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A Peptidomics Strategy To Elucidate the Proteolytic Pathways That Inactivate Peptide Hormones

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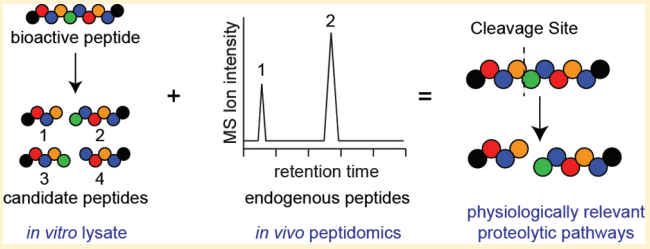
Supporting Information

ABSTRACT: Proteolysis plays a key role in regulating the levels and activity of peptide hormones. Characterization of the proteolytic pathways that cleave peptide hormones is of basic interest and can, in some cases, spur the development of novel therapeutics. The lack, however, of an efficient approach to identify endogenous fragments of peptide hormones has hindered the elucidation of these proteolytic pathways. Here, we apply a mass spectrometry (MS) based peptidomics approach to characterize the intestinal fragments of peptide histidine isoleucine (PHI), a hormone that promotes glucose-stimulated insulin secretion (GSIS). Our approach reveals a proteolytic pathway in the intestine that truncates PHI at its C-terminus to produce a PHI fragment that is inactive in a GSIS assay, a result that provides a potential mechanism of PHI regulation *in vivo*. Differences between these *in vivo* peptidomics studies and *in vitro* lysate experiments, which showed N- and C-terminal processing of PHI, underscore the effectiveness of this approach to discover physiologically relevant proteolytic pathways. Moreover, integrating this peptidomics approach with bioassays (i.e., GSIS) provides a general strategy to reveal proteolytic pathways that may regulate the activity of peptide hormones.



Peptide hormones control a number of vital physiological processes,[1,2] and the dysregulation of these signaling pathways can lead to prevalent diseases such as diabetes.[3] Understanding the molecular pathways that regulate peptide hormones can therefore provide insight into disease mechanisms and new opportunities for therapeutic intervention. Proteolysis of the hormones angiotensin[4,5] and glucagon-like peptide 1 (GLP-1)[6,7] modulates the levels and activities of these peptides. Additionally, both of these proteolytic pathways have been targeted in the development of drugs[4−6] by using peptidase inhibitors to regulate endogenous hormone levels. The characterization of the proteolytic pathways that cleave other peptide hormones would identify key regulatory mechanisms and may eventually lead to the development of novel therapeutics.

We present a general peptidomics-based approach that relies on measurements of endogenous fragments of peptide hormones to elucidate the proteolytic pathways that cleave these peptides in tissues. We develop this approach by investigating the proteolytic pathways that process the intestinal peptide hormone peptide histidine isoleucine-27 (PHI(1−27)).[8] PHI(1−27) and vasoactive intestinal peptide (VIP) are both products of the VIP gene,[9] which is expressed in the gut and other tissues. PHI(1−27) was originally discovered from porcine intestine using an innovative biochemical strategy that detected peptides with C-terminal amides,[8,10] a hallmark of peptides derived from the secretory pathway. Since its discovery PHI(1−27) has been linked to a number of biological functions including prolactin secretion,[11] glucose-stimulated insulin secretion (GSIS),[12] and the regulation of physiological glucose tolerance,[9] which led to our interest in the regulation of this hormone.

Our peptidomics approach revealed that proteolysis of PHI-(1−27) in the intestine occurs through C-terminal processing. This result differed from initial experiments with intestinal lysates, which identified proteolytic pathways that truncate PHI-(1−27) at its N- and C-termini, to demonstrate the difficulty in using *in vitro* proteolysis experiments to predict physiologically relevant pathways. Through the integration of peptidomics studies with bioassays we can identify the potential impact of proteolysis on

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the bioactivity of a peptide. In this case, C-terminal proteolysis of PHI(1−27) abrogates the activity of this peptide hormone in a glucose-stimulated insulin secretion (GSIS) assay,[13] which suggests that the intestinal proteolytic pathway discovered through peptidomics may be involved in the inactivation of PHI(1−27) *in vivo*. These results demonstrate the ability of peptidomics to discover relevant proteolytic pathways involved in the processing, and possibly the regulation, of peptide hormones.

EXPERIMENTAL PROCEDURES

Animal Studies. Wild-type (strain C57BL/6, $DPP4^{+/+}$) mice used in this study were either purchased (Jackson Laboratories, Bar Harbor, ME) or taken from a breeding colony. The $DPP4^{-/-}$ mice used in this study (a generous gift from Dr. Didier Marguet) have previously been described[7] and are on a C57BL/6 background. Mice in these studies were not littermates from het x het crosses but were obtained from separate colonies of $DPP4^{-/-}$ and $DPP4^{+/+}$ mice. Animals were kept on a 12 h light, 12 h dark schedule and fed *ad libitum*. For intestinal tissue collection, animals were euthanized with CO_2 and their tissue was dissected, flash frozen in liquid N_2, and stored at −80 °C. All animal care and use procedures were in strict accordance with the standing committee on the use of animals in research and teaching at Harvard University and the National Institutes of Health guidelines for the humane treatment of laboratory animals.

Preparation of Intestinal Tissue Lysates for *in Vitro* Lysate Experiments. The first 3 in. of the mouse intestine extending from the stomach (duodenum) was Dounce-homogenized in 1× phosphate-buffered saline (PBS), and the homogenates were then centrifuged at 1000*g* at 4 °C for 5 min to remove cellular debris. The supernatant from this spin was transferred to a thick-walled centrifuge tube and then centrifuged at 100000*g* at 4 °C for 45 min using a TLX ultracentrifuge (Beckman Instruments). The resulting supernatant was transferred to a new Eppendorf tube and used as the soluble fraction. The pellet was washed twice with 1× PBS and then suspended in 100 μL of 1× PBS, which was used as the membrane fraction. The protein concentrations of these lysates were determined using a Bradford assay. All lysates were diluted using 1× PBS to a final concentration of 1 mg/mL for all subsequent experiments.

***In Vitro* Lysate Experiments with PHI(1−27).** The concentrations of the peptide stocks were determined by amino acid analysis (Microchemistry Facility, Harvard University). Peptides (100 μM) were incubated with soluble and membrane intestinal lysates (1 mg/mL) at 37 °C for 15 min. These reactions were then quenched using 8 M GndHCl and desalted using a Zip-Tip (Millipore) prior to LC-MS or MALDI analysis.

LC-MS Analysis of *in Vitro* Lysate Experiments. Lysate samples were analyzed using a nano flow LC (Nano LC-2D; Eksigent Technologies) system coupled to a linear ion trap mass spectrometer (LTQ; ThermoFinnigan). The analytical column (Self-pack picofrit column, 75 μm i.d.; New Objective) was packed 15 cm with 3 μm of C18 (Magic C18 AQ 200A 3U; Michrom Bioresources Inc.). The trap column was obtained prepacked from New Objective Inc. (Integrafrit sample trap, C18 5 μm, 100 μm column i.d.). The samples were trapped at an isocratic flow rate of 2 μL/min for 10 min and eluted at a flow rate of 300 nL/min via a mobile phase gradient of 5−40% B in 145 min (mobile phase A, 0.1% formic acid in water; mobile phase B, 0.1% formic acid in acetonitrile). The peptides were detected in the positive mode, and the LC/MS/MS experiment was performed in a data-dependent acquisition (DDA) mode. The mass range for data acquisition was set from m/z 400 to m/z 1600. The MS/MS data were collected for the most intense peak from the MS chromatogram. Dynamic exclusion was set for 30 s, the exclusion size list was set to 200, and the normalized collision energy for CID was 35%. The capillary spray voltage was set to 2.5 kV. Peptide identification was performed using SEQUEST with differential modification of methionine to its sulfoxide. Peptide identification was performed using SEQUEST against the mouse subset of the Uniprot Knowledgebase with differential modification of methionine to its sulfoxide. A reversed decoy database strategy served to estimate a false discovery rate (FDR). Peptides were accepted within 1 Da of the expected mass, meeting a series of custom filters on ScoreFinal (S_f), −10 Log P, and charge state that attained an average peptide FDR of <2% across data sets. Manual inspection of spectra, FDR calculation, and protein inference were performed in Proteomics Browser Suite 2.23 (ThermoFisher Scientific). The peak areas served to quantify the different peptides generated during the incubation with the soluble and membrane lysates.

MALDI Analysis of *in Vitro* Lysate Experiments. MALDI spectra of the peptides were obtained with a Waters MALDI micro MX (Waters MS Technologies, Milford, MA) operated in reflectron positive mode. Operating conditions were as follows: source voltage, 12 kV; matrix suppression delay, 800 amu; pulse voltage, 1.9 kV; reflectron voltage, 5.2 kV; laser firing rate, 5 Hz. Instrument control, spectral acquisition, and processing were performed with MassLynx software (version 4.1). Samples were desalted by a Zip-Tip, mixed with a solution of peptides (0.5 μL) on the stainless steel MALDI target together with 0.5 μL of matrix (saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile), and dried under ambient conditions. The peptide identification were also confirmed by LC-tandem MS experiments as described above.

Protease Inhibitor Assays. The protease inhibitors used were e-64 (L-*trans*-epoxysuccinylleucylamido(4-guanidino)butane) (10 μM) and iodoacetamide (1 mM) for cysteine proteases; EDTA (ethylenediaminetetraacetic acid, 1 mM) and *o*-phenanthroline (1 mM) as general metalloprotease inhibitors; captopril (1 mM) and enalapril (1 mM) as ACE inhibitors; LAF-237 (1 μM) as a DPP4 inhibitor; phosphoamidon (10 μM) as a neprylsin inhibitor; pepstatin A (10 μM) for aspartyl proteases; PMSF (phenylmethanesulfonyl fluoride, 1 mM) and DFP (diisopropyl fluorophosphonate, 1 mM) as general serine protease inhibitors. PHI(1−27) (100 μM) was added along with these inhibitors to membrane lysate (1 mg/mL, diluted with 1× PBS) in a total volume of 40 μL. The incubation was allowed to proceed at 37 °C for 15 min prior to the addition of 40 μL of 8 M GdnHCl to quench the reaction. Samples were then desalted using a Zip-Tip and subsequently analyzed by MALDI (qualitative) and LC-MS (quantitive) assay.

Intestinal Peptide Isolation for Peptidomics Analysis. Tissue peptide isolation and fractionation followed the recently developed protocol.[14] Briefly, frozen intestines were placed in 500 μL of water and boiled for 15 min to inactivate any residual proteolytic activity prior to tissue homogenization. The aqueous fraction was separated and saved, and the tissue was Dounce-homogenized in ice-cold 0.25% aqueous acetic acid. The aqueous fraction and the homogenate were combined and centrifuged at 20000*g* for 20 min at 4 °C. The supernatant was then sent through a C18 Sep Pak cartridge (HLB 1 cm^3, 30 mg; Oasis), which was washed thoroughly with water (10 mL) to desalt the sample.

The peptides were then eluted with 1 mL of 70:30 H_2O/ACN and concentrated under vacuum using a Speedvac prior to fractionation by strong cation exchange (SCX).

Peptide Fractionation for Peptidomics Analysis. SCX was performed using a PolySULFOETHYL A column (200 × 2.1 mm, 5 μm, 300 Å; PolyLC Inc.) connected to an Agilent Technologies 1200 series LC. All runs were operated at 0.3 mL/min. The SCX buffers consisted of (A) 7 mM KH_2PO_4, pH 2.6, 25% ACN (v/v); (B) 40 mM KCl, 7 mM KH_2PO_4, pH 2.6, 25% ACN (v/v); (C) 100 mM KCl, 7 mM KH_2PO_4, pH 2.6, 25% ACN (v/v); (D) 600 mM KCl, 7 mM KH_2PO_4, pH 2.6, 25% ACN (v/v). Prior to the SCX runs, all samples (N = 4) were dissolved in 100 μL of buffer A (100 μL injections). A step gradient was applied that included 60 min with buffer A, 40 min with buffer B, 40 min with buffer C, and 40 min with buffer D, with 20 min transitions between the different buffer conditions. Fractions were collected separately for each of the different buffer conditions (e.g., a buffer A fraction, a buffer B fraction, and so on). All four salt fractions (A, B, C, and D) were then applied to a C18 Sep Pak cartridge, washed with water (25 mL) to desalt the samples, and then eluted with 1 mL of 70:30 H_2O/ACN and concentrated using a Speedvac. The peptides were dissolved in 0.1% aqueous formic acid (75 mg of tissue/40 μL), normalized according to the original tissue weight, prior to LC-MS analysis.

Intestinal Peptidomics Analysis. Fractionated intestinal samples were analyzed using a nano flow LC (Nano LC-2D; Eksigent Technologies) system coupled to a linear ion trap mass spectrometer (LTQ; ThermoFinnigan). The analytical column (Self-pack picofrit column, 75 μm i.d.; New Objective) was packed 15 cm with 3 μm of C18 (Magic C18 AQ 200A 3U; Michrom Bioresources Inc.). The trap column was obtained prepacked from New Objective Inc. (Integrafrit sample trap, C18 5 μm, 100 μm column i.d.). The samples were trapped at an isocratic flow rate of 2 μL/min for 10 min and eluted at a flow rate of 300 nL/min via a mobile phase gradient of 5−40% B in 227 min (mobile phase A, 0.1% formic acid in water; mobile phase B, 0.1% formic acid in acetonitrile). The peptides were detected in the positive mode, and the mass range for data acquisition was set from m/z 400 to m/z 1600. The data were collected in Full MS mode (N = 4) for quantitation and then in Top 6 MS2 mode (N = 2) for sequencing.[14] For the Top 6 analysis dynamic exclusion was set for 30s, the exclusion size list was set to 200, and the normalized collision energy for CID was 35%. The capillary spray voltage was set to 2.5 kV. We utilized an algorithm written in-house that reveals related MSMS spectra (MuQuest; Harvard Proteomics Browser Suite). To analyze the intestinal proteome to search for PHI(1−27) fragments, we used the MSMS data from the *in vitro* lysate experiments. MuQuest is then applied to compare the *in vitro* MSMS spectra with those of the *in vivo* data set to determine which *in vitro* MSMS spectra (i.e., which peptides) are present in the *in vivo* samples. The output files are filtered based on charge state, mass to charge values, and statistical scores.

Synthesis of PHI(1−27) Cleavage Products. Automated (PS3 Protein Technology, Inc.) FMOC solid-phase peptide synthesis (SPPS) was carried out with Rink Amide MBHA resin for PHI(3−27) and PHI(1−27) and with an aspartic acid loaded Wang resin for PHI(1−22). Cleavage from the resin was accomplished using a standard FMOC cleavage conditions (TFA, water, mercaptoethanol), and successful synthesis was assessed by MALDI-TOF (Waters) measurements of the crude peptide mixture. The crude peptides were purified by RP-HPLC (Shimadzu) using a C18 column (150 mm × 20 mm, 10 μm particle size; Higgins Analytical). Mobile phase A consisted of 99% H_2O, 1% acetonitrile, and 0.1% TFA, and mobile phase B consisted of 90% acetonitrile, 10% H_2O, and 0.07% TFA. The HPLC gradient proceeded from 20% to 50% B over 40 min. HPLC fractions were analyzed by MALDI-TOF (Waters) using α-cyano-4-hydroxycinnamic acid as the matrix to isolate fractions containing the pure peptide, which were then combined and lyophilized.

Recombinant DPP4 Expression [15]**.** A plasmid containing the mouse DPP4 gene was obtained from Open Biosystems, and the extracellular domain (residues 37−760) was subcloned into the *Eco*RI and *Xho*I site of a modified pFastBac vector (Invitrogen). The final construct contains a baculovirus gp64 signal peptide followed by a His6 tag fused to the coding sequence corresponding to residues 37−760 of DPP4. Recombinant baculovirus was generated by transposition using the Bac-to-Bac system (Invitrogen). Mouse recombinant DPP4 was expressed using a baculovirus expression system with Sf9 cells in liquid culture and purified by Ni(II)-affinity chromatography.

Degradation of PHI(1−27) by Recombinant DPP4. PHI-(1−27) (300 μM) was incubated with recombinant DPP4 (200 nM) in PBS. The degradation products were monitored by MALDI at 30 min, 1 h, and 2 h time intervals.

Glucose-Stimulated Insulin Secretion Assay. Insulin release in primary mouse islets was examined using the batch release method as previously described.[4] Peptides (GLP1, PHI-(1−27), PHI(3−27), and PHI(1−22)) were directly added at 100 nM final concentration to 1 mL of Krebs assay buffer[13] containing basal (1.67 mM) or stimulatory (16.7 mM) glucose concentrations. Islets were incubated for 1 h in the Krebs assay buffer at 37 °C. The supernatant was then collected for insulin measurement using an insulin ELISA kit (Crystal Chem Inc., IL, USA) according to the manufacturer's instructions for nonserum/plasma samples using half the amount of sample than suggested. The insulin concentration was calculated by using KaleidaGraph 4.0 software (Synergy Software, PA, USA) with four-parameter sigmoidal logistic curve fitting.

GLP-1R Assays. This assay was performed as a service with Shanghai ChemPartner Co., Ltd. Breifly, 4 × 10^4 cells from a reporter cell line (GLP-1R/CRE-HEK293, clone 9-5) were plated into a 96-well plate and then incubated overnight in a CO_2 incubator at 37 °C. In parallel, a 10-point serial dilution of PHI (1−27) and GLP-1 was prepared from a DMSO stock with concentrations ranging from 0.2 to 2 × 10^{-10} mM, and 0.5 μL of these stock solutions was transferred to the cell plates. The cells and peptides were then incubated for 6 h in a CO_2 incubator at 37 °C. Steady-Glo assay kit (from Promega, Cat. No. E2520, Lot No. 256974) was then prepared according to the manufacturer's instructions, and 100 μL of the Steady-Glo reagent was added to each well and then mixed for 5 min to induce cell lysis. The luminescence of each well was then read using a Flexstation 3 with an integration time of 500 ms. The data are plotted by normalizing the luminescence response and fitted to the dose−response logistic equation using KaleidaGraph 4.0 to generate an EC_{50} curve.

RESULTS AND DISCUSSION

Rationale for a Peptidomics-Based Approach To Investigate the Proteolysis of Peptide Hormones. *In vitro* experiments with synthetic peptide hormones and recombinant enzymes are commonly used in an attempt to identify a proteolytic pathway that

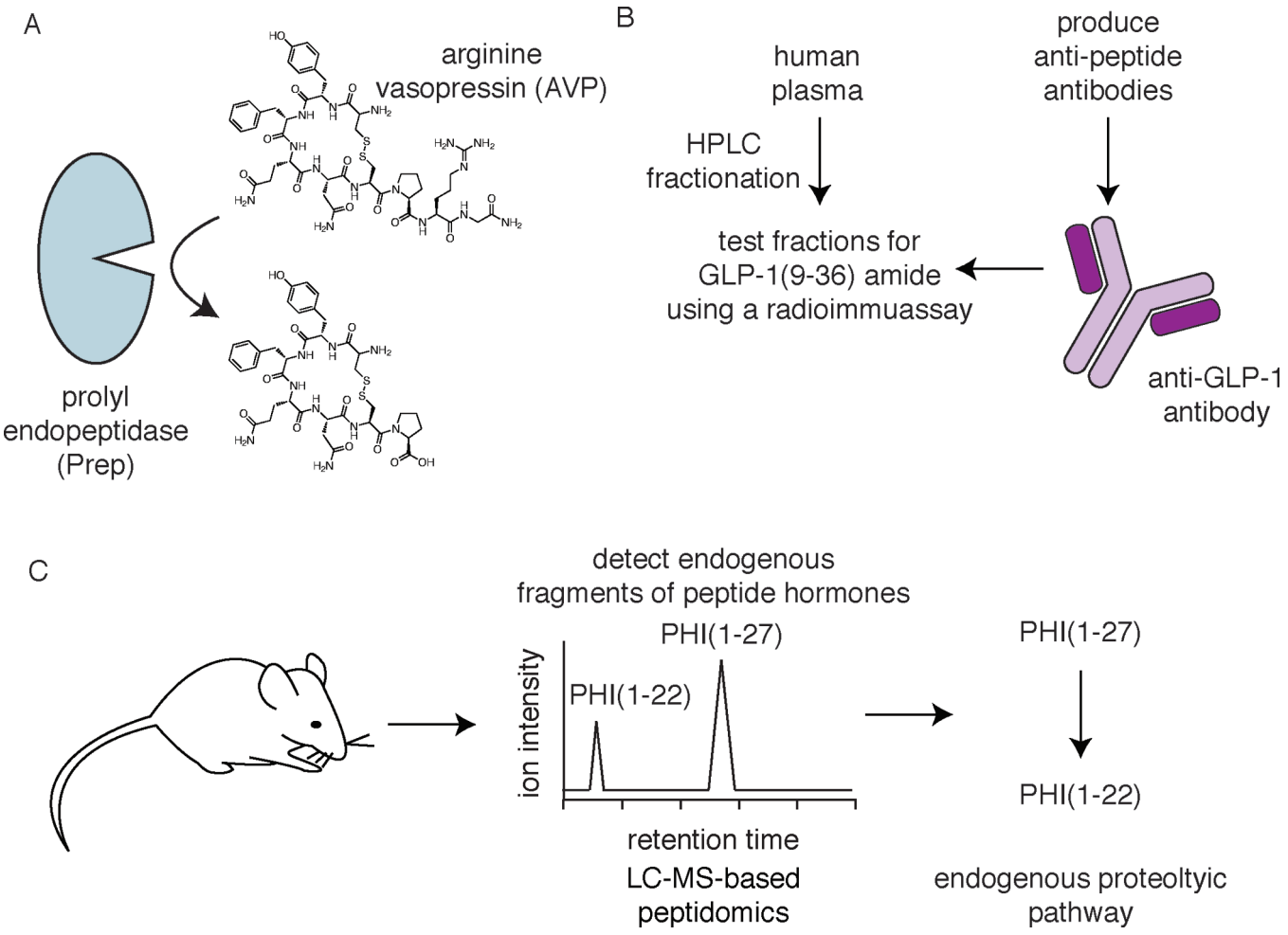

Figure 1. Methods for discovering proteolytic pathways that process peptide hormones. (A) *In vitro* assays with recombinant enzymes can reveal candidate pathways that break down a peptide hormone. The example shown here demonstrates that the hormone arginine vasopressin is a prolyl endopeptidase substrate. (B) An immunoassay can be used to detect fragments of a peptide hormone *in vivo* to assess the physiological relevance of a particular proteolytic pathway. In the case of GLP-1(7−36) the relevance of a dipeptidyl peptidase 4 (DPP4) mediated pathway was confirmed through the detection of GLP-1(9−36) in plasma. (C) Here, we present a strategy that relies on an LC-MS-based peptidomics approach to define physiologically relevant fragments of the hormone PHI(1−27), which can be used to infer the endogenous proteolytic pathway for the cleavage of this peptide hormone.

cleaves the peptide hormone.[16,17] This approach relies on using the known substrate preferences of an enzyme to identify candidate substrates from the pool of known peptide hormones. For example, the hormone arginine-vasopressin (AVP), which contains a single proline residue, was tested as a prolyl endopeptidase (Prep) substrate (Figure 1A). AVP is an excellent *in vitro* Prep substrate,[17] but subsequent tissue measurements indicate that AVP is not a Prep substrate *in vivo*.[18] This example highlights the shortcomings of *in vitro* assays that neglect important features of physiological regulation (e.g., competitive pathways and localization effects) when trying to predict endogenous proteolytic pathways.

To create improved approaches, *in vitro* and *in vivo* experiments were combined to elucidate physiologically relevant proteolytic pathways.[19] This integrated approach relies on *in vitro* lysate experiments to generate a list of candidate peptide fragments, followed by immunoassays (i.e., ELISA, RIA, and EIA) of tissues to test whether these fragments are present *in vivo* (Figure 1B). The presence of a peptide fragment in tissues is then used to infer the physiological relevance of a particular proteolytic pathway. For example, incubation of the bioactive form of the insulinotropic hormone GLP-1, GLP-1(7−36), with human plasma produced an inactive N-terminal truncated fragment, GLP-1(9−36).[19,20] Next, an effective, albeit labor-intensive, strategy that combines HPLC fractionation of plasma with a radioimmunoassay (RIA) of the resulting fractions[19] was used to confirm the physiological relevance of this proteolytic pathway by characterizing GLP-1(9−36) as the major endogenous GLP-1 fragment[19] (Figure 1B).

The determination of relevant proteolytic pathways and their impact on the bioactivity of a peptide hormone is a pivotal step in defining a role for proteolysis in peptide hormone regulation. With GLP-1(7−36), for example, subsequent experiments, which relied on the prior identification of the endogenous proteolytic pathway, established dipeptidyl peptidase 4 (DPP4) as the enzyme that cleaves and inactivates this hormone *in vivo*.[7] Moreover, this finding prompted the development of DPP4 inhibitors as a new class of antidiabetic drugs.[6] While useful at defining these pathways through the identification of peptide hormone fragments, targeted immunoassays suffer from shortcomings, including the need for specialized reagents (antibodies)[19] and cross-reactivity,[21] and can only be used to confirm, not discover, endogenous peptides. These challenges, in particular the need for antibodies,

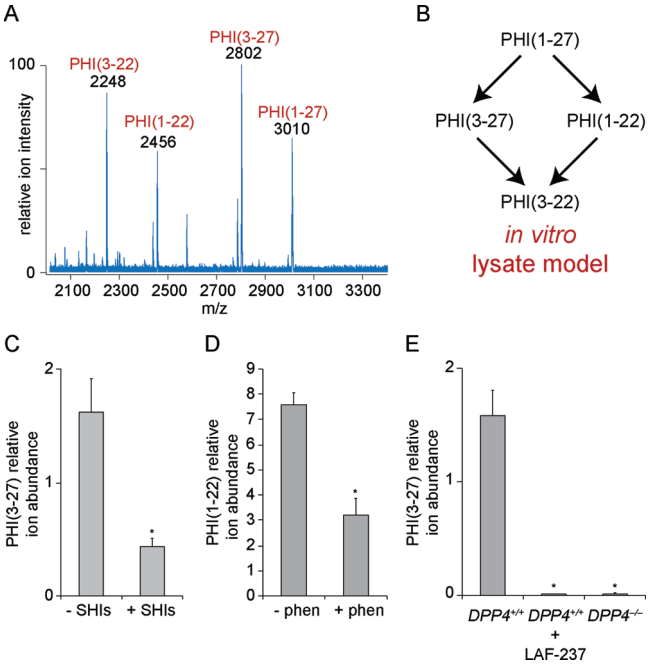

Figure 2. *In vitro* processing of PHI(1−27) with intestinal lysates. (A) Incubation of PHI(1−27) (100 μM) with intestinal lysates results in the production of three predominant PHI(1−27) fragments, PHI(1−22), PHI(3−27), and PHI(3−22), as detected by MALDI-MS. (B) A model for the *in vitro* proteolysis of PHI(1−27) based on peptide fragments produced in intestinal lysates. (C) Serine hydrolase inhibitors (SHIs, PMSF (1 mM) and DFP (1 mM)) prevents the production of the N-terminal degradation product PHI(3−27) when added to hydrolysis reactions in intestinal lysates (Student's *t* test; *, *p*-value ≤0.05, *N* = 3). (D) The metalloprotease inhibitor 1,10-phenanthroline (phen) inhibits the production of the C-terminal degradation product PHI(1−22) when added to hydrolysis reactions in intestinal lysates (Student's *t* test; *, *p*-value ≤0.05, *N* = 3). (E) The addition of the DPP4 inhibitor LAF-237 to intestinal lysates or the use of lysates from *DPP4*$^{-/-}$ mice prevent the production of PHI(3−27) to implicate DPP4 in the N-terminal processing of PHI(1−27) in these tissue lysates (Student's *t* test; *, *p*-value ≤0.05, *N* = 3).

limit the rate at which peptide hormone proteolysis can be studied and indicate the necessity for a better approach to detect physiologically relevant bioactive peptide fragments.

We reasoned that a liquid chromatography−mass spectrometry (LC-MS) based peptidomics method[14,22,23] could be used to identify fragments of peptide hormones in tissues and circumvent some of the challenges associated with immunoassays (Figure 1C). In particular, peptidomics does not require specialized reagents (i.e., species-specific antibodies) and will not suffer from cross-reactivity due to the specificity of mass spectrometry. Our peptidomics-based approach, which combines *in vitro* lysate experiments with *in vivo* peptidomics, should provide a straightforward, general, and rapid method for the discovery of the proteolytic pathways that cleave peptide hormones *in vivo*. We develop and test this strategy through investigation of the intestinal proteolysis of peptide histidine isoleucine (PHI(1−27)),[8,10] a peptide hormone with insulinotropic activity[12] similar to that of GLP-1(7−36).

***In Vitro* Tissue Lysate Experiments with PHI(1−27).** Full-length PHI(1−27) was incubated with the soluble and membrane fractions derived from intestinal lysates to identify where the majority of PHI(1−27) proteolytic activity is located. After incubation of synthetic PHI(1−27) with lysates for 15 min, the remaining amount of PHI(1−27) in each sample was quantified using the ion intensity of the resulting PHI(1−27) peak in the LC-MS chromatogram. This experiment revealed that the majority of PHI(1−27) proteolytic activity is associated with the membrane fraction (Supporting Information). This finding is consistent with the idea that peptide hormones, such as PHI(1−27), are primarily processed extracellularly after release from secretory vesicles.[24]

Next, these samples were analyzed by mass spectrometry to identify the peptide fragments that were being generated. A matrix-assisted laser desorption/ionization (MALDI)-MS spectrum of these membrane lysate samples revealed a number of new ions upon incubation of PHI(1−27) with membrane lysates. These ions corresponded to the major PHI(1−27) fragments being produced and included PHI(3−27), PHI(1−22), and PHI (3−22) (Figure 2A). All of these structural assignments were confirmed by analysis of these samples by tandem LC-MS experiments (LC-MSMS).

These particular PHI(1−27) fragments provide a wealth of information about the proteolytic pathways that are active in these intestinal tissue lysates. For example, PHI(3−27) indicates that N-terminal proteolysis of PHI(1−27) occurs through the removal of a dipeptide, which is similar to what is seen for GLP-1 in plasma. Similarly, C-terminal processing of PHI(1−27) is also taking place to generate the PHI(1−22) fragment. These experiments also reveal a fragment resulting from the N- and C-terminal processing PHI(3−22). Together, the data from these *in vitro* intestinal lysate experiments result in a model for PHI(1−27) proteolysis that degrades this peptide at both the N- and C-terminus to afford the PHI(3−27) and PHI(1−22), respectively (Figure 2B).

Candidate Peptidases Mediating PHI(1−27) Cleavage in Lysates. Class-selective protease inhibitors (e.g., metallo, cysteine, serine, and aspartyl) were added to these lysate experiments to inhibit the production of PHI(1−27) fragments (Supporting Information). After incubation, the impact of these inhibitors on PHI(1−27) processing was determined by LC-MS analysis of the samples and quantitation of the relevant ions. These experiments reveal that the serine protease inhibitors PMSF and DFP strongly inhibited the production of PHI(3−27), which indicates that N-terminal processing is being carried out by a serine protease (Figure 2C). Similarly, the metalloprotease inhibitor 1,10-phenanthroline impacted C-terminal processing of PHI (1−27) by reducing the production of PHI(1−22) (Figure 2D).

Next, we tried to identify candidate peptidases that process PHI(1−27) in these intestinal lysates by looking for enzymes that cut between amino acids 2−3 and 22−23 of the PHI(1−27) sequence. A search of the MEROPS database[25] using the cleavage sites we identified on PHI(1−27) and the enzyme class inhibitor suggested the matrix metalloproteases (MMPs) and neprilysin (Nep) as candidate C-terminal processing enzymes and dipeptidyl peptidases as potential N-terminal cleaving enzymes. Prior work investigating the *in vitro* breakdown of peptide histidine methionine (1−27) (PHM(1−27)), the human homologue of PHI(1−27), in serum implicated DPP4 in the proteolytic processing of this peptide.[20] To assess the relative contributions, if any, of these enzymes, we utilized chemical inhibitors and tissues from animals lacking these enzymes.

We found no impact of the MMP[26] inhibitor (GM6001) and Nep[27] inhibitor (phosphoramidon) on C-terminal processing of

PHI(1−27) by measuring PHI(1−22) production in lysates treated with these inhibitors (Supporting Information). These experiments highlight the difficulty in trying to use the known substrate preferences of enzymes to predict enzyme−substrate relationships. Considering that there are over 500 proteases in the mammalian genome[28] and that many of these enzymes need to be characterized, the identification of these enzyme−substrate relationships will continue to be a challenge.

In some cases, however, especially when the cleavage site is very distinctive (e.g., N-terminal dipeptide truncation), the processing of a peptide can immediately reveal a candidate enzyme. Indeed, the specific DPP4 inhibitor, LAF-237,[29] reduced the production of PHI(3−27) by greater than 100-fold, to indicate that DPP4 was responsible for N-terminal PHI(1−27) processing (Figure 2D). Furthermore, this conclusion garnered additional support from intestinal lysate experiments with tissues from DPP4 null ($DPP4^{-/-}$) mice,[7] which also showed greatly reduced production (>100-fold) of PHI(3−27) (Figure 2D). Together, these *in vitro* lysate experiments predict that PHI(1−27) undergoes N- and C-terminal processing in the intestine and that the N-terminal processing pathway is mediated by DPP4.

Intestinal Peptidomics To Characterize PHI(1−27) Fragments and Elucidate the Proteolytic Pathway That Cleaves PHI(1−27). As mentioned, *in vitro* assays are often unreliable in predicting cleavage *in vivo* because important aspects of endogenous regulation can be disrupted during lysate preparation, such as spatial organization and competitive pathways. In prior work, immunoassays were developed to identify these physiologically relevant fragments of peptide hormones. However, the shortcomings of immunoassays, including the need for specialized reagents and unwanted cross-reactivity,[21] prompted us to implement a state-of-the-art LC-MS-based peptidomics platform[14,22,23] to characterize fragments of PHI(1−27) in the mouse intestine.

The processing of these samples is identical to our previous peptidomics experiments.[14,22,23] The key step in this workflow is the heating of tissues prior to homogenization, which inactivates all proteolytic activity to maintain sample integrity for the subsequent LC-MS analysis. Indeed, an assay of DPP4 activity after tissue homogenization shows that this enzyme is completely inactive.[14] We analyzed the LC-MS data to identify PHI(1−27) and its fragments in the intestinal peptidome of wild-type (WT) mice. To simplify this complex sample, the intestinal peptidome was subjected to multidimensional fractionation. Specifically, an offline strong cation exchange (SCX) step was applied to the sample prior to LC-MS and yielded four salt fractions of reduced complexity.[14] These samples were then analyzed by LC-MSMS, which provided the data set that was searched for PHI(1−27) fragments.

Our initial attempts at identifying PHI(1−27) fragments in this data set using standard proteomics analysis software (Sequest)[30] were unsuccessful, and we did not see a single PHI(1−27) fragment. During a proteomics experiment every protein generates multiple peptides that can be used to detect the protein, and the identification of multiple peptides per protein is also used as a measure of confidence in the protein identification. One of the challenges faced in all peptidomics experiments is that each peptide results in a single MSMS spectrum that can be used to identify the peptide. As a result, the standard proteomics analysis software is not optimized for peptidomics, and we found that direct determination of PHI(1−27) and its fragments using the standard proteomics parameters for Sequest was not efficient. While lowering some of the thresholds optimized for proteomics (in particular the total intensity count) generates additional MSMS data that can be searched for the identification of PHI(1−27) peptides, these changes increase the time it takes for data analysis so much that it is no longer feasible. Additionally, even when these parameters are changed, it still results in poor identification of the additional MSMS spectra due to a higher frequency of MSMS spectra with low information content.

To circumvent this limitation, we utilized an algorithm written in-house that reveals related MSMS spectra (MuQuest; Harvard Proteomics Browser Suite). MuQuest compares MSMS spectra between different data sets (or within the same data set) to identify the same or related peptides in two (or more) different samples based on similar or related MSMS spectra. It is based on the principle that MSMS spectra derived from peptides of identical or shared sequence, independent of charge state or modifications, should have a subset of identical product ions. If one data set has a series of known peptides (i.e., standards), then it becomes an easy search for those peptides in a second sample using MuQuest. To analyze the intestinal proteome to search for PHI(1−27) fragments, we used the MSMS data from the *in vitro* lysate experiments. Sequest identified PHI(1−27) and its fragments PHI(1−22) and PHI(3−27) with high statistical confidence in this *in vitro* sample to ensure that we were looking for the correct peptides. MuQuest is then applied to compare the *in vitro* MSMS spectra with those of the *in vivo* data set to determine which *in vitro* MSMS spectra (i.e., which peptides) are present in the *in vivo* samples. The output files are filtered based on charge state, mass to charge values, and statistical scores.

The analysis of the intestinal peptidome samples revealed the presence of PHI(1−27) and PHI(1−22) (both +5 charged peptides at pH <3 found in the 600 mM KCl salt fraction) as the predominant PHI(1−27) fragments (Figure 3A). PHI(1−22) was one of the major fragments generated during the *in vitro* intestinal lysate experiments, and the detection of this peptide *in vivo* indicates that a C-terminal pathway for processing PHI(1−27) is active in the intestine. More generally, this example also highlights the utility of MuQuest in peptidomics analysis of complex biological samples and indicates that this should be the standard approach to these experiments.

Interestingly, these experiments did not detect PHI(3−27) in the intestine, one of the other major *in vitro* fragments that results from DPP4 processing of PHI(1−27) (Figure 2A). We confirmed that the absence of a detectable PHI(3−27) ion was not due to the inability of this peptide to ionize efficiently by measuring the ionization of synthetic PHI(1−27), PHI(1−22), and PHI(3−27) (2 nmol each) spiked into intestinal lysate samples. All three peptides produced peaks of comparable intensity that indicates that we would have detected PHI(3−27) if it was naturally occurring in the intestine (Supporting Information). The absence of a PHI(3−27) fragment in these samples demonstrates the importance of making *in vivo* measurements when elucidating physiologically relevant proteolytic pathways. Moreover, these experiments highlight the value of peptidomics experiments since we did not have to develop a specific assay to measure PHI(1−27) but simply relied on our standard peptidomics protocol[14] combined with an optimized data analysis approach using MuQuest (Harvard Proteomics Browser Suite, v3.2).

Investigating a Role for DPP4 in PHI(1−27) through Comparative Peptidomics. The absence of PHI(3−27) indicates that DPP4 does not process PHI(1−27) in the intestine. This

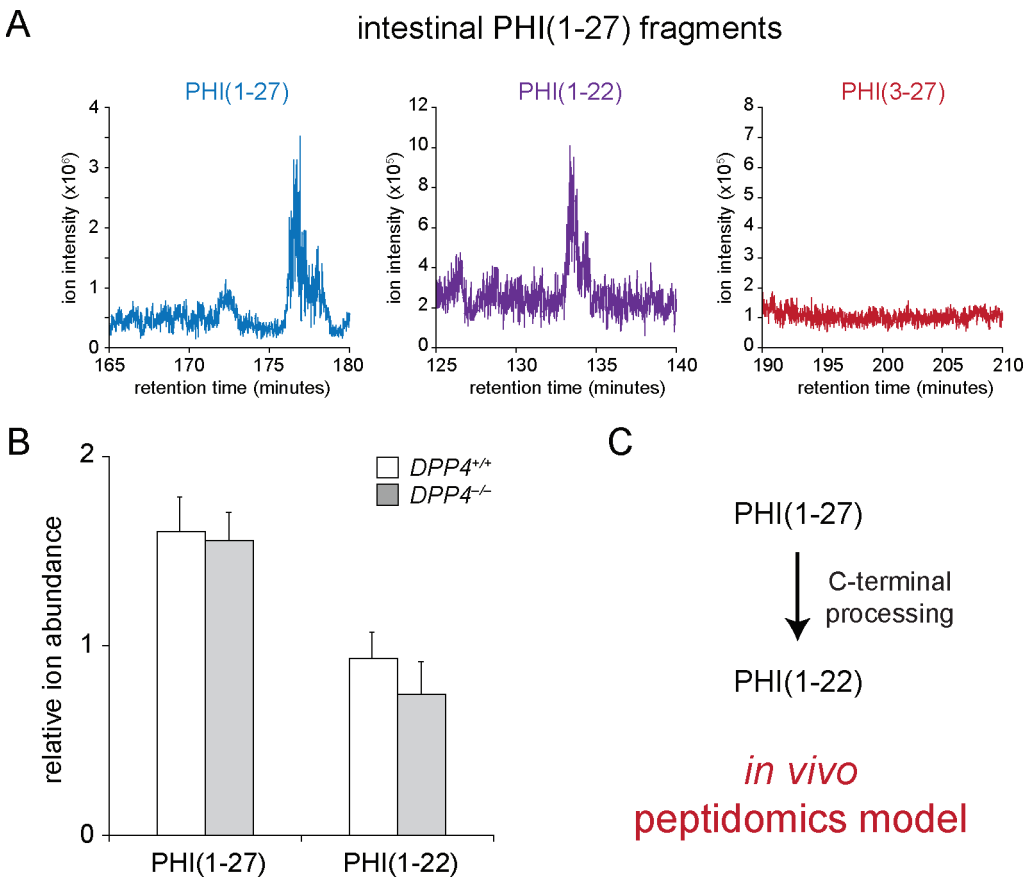

Figure 3. Intestinal processing of PHI(1−27). (A) Peptidomics of the intestine revealed the presence of PHI(1−27) (blue) and PHI(1−22) (purple) but not PHI(3−27) (red; retention time region was selected based on the known retention time of a standard (Supporting Information)). (B) Comparative peptidomics reveals that $DPP4^{+/+}$ and $DPP4^{-/-}$ intestines have similar levels of PHI(1−27) and PHI(1−22) (Student's *t* test; *p*-value is not significant, $N = 3$). (C) A model for the *in vivo* proteolysis of PHI(1−27) based on physiological fragments of PHI(1−27) detected by peptidomics.

differs from the conclusion reached from *in vitro* lysate experiments, which clearly showed a role for DPP4 in the N-terminal processing of PHI(1−27) to produce PHI(3−27). This is an important finding because it highlights the necessity for *in vivo* measurements to truly understand the physiological pathways that break down a peptide hormone. In our previous comparative peptidomics studies DPP4 substrates were discovered by identifying peptides that were elevated in $DPP4^{-/-}$ mice.[14,23] To confirm that DPP4 is not regulating PHI(1−27), we quantitated PHI(1−27) and PHI(1−22) levels in the intestines from wild-type ($DPP4^{+/+}$) and $DPP4^{-/-}$ mice using our comparative peptidomics approach.[14] If DPP4 is processing PHI(1−27) or PHI(1−22) levels *in vivo*, the prediction would be that PHI(1−27) or PHI(1−22) would be elevated in the absence of DPP4. According to these comparative peptidomics experiments, which showed similar levels of PHI(1−27) and PHI(1−22) in $DPP4^{+/+}$ and $DPP4^{-/-}$, DPP4 does not regulate intestinal levels of PHI(1−27) (Figure 3B).

In total, these *in vivo* peptidomics experiments establish that PHI(1−27) undergoes C-terminal processing to produce PHI(1−22) (Figure 3C). The data also indicate that PHI(3−27), the product of a DPP4-mediated pathway, is not produced *in vivo*, which differs from the conclusion obtained from *in vitro* experiments. As mentioned, this discordance highlights the importance of complementing *in vitro* experiments with *in vivo* analysis, since *in vitro* experiments cannot account for important aspects of *in vivo* biochemistry.

A Competitive Substrate Model To Explain the Absence of PHI(3−27) *in Vivo*. On the basis of the *in vitro* results with tissue lysates we were surprised by the absence of PHI(3−27), the DPP4 cleavage product, *in vivo*. The discordance between *in vitro* measurements and *in vivo* analysis could be explained by a number of factors. First, DPP4 might not be able to access PHI(1−27) due to differential localization. Specifically, DPP4, which is found on the extracellular portion of the plasma membrane,[31] might not have access to PHI(1−27) if a majority of this peptide is stored in secretory vesicles. Another possibility is that DPP4 is regulated by a posttranslational modification (PTM) or protein−protein interaction (PPI) that would impact DPP4 activity; however, DPP4 is not known to have any PTMs that influence the activity of this enzyme, and known DPP4-PPIs are not thought to influence the activity or localization of this peptidase.[32]

Another possibility we considered is that substrate competition might be playing a role in the substrate selectivity we are seeing. We previously identified a number of proline-containing peptides as DPP4 substrates in the intestine through comparative peptidomics.[14] Specifically, we found 22 peptides in total that were elevated in the intestine of $DPP4^{-/-}$ mice, which indicates that they are DPP4 substrates. Of these 22 candidate substrates only a single peptide has a penultimate alanine (i.e., H_2N-Xaa-Ala-peptide) while the remainder all are proline-containing peptides (i.e., H_2N-Xaa-Pro-peptide). Importantly, other penultimate

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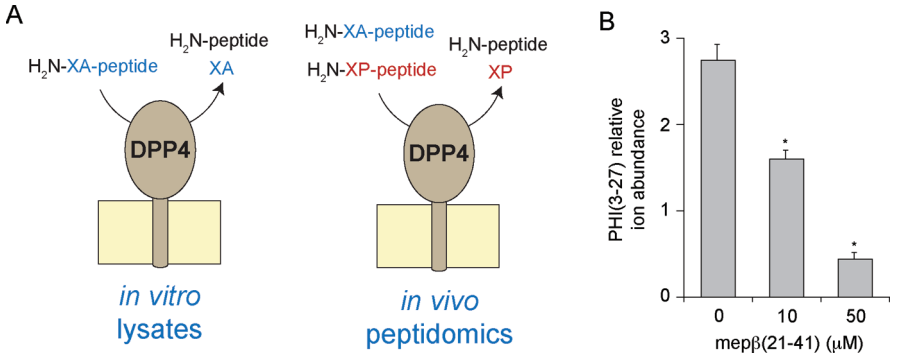

Figure 4. A competitive substrate model may explain the lack of PHI(3−27) in the intestine. (A) A hypothetical model to explain why PHI(1−27), an alanine-containing peptide, is efficiently processed *in vitro* but not *in vivo*. In this model the presence of proline-containing peptides, which are superior DPP4 substrates, suppresses the cleavage of alanine-containing peptides, like PHI(1−27). (B) N-Terminal processing of PHI(1−27) in tissue lysates is inhibited by increasing concentrations of the proline-containing peptide, mepβ(21−41), which supports a competitive substrate model (Student's *t* test; *, p <0.01, N = 3).

alanine-containing peptides could be found in this sample, but these were unchanged between DPP4$^{+/+}$ and DPP4$^{-/-}$ mice. This is consistent with *in vitro* experiments that show that proline-containing peptides are superior DPP4 substrates.[23,33]

The observation that DPP4 is cleaving a number of proline-containing substrates in the intestine suggests that the difference between our *in vitro* observations and our *in vivo* measurements might be due to differences in the available substrate pool. The preparation of lysates necessarily removes all endogenous peptide substrates. When PHI(1−27) is added in these *in vitro* lysate experiments, it is clearly processed by DPP4 to remove a dipeptide from the N-terminus of this hormone. In this experiment the only substrate available to DPP4 is exogenously added PHI(1−27). *in vivo* there is no evidence for DPP4 processing of PHI(1−27), either through the detection of PHI(3−27) or the regulation of PHI(1−27) or PHI(1−22) levels when comparing DPP4$^{+/+}$ and DPP4$^{-/-}$ intestinal tissue. We hypothesize that the presence of a large number of proline-containing peptides in the intestine leads to a situation where substrates compete for the DPP4 active site. As a result, this competition leads to preferential cleavage of proline-containing peptides, which are the best substrates of DPP4,[34] instead of alanine-containing peptides (Figure 4A).

To test this possibility, we incubated PHI(1−27) with membrane lysates and added increasing concentrations of a proline-containing DPP4 substrate meprinβ (21−41) (mepβ(21−41)), which is a *bona fide* DPP4 substrate in the kidney[14,23] (Table 1). The addition of mepβ(21−41) inhibits DPP4 processing of PHI(1−27) in a dose-dependent manner to demonstrate that proline-containing substrates may slow the cleavage of alanine-containing substrates in a competitive manner in tissue lysates (Figure 4B). Admittedly, we do not know the ratio of proline-to-alanine-containing substrates *in vivo*; however, this experiment in conjunction with our *in vivo* comparative peptidomics experiments supports a model where proline-containing peptides may be inhibiting the processing of the alanine-containing peptides. Moreover, these results are also supported by measurements in the kidney where we used SCX and peptidomics to identify 67 proline-containing DPP4 substrates, but not a single alanine-containing peptide substrate was found.[14,23] More generally, this experiment suggests another possibility for differences between *in vitro* and *in vivo* experiments, the presence of competitive substrates, that impact our ability to predict endogenous substrates from *in vitro* experiments.

Table 1. Sequences of Peptides Used and/or Detected in This Study

peptide	sequence
GLP-1(7−36)	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG-CONH2
PHI(1−27)	HADGVFTSDYSRLLGQISAKKYLESLI-CONH2
PHI(1−22)	HADGVFTSDYSRLLGQISAKKY-COOH
PHI(3−27)	DGVFTSDYSRLLGQISAKKYLESLI-CONH2
PHI(3−22)	DGVFTSDYSRLLGQISAKKY-COOH
mepβ(21−41)	LPAPEKFVKDIDGGIDQDIFD-COOH

Impact of Proteolysis on PHI(1−27) Bioactivity. On the basis of the data obtained from the *in vivo* peptidomics experiments, where we detect PHI(1−22) as a physiologically relevant fragment of PHI(1−27), we conclude that the proteolytic pathway that processes PHI(1−27) relies on C-terminal degradation (Figure 3C). We wondered whether this pathway could influence any of the known bioactivities of this hormone. PHI(1−27) has been shown to stimulate prolactin and insulin secretion.[11,12,35] To assess the functional impact of the newly discovered intestinal pathway for C-terminal processing of PHI(1−27), we tested the PHI fragments PHI(1−27) and PHI(1−22) in a glucose-stimulated insulin secretion (GSIS) assay[13] using mouse pancreatic islets. GLP-1 has been reported to stimulate insulin secretion in these experiments, and we used this peptide as a positive control.[36]

Mouse pancreatic islets were freshly isolated from whole pancreases for these experiments.[13] These islets were then incubated with glucose and GLP-1, PHI(1−27), or PHI(1−22). The conditioned media from these islets were then isolated, and insulin levels were measured. In these experiments, PHI(1−27) is able to promote insulin secretion to demonstrate the insulinotropic activity of this hormone (Figure 5A). Importantly, PHI(1−22) is inactive in this GSIS assay at the same dose as PHI(1−27) to indicate that the proteolytic processing that we see *in vivo* can regulate the biological activity of this peptide hormone (Figure 5A). Thus, this newly discovered C-terminal degradation pathway that processes PHI(1−27) *in vivo* attenuates the bioactivity of this peptide (Figure 5B).

Interestingly, we tested and found that PHI(1−27) does not activate the GLP-1 receptor (GLP-1R) (Supporting Information), despite a large degree of N-terminal homology between GLP-1(7−36) and PHI(1−27) (Table 1). The inactivity of PHI

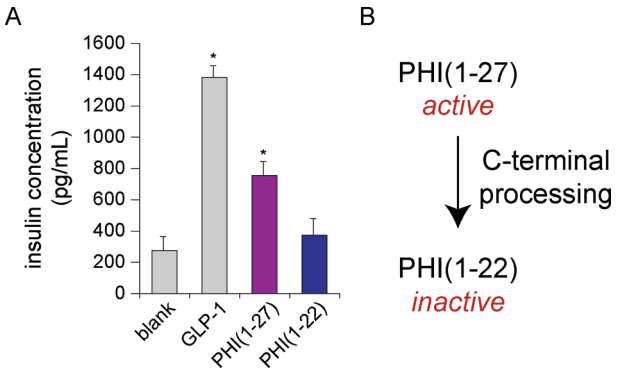

Figure 5. C-Terminal processing of PHI(1−27) inactivates this peptide in a GSIS assay. (A) Testing the different physiologically relevant PHI(1−27) fragments, PHI(1−27) (purple) and PHI(1−22) (blue) (100 nM each), in a glucose-stimulated insulin secretion (GSIS, 16.7 mM glucose) assay using freshly isolated mouse pancreatic islets (Student's *t* test; *, p <0.01, N = 7−8). (B) A pathway diagram describing the processing of PHI(1−27) *in vivo* and the impact of this processing on the GSIS activity (red) of PHI(1−27).

(1−27) with the GLP-1R indicates that PHI(1−27) and GLP-1 would operate differently in a biological setting and suggests that subsequent efforts to define the peptidase(s) that regulate PHI-(1−27) would be of benefit in studying the regulation and activity of this hormone, because it might lead to the discovery of an alternative pathway for regulating physiological insulin levels.

CONCLUSIONS

This work demonstrates the utility of peptidomics as a general method for elucidating the endogenous pathways involved in the proteolysis of peptide hormones. We discovered an intestinal proteolytic pathway that converts PHI(1−27) into PHI(1−22) and, in the process, abolishes the activity of this peptide in a GSIS assay. Obtaining this information required an optimized computational workflow that relied on MuQuest for the discovery of peptide fragments in complex biological matrices. Since we detected some, but not all, of the same peptides we saw *in vitro*, we believe that our method can also be improved to increase the sensitivity and quantitation by using multiple reaction monitoring (MRM) methods.[37] Compared with other standard immunoassay approaches, peptidomics offers some distinct advantages that accelerate the rate at which endogenous proteolytic fragments of bioactive peptides can be discovered. Furthermore, integrating this peptidomics strategy with bioassays, such as GSIS, will enrich our understanding of bioactive peptide function and regulation. Looking forward, the use of this peptidomics strategy with additional bioactive peptides, and improved methods for characterizing the peptidases that mediate these proteolytic pathways, will enrich our understanding of proteolytic regulation of bioactive peptides and might eventually provide new targets for drug development. In this case, we will begin efforts to identify the enzyme(s) responsible for the C-terminal processing of PHI(1−27) in the intestine, which may eventually lead to a better understanding of the regulation and bioactivity of this peptide hormone *in vivo*.

ASSOCIATED CONTENT

Supporting Information. Bar graph showing the increased degradation of PHI(1−27) by membrane fraction (Figure S1), bar graph showing the inhibition of C-terminal degradation of PHI-(1−27) by phenanthroline (Figure S2), intestinal peptidomics demonstrating the ability to detect PHI(1−27) and PHI(1−22), but not PHI(3−27), *in vivo* (Figure S3), and GLP-1 receptor assay showing that GLP-1 but not PHI(1−27) is a GLP-1 receptor agonist (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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