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Neuropeptide Y, B-type natriuretic peptide, substance P and peptide YY are novel substrates of fibroblast activation protein-α

Fiona M. Keane[1], Naveed A. Nadvi[1,2], Tsun-Wen Yao[1] and Mark D. Gorrell[1]

1 Centenary Institute, Sydney Medical School, University of Sydney, NSW, Australia
2 Pharmaceutical Chemistry, Faculty of Pharmacy, University of Sydney, NSW, Australia

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Correspondence
M. D. Gorrell, Molecular Hepatology, Centenary Institute, Locked Bag No. 6, Newtown, NSW 2042, Australia
Fax: +61 2 95656101
Tel: +61 2 95656156
E-mail: m.gorrell@centenary.usyd.edu.au

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Fibroblast activation protein-α (FAP) is a cell surface-expressed and soluble enzyme of the prolyl oligopeptidase family, which includes dipeptidyl peptidase 4 (DPP4). FAP is not generally expressed in normal adult tissues, but is found at high levels in activated myofibroblasts and hepatic stellate cells in fibrosis and in stromal fibroblasts of epithelial tumours. FAP possesses a rare catalytic activity, hydrolysis of the post-proline bond two or more residues from the N-terminus of target substrates. α_2-antiplasmin is an important physiological substrate of FAP endopeptidase activity. This study reports the first natural substrates of FAP dipeptidyl peptidase activity. Neuropeptide Y, B-type natriuretic peptide, substance P and peptide YY were the most efficiently hydrolysed substrates and the first hormone substrates of FAP to be identified. In addition, FAP slowly hydrolysed other hormone peptides, such as the incretins glucagon-like peptide-1 and glucose-dependent insulinotropic peptide, which are efficient DPP4 substrates. FAP showed negligible or no hydrolysis of eight chemokines that are readily hydrolysed by DPP4. This novel identification of FAP substrates furthers our understanding of this unique protease by indicating potential roles in cardiac function and neurobiology.

Structured digital abstract

- **FAP** cleaves **GLP-1-amide** by protease assay (View interaction)
- **DPP4** cleaves **PACAP** by protease assay (View interaction)
- **FAP** cleaves **PYY** by protease assay (View interaction)
- **FAP** cleaves **NPY** by protease assay (View interaction)
- **DPP4** cleaves **Substance P** by protease assay (View interaction)
- **DPP4** cleaves **GIP** by protease assay (View interaction)
- **DPP4** cleaves **CCL11-Eotaxin** by protease assay (View interaction)
- **DPP4** cleaves **GLP-1-amide** by protease assay (View interaction)
- **DPP4** cleaves **CXCL12-SDF1α** by protease assay (View interaction)

Abbreviations

BNP, B-type natriuretic peptide; CCL3/MIP1α, C-C motif chemokine 3/macrophage inflammatory protein 1α; CCL5/RANTES, C-C motif chemokine 5/RANTES; CCL11/eotaxin, C-C motif chemokine 11/eotaxin; CCL22/MDC, C-C motif chemokine 22/macrophage-derived chemokine; CXCL2/Groβ, C-x-C motif chemokine 2/Groβ; CXCL6/GCP2, C-x-C motif chemokine 6/granulocyte chemotactic protein-2; CXCL9/MIG, C-x-C motif chemokine 9/monokine induced by interferon-γ; CXCL10/IP10, C-x-C motif chemokine 10/interferon-γ-induced protein 10; CXCL11/ITAC, C-x-C motif chemokine 11/interferon-inducible T-cell alpha chemoattractant; CXCL12/SDF-1α, C-x-C motif chemokine 12/stromal cell-derived factor-1α; DPP4, dipeptidyl peptidase 4; DPP8, dipeptidyl peptidase 8; DPP9, dipeptidyl peptidase 9; ECM, extracellular matrix; FAP, fibroblast activation protein-α; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; GRF, growth hormone-releasing factor; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase-activating peptide; PEP, prolyl endopeptidase; PHM, peptide histidine methionine; PYY, peptide YY; VIP, vasoactive intestinal peptide; Z, benzyloxycarbonyl.

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- **FAP** cleaves **GRF** by protease assay (View interaction)
- **FAP** cleaves **GLP-2** by protease assay (View interaction)
- **DPP4** cleaves **Glucagon** by protease assay (View interaction)
- **FAP** cleaves **BNP** by protease assay (View interaction)
- **DPP4** cleaves **CXCL2-GROβ** by protease assay (View interaction)
- **DPP4** cleaves **CCL22-MDC** by protease assay (View interaction)
- **DPP4** cleaves **CXCL9-MIG** by protease assay (View interaction)
- **FAP** cleaves **GIP** by protease assay (View interaction)
- **DPP4** cleaves **GRF** by protease assay (View interaction)
- **DPP4** cleaves **CXCL11 ITAC** by protease assay (View interaction)
- **FAP** cleaves **Substance P** by protease assay (View interaction)
- **DPP4** cleaves **VIP** by protease assay (View interaction)
- **DPP4** cleaves **CCL5 -RANTES** by protease assay (View interaction)
- **DPP4** cleaves **PHM** by protease assay (View interaction)
- **DPP4** cleaves **Oxyntomodulin** by protease assay (View interaction)
- **DPP4** cleaves **CXCL10-IP10** by protease assay (View interaction)
- **DPP4** cleaves **PYY** by protease assay (View interaction)
- **DPP4** cleaves **BNP** by protease assay (View interaction)
- **DPP4** cleaves **GLP-2** by protease assay (View interaction)
- **DPP4** cleaves **NPY** by protease assay (View interaction)

Introduction

The dipeptidyl peptidase 4 (DPP4) enzyme family contains two pairs of closely related proteases, namely the cell surface glycoproteins DPP4 (EC 3.4.14.5) and fibroblast activation protein-α (FAP), and the intracellular proteases dipeptidyl peptidase 8 (DPP8) and dipeptidyl peptidase 9 (DPP9). This family of enzymes has clinical importance, as DPP4 is a target for type 2 diabetes treatment [1,2], and FAP has emerged as a potential fibrosis, metabolic syndrome and cancer therapeutic target [3–6]. All four enzymes are members of the larger prolyl oligopeptidase family, characterized by a catalytic triad of serine, aspartic acid and histidine, which is the reverse order of that seen in typical serine proteases. These proteases have the unique ability to cleave a post-proline bond, which differs from all other amino acid bonds, because of the cyclical nature of proline. This gives a specialized function to members of the DPP4 enzyme family, as they can degrade proline-containing substrates that would otherwise resist cleavage. FAP, DPP4, DPP8 and DPP9 have dipeptidyl peptidase activity, exhibiting an ability to hydrolyse the prolyl bond two residues from the N-terminus of substrates [7–10]. In addition, FAP has endopeptidase activity, favouring cleavage after Gly–Pro [11–14]. Prolyl endopeptidase (PEP) is the only other prolyl oligopeptidase family member that has endopeptidyl peptidase activity. However, PEP is a soluble cytoplasmic enzyme and has a broader substrate specificity than FAP. PEP has important functions in the brain [15].

Although FAP (Protein Data Bank ID: 1Z68) [12] shares a similar tertiary and quaternary structure and 52% sequence identity with DPP4 (Protein Data Bank ID: 1R9M) [16], these two proteins differ in two main respects: their enzyme activities and their expression profiles. FAP exhibits dipeptidyl peptidase activity on synthetic fluorogenic substrates, but the only known natural substrates of FAP are cleaved at endopeptidase sites. Denatured type 1 collagen [9,14] and α_2-antiplasmin [11,17] are the only two natural FAP substrates reported. In contrast to DPP4, FAP has a limited expression profile and is not expressed in normal adult tissue [18]. Its expression is restricted to sites of tissue remodelling and activated stroma. Given that FAP expression is associated with wound healing, malignant tumour growth and chronic inflammation, which all involve extracellular matrix (ECM) degradation, the gelatinase activity of FAP may contribute to ECM degradation. FAP is associated with fibrosis, cell migration and apoptosis [19], and it may also be a marker for certain cancers [20–22]. FAP's role in liver disease has been recently reviewed [23]. Despite numerous studies on the roles of FAP in human diseases, its range of natural substrates is poorly characterized.

Identifying substrates is a crucial step in gaining insights into the precise functions of proteases and their mechanisms of action in biology and disease. DPP4 is the prototype member of this family, and over 30 different substrates have been identified. The insulin-secreting hormones are among the most well-characterized DPP4 substrates [8]. The inhibition of DPP4-mediated glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP)

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degradation is the basis for targeting this enzyme in the treatment of type 2 diabetes [24]. GLP-1, glucagon-like peptide-2 (GLP-2), glucagon and oxyntomodulin all have roles in glucose homeostasis [25]. Growth hormone-releasing factor (GRF) is released from nerve terminals and stimulates growth hormone secretion. Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) both bind to the VIP receptor expressed by the liver, pancreas and intestine. PACAP is a neurotransmitter that results in increased cytoplasmic cAMP levels. VIP is produced by the gut and pancreas, and also by the hypothalamus. Peptide histidine methionine (PHM) functions in vasodilation. All of the above peptides, termed gastrointestinal hormones in this study, have an N-terminal sequence beginning with His-Ala, His-Ser or Tyr-Ala, and all are known substrates of DPP4 [8,25–31].

In addition to gastrointestinal hormones, neuropeptides are among the most efficient of the DPP4 substrates. Neuropeptide Y (NPY) is found throughout the brain, and is involved in the regulation of energy balance by stimulating increased food intake. Peptide YY (PYY) is produced by the gastrointestinal tract, and, via NPY receptor binding, functions to reduce appetite and slow gastric emptying. Substance P is a neurotransmitter released from sensory nerves [32]. B-type natriuretic peptide (BNP) was originally isolated from brain [33], but is predominantly produced by cardiac ventricles in response to cardiomyocyte stretching. These four neuropeptides have an N-terminal dipeptide of Tyr-Pro, Ser-Pro or Arg-Pro, and all are cleaved efficiently by DPP4, resulting in altered functions [7,34,35].

Chemokines are important cytokines that activate and direct the migration of different types of leukocytes from the bloodstream into sites of infection and inflammation. Some chemokines have previously been shown to be DPP4 substrates [36–39], a subset of which is also cleaved by DPP8 [40].

This study investigated the relative abilities of recombinant human FAP to catalyse the degradation of known DPP4 substrates of the gastrointestinal hormone, neuropeptide and chemokine classes by MALDI-TOF MS analysis.

Results

Enzyme activity of recombinant soluble human FAP and DPP4

Recombinant human FAP and DPP4 were highly purified and active. The specific activities of FAP and DPP4 were > 1800 pmol·min^{-1}·μg^{-1} on benzyloxycarbonyl (Z)-Gly-Pro-AMC and 1830 nmol·min^{-1}·μg^{-1} on H-Gly-Pro-*p*-nitroanilide, respectively. To assay the substrate specificity of each protease, enzyme activity assays were carried out on synthetic fluorogenic substrates. FAP acts as both a dipeptidyl peptidase and an endopeptidyl peptidase, and this was shown by hydrolysis of both H-Ala-Pro-AMC and Z-Gly-Pro-AMC. FAP is known to poorly hydrolyse H-Gly-Pro-containing substrates [12], as was observed (Fig. 1A). It was also shown that there was no PEP contamination of the purified FAP by the absence of

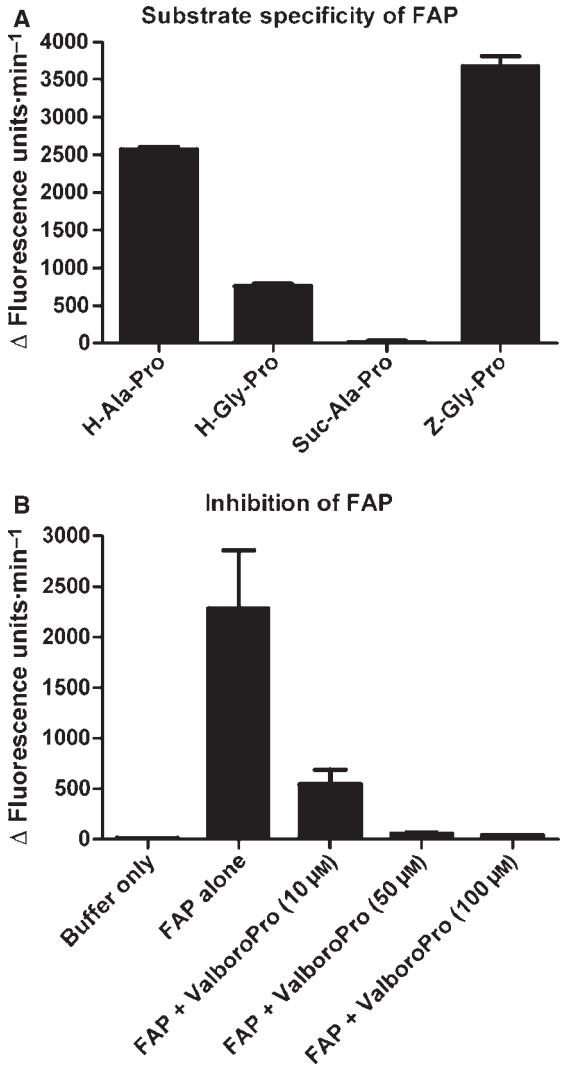


Fig. 1. FAP enzyme activity. (A) Purified soluble recombinant human FAP was incubated with H-Ala-Pro-AMC, H-Gly-Pro-AMC, succinyl-Ala-Pro-AMC and Z-Gly-Pro-AMC fluorescent substrates. (B) Inhibition profile of FAP hydrolysis of Z-Gly-Pro-AMC. Various concentrations of ValboroPro showed dose-dependent inhibition of FAP as compared with buffer alone. Enzyme activity was detected as change in fluorescence units over time.

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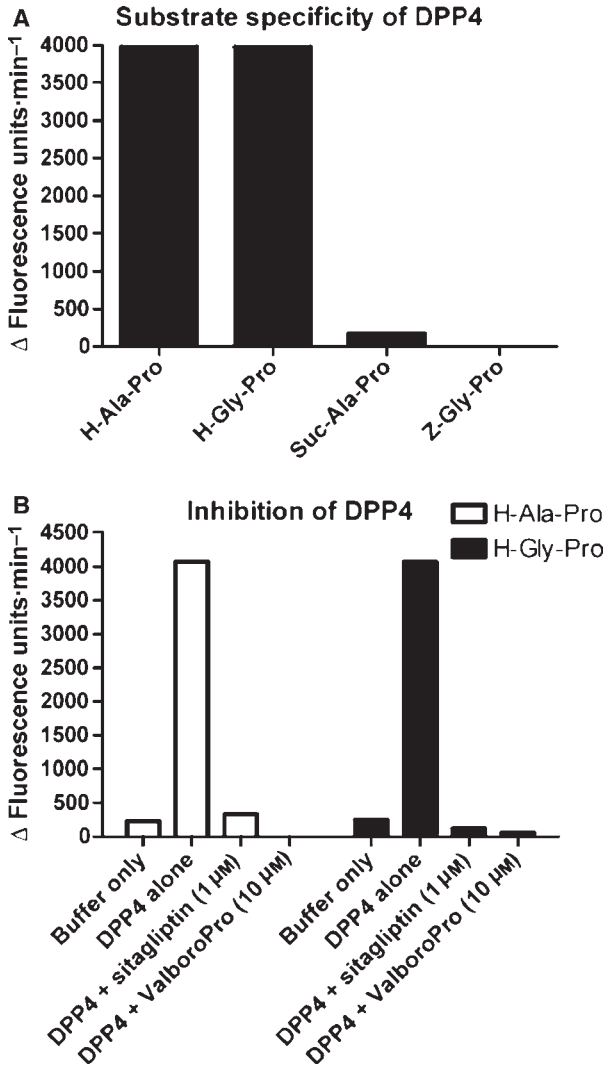


Fig. 2. DPP4 enzyme activity. (A) Purified soluble recombinant human DPP4 was incubated with H-Ala-Pro-AMC, H-Gly-Pro-AMC, succinyl-Ala-Pro-AMC and Z-Gly-Pro-AMC fluorescent substrates. DPP4 had dipeptidase activity, and no endopeptidase contamination was detected. (B) Inhibition of DPP4 cleavage of H-Ala-Pro-AMC and H-Gly-Pro-AMC substrates. Final concentrations of 1 μM sitagliptin and 10 μM ValboroPro were incubated with DPP4. Enzyme activity was detected as change in fluorescence units over time.

detectable succinyl-Ala-Pro-AMC cleavage (Fig. 1A). PEP hydrolyses both succinyl-Ala-Pro-AMC and Z-Gly-Pro-AMC, whereas FAP can hydrolyse only Z-Gly-Pro-AMC. FAP was also inhibited by the dipeptidyl peptidase peptidase inhibitor ValboroPro, in a dose-dependent manner (Fig. 1B). Recombinant DPP4 hydrolysed H-Ala-Pro-AMC and H-Gly-Pro-AMC equally, as expected, and no hydrolysis of the endopeptidase substrates succinyl-Ala-Pro-AMC and Z-Gly-Pro-AMC occurred, which showed that there was no endopeptidase contamination (Fig. 2A). On further investigation of the action of DPP4 on H-Ala-Pro-AMC and H-Gly-Pro-AMC, it was shown that both the selective DPP4 inhibitor sitagliptin and the nonselective dipeptidyl peptidase inhibitor ValboroPro inhibited the activity of DPP4 on both substrates (Fig. 2B).

Substrate cleavage by FAP and DPP4

After the integrity of both recombinant enzymes had been verified, the ability of FAP to cleave known natural DPP4 substrates (Table 1) was then tested at least three times with a MALDI-TOF MS-based assay. Representative samples were taken at various relevant times of peptide–enzyme coincubation. A control incubation was also set up, containing each substrate in enzyme buffer to monitor any potential natural breakdown of substrates over time at 37 °C. None of the substrates tested broke down in buffer alone. Within minutes of DPP4 incubation, all previously reported DPP4 substrates tested exhibited size reductions consistent with removal of two N-terminal amino acids. FAP cleaved the neuropeptides NPY, BNP, substance P and PYY most efficiently. For each protease, a hierarchy of peptide cleavage was determined. DPP4 is very active on these peptides, so more FAP than DPP4 was used, in order to increase the probability of detecting cleavage by FAP. The half-lives of all substrates, upon FAP and DPP4 coincubation, are given in Table 2.

Neuropeptides – PYY, NPY, substance P and BNP

PYY had an average observed molecular mass of 4307 Da. This peptide was an efficient FAP substrate, with the dipeptide, Tyr-Pro, being cleaved off with a half-life of 60 min. Cleavage resulted in a predominant peak of 4047 Da. Intact NPY had an average observed molecular mass of 4265 Da. NPY was an efficient substrate of FAP, with the Tyr-Pro dipeptide being cleaved off with a half-life of 6 min to yield a peptide of 4007 Da. Substance P had an average observed molecular mass of 1348 Da. Upon FAP coincubation, two amino acids (Arg-Pro) followed by a further two amino acids (Lys-Pro) were cleaved off substance P to yield peptides of 1095 Da and 870 Da, respectively. The half-life of the full-length peptide was calculated to be 8 min. No further breakdown of substance P occurred with FAP incubation up to 72 h. BNP had an average observed molecular mass of 3466 Da. Upon FAP coincubation, the N-terminal dipeptide, Ser-Pro, was cleaved off BNP, displaying a half-life of 6 min and no further cleavage event occurred up to 72 h. Similar dipeptidyl peptidase cleavage of all four

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Table 1. Substrate properties. N-terminal amino acid sequences, in single-letter code, were obtained from the UniProt accession numbers listed at http://www.uniprot.org. Observed masses were calculated from six individual MALDI-TOF MS spectra.

Category	Name	UnipProt number	N-terminal sequence	No. of amino acids	Theoretical full-length mass (Da)	Average observed full-length mass (Da)	Average observed cleaved mass (Da)	Average mass loss (Da)
Gastrointestinal hormone	GLP-1-amide	P01275	HAEGTF	30	3299	3299	3090	209
	GLP-2	P01275	HADGSF	33	3766	3768	3559	209
	GIP	P09681	YAEGTF	42	4983	4982	4749	233
	Glucagon	P01275	HSQGTF	29	3483	3484	3260	224
	PHM	P01282	HADGVF	27	2986	2986	2778	208
	GRF-amide	P01286	YADAIF	29	3359	3359	3125	234
	Oxyntomodulin	P10275	HSQGTF	37	4449	4453	4227	226
	VIP	P01282	HSDAVF	28	3327	3327	3104	223
	PACAP-amide	P18509	HSDGIF	38	4535	4531	4308	223
Neuropeptide	PYY	P10082	YPIKPE	36	4311	4307	4047	260
	BNP	P16860	SPKMVQ	32	3466	3466	3281	185
	NPY	P01303	YPSKPD	36	4273	4266	4007	259
	Substance P	P20366	RPKPQQ	11	1348	1348	1095, 886	253, 209
Chemokine	CCL3/MIP1α	P10147	ASLAAD	70	7788	7783	7631	152
	CCL5/RANTES	P13501	SPYSSD	68	7851	7863	7668	195
	CCL11/eotaxin	P61671	GPASVP	74	8365	8364	8198	166
	CCL22/MDC	O00626	GPYGAN	69	8090	8060	7915, 7684	145, 231
	CXCL2/Groβ	P19875	APLATE	73	7892	7886	7717	169
	CXCL6/GCP2	P80162	VLTELR	72	7904	7899	–	–
	CXCL9/MIG	Q07325	TPVVRK	104	11 725	11 720	11 550	170
	CXCL10/IP10	P02778	VPLSRT	77	8646	8601	8398	203
	CXCL11/ITAC	O14625	FPMFKR	73	8307	8332	8076	256
	CXCL12/SDF-1α	P48061	KPVSLS	67	7835	7830	7599	231

neuropeptides was seen with DPP4 coincubation (Figs 3 and 4). The order of neuropeptide substrate preference for FAP was NPY ≈ BNP > substance P > > PYY, whereas that for DPP4 was NPY ≈ BNP > PYY > substance P.

Gastrointestinal hormones – GLP-1, GLP-2, PHM, GRF and GIP

GLP-1 and GLP-2 are similar peptides, with molecular masses of 3299 Da and 3768 Da, respectively. Both peptides have His-Ala as the N-terminal dipeptide, and DPP4 cleavage of these substrates has been studied extensively [8,28]. Here, we showed that FAP is capable of the same cleavage event, producing peptides of 3090 Da and 3558 Da for GLP-1 and GLP-2, respectively. Both peptides were inefficient FAP substrates, with half-lives of 22 h and 19 h, respectively (Fig. 5). PHM and GRF were also inefficient substrates of FAP. PHM had an average observed molecular mass of 2986 Da, and, upon FAP coincubation, two amino acids (His-Ala) were cleaved off, with the half-life calculated to be 16 h. GRF was detected as a peak of 3359 Da that was degraded to 3124 Da upon FAP coincubation. This size change is consistent with the loss of the N-terminal dipeptide Tyr-Ala from GRF. No further breakdown of either peptide was observed up to 72 h, and neither peptide showed breakdown at 37 °C in the absence of protease (Fig. 6). GIP had an average observed molecular mass of 4982 Da. Upon FAP coincubation, dipeptidyl cleavage of Tyr-Pro from GIP was observed after prolonged incubation (half-life of 39 h), yielding a peptide of 4748 Da (Fig. 7). In contrast, efficient dipeptidyl peptidase cleavage of these five gastrointestinal hormones was seen with DPP4 coincubation (Figs 5–7). The order of substrate preference for FAP was PHM ≈ GRF > GLP-2 > GLP-1 > > GIP, whereas the order of preference for DPP4 was GRF > PHM ≈ GLP-1 ≈ GIP > > GLP-2.

Gastrointestinal hormones – VIP, glucagon, PACAP and oxyntomodulin

The remaining gastrointestinal hormones tested all showed poor dipeptidyl peptidase cleavage by FAP, with half-lives for full-length VIP, glucagon, PACAP and oxyntomodulin not being calculated, as 50%

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Table 2. Substrate cleavage by DPP4 and FAP. The CCL3/MIP1α and CXCL6/GCP2 used in this study are not DPP4 substrates. CCL3/MIP-1α (LD-78α) (ASLAADTPTACCFSYTSRQIPQNFIADYFETSSQCSKPGVIFLTKRSRQVCADPSEEWVQKYVSDLELSA) has been found not to be a DPP4 substrate [38]. Concordantly, CCL3/MIP-1α was very inefficiently cleaved by DPP4, with slight cleavage detected after 78 h of incubation (Fig. S3B). Full-length CXCL6/GCP2 has 77 residues, with an N-terminal sequence of GPVSAVLTELR, but the commercially available CXCL6/GCP2 used here lacks the N-terminal five amino acids, so it begins with VLTELR and is thus not a DPP4 substrate. *n*, number of replicate experiments; NM, not measured, owing to there being less than 50% cleavage of the peptide during the indicated incubation time; SD, standard deviation.

		Incubation with DPP4 (0.1 μM)			Incubation with FAP (0.2 μM)		
Substrate category	Name	Half-life ± SD	Unit	*n*	Half-life ± SD	Unit	*n*
Gastro intestinal hormone	GLP-1-amide	8.63 ± 0.92	min	3	21.8 ± 12.7	h	7
	GLP-2	38.2 ± 7.8	min	3	18.76 ± 13.4	h	4
	GIP	8.02 ± 2.19	min	4	39.1 ± 14.7	h	4
	Glucagon	90.9 ± 36.8	min	3	NM (> 72 h)	–	2
	PHM	8.44 ± 2.84	min	5	15.5 ± 4.7	h	4
	GRF-amide	2.02 ± 1.03	min	4	16.14 ± 4.5	h	3
	Oxyntomodulin	133.1 ± 23.5	min	3	NM (> 72 h)	–	2
	VIP	173.3 ± 30.2	min	3	NM (> 72 h)	–	2
	PACAP-amide	18.5 ± 8.5	min	4	NM (> 72 h)	–	2
Neuropeptide	PYY	24.3 ± 3.97	min	5	60.2 ± 16.9	min	4
	BNP	4.04 ± 0.63	min	5	6.24 ± 1.85	min	4
	NPY	2.96 ± 0.94	min	4	5.78 ± 1.62	min	4
	Substance P	28.5 ± 5.4	min	3	8.24 ± 1.95	min	3
Chemokine	CCL3/MIP1α	NM (> 78 h)	–	1	No cleavage	–	1
	CCL5/RANTES	55.6 ± 0.5	min	2	No cleavage	–	2
	CCL11/eotaxin	58.5 ± 3.29	min	2	No cleavage	–	2
	CCL22/MDC	1.48 ± 0.54	min	5	NM (> 78 h)	–	2
	CXCL2/Groβ	24.0 ± 3.83	min	2	NM (> 24 h)	–	2
	CXCL6/GCP2	No cleavage	–	1	No cleavage	–	1
	CXCL9/MIG	72.9 ± 1.79	min	2	No cleavage	–	2
	CXCL10/IP10	15.8 ± 1.82	min	2	No cleavage	–	2
	CXCL11/ITAC	5.64 ± 1.31	min	2	No cleavage	–	2
	CXCL12/SDF-1α	2.33 ± 0.54	min	4	NM (> 24 h)	–	2

degradation was not achieved during the long coincubation time periods that were evaluated (Figs S1 and S2). The maximum detected extents of degradation of VIP, glucagon, PACAP and oxyntomodulin were 20%, 15%, 13% and 38%, respectively, after 72 h. As expected, however, these four substrates were cleaved by DPP4, with PACAP, glucagon, oxyntomodulin and VIP showing half-lives of 18.5 ± 8.46, 90.85 ± 36.83, 133.13 ± 23.51 and 173.33 ± 30.19 min, respectively (Table 2).

Chemokines

Chemokines are a family of small cytokines secreted to induce chemotaxis in nearby responsive cells. They are larger peptides than the incretins and neuropeptides that were tested here. The chemokines in this study varied from 7700 to 11 700 Da. A subset of chemokines have previously been shown to be DPP4 substrates [37]. We tested 10 chemokines for FAP cleavage. These 10 chemokines included eight that are known to be cleaved by DPP4 [C-C motif chemokine 5/RANTES (CCL5/RANTES), C-C motif chemokine 11/eotaxin (CCL11/eotaxin), C-C motif chemokine 22/macrophage-derived chemokine (CCL22/MDC), C-x-C motif chemokine 2/Groβ (CXCL2/Groβ), C-x-C motif chemokine 9/granulocyte chemotactic protein-2 (CXCL9/GCP2), C-x-C motif chemokine 10/interferon-γ-induced protein 10 (CXCL10/IP10), C-x-C motif chemokine 11/interferon-inducible T-cell alpha chemoattractant (CXCL11/ITAC) and C-x-C motif chemokine 12/stromal cell-derived factor-1α (CXCL12/SDF-1α)] and two that are not DPP4 substrates [C-C motif chemokine 3/macrophage inflammatory protein 1α (CCL3/MIP-1α)/LD78α and an N-terminally truncated variant of C-x-C motif chemokine 6/granulocyte chemotactic protein-2 (CXCL6/GCP2)]. All 10 chemokines showed little or no cleavage upon FAP coincubation (Figs S3–S7). Three chemokines showed slight dipeptidyl peptidase cleavage upon prolonged FAP coincubation: the extents of cleavage of CCL22/MDC, CXCL2/Groβ and

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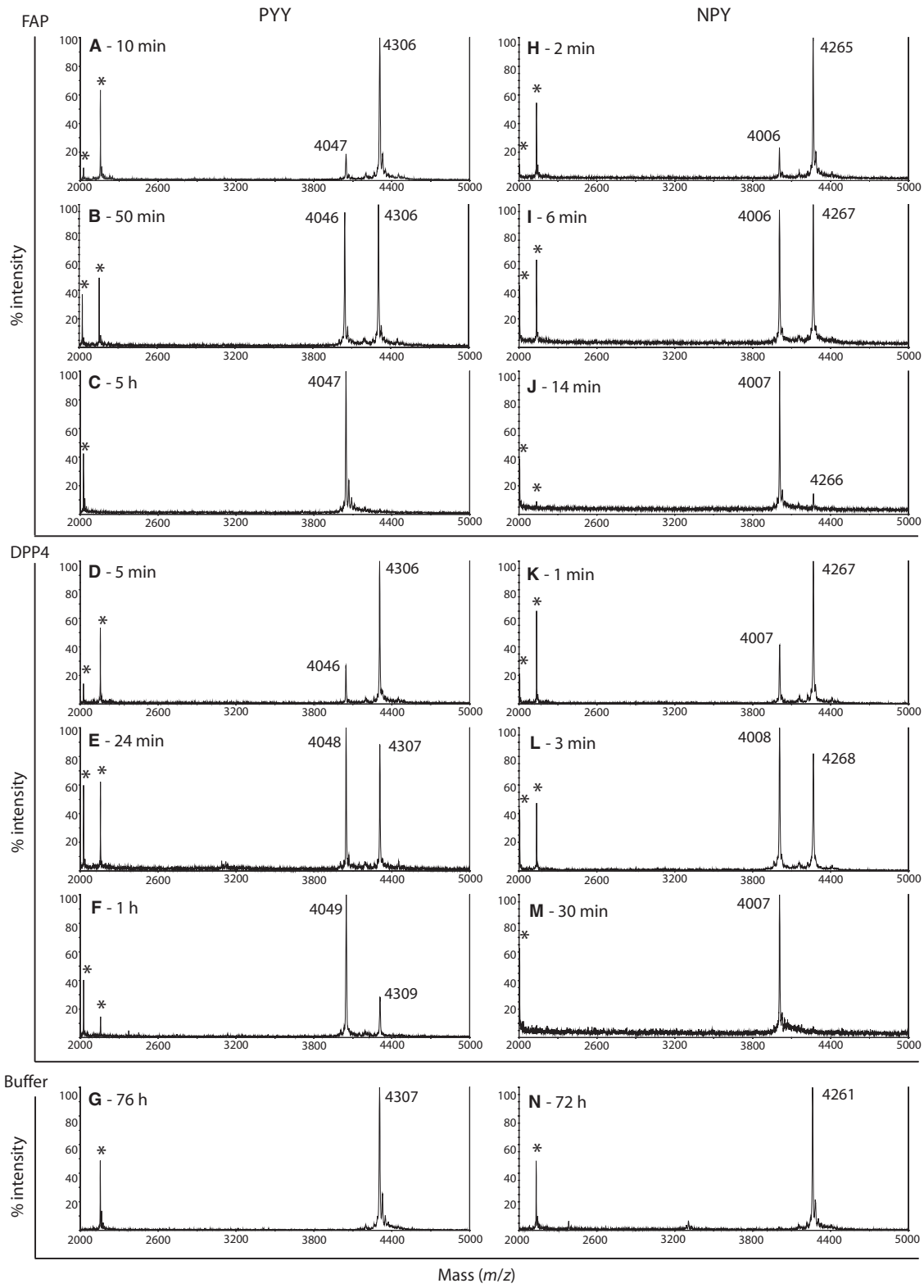


Fig. 3. PYY and NPY cleavage by FAP and DPP4. FAP (0.2 μM) (A, B, C, H, I, J) and DPP4 (0.1 μM) (D, E, F, K, L, M) were incubated with PYY (A–G) and NPY (H–N) for various lengths of time. The control incubation of peptide in buffer alone is also shown (G, N). Representative MALDI-TOF MS analyses of substrate at early (A, D, H, K), middle (B, E, I, L) and late (C, F, J, M) stages of cleavage are shown. Peaks are labelled with their molecular masses. Asterisks denote double charged peaks.

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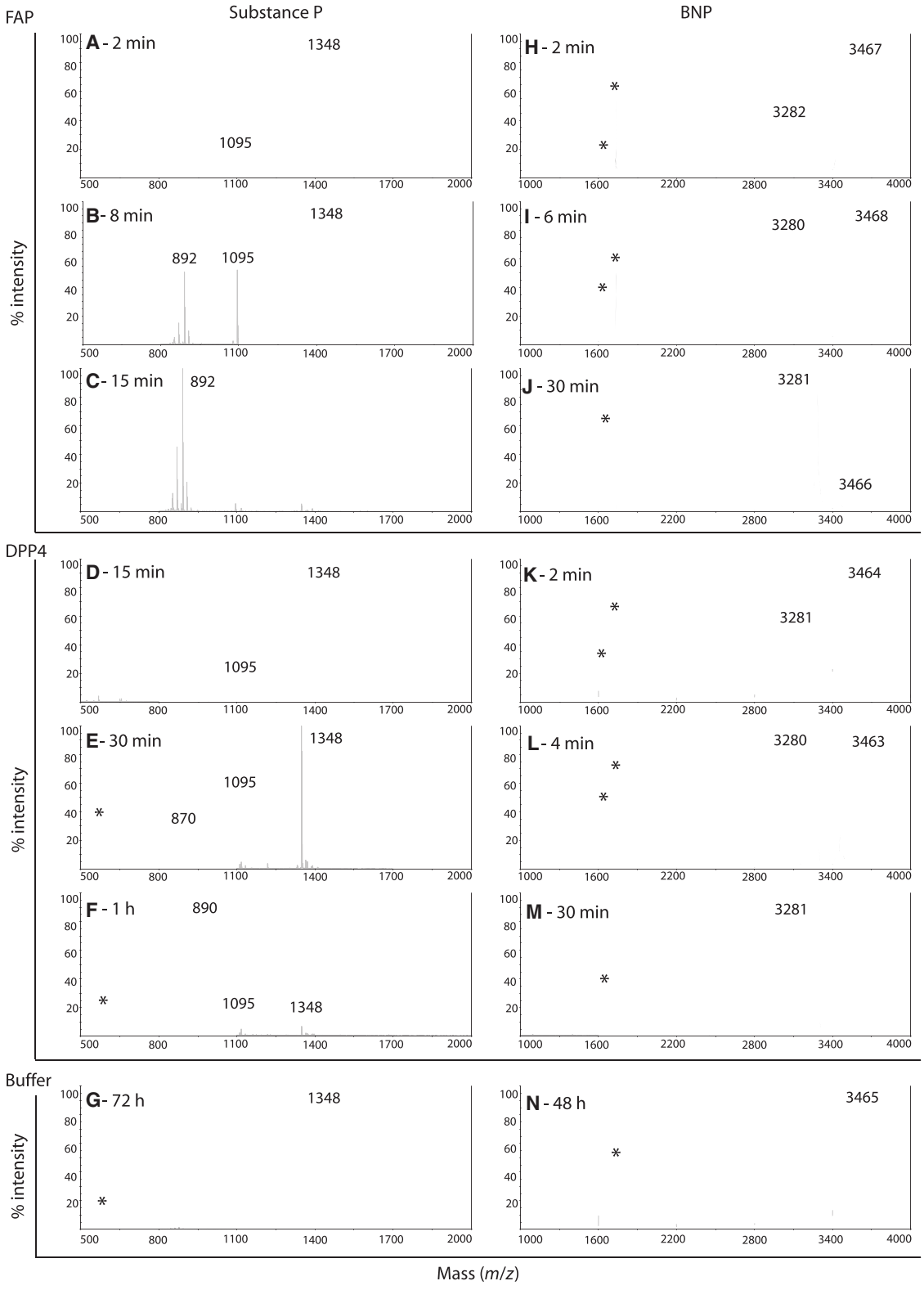


Fig. 4. Substance P and BNP cleavage by FAP and DPP4. FAP (0.2 μM) (A, B, C, H, I, J) and DPP4 (0.1 μM) (D, E, F, K, L, M) were incubated with substance P (A–G) and BNP (H–N) for various lengths of time. The control incubation of peptide in buffer alone is also shown (G, N). Representative MALDI-TOF MS analyses of substrate at early (A, D, H, K), mid (B, E, I, L) and late (C, F, J, M) stages of cleavage are shown. Peaks are labelled with their molecular masses. Asterisks denote double charged peaks.

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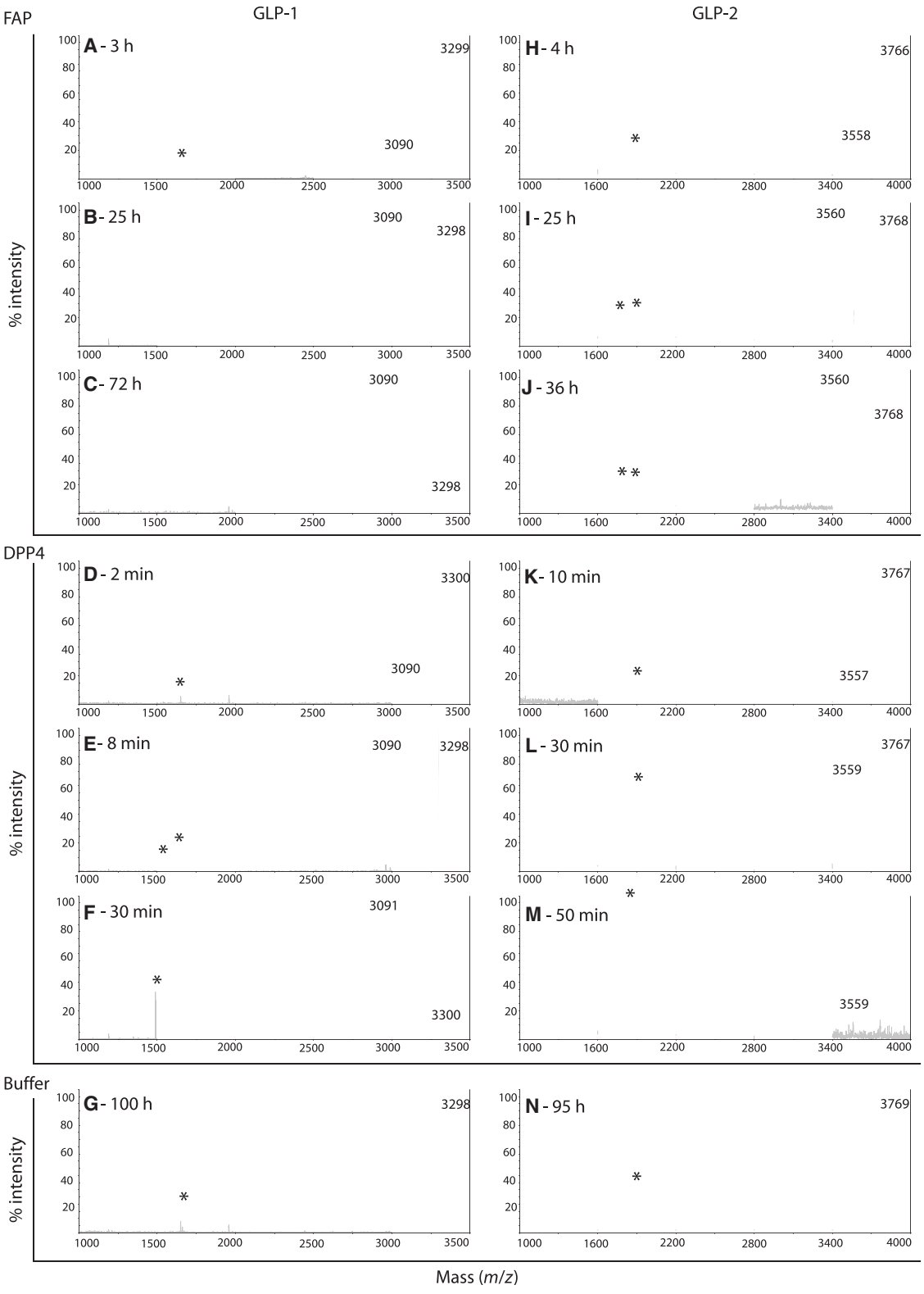


Fig. 5. GLP-1 and GLP-2 cleavage by FAP and DPP4. FAP (0.2 μM) (A, B, C, H, I, J) and DPP4 (0.1 μM) (D, E, F, K, L, M) were incubated with GLP-1 (A–G) and GLP-2 (H–N) for various lengths of time. The control incubation of peptide in buffer alone is also shown (G, N). Representative MALDI-TOF MS analyses of substrate at early (A, D, H, K), middle (B, E, I, L) and late (C, F, J, M) stages of cleavage are shown. Peaks are labelled with their molecular masses. Asterisks denote double charged peaks.

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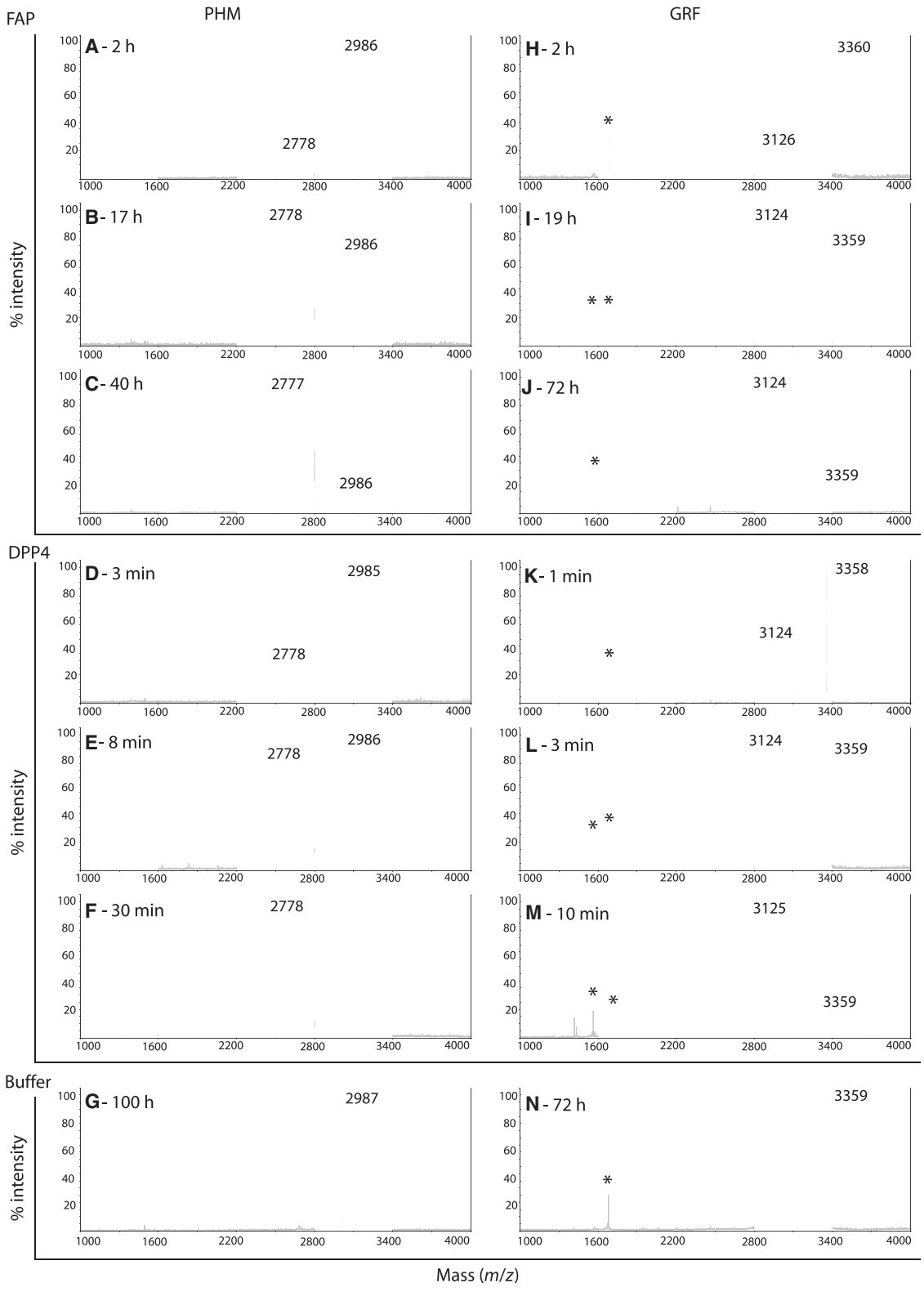


Fig. 6. PHM and GRF cleavage by FAP and DPP4. FAP (0.2 μM) (A, B, C, H, I, J) and DPP4 (0.1 μM) (D, E, F, K, L, M) were incubated with PHM (A–G) and GRF (H–N) for various lengths of time. The control incubation of peptide in buffer alone is also shown (G, N). Representative MALDI-TOF MS analyses of substrate at early (A, D, H, K), middle (B, E, I, L) and late (C, F, J, M) stages of cleavage are shown. Peaks are labelled with their molecular masses. Asterisks denote double charged peaks.

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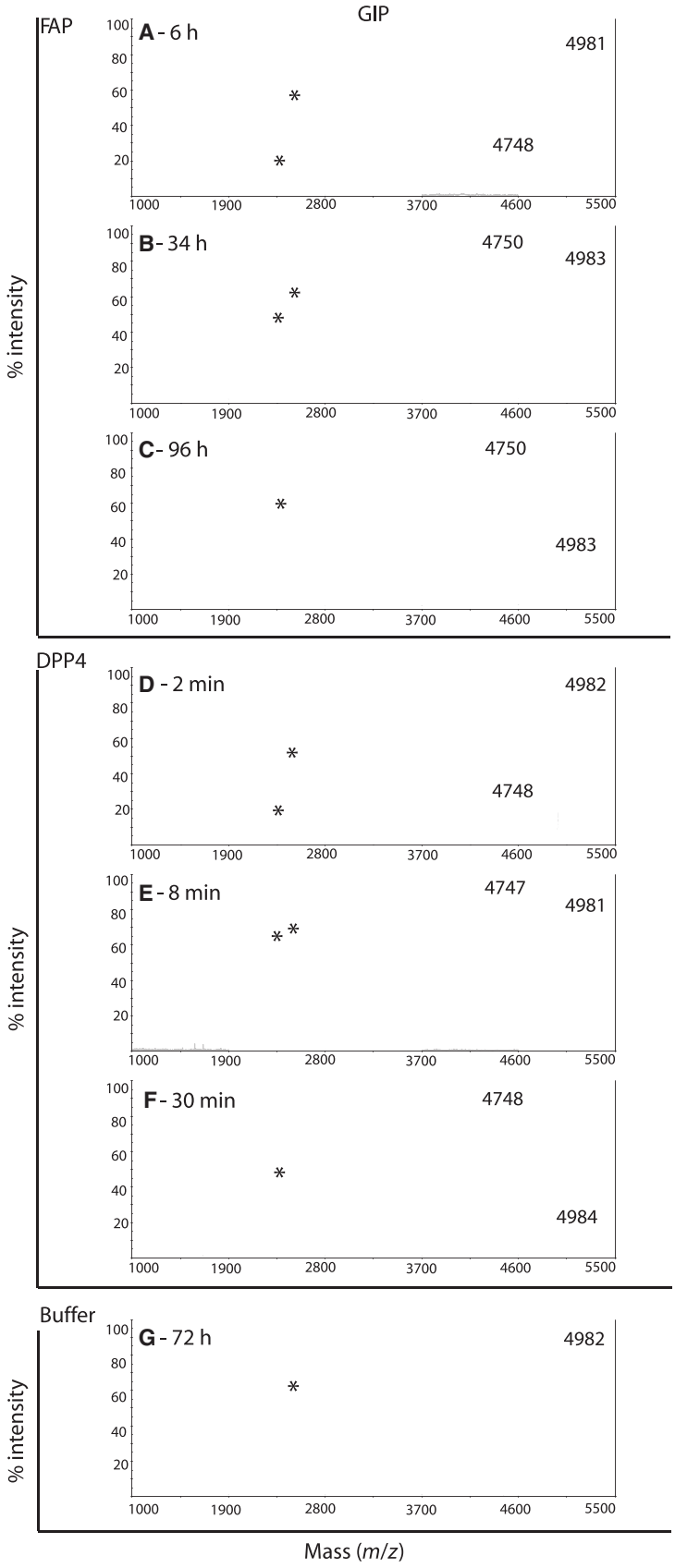


Fig. 7. GIP cleavage by FAP and DPP4. FAP (0.2 μM) (A, B, C) and DPP4 (0.1 μM) (D, E, F) were incubated with GIP for various lengths of time. The control incubation of GIP in buffer alone is also shown (G). Representative MALDI-TOF MS analyses of substrate at early (A, D), middle (B, E) and late (C, F) stages of cleavage are shown. Peaks are labelled with their molecular masses. Asterisks denote double charged peaks.

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CXCL12/SDF-1α were 34% after 78 h, 25% after 24 h and 18% after 24 h, respectively. In contrast, DPP4 showed efficient dipeptidyl peptidase cleavage of its known substrates (Table 2), with an order preference of CCL22/MDC ≈ CXCL12/SDF-1α > CXCL11/ITAC > CXCL10/IP10 > CXCL2/Groβ > CCL5/RANTES ≈ CCL11/eotaxin > CXCL9/MIG.

Discussion

This is the first identification of natural substrates of FAP dipeptidyl peptidase activity. NPY, BNP, substance P and PYY were the most efficient FAP substrates, and the first hormone substrates of FAP to be identified. These peptides are also known substrates of DPP4, and cleavage by DPP4 was also shown in this study. Unlike DPP4, FAP showed poor cleavage rates on the non-neuropeptide hormones, including the incretins GLP-1 and GIP, which are known to be efficient DPP4 substrates. The FAP hormone degradome appears to be more restricted than that of DPP4, and this possibly indicates a narrower P2–P1 substrate specificity of FAP than of DPP4. Notably, FAP exhibited very little or no hydrolysis of chemokines, even though efficient hydrolysis by DPP4 was observed.

FAP and DPP4 have the rare ability to cleave the post-proline bond. Indeed, the four most efficient FAP substrates from this study contain a proline at P1. None of the gastrointestinal hormone peptides tested contain a proline at P1, which may be a cause of the poor FAP cleavage of these peptides (all had a half-life of greater than 15 h). These new data on natural peptide substrates provide a new perspective on the dipeptidyl peptidase cleavage site specificity of FAP. Previous reports have shown a preference for isoleucine, arginine and proline at P2 for efficient FAP dipeptidyl peptidase cleavage of artificial synthetic substrates [41]. In the present study, the presence of polar residues (tyrosine, serine and arginine) at P2 along with a charged lysine at P1′ (BNP and substance P) or P2′ (NPY and PYY) may be involved in the greater affinity of these four neuropeptide hormones for FAP. No other peptide substrate of FAP identified here contains a positively charged residue at P1′ or P2′ (Table 1). FAP seems to have no preference at P2; of the four neuropeptides, the dipeptide Tyr-Pro is present in both the fastest (NPY) and the slowest (PYY) substrates.

Previously reported data on the endopeptidyl substrates of FAP, α_2-antiplasmin and denatured type I collagen, show a preference for the Gly-Pro sequence to be at P2-P1 [9,13,42]; however, all four efficient dipeptidyl peptidase substrates described in this study do not contain glycine at P2 but rather have tyrosine, serine, arginine or lysine (in the case of the sequential cleavage of substance P). The poor dipeptidyl peptidase cleavage of Gly-Pro, when presented to FAP as an artificial dipeptide substrate such as H-Gly-Pro-AMC, was shown here (Fig. 1A), and has been shown previously [12]. Indeed, the only peptides tested that do contain N-terminal Gly-Pro were CCL11/eotaxin and CCL22/MDC, which FAP did not cleave. These results have important implications for the design of FAP inhibitors based on substrate cleavage sites. It is possible that the preferred cleavage sites for dipeptidyl peptidase and endopeptidyl hydrolysis may differ. The molecular basis for this is unknown, as the same catalytic serine is involved in both enzymatic activities [9]. Moreover, FAP cleaves the H-Ala-Pro-AMC synthetic substrate very efficiently (Fig. 1A) but, despite this dipeptide occurring in three of the gastrointestinal hormones (GLP-1, GLP-2 and PHM), FAP produced long half-lives of 15–22 h for these peptides, which represent very poor cleavage rates. In contrast, DPP4 cleaves His-Ala, Tyr-Ala and His-Ser dipeptides efficiently.

Despite its clear preference for proline at P1, FAP did not cleave any of the eight known DPP4 chemokine substrates, which all contain proline at P1. P2 of these chemokines is occupied by a variety of amino acids, most of which are hydrophobic (alanine, valine and phenylalanine). However, many chemokines that contain proline at P1 are not cleaved by DPP4 or the closely related protease DPP8 [40]. Therefore, a proline at P1 is not sufficient for hydrolysis by FAP, DPP4 or DPP8. Perhaps peptide length has a role in FAP dipeptidyl peptidase cleavage. All of the chemokines are at least twice the length of the other peptides tested, and, although FAP does not cleave Ser-Pro or Lys-Pro in CCL5/RANTES or CXCL12/SDF-1α, respectively, it cleaved these same dipeptides from BNP and substance P, respectively. Peptide length has been shown to affect DPP4 cleavage. The rate of hydrolysis by DPP4 of several cytokine-derived oligopeptides has been found to be negatively correlated with peptide chain length [43]. However, in contrast to this, in the case of GRF, the rate of DPP4 hydrolysis of longer peptides (44 amino acids) is higher than that of shorter peptides (three and 11 amino acids) [44]. Moreover, the longer version of PACAP (PACAP-38) is more readily cleaved by DPP4 than is the shorter form (PACAP-27) [26], but this may be because of the positively charged C-terminal extension of PACAP-38. This provides further evidence for the need to consider residues distal to the scissile bond when examining substrate specificity in the DPP4 enzyme family. The small catalytic pocket of DPP4 (~ 8 Å in diameter) is

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thought to limit substrate size, but, although the substrate entry channel of FAP is larger than that of DPP4 [16], FAP was unable to cleave the longer chemokine DPP4 substrates. Therefore, although FAP can accommodate larger substrates in its active site than DPP4, peptide length alone does not appear to be an important consideration in predicting cleavage by FAP.

The consequences of removing the N-terminal dipeptide from NPY have been studied. NPY is released during intense stress, and is ubiquitously expressed and functions in the nervous system, endothelium, immune cells, megakaryocytes, adipose tissue and gut [45,46]. Dipeptidyl peptidase truncation of NPY by FAP or DPP4 yields $NPY_{3–36}$. $NPY_{3–36}$ is inactive on the Y1 receptor, and has greater affinity for the Y2 receptor, which abolishes its vasoconstrictive properties and converts it into a vascular growth factor [46]. Therefore, the role of FAP in promoting blood vessel density in tumours [22] might involve its NPY-cleaving activity.

BNP is a cardiac hormone, and has an important role in maintaining cardiovascular homeostasis by activating guanylyl cyclase A [47]. In addition, BNP has been shown to inhibit liver fibrosis by attenuating stellate cell activation [48]. Whether the truncation of BNP by FAP has physiological implications remains to be elucidated. Little is known about the importance of the N-terminal dipeptide of BNP for receptor binding, and widely used commercial assays for BNP do not clearly differentiate between the full-length peptide and its processed forms [49].

Substance P is an undecapeptide hormone belonging to the tachykinin family and is released during the activation of sensory nerves, causing vasodilation, oedema and pain through activation of neurokinin 1 receptors. Substance P mediates multiple activities in various cell types, including cell proliferation, antiapoptotic responses, and inflammatory processes. The proinflammatory effects of substance P are known to be terminated by proteases such as angiotensin-converting enzyme and neutral endopeptidase. The sequential dipeptidyl peptidase cleavage of substance P by FAP (and DPP4) might similarly be anti-inflammatory.

PYY was the least efficient FAP substrate detected here; however, a half-life of approximately 1 h could still be biologically relevant. As with NPY, removing the N-terminal dipeptide from PYY alters its tertiary structure, preventing it from stimulating its Y1 receptor, and thereby altering its function. PYY regulates glucose homeostasis. Specifically, PYY is important for acylethanolamine receptor Gpr119-activated responses in the gastrointestinal tract, and this PYY function is unaltered by DPP4 inhibition [50]. However, our data showed that FAP can also truncate PYY to $PYY_{3–36}$, so the potential role of FAP in PYY function should be investigated.

Discovering the repertoire of substrates of a protease is crucial to understanding its functions and biological roles. Because of FAP's cleavage of α_2-antiplasmin and denatured type I collagen, it has been proposed to be involved in fibrinolysis and ECM degradation and remodelling. The numerous substrates discovered in this study indicate the possibility that FAP is involved in additional biological processes. It is possible that the dipeptidyl peptidase activity of FAP acts in concert with aminopeptidases to further truncate the N-termini of peptides [51], which would widen the range of potential FAP roles *in vivo*.

The extracellular location of FAP and DPP4 means that these proteases can access small biomolecules such as the peptides tested in this study. To evaluate the biological significance of this, more detailed *in vivo* investigations are required. The hormones GLP-1, GIP, PHM and PACAP have been shown to be physiological DPP4 substrates *in vivo* [8,25]. Therefore, further studies need to establish where FAP sits in the hierarchy of proteases that hydrolyse NPY, BNP, substance P and PYY *in vivo*. It is tempting to speculate about the roles of FAP in the processing of these important neuropeptide hormones. FAP is expressed by stromal fibroblasts and pericytes in tumours [6], and by activated hepatic stellate cells in liver disease [5,19,52]. Activated hepatic stellate cells express several neural molecules [53]. Liver innervation has been studied in some detail, showing the presence of NPY, substance P and VIP [54,55]. Therefore, the potential physiological relevance of neuropeptide cleavage by FAP should be examined *in vivo*.

In summary, this is the first report that FAP has natural dipeptidyl peptidase substrates, and provides novel insights into the differential substrate specificity between FAP and DPP4. It is clear that few substrates are cleaved efficiently by both FAP and DPP4, consistent with diverse functions for these proteases.

Experimental procedures

Reagents

Cloning, expression and purification of the recombinant human soluble DPP4 have been described previously [40,56]. This form of DPP4 lacks the cytoplasmic and transmembrane domains, and was purified by immobilized metal affinity chromatography, followed by Superose 12 (GE Healthcare, Uppsala, Sweden), dialysed against 10 mM Tris (pH 8.0), and then stored at 4 °C. Purified soluble recombinant human FAP (26–760) was from R&D Systems

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(Minneapolis, MN, USA). This soluble form of FAP lacks the cytoplasmic and transmembrane domains (amino acids 1–25). Purified synthetic human gastrointestinal hormones (GLP-1, GLP-2, GIP and PHM) and neurological peptides (NPY, PYY, BNP and substance P) were all from Bachem (Bubenhof, Switzerland). Purified recombinant human chemokines (CCL3/MIP1α, CCL5/RANTES, CCL11/eotaxin, CCL22/MDC, CXCL2/Groβ, CXCL9/MIG, CXCL10/IP10, CXCL11/ITAC and CXCL12/SDF-1α) were from PeproTech (Rocky Hill, NJ, USA). Purified synthetic human GRF, oxyntomodulin, VIP, PACAP and glucagon, as well as the synthetic substrates H-Ala-Pro-AMC, H-Gly-Pro-AMC, succinyl-Ala-Pro-AMC and Z-Gly-Pro-AMC, were from Mimotopes (Clayton, Vic., Australia). Only GLP-1, GRF and PACAP were used in the amidated form.

Enzyme assays

Enzyme assays were carried out in black 96-well plates (Greiner Bio One, Frickenhausen, Germany), with the fluorescent substrates H-Ala-Pro-AMC, H-Gly-Pro-AMC, succinyl-Ala-Pro-AMC and Z-Gly-Pro-AMC (Mimotopes). H-Ala-Pro-AMC, H-Gly-Pro-AMC and succinyl-Ala-Pro-AMC were used at a final concentration of 1 mM in TE buffer (pH 7.6) with 10% methanol, whereas Z-Gly-Pro was made up to a final concentration of 100 μM in 25 mM Tris and 250 mM NaCl (pH 7.6) with 1% dimethylformamide. Fifty microlitres of substrate was added to 50 μL of enzyme sample in each well, and the fluorescence produced was monitored every 5 min for 1 h in a microplate reader (BMG Labtech, Offenburg, Germany), with excitation at 355 nm and emission at 450 nm. Control wells contained substrate only, to measure background fluorescence. Enzyme activity was converted to change in fluorescence units per minute.

Enzyme assays with inhibitors

Enzyme assays with inhibitors were carried out with substrates as described above. Inhibitors were added to the enzyme to give a final volume of 50 μL. The DPP4-selective inhibitor sitagliptin (Merck, Rahway, NJ, USA) was used at a final concentration of 1 μM, and the nonselective dipeptidyl peptidase inhibitor ValboroPro was used at final concentrations of 10 μM, 50 μM and 100 μM. The assay plate was read in a microplate reader as above. Control wells contained substrate only, and enzyme activity was converted to change in fluorescence units per minute.

Kinetic constants

The specific activity of FAP cleavage of Z-Gly-Pro-AMC in 50 mM Tris and 1 M NaCl was given by the manufacturer as > 1800 pmol·min^{-1}·μg^{-1}. DPP4 was assayed in 100 mM Tris/HCl buffer, with 5 μL of undiluted DPP4 added to 420 μL of H-Gly-Pro-*p*-nitroanilide (Bachem) in a 0.5-mL cuvette, and measurement of the absorbance at 392 nm for 10 min. The specific activity was calculated to be 1830 nmol·min^{-1}·μg^{-1}, with an extinction coefficient of *p*-nitroanilide at 395 nm of 11.5 M^{-1}·cm^{-1}.

Substrate cleavage by FAP and DPP4

All substrates (1 μg) were incubated with 0.25 μg/0.2 μM FAP in 25 mM Tris and 0.25 M NaCl (pH 8.0) or 0.14 μg/0.112 μM DPP4 in 50 mM Tris/HCl (pH 7.6) for up to 100 h at 37 °C in a total volume of 15 μL. One microgram of each substrate was also incubated with 25 mM Tris and 0.25 M NaCl (pH 8.0) buffer alone to check for FAP/DPP4-independent breakdown over time at 37 °C. One-microlitre aliquots of the reaction mixture were taken at relevant intervals for analysis by MS.

MALDI-TOF MS analysis

MALDI-TOF MS analysis was performed as previously described [40]. MALDI-TOF MS was performed on a Voyager-DE STR Biospectrometry Workstation (Perseptive Biosystems, Framingham, MA, USA) equipped with a nitrogen laser (337 nm) running in reflector or linear mode with delayed extraction and ion acceleration at 25 000 V. At each time point, a 1-μL sample of enzyme/substrate solution was spotted onto a standard stainless steel MALDI sample plate, and was then overlaid with 1 μL of matrix solution (10 mg·mL^{-1} α-cyano-4-hydroxycinnamic acid, 70% acetonitrile, 0.1% trifluoroacetic acid) and allowed to air evaporate. Calibration was performed with calibration mixture 3 from the Sequazyme Peptide Mass Standards Kit (Applied Biosystems, Foster City, CA, USA).

In vitro relative half-lives of FAP-cleaved substrates

MALDI-TOF MS was used as described above to measure cleavage rates of substrates by FAP and DPP4. To estimate cleavage rates, substrate (1 μg) was incubated with purified FAP (0.2 μM) or purified DPP4 (0.112 μM) in a total volume of 15 μL at 37 °C. Reaction samples were taken at relevant intervals. The percentage of full-length peptide was calculated and plotted over time. Relative *in vitro* half-lives were estimated from ratios between the MS intensities of intact and cleaved substrates after baseline correction and noise-filter/smoothing.

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Supporting information

The following supplementary material is available:
Fig. S1. Representative MALDI-TOF MS analyses of VIP and glucagon incubation with FAP and DPP4.
Fig. S2. Representative MALDI-TOF MS analyses of PACAP and oxyntomodulin incubation with FAP and DPP4.
Fig. S3. Representative MALDI-TOF MS analyses of CCL3/MIP1α and CCL5/RANTES incubation with FAP and DPP4.
Fig. S4. Representative MALDI-TOF MS analyses of CCL11/eotaxin and CCL22/MDC incubation with FAP and DPP4.
Fig. S5. Representative MALDI-TOF MS analyses of CXCL2/Groβ and CXCL6/GCP2 incubation with FAP and DPP4.
Fig. S6. Representative MALDI-TOF MS analyses of CXCL9/MIG and CXCL10/IP10 incubation with FAP and DPP4.
Fig. S7. Representative MALDI-TOF MS analyses of CXCL11/ITAC and CXCL12/SDF-1α incubation with FAP and DPP4.

This supplementary material can be found in the online version of this article.

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Neuropeptide Y, B-type natriuretic peptide, substance P and peptide YY are novel substrates of fibroblast activation protein-α

Fiona M. Keane, Naveed A. Nadvi, Tsun-Wen Yao and Mark D. Gorrell

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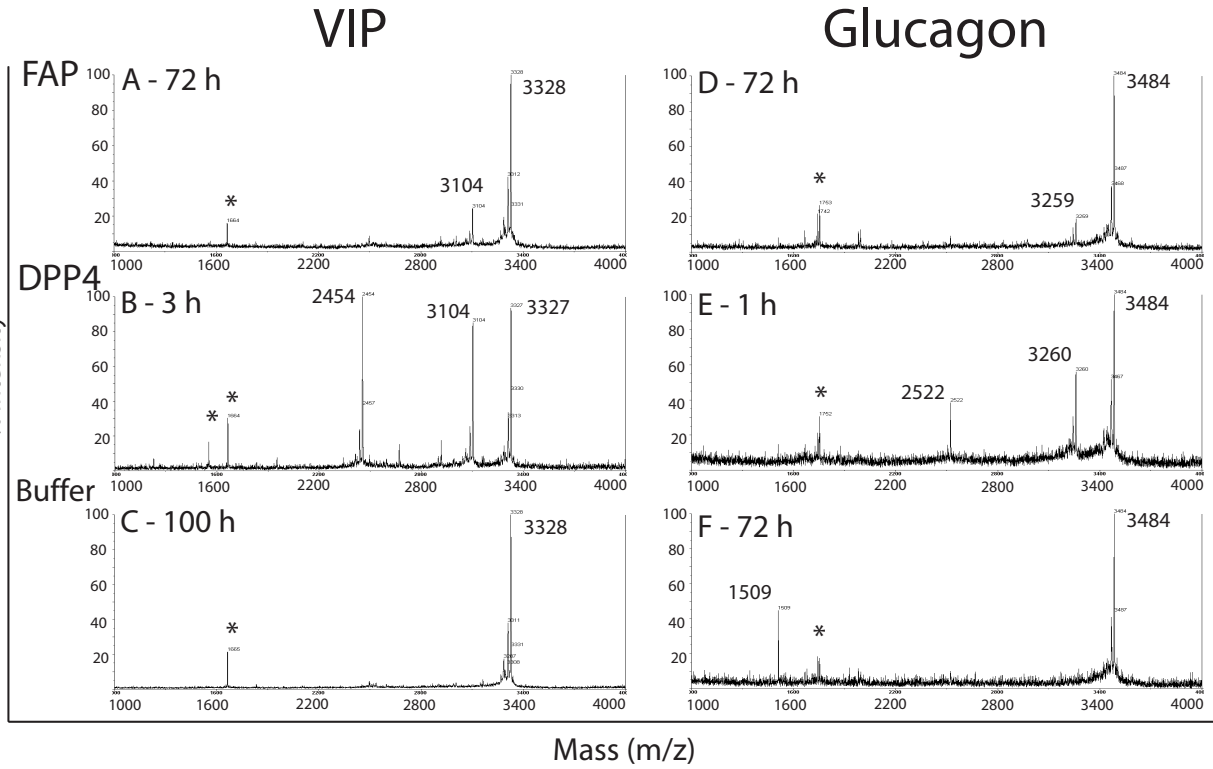


Fig. S1: Representative MALDI-TOF MS analyses of VIP and glucagon incubation with FAP and DPP4. 0.2 μM recombinant FAP (A and D) and 0.1 μM DPP4 (B and E) were incubated with VIP (A, B and C) and glucagon (D, E and F) for the time periods indicated. The control incubation of peptides with buffer alone is also shown (C and F). Peaks are labelled with their molecular masses. Asterisks denote double charged peaks.

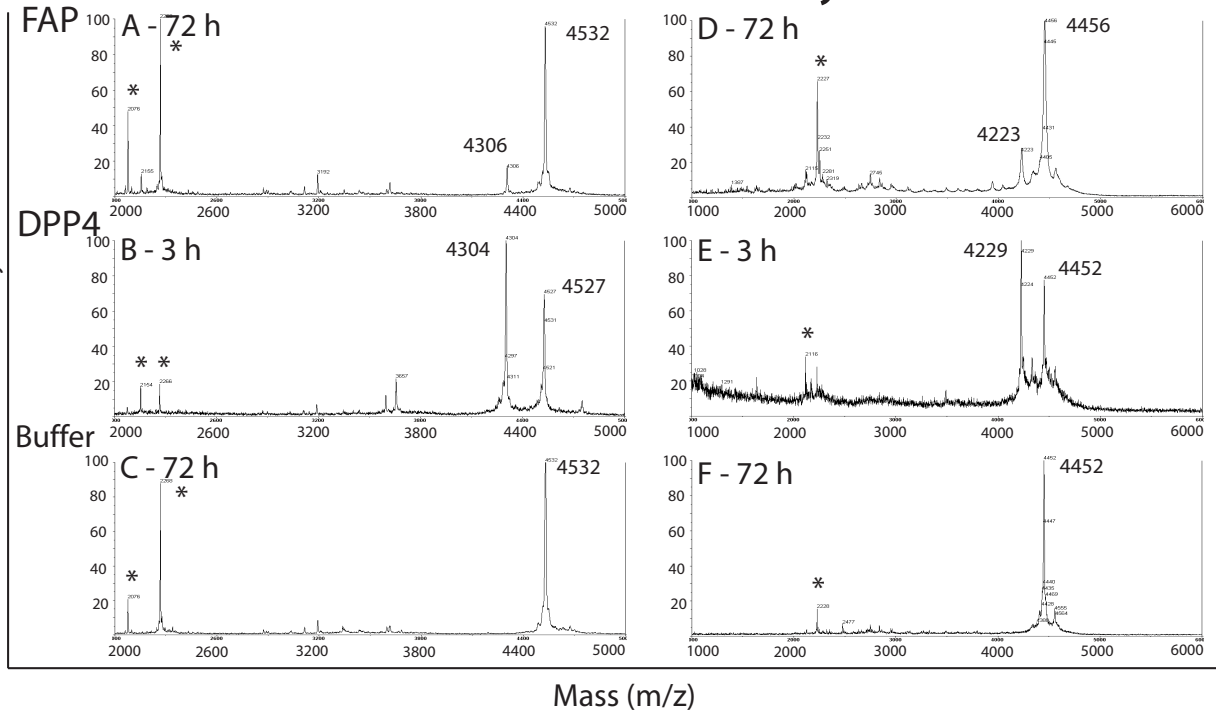


Fig. S2: Representative MALDI-TOF MS analyses of PACAP and oxyntomodulin incubation with FAP and DPP4. 0.2 μM recombinant FAP (A and D) and 0.1 μM DPP4 (B and E) were incubated with PACAP (A, B and C) and oxyntomodulin (D, E and F) for the time periods indicated. The control incubation of peptides with buffer alone is also shown (C and F). Peaks are labelled with their molecular masses. Asterisks denote double charged peaks.

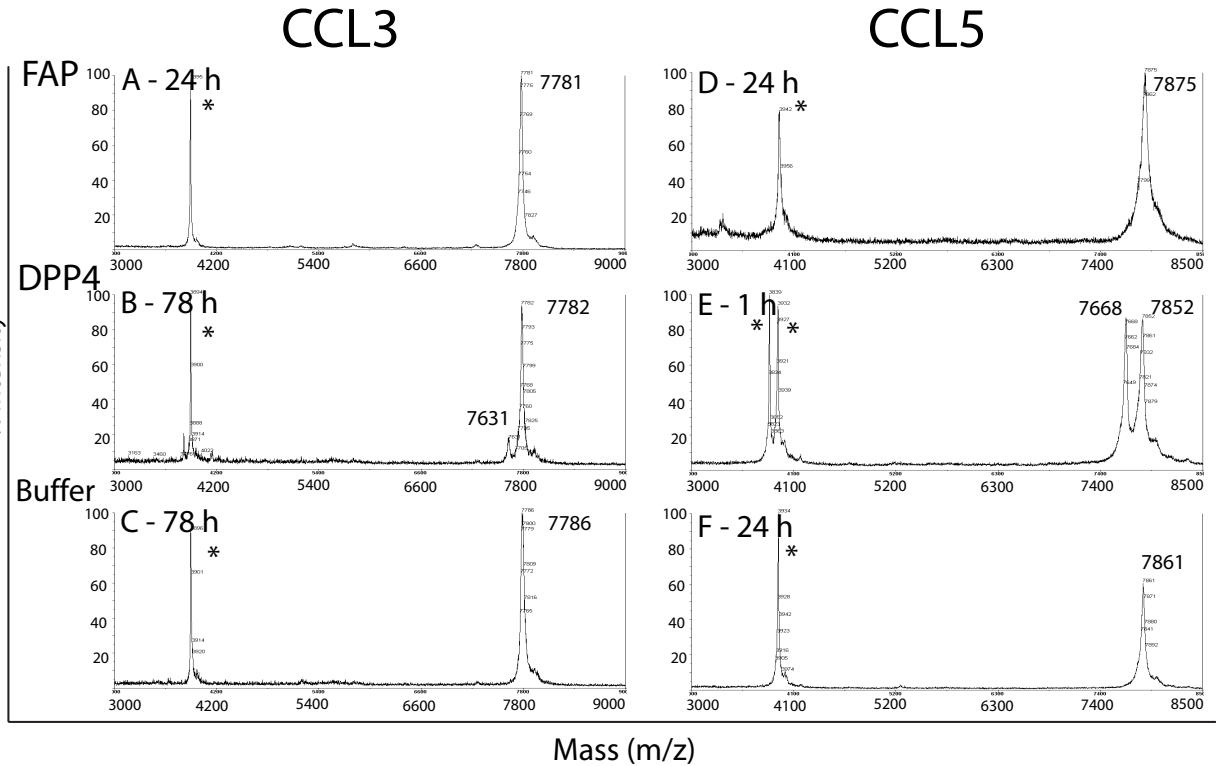


Fig. S3: Representative MALDI-TOF MS analyses of CCL3/MIP1α and CCL5/RANTES incubation with FAP and DPP4. 0.2 µM recombinant FAP (A and D) and 0.1 µM DPP4 (B and E) were incubated with CCL3 (A, B and C) and CCL5 (D, E and F) for the time periods indicated. The control incubation of peptides with buffer alone is also shown (C and F). Peaks are labelled with their molecular masses. Asterisks denote double charged peaks.

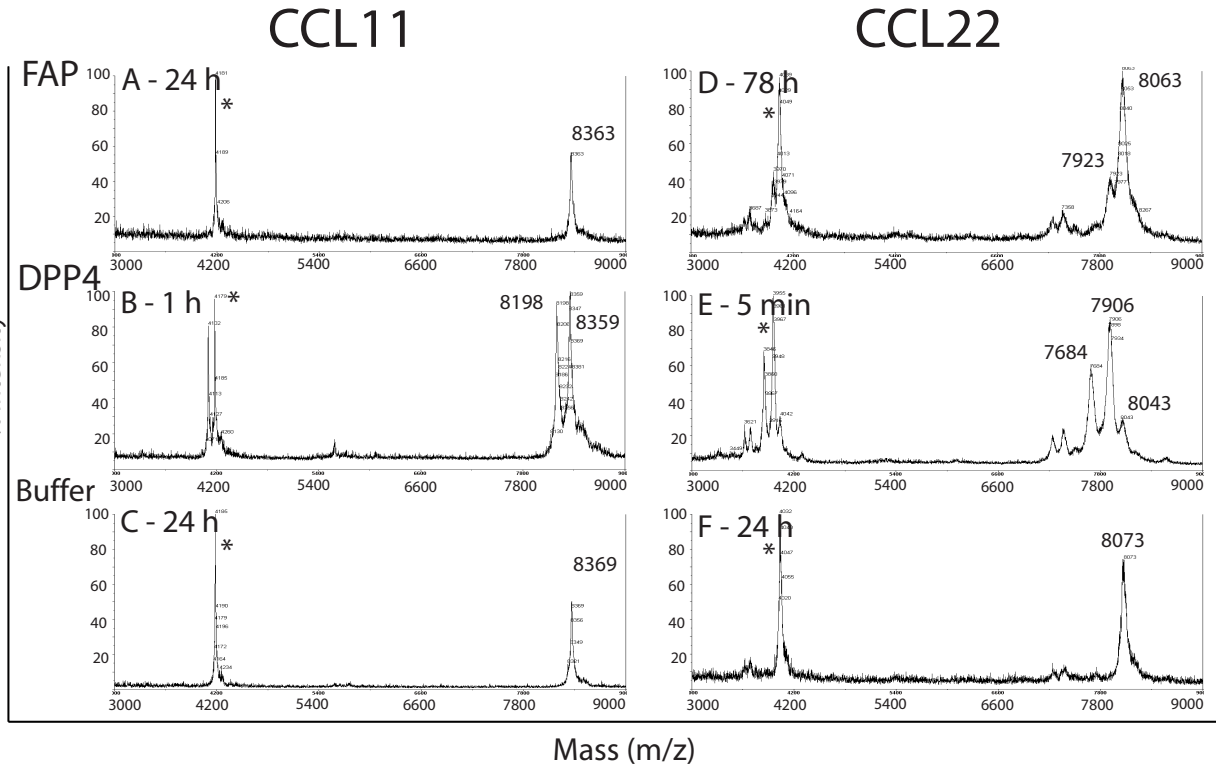


Fig. S4: Representative MALDI-TOF MS analyses of CCL11/eotaxin and CCL22/MDC incubation with FAP and DPP4. 0.2 μM recombinant FAP (A and D) and 0.1 μM DPP4 (B and E) were incubated with CCL11 (A, B and C) and CCL22 (D, E and F) for the time periods indicated. The control incubation of peptides with buffer alone is also shown (C and F). Peaks are labelled with their molecular masses. Asterisks denote double charged peaks.

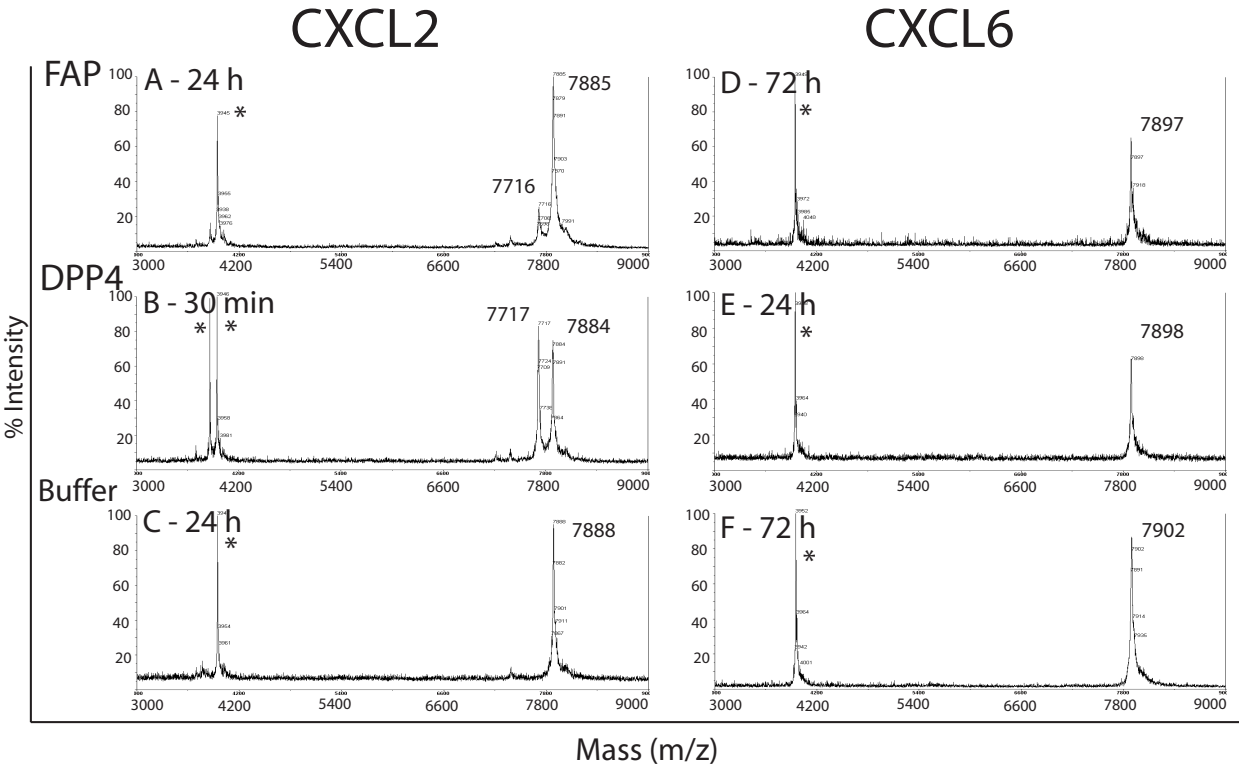


Fig. S5: Representative MALDI-TOF MS analyses of CXCL2/Groβ and CXCL6/GCP2 incubation with FAP and DPP4. 0.2 μM recombinant FAP (A and D) and 0.1 μM DPP4 (B and E) were incubated with CXCL2 (A, B and C) and CXCL6 (D, E and F) for the time periods indicated. The control incubation of peptides with buffer alone is also shown (C and F). Peaks are labelled with their molecular masses. Asterisks denote double charged peaks.

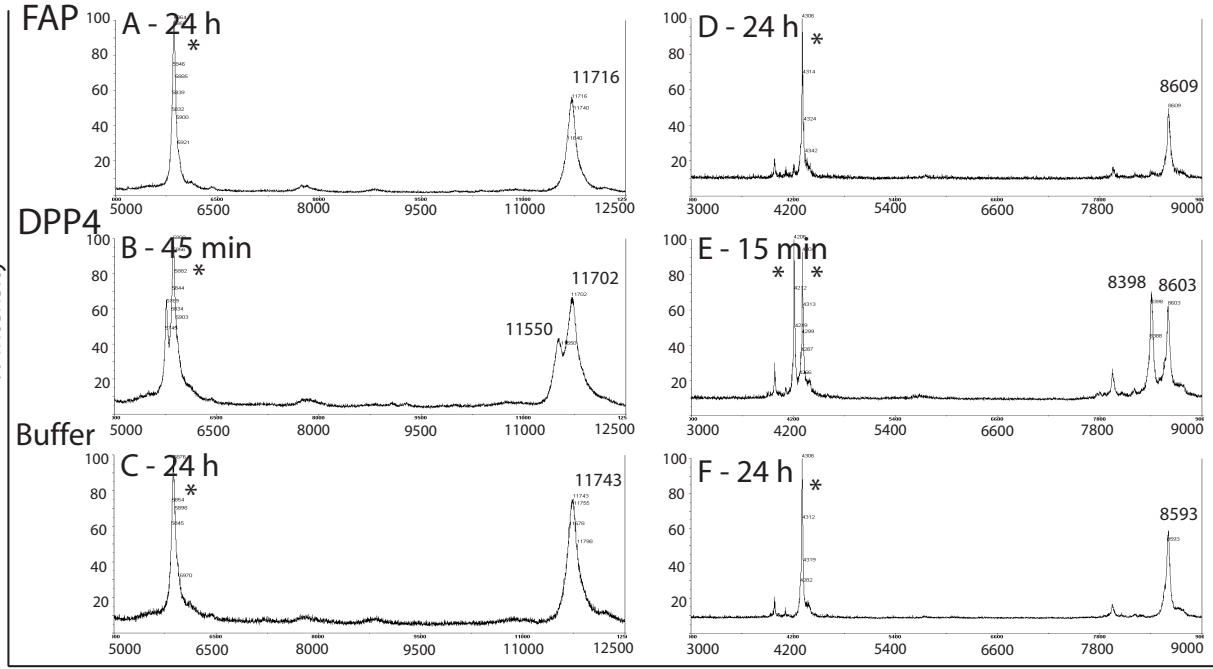


Fig. S6: Representative MALDI-TOF MS analyses of CXCL9/MIG and CXCL10/IP10 incubation with FAP and DPP4. 0.2 µM recombinant FAP (A and D) and 0.1 µM DPP4 (B and E) were incubated with CXCL9 (A, B and C) and CXCL10 (D, E and F) for the time periods indicated. The control incubation of peptides with buffer alone is also shown (C and F). Peaks are labelled with their molecular masses. Asterisks denote double charged peaks.

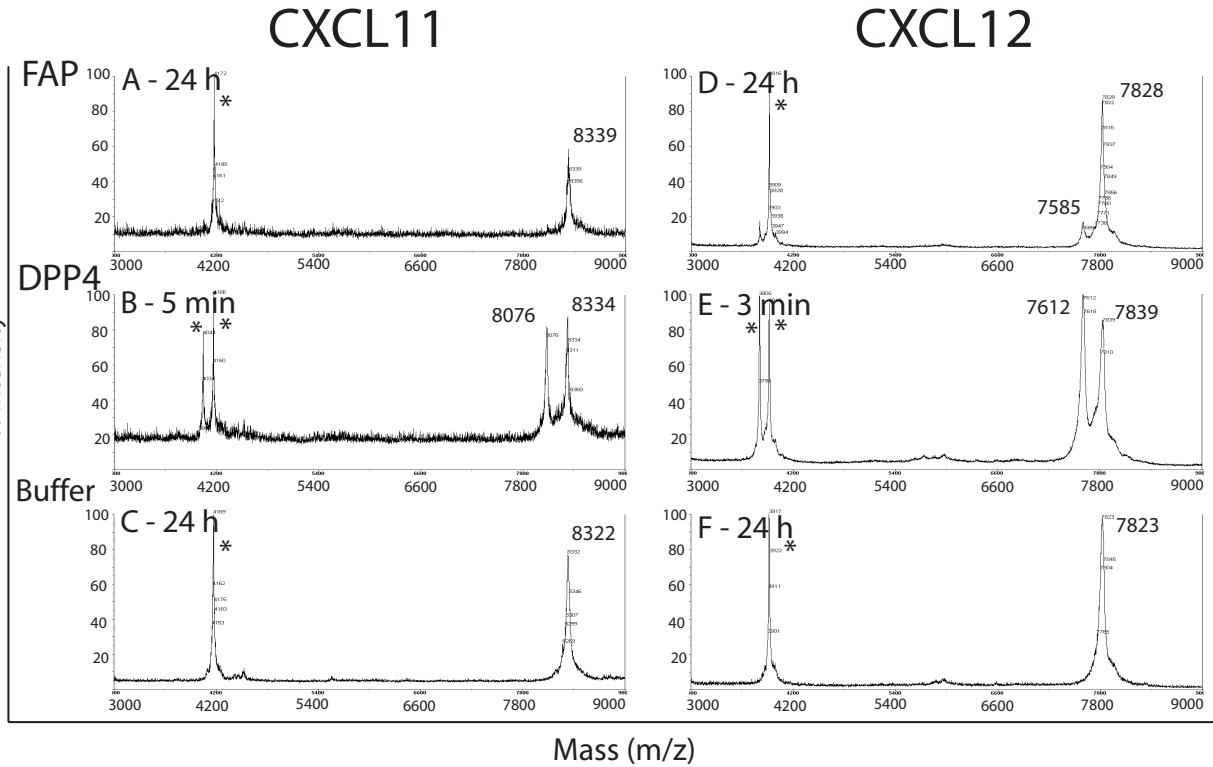


Fig. S7: Representative MALDI-TOF MS analyses of CXCL11/ITAC and CXCL12/SDF-1α incubation with FAP and DPP4. 0.2 μM recombinant FAP (A and D) and 0.1 μM DPP4 (B and E) were incubated with CXCL11 (A, B and C) and CXCL12 (D, E and F) for the time periods indicated. The control incubation of peptides with buffer alone is also shown (C and F). Peaks are labelled with their molecular masses. Asterisks denote double charged