Peptidase Substrates via Global Peptide Profiling

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Abstract

Peptide metabolism is a complex process involving many proteins working in concert. Mass spectrometry (MS)-based global peptide profiling of mice lacking dipeptidyl peptidase 4 (DPP4) identified endogenous DPP4 substrates and revealed an unrecognized pathway during proline peptide catabolism that interlinks aminopeptidase and DPP4 activities. Together, these studies elucidate specific aspects of DPP4-regulated metabolism and, more generally, highlight the utility of global peptide profiling for studying peptide metabolism in vivo.

The prolyl peptidase family has emerged as an intriguing class of enzymes due to the development of DPP4 inhibitors as anti-diabetic drugs1. DPP4 regulates physiological processes through the regulation of peptide hormones, such as the insulinotropic hormone glucagon-like peptide 1 (GLP-1), by cleaving a dipeptide from the N terminus. There has been a great deal of recent interest in finding additional physiological roles for DPP4 through the identification of new endogenous substrates2. We reasoned that the application of a liquid chromatography-mass spectrometry (LC-MS)-based global peptide profiling approach could improve on existing in vitro methods by directly measuring changes in tissue peptide levels as a consequence of peptidase inhibition to identify relevant in vivo substrates3. Here, we develop and apply such an approach to assign DPP4-regulated peptides in the kidney, a tissue with high levels of DPP4 activity, and in the process gain mechanistic insights into the biochemical and physiological roles of this biomedically relevant enzyme.
DPP4 is a broadly distributed peptidase found in two major forms: a membrane-bound extracellular protein and a secreted form found at high levels in the blood. Currently, some DPP4 plasma substrates are known but the substrate profile in other tissues that express DPP4, such as the kidney (Supplementary Fig. 1), has not been determined. Using DPP4 null (DPP4<sup>−/−</sup>) mice, we isolated physiological peptides from wild-type (DPP4<sup>+/+</sup>) and DPP4<sup>−/−</sup> kidneys for subsequent analysis by nanospray LC-MS/MS (Supplementary Fig. 2). Differences between the DPP4<sup>+/+</sup> and DPP4<sup>−/−</sup> tissues were identified by spectral counting using the MS/MS data and subsequent integration of the corresponding peaks in the LC-MS chromatograms to quantitate these changes (Fig. 1a). This analysis resulted in a final list of ten significantly different DPP4-regulated peptides between the two genotypes (Table 1).

The DPP4-regulated peptides ranged from 10-22 residues long, with five peptides elevated in the DPP4<sup>−/−</sup> sample and five peptides elevated in the DPP4<sup>+/+</sup> samples (Table 1). Comparison of the proteins from which these peptides are derived reveals that they are expressed in the kidney but there are no other obvious trends in their function or cellular localization and none of these peptides have any known physiological role. All the peptides elevated in the DPP4<sup>−/−</sup> samples contain a preferred DPP4 cleavage site (H<sub>2</sub>N-Xaa-Pro), suggesting that these peptides are endogenous DPP4 substrates. Indeed, for two of the peptides elevated in the DPP4<sup>−/−</sup> sample, Mepβ(21-41) and DBI(92-105), the corresponding DPP4 cleavage products are at higher levels in the DPP4<sup>+/+</sup> sample (Fig. 1a and Table 1). The existence of these “substrate-product pairs” establishes these peptides as physiological substrates of DPP4.

The absence of any H<sub>2</sub>N-XaaAla-peptides (e.g., GLP-1) on our list could result from incomplete coverage of the peptidome during MS analysis or the presence of other enzymatic activities that are able to efficiently process H<sub>2</sub>N-XaaAla motifs in the absence of DPP4 activity. In support of this latter idea, GLP-1 has been shown to be regulated in a DPP4-independent manner in the kidney using radiolabeled immunoassays. Additionally, for DPP4<sup>+/+</sup> elevated peptides that lack a corresponding substrate peptide in the DPP4<sup>−/−</sup> sample, we cannot conclusively establish these peptides as endogenous DPP4 products, but the presence of DPP4 cleavage sites proximal to their N terminus (3-9 residues upstream in the full length protein sequence) suggests the possibility that these peptides are downstream products of a DPP4 cleavage event (Supplementary Table 1).

We also examined the effects of DPP4 inhibitors on the peptide profile in DPP4<sup>+/+</sup> mice. After treatment of DPP4<sup>+/+</sup> mice with the DPP4-selective inhibitor LAF-2379 (1), two of the DPP4-regulated peptides, Mepβ(21-41) and EF-1α(281-291), were clearly increased in the LAF-237 treated mice, providing additional evidence that these peptides are regulated by DPP4 activity in vivo (Table 1). These pharmacological studies complement the knockout studies and also rule out any possible artifacts due to non-catalytic functions of DPP4. The disparity in the total number of peptides between DPP4<sup>−/−</sup> and LAF-237-treated samples is likely the result of inherent differences in the kinetics of peptide production, where only a subset of the peptides are produced quickly enough to substantially change their levels after acute inhibition.
In vitro assays with recombinant DPP4 and synthetic peptides were used to test DPP4−/− elevated peptides as substrates for the enzyme. These in vitro assays consisted of qualitative MALDI experiments as well as quantitative specific activity measurements using an LC-MS-based assay (Supplementary Methods). These experiments demonstrated that all substrates containing a penultimate proline residue were efficiently cleaved by DPP4, including unaffected XP motif peptides, which were not regulated by DPP4 in vivo (Supplementary Fig. 3). A similar discordance between in vivo and in vitro measurements was previously observed during the global metabolite profiling of the lipase, fatty acid amide hydrolase (FAAH)10, which highlights the general difficulty in predicting endogenous substrates from in vitro measurements, regardless of the enzyme class.

The presence of multiple peptide fragments from the diazepam-binding inhibitor (DBI) protein (Supplementary Fig. 4), including the DPP4 substrate peptide DBI(92-105), suggests that proteolytic catabolism of DBI is the source of DBI(92-105). Notably, no changes were seen in the abundance of the other DBI peptide fragments, which indicates that changes in global DBI protein levels are not responsible for the quantitative differences in the DBI(92-105) peptide (Supplementary Table 1). Similarly, additional peptide fragments were also seen for meprin-β (Mepβ(693-704)) (Supplementary Table 1) suggesting that all of the DPP4 regulated peptides might be products of protein catabolism. This data supports a role for DPP4 in renal catabolism of proline-containing peptides, as previously demonstrated using radiolabeled peptides11, and identify, for the first time, the endogenous peptides regulated by DPP4 in the kidney.

We studied the processing of DPP4-regulated peptides in greater detail using an LC-MS-based assay and kidney brush border membranes derived from DPP4+/+ and DPP4−/− mice. The distribution of peptide cleavage products in the MS profile after incubation of synthetic peptides with brush border membranes reveals the proteolytic activities present in the sample. For example, addition of the Mepβ(25-41) peptide, which lacks proline, to DPP4+/+ or DPP4−/− brush border membranes generates an N-terminal truncation ladder that is a hallmark of aminopeptidase (AP) activity (Fig. 1b). In contrast, the N-terminal processing of peptides that contain a proline residue, such as Mepβ(21-41), in DPP4+/+ membranes results in dipeptide truncated products that are indicative of DPP4 activity (Fig. 1c). The generation of these dipeptide cleavage products is strongly diminished in DPP4−/− membranes, which implies a specific and essential role for DPP4 in the processing of proline-containing peptides in the kidney.

Interestingly, only minor amounts of proline-terminated peptides (HN-Pro-peptide) are generated in these experiments (Fig. 1c), which is consistent with our in vivo measurements. These data suggest that penultimate proline substrates are poor AP substrates in the kidney. Not surprisingly, two aminopeptidases that are highly expressed in the kidney12, aminopeptidase N (APN) and aminopeptidase A (APA), exhibit in vitro substrate specificities that restrict cleavage adjacent to a penultimate proline13. One consequence of this substrate specificity is the possibility that aminopeptidase activity generates DPP4 substrates by successive cleavage of peptides until a penultimate proline is reached. While our earlier data clearly shows that DPP4 products (e.g., Mepβ(25-41)) are AP substrates, this model suggests that AP activity is equally important for the production of DPP4 substrates.
A similar idea was previously postulated, but has never been evaluated, based on the presence of APN, APA, and DPP4 in intestinal brush border membranes13.

The ability of membrane AP activity to generate DPP4 substrates was tested by using model peptides that contain proline residues at positions 3 and 5, termed 3P and 5P, respectively (Fig. 1d). A survey of the peptide distribution after incubation of the 3P and 5P peptides with the DPP4−/− membranes shows the accumulation of H2N-Xaa-Pro-peptides (e.g., LPVWHLDQS) when compared to a similar experiment with DPP4+/+ membranes (Fig. 1c). These results establish that total kidney brush border membrane AP activity can generate, but not further process, peptides with penultimate proline residues.

Furthermore, similar experiments using kidney brush border membranes from double-knockout mice lacking both APN14 and APA15 (APN/A−/− mice, Supplementary Fig. 5) resulted in reduced levels of 3P and 5P truncated products (Fig. 1e and Supplementary Fig. 6). These experiments indicate a direct role for AP activity in the production of DPP4 substrates. In total, the data reveals an unappreciated pathway that couples AP and DPP4 activities to generate amino acids and dipeptides from proline-containing peptides (Fig. 1f).

In turn, these amino acids and dipeptides can be reabsorbed via active transport mechanisms mediated by amino acid and peptide transporters11. Because these membrane lysates should faithfully represent the biochemical activities present on the surface of kidney brush border, it is likely that aminopeptidase and DPP4 activities are similarly coupled in vivo. In total, these findings reveal the utility of MS-based approaches in defining the physiological functions of peptidases and elucidating endogenous pathways involved in peptide metabolism.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

In vivo and in vitro MS-based peptide profiling experiments. (a) In vivo global peptide profiling reveals that the DPP4 cleavage product Mepβ(25-41) is elevated in the DPP4^{+/+} sample, while higher levels of the DPP4 substrate, Mepβ(21-41), are detected in the DPP4^{−/−} samples. (b) In vitro incubation of the Mepβ(21-41) peptide with kidney brush border membranes from DPP4^{+/+} and DPP4^{−/−} results in a series of aminopeptidase cleavage products. (c) In contrast, incubation of Mepβ(21-41), a proline containing DPP4 substrate, with DPP4^{+/+} membranes results in dipeptide cleavage products that are not produced with DPP4^{−/−} membranes. The data also indicates a lack of aminopeptidase activity adjacent to penultimate proline containing peptides. (d) Brush border membrane experiments with the model peptide substrates 3P and 5P show that brush border membrane peptidase activity is able produce DPP4 substrates. (e) Incubation of 3P and 5P with brush border membranes from APN/A^{+/+} and APN/A^{−/−} mice indicate a direct role for brush border membrane AP activity in the production of DPP4 substrates. (f) A model showing the interlinked relationship between AP and DPP4 activities during the degradation of proline-containing peptides. For all graphs, data represent mean values ± s.e. (*, p <0.05; **, p<0.01), Student’s t-test.
Table 1
Precuror proteins and absolute fold changes of DPP4-regulated peptides in DPP4<sup>−/−</sup>, DPP4<sup>+/+</sup>, LAF-237-treated, and vehicle-treated mice.

| Protein (peptide region) | Peptide Sequence | Fold Change |
|--------------------------|------------------|-------------|
| **DPP4<sup>−/−</sup> Elevated Peptides** | | |
| Diazepam-Binding Inhibitor (92-105) (DBI(92-105)) | RPGLDLKAGKAKWD | 38.1* |
| Meprin-β (21-41) (Mepβ(21-41)) | LPAPFVKDGGIDQIDF | 36.4** |
| Sorbitol Dehydrogenase (25-40) | YPIPELGNDVLLLKH | 13.4* |
| Elongation Factor 1 alpha1 (281-291) (EF-1α(281-291)) | APVNTTEVKS | 8.6** |
| Elongation Factor 2 (848-858) | IPALDNFLDKL | 7.4** |
| **DPP4<sup>+/+</sup> Elevated Peptides** | | |
| DBI(94-103) | GLLDLKGA | 3.0* |
| Mepβ(25-41) | EKFVKDGGIDQIDF | 8.2** |
| Histidine-triad nucleotide binding protein 1 (42-63) | HDISPQAPTHFLVIPKKHISQ | 3.0* |
| Vimentin (440-460) | RLTLIKTVERDQQVINESQ | 3.8* |
| Mitochondrial 28S ribosomal protein S36 (87-101) | PMSQEEMFIRQGIE | 2.5** |
| **Unaffected XP motif Peptides** | | |
| Heat Shock Protein 1(10-19) (HSP1(10-19)) | LPLFDRVLVE | 1.2 |
| Acid Sphingomyelinase-like Phosphodiesterase(31-43) (ASML-PDE(31-43)) | APAVGQFWHTDL | 1.0 |
| **LAF-237 Elevated Peptides** | | |
| Mepβ(21-41) | LPAPFVKDGGIDQIDF | 12.8* |
| EF-1α(281-291) | APVNTTEVKS | 10.3* |
| **Vehicle Elevated Peptides** | | |
| Mepβ(25-41) | EKFVKDGGIDQIDF | 2.3* |