Published in final edited form as:

Peptides. 2008 June ; 29(6): 904–911.

REGULATION OF GHRELIN STRUCTURE AND MEMBRANE BINDING BY PHOSPHORYLATION

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Abstract

The peptide hormone ghrelin requires Ser-3 acylation for receptor binding, orexigenic and anti-inflammatory effects. Functions of desacylghrelin are less well understood. *In vitro* kinase assays reveal that the evolutionarily conserved Ser-18 in the basic C-terminus is an excellent substrate for protein kinase C. Circular dichroism reveals that desacylghrelin is ~12 % helical in aqueous solution and ~ 50 % helical in trifluoroethanol. Ser-18-phosphorylation, Ser-18-Ala substitution, or Ser-3-acylation reduces the helical character in trifluoroethanol to ~ 24 %. Both ghrelin and desacylghrelin bind to phosphatidylcholine:phosphatidylserine sucrose-loaded vesicles in a phosphatidylserine-dependent manner. Phosphoghrelin and phosphodesacylghrelin show greatly diminished phosphatidylserine-dependent binding. These results are consistent with binding of ghrelin and desacylghrelin to acidic lipids via the basic face of an amphipathic helix with Ser-18 phosphorylation disrupting both helical character and membrane binding.

Keywords

Ghrelin structure; ghrelin phosphorylation; peptide-membrane binding; PKC substrate

1. INTRODUCTION

The peptide hormone ghrelin has generated a lot of interest, especially for its orexigenic effects [28,2]. Highest concentrations are produced by the stomach before meals and concentrations are decreased after eating [28,2] --strongly by protein, weakly by fats, and biphasically by carbohydrates [18]. In addition to its central effects to stimulate appetite, growth hormone (GH) ¹ production and decreased blood pressure [28,2,35,32], ghrelin also has anti-apoptotic effects in many peripheral tissues [e.g., 3] and significant anti-inflammatory effects [31,16]. Activated lymphocytes produce ghrelin as part of their response to stimulation by antigens [16]. Disease states causing cachexia and inflammation are associated with increases in circulating ghrelin

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¹The abbreviations used are: GH, growth hormone; GHSR-1a, GH secretagogue receptor-1a; PKC, Protein kinase C; PC, phosphatidylcholine; PS, phosphatidylserine; DOPS, dioleoyl-PS; DOPC, dioleoyl-PC; DO, dioleoin; CD, circular dichroism; TFE, trifluoroethanol; TBS, Tris-buffered saline; MOPS, 4-morpholinepropanesulfonic acid.

concentrations [e.g., 19, 38, 25]. Those associated with gastric atrophy or removal [25,24] or the metabolic syndrome - obesity, hypertension and diabetes - are associated with decreased ghrelin [40,39].

Ghrelin is produced from a precursor, preproghrelin, of 117 amino acids [28] by the endoprotease prohormone convertase 1/3 [54]. Recently, preproghrelin was found also to encode an additional peptide, obestatin, which was reported to have anorexigenic actions [53], although that finding remains controversial [reviewed in 20]. More recently, an alternatively spliced preproghrelin has been identified in which the third of four coding exons (that encoding obestatin) has been omitted, resulting in an altered reading frame at the C-terminus [26]. Accumulating evidence suggests increased expression of this splice form in some cancers [26,51]. A few mutations have been noted in the ghrelin gene [46,49,30] but whether these are over-represented in some disease states remains controversial.

Ghrelin is unique among circulating hormones in requiring acylation for its actions at the growth hormone secretagogue receptor (GHSR-1a; the ghrelin receptor), which mediates GH production [28] appetite stimulation [2] and anti-inflammatory effects [16]. Acylated ghrelin circulates bound to high density lipoprotein [40,5] and is eliminated by the kidney [52]. It is rapidly desacylated by serum or tissue esterases [14] such that desacylghrelin concentrations usually are higher than concentrations of the acylated peptide. Acylation and secretion seem to be regulated independently [34].

The GHSR1a ghrelin receptor is a fairly widely expressed seven transmembrane G-protein-coupled receptor that activates calcium transients [28,16,23]. It undergoes a high level of constitutive internalization and this internalization is enhanced by ghrelin binding [23]. Protein kinase C (PKC) pathways are implicated strongly in many effects [23,22] and protein kinase A, mitogen-activated protein kinase, and other pathways may be involved in some responses [3, reviewed in 47,11]. Desacylated ghrelin exhibits some shared anti-apoptotic and other effects, and has some unique effects, which sometimes oppose those of ghrelin, suggesting possible existence of additional receptors [reviewed in 3,47].

Thus, regulation of ghrelin by various hormonal, metabolic, and inflammatory states may involve transcription, alternative splicing, excision of peptides from the precursor, acylation, desacylation, protein binding, peptide degradation, and renal excretion. Here we report yet another potential means of ghrelin regulation: phosphorylation. Although the N-terminus is required for interaction at the ghrelin receptor, the C-terminal 2/3 of the peptide also is highly conserved over 350 million years of evolution. This region is highly basic and contains a highly conserved Ser at position 18 in most mammals (or Ser-22 in ungulates), suggestive of a potential PKC phosphorylation site. Lower terrestrial vertebrates have a Thr at one or both of these positions, also expected to be good PKC target sites. Here we show that ghrelin serves as an excellent PKC substrate *in vitro* and that phosphorylation causes significant changes in the ghrelin structure and membrane binding.

2. EXPERIMENTAL

2.1. Materials

³²P-ATP (7000Ci/mmol) and 4(aminoethyl) benzenesulfonyl fluoride were purchased from MP Biochemicals, LLC. 2,2,2-Trifluoroethanol (TFE) and bovine brain phosphatidylserine (PS) were from Sigma. Dioleoylphosphatidylserine (DOPS), dioleoylphosphatidylcholine (DOPC) and diolein (DO) were obtained from Avanti Polar Lipids, Inc. Ghrelin and des-n-octanoyl ghrelin were obtained from Global Peptide Services, LLC. Ser-18-phosphoghrelin was custom synthesized by AnaSpec, Inc. or synthesized in the Chemistry department at University of VA as described below. A rabbit polyclonal antiserum to ghrelin was generated

using full length human acyl-ghrelin as antigen (gift from Bristol-Myers Squibb) and then was affinity purified against ghrelin to generate the antiserum used for Western blotting. In some cases, it was purified against ghrelin residues 21–27 to generate a C-terminal-specific antiserum.

2.2. Methods

Purification of Protein Kinase C—PKC α , β_{II} and δ were purified through ion exchange and hydrophobic columns from Sf-9 cells infected with recombinant baculovirus expressing the various PKC isoforms as described previously [42,43]. Purity was assessed by gel electrophoresis followed by silver staining and Western blotting. PKC was usually the only band as shown in [43].

In vitro phosphorylation of ghrelin—PKC peptide phosphorylation reactions were carried out as described in [48] in polypropylene tubes in order to minimize loss of ghrelin due to nonspecific adsorption to tube walls. A typical reaction mixture contained 10 μ M substrate peptide; 20 mM 4-morpholinepropanesulfonic acid (MOPS), pH 7.4; 5 mM $MgCl_2$; 133 μ M $CaCl_2$ (for PKC α and β_{II}); 1–2 μ Ci [γ - ^{32}P]ATP; 40 μ M ATP; 200 μ M bovine brain PS with 5 mol % DO and 25 nM PKC α , β_{II} or δ . Reactions were incubated at 30°C. At indicated time points, 60 μ l samples were removed from the reaction mixture and spotted onto P-81 ion exchange paper (Whatman). The papers were washed three times in 50 mM NaCl to remove unincorporated ATP and dried. PKC-mediated phosphorylation of ghrelin peptides was determined by counting the papers in a scintillation counter.

Western blot analysis of ghrelin—Western blot analysis was performed as described earlier [29]. In brief, peptides samples (25–50 ng/lane) were separated by electrophoresis through 15 % Tris-Tricine gels and electroblotted onto a 0.2 μ m polyvinylidene fluoride membrane. After transfer, the peptides were cross-linked to the membrane with 0.5% (v/v) glutaraldehyde in phosphate-buffered saline, washed with 50 mM glycine to stop the cross-linking reaction and finally washed with Tris-buffered saline (TBS). After blocking with 5% non-fat milk in TBS-Tween for 1 h at room temperature, the membranes were sequentially incubated with affinity purified rabbit anti-human ghrelin serum (diluted 1:15 000 in 1% milk/TBS-Tween) overnight at 4°C and horse radish peroxidase-conjugated anti-rabbit IgG (diluted 1:10 000 in 5% milk/TBS-Tween) for 2 h at room temperature. Immuno-reactive bands were visualized with SuperSignal West Pico detection reagents (Pierce) followed by autoradiography.

Membrane binding assay—Binding of ghrelin peptides to lipid vesicles was measured via a centrifugation assay using sucrose-loaded vesicles [41,37,21]. Large unilamellar lipid vesicles were prepared by vortexing lipid mixtures after several freeze-thaw cycles in 170 mM sucrose, 20 mM MOPS, pH 7.4 to prepare multilamellar vesicles. These were extruded through 0.1 μ m polycarbonate filters, diluted in sucrose-free buffer and pelleted at 100 000 $\times$ g as described by Rebecchi et al., [41] and modified by Mosior and Epand [37] and Giorgione and Newton [21]. Binding experiments were performed in 1.5 ml polypropylene tubes to minimize loss of ghrelin. The various ghrelin peptides (15 pmol) were incubated for 15 minutes with 200 μ M sucrose-loaded DOPC:DOPS vesicles containing 0, 10, 30, 60 or 90 mol % DOPS in 20 mM MOPS (pH 7.4), followed by centrifugation at 16 000 $\times$ g for 145 min. at 20°C. Based on recovery of ^{14}C in ^{14}C -spiked vesicles, approximately 70–80 % of the vesicles were pelleted after this centrifugation. The vesicle-containing pellets were washed with 500 μ l of 20 mM MOPS (pH 7.4) to remove traces of unbound ghrelin and re-centrifuged as above. The supernatant was carefully removed and the vesicles were resuspended in Na-dodecyl-sulfate sample buffer. Half of each sample was loaded onto a 15% Tris-Tricine gel, and separated peptides were transferred to polyvinylidene membrane and subjected to Western blot analysis

as described above. Vesicle-bound peptides were quantified by densitometric analysis (ImageQuant 5.2) of the autoradiographs as % of the amount of peptide that was initially added to the vesicles in the binding reaction.

Purification by C18 reverse phase chromatography—Purification of phosphoghrelin species was via high performance liquid chromatography using a Polaris C-18-ether reverse phase column (Varian, Inc.) specifically designed for improved resolution of phosphorylated peptides.

Assay of ghrelins—Ghrelin and desacylghrelin were assayed via in-house sandwich immunoassays specific for the acylated and desacylated full-length peptides as described [34].

Mass spectrometry of phosphoghrelin fragments—Identification of the sites of ghrelin phosphorylation was performed in the University of VA W.M. Keck Biomedical Mass Spectrometry Lab. The appropriate fractions of phosphorylated ghrelin and desacylghrelin purified via C18 HPLC were dried, resuspended in 100 mM ammonium bicarbonate, pH 8.0 and digested with 0.1 µg of Arg-C peptidase for 12 h at room temperature. The samples were acidified with acetic acid and ~1% was analyzed on a Finnigan LTQ ion trap mass spectrometer system with a Protana nanospray ion source interfaced to a self-packed 8 cm × 75 µm id Phenomenex Jupiter 10 µm C18 reversed-phase capillary column.

Volumes (0.5 – 5 µl) of the digest were injected and the peptides were eluted from the column with an acetonitrile/0.1 M acetic acid gradient at a flow rate of 0.25 µl/min. The nanospray ion source was operated at 2.8 kV. The digests were analyzed using the double play capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in sequential scans. This mode of analysis produces approximately 400 collisionally activated dissociation spectra of ions ranging in abundance over several orders of magnitude. Not all spectra were derived from peptides. The data were analyzed manually against the sequence.

Synthesis of ghrelin analogs—Ghrelin peptides were synthesized using a 9-fluorenylmethoxycarbonyl strategy on tentagel resin (0.27 mmol/g) with a rink linker, 2-(1H-benzotriazol-1-yl) – 1,1,3,3-tetramethyluroniumhexafluorophosphate, and N,N-diisopropylethylamine in dimethylformamide. If a double coupling was needed, N, N'-diisocarbodiimide and 1-hydroxybenzotriazole were used. Ninhydrin and chloranil tests were used to monitor the presence of primary and secondary amines. Phosphorylation was introduced using pre-phosphorylated serine: 9-fluorenylmethoxycarbonyl-Ser(PO(OBzl)OH)-OH with 2-(1H-benzotriazol-1-yl) – 1,1,3,3-tetramethyluroniumhexafluorophosphate, 1-Hydroxybenzotriazole and N,N-diisopropylethylamine as activating agents. O-octanoylated serine 3 was synthesized by using 9-fluorenylmethoxycarbonyl-Ser-OH at position 3. Following the coupling of the last amino acid, O-Octanoylation was carried out using n-octanoyl chloride and N,N-diisopropylethylamine in dichloromethane overnight. Peptides were cleaved from the resin with Reagent K (trifluoroacetic acid 82.5%, H₂O 5%, phenol 5%, thioanisole 5%, ethanedithiol 2.5%). The cleavage solutions were filtered, concentrated under pressure, and precipitated in cold ether. The resulting precipitates were dissolved in water and lyophilized. The product peptides were purified via HPLC and verified by mass spectrometry.

Circular Dichroism—CD spectra were acquired for ghrelin, DA-ghrelin, and the various S-18 analogs (10 or 15 µM) in 20 mM Tris buffer, pH 7.4 or in trifluoroethanol (TFE) in an AVIV spectropolarimeter model 215 using a 2 mm cell at 4 °C. Settings used were: band width 1.0 nm, wavelength range from 190 nm to 260 nm, scan speed 2–20 nm/min, step resolution

1.0 nm. Three spectra per condition were averaged after subtraction of an average background scan. The spectra were deconvoluted with the CDNN computer algorithm [10].

3. RESULTS

The C-terminal 2/3 of ghrelin is highly conserved over evolution [28, reviewed in 47,11] with multiple basic residues and, in most mammals, a Ser at position 18 that fits a PKC phosphorylation motif. As shown in Fig. 1, ghrelin serves as an excellent *in vitro* substrate for the widely expressed calcium-dependent PKCs α and β as well as for the ubiquitous calcium-independent PKC δ (Fig. 1). Similar results were observed for desacylghrelin (data not shown). Most of the phosphorylation is lipid-dependent. PKC α exhibits some lipid-independent activity with peptide substrates and the PKC δ preparation contained some lipid-independent catalytic fragment; but in both cases, the lipid-dependent phosphorylation is much faster.

Purification of the phosphorylated ghrelin and desacylghrelin through C18 reverse phase columns revealed co-elution with the respective unphosphorylated forms (data not shown). The site of phosphorylation was determined by digesting the peptides with Arg C peptidase and subjecting the fragments to tandem electrospray mass spectrometry. As shown in Table 1, the C-terminal fragment containing Ser-18 as the only potential phosphorylation site is 85 % phosphorylated in phospho-ghrelin and 90 % phosphorylated in phospho-desacylghrelin. Only 5–6 % phosphate incorporation was noted at Ser-2 (phospho-ghrelin) or Ser-3 (phospho-desacylghrelin).

The pattern of basic amino acids in ghrelin at approximately every 4 residues is suggestive of helix formation with the charged residues on one face. Submission of the sequence to a secondary structure prediction program using a type-2 (minimal assumptions) Discrete State-space Model library [44] returned a prediction of 20–60 % probability of helix between Pro-7 and Pro-21. Although nuclear magnetic resonance analysis in aqueous solution at low pH revealed only random coil behavior [17], we examined the secondary structure of ghrelin and desacylghrelin both in 20 mM Tris buffer, pH 7.4 and in 100 % TFE, a helix stabilizing solvent, by CD. As shown in Table 2, acylated and unacylated forms exhibited about 12 % helical behavior in aqueous solution. The helicity is increased in the presence of TFE to ~ 23 % for ghrelin and to 49 % for desacyl-ghrelin. As shown in Fig. 2A, the % helical character of desacylghrelin increases from 12 to 47 % over TFE concentrations from 5 – 80 %.

In consideration of the possibility that phosphorylation at Ser-18 might alter the helicity of the peptides, we synthesized Ser-18-Ala, Ser-18-Asp, and phospho-Ser-18 analogs of both ghrelin and desacyl-ghrelin and compared their secondary structure via CD. As shown in Fig. 3, the helical content of the ghrelin analogs in 100 % TFE was about 25–28 % and did not vary significantly as a function of alterations at Ser-18. Ser-18-Asp substitution in desacylghrelin still showed about 50 % helical character, like the native desacyl peptide. Ser-18-Ala substitution disrupted the helix and so did phosphorylation at Ser-18.

In consideration of the possibility that an amphipathic helix might contribute to binding of ghrelin to acidic lipids, we examined binding to sucrose-loaded vesicles composed of increasing molar percentages of DOPS in a DOPC background. As shown in Fig. 4, both ghrelin and desacylghrelin bind to PC:PS sucrose-loaded vesicles in a PS-dependent manner. Ghrelin exhibits some binding to PC (20 %) and requires less PS (10 % vs. 30 %) for maximal binding than does desacylghrelin; but maximal binding of desacylghrelin is greater (~85 %) than that of ghrelin (~60 %) at optimal PS. Phosphoghrelin and phosphodesacylghrelin show greatly diminished PS-dependent binding.

4. DISCUSSION

Orexigenic, pituitary, and anti-inflammatory effects of ghrelin have been shown to depend on Ser-3 acylation and interaction with the GHSR-1a ghrelin receptor [28,16,47,11,6,45]. However, both ghrelin and desacylghrelin exhibit antiapoptotic and other effects independent of this receptor [reviewed in 47,11]. These observations, coupled with the strong evolutionary conservation of the entire ghrelin sequence, suggest that other parts of the peptide are critical for some functions. The C-terminal 2/3 of ghrelin is highly basic and contains a potential PKC phosphorylation site at Ser-18. Here we show that both ghrelin and desacylghrelin can be phosphorylated at this site *in vitro* (Fig. 1, Table 1) and that this phosphorylation has significant structural and membrane binding consequences.

The pattern of basic residues in the C-terminal portion of ghrelin suggested possible formation of an α -helix. Earlier NMR and CD studies revealed only random coil behavior in solution [17] at pH 1.0–1.5. However, we observed 12–13 % helical content of ghrelin and desacylghrelin in neutral aqueous solution via CD (Table 2). Our results are consistent with a molecular dynamics study by Beevers and Kukol showing a short stretch of α -helix from His-9 to Gln-13 after 2 ns of simulation and a longer stretch involving residues Pro-7 to Glu-13 plus a loop from Ser-18 - Lys-20 after 10 sec of simulation [7]. The helical character can be enhanced in the helix-stabilizing solvent TFE to 24 % for ghrelin and to ~ 50 % for desacylghrelin (Table 2) and the increase is a function of the concentration of TFE (Fig. 2).

Phosphorylation of ghrelin does not change significantly the helical character of ghrelin as measured via CD in 100 % TFE, nor does Ser-18-Ala or Ser-18-Asp substitution (Fig. 3). However phosphorylation of Ser-18 or Ser-18-Ala substitution decreases the helicity of desacylghrelin to that observed for acylated ghrelin. Thus it seems that a negative charge or free hydroxyl group at amino acid 18 is required for stabilization of a longer stretch of helix. Apparently the phosphate group is able to disrupt the helix. Somehow, acylation at the other end of the molecule also disrupts the longer helix. Beevers and Kukol observed a significant loop in ghrelin involving Ser-18 and Lys-20 in molecular dynamics simulation of ghrelin [7]. It would be most interesting to learn whether desacylghrelin also exhibits this bend in molecular dynamics simulations. Our data suggest that this region may be incorporated into a longer helical extension in the unacylated and unphosphorylated peptide. Consistent with our CD evidence that acylation affects ghrelin structure, we (data not shown) and others [15] find that desacylghrelin is recognized with greater affinity by antisera specific for the common C-terminal end of the peptides (far from the acylation site). These results are compatible with the possibility that ghrelin, but not desacylghrelin, assumes a folded structure that interferes with antibody interaction.

Phosphorylation at Ser-18 or acylation at Ser-3 causes a large decrease in the helical character of the basic desacylghrelin peptide. We observed a similar change in helical character of a PKC substrate peptide derived from the PKC phosphorylation site in neuromodulin [48]. The neuromodulin peptide is helical in TFE and in the presence of acidic PS lipid vesicles but not in aqueous buffer or in the presence of short chain zwitterionic PC micelles [48], which can substitute for PS in activating PKC [50]. Thus, membrane binding may convert a poor PKC substrate into a good one by virtue of stabilizing a helical conformation. Modeling suggested that the PKC active site could accommodate the helical neuromodulin substrate [48].

Ghrelin may provide a second example of a helical PKC substrate. As shown in Fig. 4, ghrelin and desacylghrelin also bind to lipid vesicles in a PS-dependent manner. The greater binding of the more helical desacylghrelin with respect to ghrelin is consistent with binding of desacylghrelin via the basic surface of the amphipathic helix to the acidic lipid surface. This hypothesis is supported by the observation that phosphorylation of desacylghrelin, which

greatly diminishes its helical character, eliminates its membrane binding. Phosphoghrelin does not show any PS-dependent membrane binding either; however it does show some binding to PC. This observation suggests that with the helix disrupted by Ser-18-phosphorylation, any membrane binding depends on the Ser-3-acylation and proceeds by a charge-independent mechanism. Phosphorylation of the myristoylated Ala-rich C-kinase substrate similarly decreases its binding to acidic lipid vesicles [27]. Beevers and Kukol modeled binding of their energy-minimized ghrelin structure to PC vesicles and the modeling suggested binding via the Ser-18-Lys-20 loop region of the molecule rather than via the acyl chain [7]. That observation serves to emphasize the potential importance of the Ser-18 PKC site in ghrelin; but the lack of involvement of the acyl chain in membrane binding in the molecular dynamics model is counter-intuitive. It seems to us more likely that the acyl chain inserts into the membrane rather than into the aqueous phase – or perhaps is involved in the folding of the molecule to form the loop involving Ser-18. In the latter case, it is conceivable that phosphorylation at Ser-18 might free the acyl chain and alter the binding to PC.

In activated, ghrelin-producing lymphocytes, the ghrelin is enriched in PS-rich lipid rafts [16], which have been shown to concentrate signaling components including PKC [e.g., 8]. The faster phosphorylation of ghrelin and desacylghrelin with lipid-dependent PKC vs. lipid-independent PKC or catalytic fragment (Fig. 1) is consistent with binding of both to the same PS-enriched regions to generate a reduction in dimensionality as with other basic-rich PKC substrates [e.g. 27]. McCabe and Berthiaume have reported that acylation is not sufficient for selective targeting of proteins to lipid rafts [36]. Raft binding often requires electrostatic interactions between the PS and basic amino acids. If ghrelins already are bound to PS when they encounter PKC, it is likely that PKC phosphorylates them in their helical forms. In that case, phosphorylation, especially of desacylghrelin, would serve to disrupt ghrelin structure and membrane binding and alter the subcellular localization of the peptide.

Although perhaps less likely, the possibility exists that ghrelin may encounter PKC after uptake into cells via ghrelin receptors [23]. Although this would not be a mechanism for internalization of desacylghrelin, other basic amphipathic peptides can be taken up by cells through less well characterized mechanisms [33,9] that also might accommodate desacylghrelin. Finally, one report shows that active PKC can become externalized in intestinal cells undergoing apoptosis induced by enteropathogenic *E. coli* and that its action might abort the apoptotic process [12]. Thus it is conceivable that extracellular phosphorylation of ghrelin also could occur.

5. CONCLUSIONS

In summary, we have observed phosphorylation of ghrelin and desacylghrelin at Ser-18 by the widely expressed PKCs α , β , and δ . PKC isozymes and both acyl- and desacylghrelins bind to membranes rich in acidic lipids. Indeed, both have been identified in ghrelin-producing lymphocytes in association with PS-rich lipid rafts [16,8]. Phosphorylation decreases both the helicity of desacylghrelin and its binding to acidic lipids. The cellular role of phosphoghrelin remains to be determined; however the finding that ghrelin structure and membrane binding can be altered by phosphorylation suggests a new level at which this critical peptide may be regulated. Disease-associated alterations in PKC isozymes in ghrelin-producing or ghrelin-responsive cells – such as the PKC α mutation in some pituitary tumors [1], the decreased PKC δ associated with some cancers [e.g., 13], the increased PKC δ associated with loss of intestinal epithelial barrier function [4] – may impact ghrelin function as well.

Acknowledgements

We thank Dr. Nick Sherman of the University of Virginia Biomolecular Research Facility for the ghrelin phosphopeptide analysis via mass spectrometry, Dr. David Cafiso for advice on deconvolution of CD spectra, and Tamara Stoops and Vanessa King-Mangard for technical assistance at the early stages of this work. This work was

supported by HHS grant GM31184 (to JJS.) and by a pilot feasibility grant from the University of Virginia Silvio O. Conte Digestive Disease Research Center.

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Fig. 1. Phosphorylation of ghrelin by PKC isozymes

Ghrelin was phosphorylated as described in the Methods by calcium-dependent PKCs α (●) or β (▲) or by calcium-independent PKC δ (■) both with (+PL, solid symbols) and without (–PL, open symbols) PS and DO. Symbols represent means of two determinations that varied by less than 10 %. A representative of at least three independent experiments is shown.

Fig. 2. Effect of TFE concentration on desacylghrelin structure

Desacylghrelin (15 μ M in 2 ml samples) was dissolved in the indicated concentrations of TFE and CD spectra were obtained and deconvoluted as described in Methods. The % helical content is plotted as a function of % TFE.

Fig. 3. Helical character of ghrelin and desacylghrelin analogs in TFE

CD spectra were collected as described in Methods on synthetic analogues of ghrelin or desacylghrelin that varied at amino acid residue 18: S, Ser; Ph-S, phospho-Ser; A, Ala; D, Asp. Spectra were deconvoluted and the % helical content was averaged for 2–4 independent preparations depending on the analog except for the Ser-18-Asp samples, which were made up only once due to limiting quantity.

Fig. 4. Effect of phosphorylation on binding of ghrelin (A, B) or desacylghrelin (A, C) to sucrose-loaded vesicles

Binding of ghrelin, desacylghrelin and their phospho-derivatives to sucrose-loaded DOPC:DOPS vesicles as a function of mol % PS in the lipid was determined by pelleting the vesicles and assessing bound ghrelins via Western blotting as described in the Methods. (A), Western blots of indicated samples; (B), quantification by densitometry of ghrelin ($\circ$) and phosphoghrelin ($\bullet$) binding as a percentage of input (in); (C) quantification of desacylghrelin (Δ) or phosphodesacylghrelin ($\blacktriangle$) binding as a percentage of input. Representative data from one of four independent experiments are shown.

Table 1Identification of phosphorylated residues by mass spectrometry^a

Ghrelin fragments:	GSS(ocatnoyl)FLSPEHQR	VQQR	KESKKPPAKLQPR
% phosphorylation	6 % (<i>Ser-2</i>)	0 %	85 % (<i>Ser-18</i>)
Desacylghrelin fragments:	GSSFLSPEHQR	VQQR	KESKKPPAKLQPR
% phosphorylation	1 % (<i>Ser-2</i>) + 5 % (<i>Ser-3</i>)	0 %	90 % (<i>Ser-18</i>)

^a Ghrelin and desacylghrelin were phosphorylated by PKC β II as illustrated in Fig. 1, separated from reaction components by HPLC over C18 columns, and subjected to hydrolysis with Arg C peptidase. The cleavage sites are represented by spaces between the peptide fragments. Analysis of each peptide fragment for % phosphorylation was determined by mass spectrometry as described in the Methods.

Table 2

Circular dichroism of ghrelin and desacylghrelin in buffer and TFE

	(n)	Helix	% Secondary Structure in different solutions (195–260 nm) ^a			β-turn	random
			antiparallel	parallel			
Ghrelin							
in Tris	(2)	12	28	11	17	32	
in TFE	(2)	23	16	10	18	33	
Desacylghrelin							
in Tris	(3)	12	30	11	18	28	
in TFE	(2)	48	8	6	17	21	

^a CD spectra were collected in 20 mM Tris buffer, pH 7.4 or in 100 % TFE as indicated and deconvoluted as described in Methods. Standard errors, omitted for clarity, did not differ by more than 2 % in the replicate experiments.