lead to a new therapy.

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Abbreviations: **α_2-AP**, alpha-2-antiplasmin; **APP**, amyloid precursor protein; **CAR**, chimeric antigen receptor; **CN**, collagen; **DPP**, dipeptidyl peptidase; **ECM**, extracellular matrix; **FAP**, fibroblast activation protein; **NPY**, neuropeptide Y; **PREP**, prolyl endopeptidase; **SRM**, selected reaction monitoring; **TAILS**, terminal amine isotopic labelling of substrates; **TG**, thapsigargin

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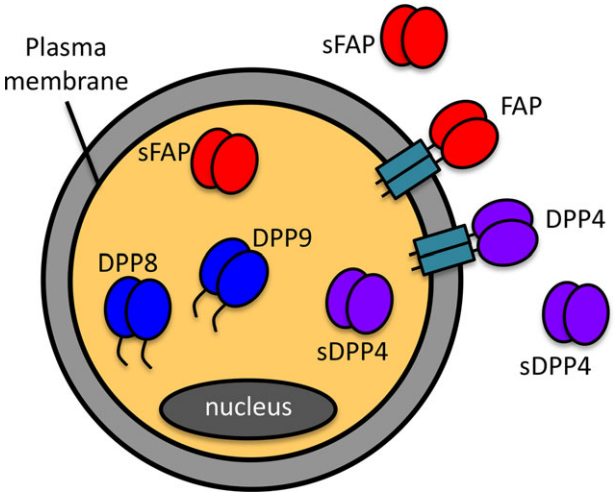

Figure 1. Enzymatic members of the DPP4 family. FAP and DPP4 are type II integral membrane proteins that have intracellular and extracellular soluble, truncated forms. DPP8 and DPP9 are intracellular proteins.

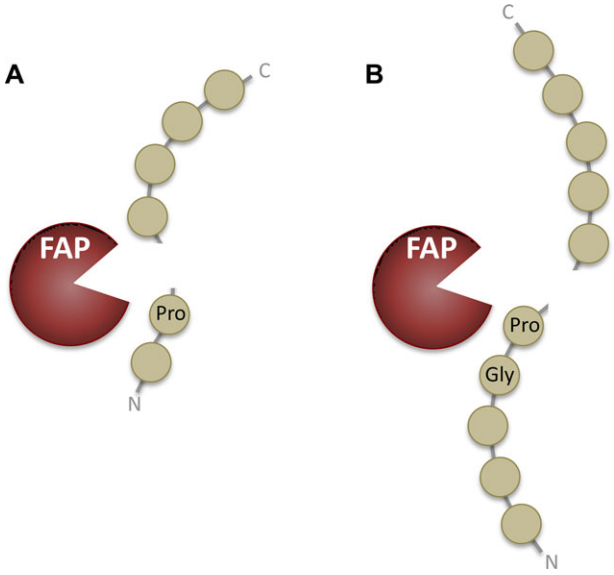

Figure 2. Dual-enzyme activity of FAP. (A) Dipeptidyl peptidase activity of FAP allows it to cleave two amino acids off the N-terminus of a protein. This cleavage occurs after a proline (Pro) residue. (B) Endopeptidase activity of FAP enables cleavage that is more than two amino acids from the N-terminus of a protein. Cleavage is restricted to the post-Pro bond after glycine-Pro (Gly-Pro) (adapted from [68] with permission).

FAP is closely related to DPP4, sharing 52% amino acid sequence identity [3]. However, unlike DPP4, the substrate repertoire and the patterns of in vivo FAP expression are poorly defined. Despite this, FAP is attracting attention in cancer, cardiology and fibrosis research because its expression is greatly upregulated in disease. A number of tools have thus been developed to target and exploit this protease for therapeutic intervention. The aim of this review is to emphasise the need to elucidate the roles and functions of this protease through substrate discovery in order to fundamentally progress the field.

2 Fibroblast activation protein

2.1 Enzyme activity and substrate specificity

Unique from other members of the DPP4 family, FAP has both DPP and endopeptidase activities (Fig. 2). However, despite exhibiting both activities, FAP has been shown to be a more efficient endopeptidase than DPP [4, 5]. The activities and specificities of FAP have been investigated using artificial substrates and synthetic peptide libraries [4, 6–9], which revealed a strong preference for FAP cleavage of endopeptidase substrates after glycine-proline (Gly-Pro) motifs [4, 8, 9]. This restricted cleavage site specificity was supported by results obtained using a fluorescence resonance energy transfer substrate library, which also identified endopeptidase cleavage events only after Gly-Pro motifs [8]. However, a MS-based analysis of FAP endopeptidase specificity using gelatin as a substrate proposed that cleavage can also occur C-terminal to Ala-Pro, Arg-Pro, Lys-Pro and Ser-Pro motifs as well as the reported Gly-Pro motif [10]. The disparate results from these cleavage site specificity profiling studies highlight the limitations of such biochemical approaches. Synthetic peptide libraries do not take into account native protein or peptide structure or the contribution of exosites in protease–substrate interactions. Thus, identification of novel physiological FAP substrates will assist in defining the cleavage site specificity of FAP and determining the potential contribution of exosite interactions of this protease during cleavage events.

2.2 Substrates in vitro and in vivo

Although FAP is involved in numerous (patho-) physiological processes, its substrate repertoire remains mostly unknown. However, screening known DPP4 substrates against a recombinant human soluble form of FAP has identified a number of natural FAP substrates [11]. In that study, full-length neurological peptides were cleaved by FAP and analysed by MALDI-TOF-MS. Four neuropeptides were efficiently cleaved by FAP, namely neuropeptide Y (NPY), peptide YY, B-type natriuretic peptide and substance P, with in vitro half-lives comparable to incubation with DPP4 [11]. Those results clearly show that the DPP specificity of FAP is much less restricted than previously thought, with cleavage occurring after Lys-Pro, Tyr-Pro, Arg-Pro and Ser-Pro dipeptides [11]. Interestingly, DPP cleavage was not observed for some other potential peptide substrates that were also screened and had the same N-terminal dipeptide sequence as the four cleaved neuropeptide substrates. Furthermore, in a previous study using a synthetic dipeptide fluorescent substrate library, limited levels of cleavage by FAP

were reported for these particular dipeptide sequences [6]. Together, these data strengthen the notion that exosite binding has a role in FAP cleavage events.

As the endopeptidase activity of FAP distinguishes this protease from other members of the DPP4 family, it could be speculated that this may be its predominant enzymatic role as well as its unique role. Towards this, two physiological endopeptidase substrates have so far been discovered for FAP; denatured type I collagen (CN-I) [12, 13] and α_2-antiplasmin (α_2-AP) [14, 15]. In both cases, cleavage occurs after a Gly-Pro motif [10, 14].

CN fibres are major components of the extracellular matrix (ECM), providing structural support for cells and tissues. ECM proteins also bind and sequester growth factors and bioactive peptides, thus regulating and influencing important biochemical and biomechanical processes [16]. As CN is rich in Gly-Pro residues, it is unsurprising that FAP is able to digest this ECM protein. While FAP is unable to cleave CN-I in its native form, partial digestion, for example, by matrix metalloproteinase 1, results in unwinding of the CN fibre, and this facilitates FAP cleavage [12, 17]. FAP is also able to cleave denatured CN-III in vitro [17]. CN deposition and degradation in ECM remodelling are common processes in tumourigenesis and fibrosis. The ability to recognise and digest denatured CN-I implicate FAP in ECM remodelling and the pathological processes where this is abnormal.

As well as being a cell surface protease, FAP also exists in a truncated, soluble form in human plasma, lacking the transmembrane domain [15, 18]. Soluble FAP cleaves α_2-AP at prolyl bonds Pro3-leucine4 and Pro12-Asn13 [18]. During tissue repair, fibrin is deposited to form a fibrin clot. Fibrinolysis is the natural process in which a fibrin clot is dissolved by plasmin leading to scar resolution. α_2-AP is an inhibitor of plasmin and therefore reduces the rate of lysis of the fibrin clot (Fig. 3). However, cleavage of α_2-AP by FAP converts α_2-AP into a more potent inhibitor of plasmin [18]. We have therefore hypothesised that this activity of FAP leads to a reduction in fibrinolysis and promotion of scar formation in tissues [1].

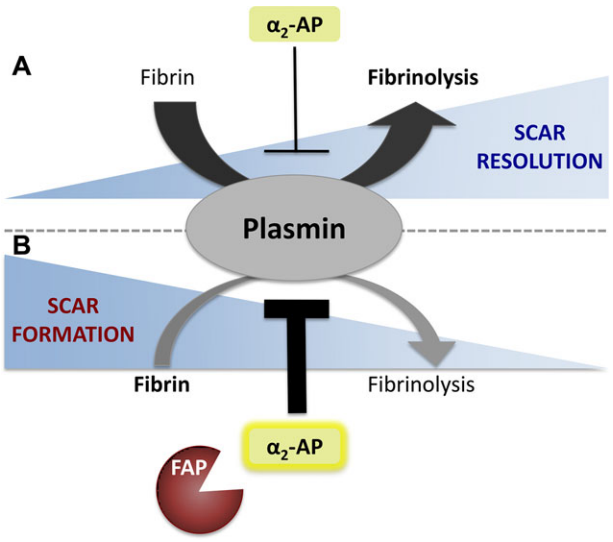

Figure 3. FAP modulates fibrinolysis. α_2-Antiplasmin (α_2-AP) becomes a more potent inhibitor of plasmin following FAP-mediated cleavage between Pro12 and Asn13. This results in a shift from increased fibrinolysis and scar resolution in the absence of FAP (A), to decreased fibrinolysis and increased scar formation when FAP is present (B) (adapted from [1] with permission).

2.3 Expression pattern

FAP is a difficult protein to study due to the limited availability of specific or selective tools to detect and target it [19]. However, recent developments have enabled more accurate and thorough characterisation of the expression pattern of this protease.

Initially it was widely accepted that FAP was either not expressed or present at insignificant levels in normal adult tissue. However, under certain biological circumstances, FAP expression has been observed, such as during mouse embryogenesis [20] and in the resorbing tadpole tail [21]. These observations suggest a role for FAP in normal developmental processes and tissue remodelling. Despite the observed expression of FAP in healthy tissue and its presence in plasma, the FAP knockout mouse has a healthy phenotype [22, 23], suggesting that the role of this protease under normal circumstances is redundant or non-essential.

FAP expression is, however, implicated in a number of pathological processes, resulting in the emergence of this protease as a potential therapeutic target. FAP expression has been observed during wound healing [24], at sites of inflammation including arthritis [25, 26] and in atherosclerotic plaques [27]. Greatly increased intrahepatic FAP expression occurs in cirrhosis and correlates with liver fibrosis severity, where it is expressed by activated hepatic stellate cells [13, 28–30]. FAP is also strongly expressed by stromal fibroblasts in over 90% of epithelial carcinomas [31]. However, despite efforts to study this protease in various cancer models [32–35], little is known about its enzymatic role in the tumour microenvironment. It is often speculated that the collagenolytic role of FAP promotes invasion of tumour cells. Supporting this idea, FAP-positive fibroblasts isolated from human breast tumour stroma co-cultured with breast cancer cells lead to increased cancer cell migration and induction of epithelial–mesenchymal transition [36]. FAP-expressing cells have been shown to have an immunosuppressive role in mice in an immunogenic cancer model [37]. In that study, depletion of FAP-expressing cells resulted in rapid hypoxic necrosis of tumour and stromal cells. The enzymatic or extra-enzymatic mechanism by which FAP is involved in these processes requires further investigation.

Several studies have linked FAP to promoting angiogenesis in the tumour microenvironment [34, 38, 39]. One such study demonstrated that inhibition of both DPP4 and FAP led

to decreased tumour vascularisation, but such a decrease was not observed when DPP4 alone was inhibited [34], thus implicating the enzyme activity of FAP in promoting angiogenesis. FAP is able to cleave NPY to generate a truncated form [11] that is proangiogenic [40], which indicates that increased vascularisation may be mediated partly by FAP cleavage of NPY.

The persistent expression of FAP in tumour stroma clearly implicates this protease in tumourigenesis [31]. However, there is controversy over this role for FAP, with some studies correlating elevated FAP expression with tumour suppression, while others show a correlation with tumour progression [33]. An important consideration when interpreting results from such studies is the source of FAP expression. Although it is well established that FAP is expressed by stromal cells [31], particularly at the invasive front [36, 41], many studies that aim to investigate this protease in the tumour microenvironment have focussed on cancer models using tumour cells that artificially express FAP [42], which is not an accurate reflection of the in vivo tumour microenvironment and results from such studies should be interpreted cautiously. In spite of this, the role of FAP in tumour stroma is still highly contextual and further investigation is required to elucidate its function in tumourigenesis.

In addition to the strong focus on FAP as a tumour stroma marker, FAP is also considered to be a protease of interest in several other pathological conditions, including atherosclerosis [27, 33], where FAP enzyme activity has been implicated via cleavage of CN-I, promoting plaque instability [27]. In this condition, FAP is expressed in human aortic smooth muscle cells and FAP expression correlates with progression of atherosclerotic plaques. Moreover, in this study, higher FAP levels were detected in thin fibrous caps compared to thick caps and treating human sclerotic plaques in vitro with FAP enzyme activity neutralising antibodies decreased FAP-associated CN degradation [27]. Thus, the collagenolytic activity of FAP may be an important driver of fibrous cap rupture. In vivo studies are needed to further evaluate FAP as a candidate therapeutic target for the treatment of patients with high-risk atherosclerotic plaques.

2.4 FAP as a biomarker

A major hurdle in the study of FAP as a potential disease biomarker is the inability to specifically measure FAP enzyme activity. However, an FAP-specific substrate has recently become available, enabling the measurement of FAP activity in fluids and organs from diseased and non-diseased samples [43]. There is a clear need to closely examine whether FAP enzyme activity levels associate with disease progression and pathogenesis to better understand roles of FAP, and to determine whether an FAP-specific enzyme assay could be an informative diagnostic tool in the clinical setting. For example, such an assay may be useful for selecting patients who may be more responsive to a FAP-targeted therapy.

However, regardless of whether FAP itself correlates with severity of disease or tumour progression, the ratio of full-length to cleaved FAP substrates may be found to change in relation to pathogenesis. If so, FAP substrates might become disease biomarkers. Consider the case of FAP-mediated cleavage of NPY, where the resulting cleaved form of NPY is proangiogenic [40]. In the normal healthy adult, FAP digestion of NPY may be negligible and potentially redundant as DPP4 is ubiquitous and cleaves NPY efficiently. However, in a patient with an aggressive epithelial tumour, the stromal fibroblasts surrounding the tumour can express high levels of cell surface FAP and thus the contribution by FAP to the N-terminal cleavage of NPY in that microenvironment may supersede the contribution of DPP4. Thus, levels of cleaved versus full-length FAP substrates may have potential as biomarkers of disease progression. Proteomics-based assays utilising selected reaction monitoring (SRM) can be designed and optimised to detect and quantify levels of peptides in complex biological samples. SRM analysis has previously been applied to not only determine levels of cleaved and full-length caspase substrates in vivo, but also the rate of cleavage [44]. Such techniques may be applicable to FAP substrates.

3 Targeting FAP

As the role of FAP seems to be highly contextual, it is important to consider the pathological situation in order to determine the best method to target FAP-expressing cells. A number of tools have therefore been designed to exploit the enzyme activity of FAP or to target this protein on cells.

3.1 Inhibitors

A major hurdle in the study of FAP enzyme activity has been the lack of selective inhibitors against this protease. FAP shares DPP specificity with the enzyme members of the DPP4 family, DPP4, DPP8 and DPP9, as well as endopeptidase specificity with prolyl endopeptidase (PREP). Thus, designing inhibitors that are selective for FAP over other DPPs and PREP is challenging. Recently, however, two independent groups have designed potent FAP-selective inhibitors [45, 46] (Table 1).

Poplawski et al. have recently developed potent FAP-selective and PREP-selective inhibitors using boronic acid-based compounds [46]. One compound, *N*-(pyridine-4-carbonyl)-D-Ala-boroPro, has a more than 350-fold selectively for FAP over PREP, and also has very strong selectivity over DPP4, DPP8 and DPP9. The development of such a potent and selective inhibitor will no doubt prove extremely useful in the investigation of the biological role of FAP.

An independent study has developed a new class of FAP inhibitors based on an *N*-(4-quinolinoyl)-Gly-(2-cyanopyrolidine) scaffold [45]. Of the 34 compounds

Table 1. Properties of potent FAP-selective inhibitors

Compound	IC_{50} (nM)					K_i (nM)		
	FAP	DPP4	DPP8	DPP9	PREP	FAP	PREP	References
N-(Pyridine-4-carbonyl)-D-Ala-boroPro, (compound 6)	36 ± 4.8	>100 000	5600 ± 1300	3400 ± 800	13 000 ± 4300	9 ± 0.9	3100 ± 260	[46]
Compound 7	10.3 ± 0.4	>100 000	N/A	>100 000	860 ± 70	3 ± 0.4	N/A	[45]

Values are expressed as ±SEM. N/A, not available.

developed in that study, compound 7 has particular selectivity towards FAP over the related proteases DPP4, DPP8 and DPP9, and is also selective over PREP. Modifications to the quinolinoyl ring improved the selectivity for FAP over PREP [45], indicating that further developments of this scaffold can yield compounds with more selectivity for FAP.

As combined inhibition of FAP and DPP4 enzyme activities attenuates tumour growth more than DPP4 inhibition alone [34, 47], a potent FAP-selective inhibitor may have potential for treating certain epithelial tumours and may be useful in a number of other clinical settings, such as atherosclerosis and arthritis [33]. The development of potent selective FAP inhibitors will be very useful in developing an understanding of the biological roles of FAP.

3.2 Prodrugs

Prodrugs consist of a cytotoxic agent coupled to a peptide that encodes the consensus sequence for cleavage by a particular protease. The cytotoxic agent becomes active upon release from the peptide, which only occurs in the presence of the specific protease (Fig. 4). Due to the restricted expression pattern and unique enzyme activity of FAP, FAP-activated prodrugs have potential as a therapeutic intervention in a number of pathological processes where FAP-expressing cells are implicated. Several FAP-activated prodrugs and protoxins have been designed.

The honeybee toxin melittin has been modified to produce an FAP-activated protoxin that causes lysis of FAP-positive tumour stromal cells [48]. Intra-tumoural injection of this protoxin into breast and prostate cancer xenographs in mice resulted in significant cell lysis and inhibition of tumour growth. However, intravenous administration of this protoxin caused haemolysis and death, indicating that use of this protoxin should be limited to situations where intra-tumoural injection is possible. Despite its limitations, this study demonstrates that FAP-activated protoxins can significantly impair tumour growth.

Doxorubicin has had limited clinical success as a chemotherapeutic agent due to cytotoxic side effects [49]. An FAP-activated doxorubicin prodrug has been shown to minimise these off-target effects [9]. This prodrug produced

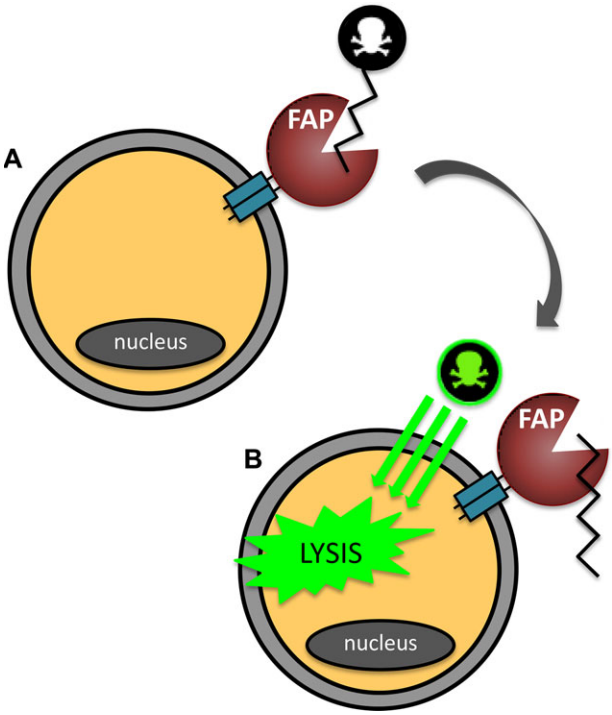

Figure 4. FAP-activated prodrugs. Prodrugs consist of a cytotoxic agent conjugated to a peptide sequence specific for a particular protease, in this case FAP. Exposure of the prodrug to FAP (A) results in cleavage of the peptide sequence thereby releasing and activating the cytotoxic agent (B), which acts on the cell.

similar anti-tumour effects to doxorubicin alone, without the severe cytotoxic side effects in normal tissue. However, low levels of non-specific activation of the prodrug were observed in normal tissue and plasma, which was attributed to the instability of the peptide bond linking the dipeptide to doxorubicin. However, the dipeptide sequence used in that study is known to be cleaved by other oligopeptidases, including PREP [8], which is ubiquitous in normal tissues. Such issues of linker instability, peptide specificity and protease distribution are commonly encountered in the development and use of protease-activated prodrugs.

Another FAP-activated prodrug contains the plant-derived toxin thapsigargin (TG) [50], which inhibits calcium pumps, leading to an intracellular build up of calcium, resulting in

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