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Structure–Activity Analysis of Human Ghrelin *O*-Acyltransferase Reveals Chemical Determinants of Ghrelin Selectivity and Acyl Group Recognition

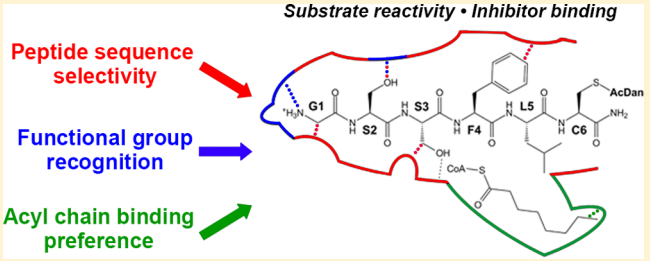
Joseph E. Darling,[†] Feifei Zhao,[‡] Rosemary J. Loftus,[†] Leslie M. Patton,[†] Richard A. Gibbs,[‡] and James L. Hougland*,[†]

[†]Department of Chemistry, Syracuse University, Syracuse, New York 13244, United States

[‡]Department of Medicinal Chemistry and Molecular Pharmacology, College of Pharmacy, Purdue University, West Lafayette, Indiana 47907, United States

Supporting Information

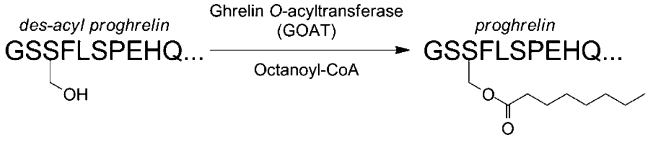
ABSTRACT: Ghrelin *O*-acyltransferase (GOAT) is an integral membrane acyltransferase responsible for catalyzing a serine-octanoylation posttranslational modification within the peptide hormone ghrelin. Ghrelin requires this octanoylation for its biological activity in stimulating appetite and in regulating other physiological pathways involved in energy balance. Blocking ghrelin acylation using GOAT inhibitors is a new potential avenue to treat health conditions impacted by ghrelin signaling, such as obesity and diabetes. Designing novel and potent GOAT inhibitors as potential therapeutics requires insight into the interactions between the ghrelin and octanoyl coenzyme A substrates and the GOAT active site. Through structure–activity investigation of ghrelin-mimetic peptide substrates and inhibitors, we have analyzed the amino acid selectivity of the enzyme as well as the functional groups involved in substrate recognition by human GOAT (hGOAT). This analysis reveals that hGOAT both prefers and tolerates a distinct set of chemical properties at each position within the N-terminal sequence of ghrelin and that sequence elements downstream of the ghrelin N-terminal sequence contribute to ghrelin binding to hGOAT. We also found that the hGOAT active site exhibits a marked preference for binding an eight-carbon acyl chain, which potentially explains the biological observation of ghrelin octanoylation in light of the acyl donor promiscuity reported for GOAT. Bioinformatics analysis, guided by our reactivity data, supports the conclusion that ghrelin is a unique substrate for hGOAT within the human proteome, providing further justification for the ghrelin–hGOAT system as a desirable drug target. By defining an array of substrate–enzyme interactions used by hGOAT to bind, recognize, and acylate ghrelin, this study yields novel insight into the character of the hGOAT active site that can serve as a guide toward the rational design of hGOAT inhibitors.



Ghrelin is a 28-residue peptide hormone that serves multiple signaling functions in humans.[1,2] Initially demonstrated to stimulate appetite, ghrelin plays a role in multiple pathways integral to energy balance, including glucose metabolism, insulin secretion and sensitivity, adaptation to starvation, and adiposity.[3−8] Elevated ghrelin levels are observed in several disorders in which appetite disregulation is exhibited, such as anorexia nervosa and Prader-Willi syndrome.[9−11] In addition, ghrelin affects neurological processes such as learning, memory, and depression, with a recent study linking ghrelin to post-traumatic stress disorder.[12−14] Ghrelin may also serve a neuroprotective role in traumatic brain injury, Parkinson's, and Alzheimer's diseases.[15−17]

Ghrelin requires multiple covalent modifications for biological activity, making ghrelin-dependent pathways attractive targets for drug development.[18] Following its expression as a 117-amino acid precursor (preproghrelin), ghrelin undergoes several maturation steps prior to secretion. One key maturation step involves the esterification of a specific serine near the N terminus of proghrelin by an octanoyl (eight-carbon) fatty acid (Scheme 1). Subsequent proteolytic processing of acyl proghrelin by a prohormone convertase yields mature ghrelin.[19,20] Although both ghrelin and des-acyl ghrelin are present in circulation, only the acylated form of ghrelin (hereafter referred to as ghrelin) can bind its cognate receptor (GHSR-1a) to activate signaling.[1] Because ghrelin is the only protein known to undergo serine

Scheme 1. Ghrelin Octanoylation by Ghrelin *O*-Acyltransferase (GOAT)



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octanoylation,[1,21] the unique and essential nature of ghrelin octanoylation makes this modification an ideal target for controlling ghrelin activity.

The enzyme that catalyzes ghrelin acylation, ghrelin *O*-acyltransferase (GOAT), was identified in 2008.[21,22] GOAT is a member of the membrane-bound *O*-acyltransferase (MBOAT) family of integral membrane acyltransferases.[23] GOAT is one of three MBOAT family members known to acylate protein substrates; the other two are hedgehog acyltransferase (Hhat) and porcupine (PORCN).[24] All three of these acyltransferases are of interest as potential drug targets, but their nature as integral membrane proteins has complicated studies aimed at the structural characterization and identification of active-site residues. Site-directed mutagenesis of conserved residues and activity analysis of truncated enzyme constructs have identified functionally essential amino acids within GOAT, Hhat, and PORCN, suggesting that the enzyme active site lies in the C-terminal region of each of these enzymes.[21−23,25−29] Complementing these functional studies, a recent study of GOAT topology by Taylor and co-workers identified 11 transmembrane regions and 1 re-entrant loop connected by 11 exposed-loop regions.[29] The predicted topology for GOAT is similar to that described for other MBOAT family members that modify small molecules, such as ACAT1 and Gup1.[30,31] The GOAT topology model maps the two putative-active-site residues within GOAT (N307 and H338) to opposite sides of the ER membrane, raising doubt as to whether both of these essential residues can participate in the GOAT active site. Although these studies have placed the potential active site in the C-terminal region of GOAT, the location and architecture of the enzyme active site and substrate-binding sites remain unknown. The lack of information with regard to how GOAT binds its substrates and catalyzes ghrelin acylation has hampered the mechanistic investigation of GOAT and has left ambiguity regarding the potential for GOAT substrates beyond ghrelin.

Targeted GOAT inhibitors have been proposed as potential treatments for a number of diseases, including obesity, diabetes, and Prader-Willi syndrome.[8,32,33] Several groups have reported GOAT inhibitors based on mimics of ghrelin or acylated ghrelin, screening of small-molecule libraries, or bisubstrate analogs.[28,34−36] Treatment with the peptide-based bisubstrate inhibitor GO-CoA-Tat reduced levels of acylated ghrelin in both cultured mammalian cells and mice, and treatment of mice with GO-CoA-Tat yielded increased glucose tolerance and reduced weight gain.[35] The success of GO-CoA-Tat at modulating ghrelin-dependent physiological processes validates GOAT inhibitors as potential therapeutics. Garner and Janda reported a set of nonpeptidomimetic GOAT inhibitors as potential leads to a new class of agents targeting ghrelin acylation.[36] However, designing novel GOAT inhibitors with increased activity and bioavailability is complicated by the lack of information regarding the number and nature of the interactions between GOAT and its substrates within the active site.

Creating efficient GOAT inhibitors requires an understanding of how GOAT recognizes ghrelin and catalyzes its acylation. Functional studies allow us to identify essential interactions between ghrelin and hGOAT for targeting by inhibitors. We previously reported the development of a fluorescent peptide-substrate mimic of ghrelin and validated its reactivity with the human orthologue of GOAT (hGOAT).[37] This substrate permits the synthesis and screening of peptides incorporating substitutions and chemical modifications within the essential N-terminal GSSF sequence of ghrelin that is required for octanoylation by GOAT.[28,38] In this work, we utilize structure−activity analysis to probe the tolerance of hGOAT for alterations of amino acids and functional groups within the N-terminal sequence of ghrelin. We also determine the optimal length of the acyl chain for binding in the hGOAT active site, as a guide for designing lipid-mimic groups in potential hGOAT inhibitors. By defining selectivity with regard to substrate sequence and chemical properties recognized by hGOAT, we can assess the potential for hGOAT substrates beyond ghrelin within the human proteome. Our analysis also offers insight into substrate-binding interactions within the hGOAT active site as a potential guide for designing novel hGOAT inhibitors.

MATERIALS AND METHODS

General Methods. Data plotting and curve fitting were carried out with Kaleidagraph (Synergy Software, Reading, PA, USA). Octanoyl coenzyme A (octanoyl-CoA) and palmitoyl coenzyme A (palmitoyl-CoA) were solubilized to 5 mM in 10 mM Tris-HCl (pH 7.0), aliquoted into low-adhesion microcentrifuge tubes, and stored at −80 °C. Acrylodan (Anaspec) was solubilized in acetonitrile, with the stock concentration determined by absorbance at 393 nm after dilution into methanol (ε = 18 483 M^{-1} cm^{-1}, per the manufacturer's data sheet). Unlabeled des-acyl ghrelin peptides (5-mer, 10-mer, 18-mer, 28-mer) were purchased from Peptides International (>97% purity); the 5-mer, 10-mer, and 18-mer des-acyl ghrelin peptides are all C-terminal amidated. Peptides for fluorescent labeling with acrylodan were synthesized by Sigma-Genosys (The Woodlands, TX, USA) in the Pepscreen format or were synthesized in-house with amidated C- termini. For the synthesis of peptides with modifications of the glycine amino group, the appropriate acid (methoxyacetic acid, *N*,*N*-dimethylglycine, or betaine) was substituted for Fmoc-glycine in the last coupling step of amino acid synthesis. The 28-mer ghrelin-mimetic peptides with substitutions at the S2, S3, and F4 position were synthesized by Pepmic (Suzhou, China) and purified to >90% purity by HPLC, with peptide mass verified by mass spectrometry. Peptides were solubilized in either absolute ethanol or a 1:1 mixture of acetonitrile/H_2O and stored at −80 °C. Peptide concentrations were determined spectrophotometrically at 412 nm by reaction of the cysteine thiol with 5,5′-dithiobis(2-nitrobenzoic acid), using ε_{412} = 14 150 M^{-1} cm^{-1}.[39]

Expression and Enrichment of hGOAT. hGOAT was expressed in insect cells (Sf9) and enriched in membrane fractions, using a previously published procedure.[37]

Peptide Substrate Fluorescent Labeling. Peptide substrates were labeled with acrylodan on a cysteine thiol and purified via HPLC as previously reported.[37] Peptide labeling with acrylodan was verified by MALDI−TOF mass spectrometry; calculated and observed masses for all substrates are reported in Table S1. Acrylodanylated peptides were solubilized in a 1:1 mixture of acetonitrile/H_2O and stored at −80 °C, with peptide concentrations determined by UV absorbance at 360 nm of the cysteine-conjugated acrylodan group in aqueous solution (ε_{360} = 13 300 M^{-1} cm^{-1}).[37,40]

Synthesis of Acylated Inhibitors. Acylated hGOAT inhibitors were synthesized by using standard solid-phase peptide-synthesis protocols with Rink resin to generate C-terminal amidated peptides. Side-chain acylation of the GS-(Dap)FL peptide was carried out using an adaptation of a published approach that employed an on-resin orthogonal deprotection of the Alloc-Dap residue side chain.[41] Inhibitors were purified by reverse-phase HPLC and verified by MALDI

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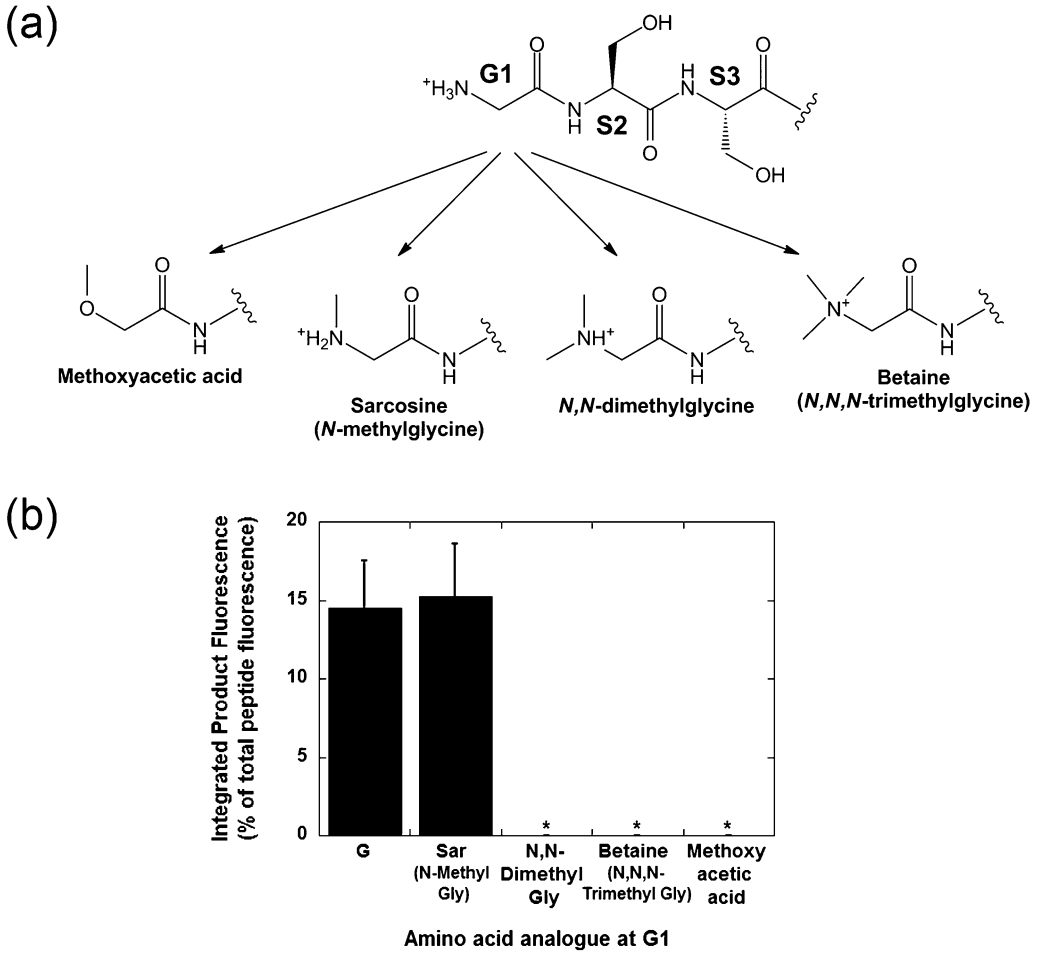

Figure 1. Recognition of the N-terminal amine of ghrelin by hGOAT. (a) Modifications of the N-terminal amine to introduce methyl groups or replace the nitrogen atom with oxygen. (b) Product formation, reported as percent of total peptide fluorescence, for the $GSSFLC_{AcDan}$ peptide (G) compared to peptides with modified N-terminal amino acids. An asterisk (*) indicates reactivity that was below the fluorescence detection limit for our assay (~0.05 AU).

mass spectrometry. Details of synthesis procedures and inhibitor characterization are contained in the Supporting Information.

hGOAT Activity Assays and Analysis. Membrane fractions from Sf9 cells expressing hGOAT were thawed on ice and passed through an 18 gauge needle 10 times. Unless noted otherwise, assays were carried out in a total volume of 50 μL with 10−50 μg of membrane protein, 1.5 μM acrylodanylated peptide substrate, 500 μM octanoyl-CoA, and 50 mM HEPES (pH 7.0). Assays were initiated by the addition of the acrylodanylated peptide substrate. Assays comparing the reactivity of peptide substrates containing sequence substitutions or chemical modifications to the $GSSFLC_{AcDan}$ substrate were carried out in parallel and used the same stock of membrane protein and other reaction components. Assays were incubated at room temperature and stopped by the addition of 50 μL of 20% acetic acid in isopropanol. Unless noted otherwise, reactions were carried out for 1 h. Assays were analyzed by reverse-phase HPLC with fluorescence detection.[37] Chromatogram analysis and integration of the peptide substrate and product peaks were carried out using Chemstation for LC (Agilent Technologies), with quantitation reported in Table S2; product formation is shown as percent of total peptide fluorescence (i.e., total fluorescence = the sum of substrate and product fluorescence). Data reported are the average of three independent determinations, with error bars representing one standard deviation. All peptide octanoylation reactions exhibited linear increases in product formation over the 1 h time course used under the standard assay conditions.

For the measurements of peptide K_m values, assays were carried out using a range of peptide substrate concentrations (0−5 μM). Following HPLC analysis, the integrated product peak was plotted versus the initial peptide substrate concentration and fit to eq 1, represented as

$$\text{product fluorescence} = (\text{maximum product fluorescence})\left(\frac{[\text{substrate}]}{[\text{substrate}] + K_m}\right) \quad (1)$$

For the majority of peptide substrates, the integrated product fluorescence did not become saturated over the experimentally accessible substrate range, leading to a K_m value >5 μM.

Determination of IC_{50} Values for Acylated Inhibitors. For inhibition experiments, the inhibitor or solvent/carrier control (3 μL) was added to the reaction mixture described above before the reaction was initiated by the addition of the acrylodanylated peptide substrate. Reactions were stopped after 1 h and analyzed as described, with percent activity at each inhibitor concentration calculated from HPLC data using eqs 2 and 3, represented as

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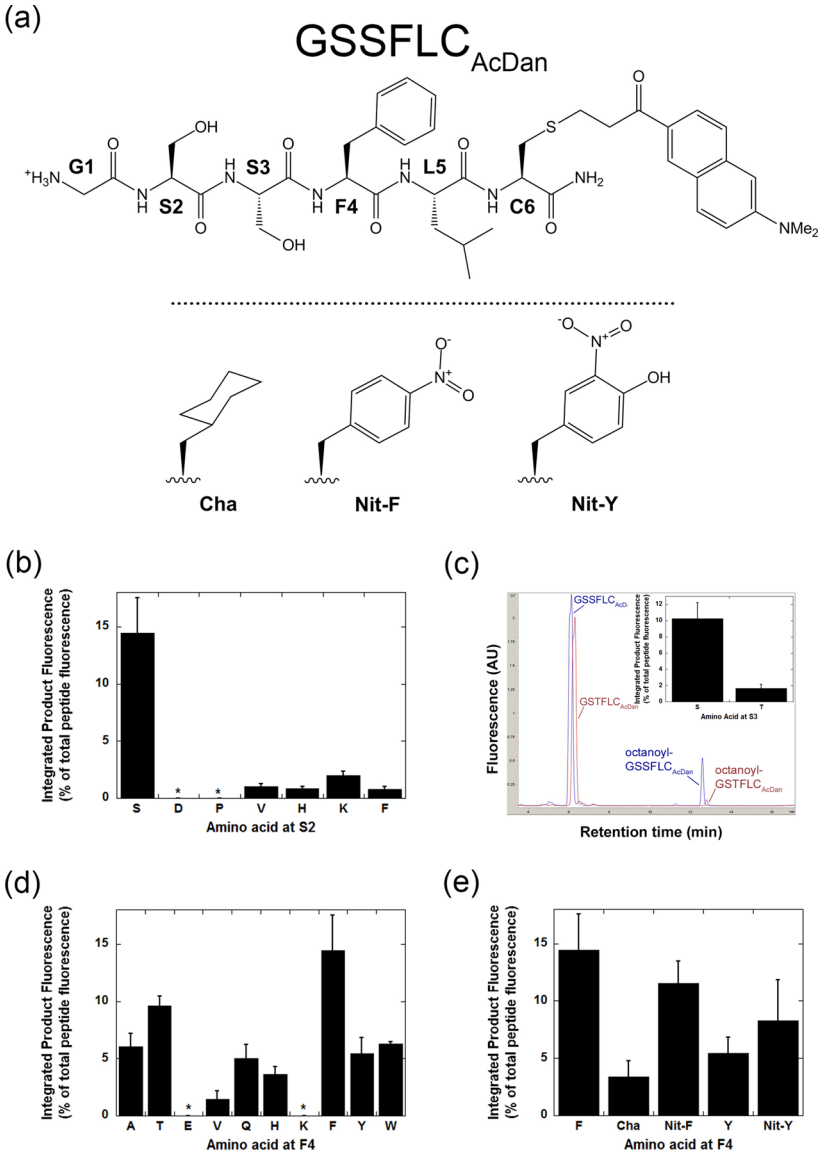

Figure 2. Sequence selectivity of hGOAT within the N-terminal sequence of ghrelin. (a) Model of the $GSSFLC_{AcDan}$ substrate, indicating the S2, S3, and F4 positions. Structures of the unnatural amino acids cyclohexylalanine (Cha), nitrophenylalanine (Nit-F), and nitrotyrosine (Nit-Y) used in studies at the F4 position are shown below the dotted line. (b) Product formation for peptide substrates with amino acid substitutions at the S2 position. (c) HPLC chromatograms of octanoylation reactions with $GSSFLC_{AcDan}$ (S3, blue) and $GSTFLC_{AcDan}$ (T3, red) substrates. Peaks for the substrates and the octanoylated peptide products are labeled. Inset shows product formation for S3 and T3 peptide substrates. (d) and (e) Product formation for peptide substrates with amino acid substitutions at the F4 position, with natural amino acids shown in panel d and unnatural amino acids shown in panel e. An asterisk (*) indicates reactivity that was below the fluorescence detection limit for our assay (~0.05 AU).

$$\%\ \text{activity} = \frac{\%\ \text{peptide octanoylation in presence of inhibitor}}{\%\ \text{peptide octanoylation in absence of inhibitor}} \quad (2)$$

$$\%\ \text{peptide octanoylation} = \frac{\text{fluorescence of octanoylated peptide}}{\text{total peptide fluorescence (octanoylated and non-octanoylated)}} \quad (3)$$

To determine the IC_{50} value, the plot of percent activity versus inhibitor concentration was fit to eq 4, with "% $activity_0$" denoting hGOAT activity in the presence of the DMSO carrier alone.

$$\%\ \text{activity} = \%\ \text{activity}_0\left(1 - \frac{[\text{inhibitor}]}{[\text{inhibitor}] + IC_{50}}\right) \quad (4)$$

Determination of IC_{50} Values for Des-Acyl Ghrelin Peptides. Inhibition assays involving inhibitors based on des-acyl ghrelin were carried out as noted above but with the following modifications to the reaction conditions: An assay mixture of 50 μM palmitoyl-CoA, 1 μM methoxy arachidonyl fluorophosphonate (MAFP), 10–100 μg of membrane protein, and 50 mM HEPES (pH 7.0) was preincubated for 10 min, followed by the addition of 100 μM octanoyl-CoA and 0–160 μM des-acyl ghrelin peptide inhibitor. The assay mixture was incubated at room temperature for 1 h, at which time the reactions were initiated by the addition of 8.5 μM $GSSFLC_{AcDan}$ peptide substrate. Assays were carried out in a total volume of 50 μL. Reactions comparing peptide inhibitors of different lengths or 28-mer peptides with sequence substitutions to the wild-type 28-mer ghrelin peptide were carried out in parallel and used the

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same stock of membrane protein and other reaction components. Following 1 h of reaction time, assays were analyzed as described above to determine IC_{50} values for each peptide inhibitor.

RESULTS

hGOAT Recognition of the N-Terminal Glycine Amino Group. We previously demonstrated that the α amino group of the essential N-terminal glycine residue of ghrelin is required for substrate recognition by hGOAT.[37] Removing this amine completely abolished peptide octanoylation by hGOAT, whereas acetylation of it led to markedly reduced reactivity. Acetylation modifies multiple properties of the N-terminal amine, such as charge, steric bulk, and hydrogen bond donor/acceptor ability. Through the probing of additional modifications at this nitrogen, we can determine which properties are recognized by hGOAT. We examined the effect of methylating this amine by substituting sarcosine, *N*,*N*-dimethylglycine, and betaine at the N- terminus of the peptide substrate (Figure 1a). Each successive methyl group adds steric bulk and maintains the positive charge of the amine at neutral pH. The sarcosine-substituted substrate exhibits comparable reactivity to the parent substrate, indicating that the incorporation of a single methyl group at the N-terminal amine does not impede recognition by hGOAT (Figure 1b). In contrast, no octanoylation was observed with the *N*,*N*-dimethylglycine- and betaine-substituted substrates. To explore the possibility that these more highly substituted substrates form potent product inhibitors upon octanoylation, we carried out coincubation reactions of the *N*,*N*-dimethylglycine- and betaine-substituted substrates with the $GSSFLC_{AcDan}$ substrate; we observed no significant decrease in formation of octanoylated $GSSFLC_{AcDan}$ (Figure S1). Together, these data suggest that hGOAT tolerates a single methyl group at the N-terminal amine of ghrelin, whereas two or more methyl groups lead to a defect in substrate binding. Because *N*-methylation can provide protection against protease degradation,[42,43] the ability of hGOAT to accept a substrate with sarcosine at the N- terminus offers the potential to design hGOAT inhibitors with increased biostability.

Depending on its protonation state, the sarcosine methylamino group can serve as either a positively charged hydrogen bond donor or a neutral hydrogen bond donor and acceptor. To determine whether positive charge and/or hydrogen bond donation ability at the N-terminal amine is required for reactivity with hGOAT, we examined a peptide substrate with methoxyacetic acid substituted for the N-terminal glycine of the GSSF sequence (Figure 1a). Replacement of the nitrogen of the sarcosine methylamino group with an oxygen (as in the methoxyacetic acid substrate) leads to a complete loss of reactivity with hGOAT (Figure 1b). The loss of reactivity upon methoxy group incorporation suggests that hGOAT recognizes the N-terminal amine of ghrelin based on positive charge and/or hydrogen bond donation.

Probing hGOAT Selectivity at the S2 Position. Our previous study revealed that hGOAT exhibits moderate steric selectivity at the S2 position, with the presence of a side-chain hydroxyl group enhancing substrate reactivity.[37] To investigate the tolerance for side-chain polarity and charge at this position, we screened peptide substrates with aspartate, histidine, and lysine substitutions at S2. We also incorporated proline to probe the effect of a conformational restriction adjacent to the site of serine octanoylation. Aspartate incorporation at S2 results in a complete loss of peptide octanoylation by hGOAT (Figure 2b). The near-isosteric substitution of threonine at S2 has little effect on reactivity (amino acid volume = 111.1 and 116.1 $Å^3$ for aspartate and threonine, respectively),[37,44] leading us to attribute the loss of reactivity upon aspartate substitution to the presence of the negatively charged carboxylate side chain. Proline substitution also completely blocks peptide modification by hGOAT, indicating peptide flexibility may be required for ghrelin recognition by hGOAT. Substrates bearing histidine and lysine substitutions at S2 exhibit considerably reduced but still detectable octanoylation activity with hGOAT, but this loss of reactivity must be interpreted in the context of the known steric selectivity at this position. When compared to the reactivity of substrates with the nonpolar amino acids valine (140 $Å^3$) and phenylalanine (189.9 $Å^3$) at S2, which increase steric bulk without introducing charge or polarity, neither basic amino acid displays a further reduction in reactivity when compared to the reactivity of substrates with nonpolar amino acids of similar size at S2. This suggests that the presence of positive charge at S2 does not interfere with hGOAT substrate recognition, which is in contrast to the deleterious effect of negative charge at this position.

Our previous and current analyses indicate that hGOAT selects against large amino acids at the S2 position, prefers a hydroxyl group, and accepts positively charged residues. These data are consistent with a moderately sized S2 binding pocket that presents a group capable of accepting a hydrogen bond from the S2 side-chain hydroxyl of ghrelin. The reduced reactivity of substrates with S2 substitutions may result from weaker binding to hGOAT and/or a reduced rate of hGOAT-catalyzed octanoylation within the enzyme−substrate complex. With the exception of valine, all of the amino acid substitutions at the S2 position lead to an increase in the observed K_m value, as determined by substrate-titration experiments (Table S3 in Supporting Information). Therefore, the loss of reactivity observed with S2 substitutions likely arises from effects on both substrate binding and catalysis of peptide octanoylation by hGOAT.

hGOAT Tolerates Threonine at the Octanoylation Site with Reduced Activity. To probe the recognition of S3 by hGOAT, we determined the reactivity of a substrate with threonine at this position ($GSTFLC_{AcDan}$). There is precedent for threonine acylation by GOAT in the context of bullfrog ghrelin and cell-based analysis of mutated mouse proghrelin,[21,45] and Rios-Esteves and co-workers report that the MBOAT family member Porcupine can acylate threonine in place of serine in its Wnt substrate.[25] Threonine substitution at S3 results in reduced but detectable peptide octanoylation by hGOAT (Figure 2c). The loss of reactivity for the T3 substrate suggests that the hGOAT active site poorly tolerates methyl group incorporation at the β carbon of S3, which is consistent with a steric contact to the threonine/serine side chain.

hGOAT Selectivity at the F4 Position. GOAT selectivity at the fourth position of the ghrelin sequence has received limited study, with a single report that alanine substitution of this phenylalanine leads to a loss of substrate octanoylation in both in vitro and cell-based ghrelin octanoylation assays that use the mouse isoform of GOAT.[28] Consequently, we explored a range of amino acids at this position to determine the effects of altered amino acid size and polarity on recognition by hGOAT. Substitution of F4 with alanine yielded reduced reactivity (Figure 2d), which is similar to the effect observed upon alanine substitution at S2 in our previous study.[37] No clear trend is evident for steric selectivity at F4. The substrate bearing a threonine substitution at F4 exhibits the largest extent of product

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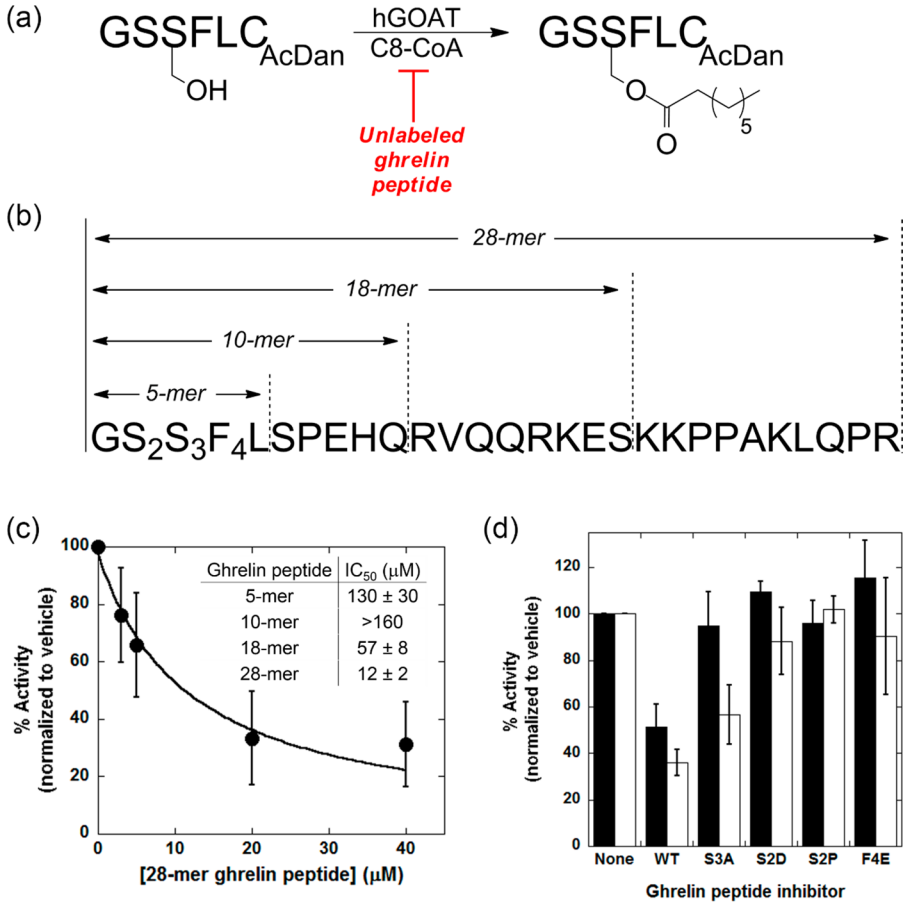

Figure 3. Length and sequence dependence of hGOAT inhibition by des-acyl ghrelin peptides. (a) Schematic hGOAT octanoylation reaction with inhibition by unlabeled des-acyl ghrelin peptides. (b) Sequence of human ghrelin with the lengths of the des-acyl ghrelin peptide used as inhibitors indicated. The S2, S3, and F4 positions are identified by subscript numbers. (c) Inhibition of hGOAT-catalyzed octanoylation of $GSSFLC_{AcDan}$ by the 28-mer des-acyl ghrelin peptide. IC_{50} values for des-acyl ghrelin peptides of varying length are given in the inset table. Percent activity was calculated as described in the Materials and Methods, with error bars indicating the standard deviation calculated from a minimum of three independent measurements. (d) Inhibition of hGOAT-catalyzed octanoylation of $GSSFLC_{AcDan}$ by 28-mer des-acyl ghrelin peptides incorporating sequence substitutions. Inhibition was measured at two peptide concentrations: 10 and 40 μM (filled and open bars, respectively). Percent activity was normalized as described in the Materials and Methods to activity in the presence of carrier/solvent alone (columns marked "None"), with error bars indicating the standard deviation calculated from a minimum of two independent measurements.

formation of the mutated substrates, within 2-fold of the wild-type substrate, and a wide range of amino acid substitutions at this position exhibit reactivity that was further reduced but still detectable. Incorporation of the ionizable amino acids glutamate and lysine leads to a complete loss of peptide octanoylation. In substrate-titration experiments, valine substitution at F4 led to a decrease in K_m, whereas the other substitutions exhibited weaker apparent binding (Table S3).

The broad tolerance for uncharged amino acids at F4 is consistent with a nonpolar, sterically permissive binding site for this amino acid within the hGOAT active site. This steric flexibility at the F4 position, in contrast to the steric discrimination observed at the G1 and S2 positions in the ghrelin N-terminal sequence,[28,37] suggests that this site may provide an opportunity for introducing chemical diversity into potential hGOAT inhibitors. To assess the potential for chemical diversification at F4, we incorporated a number of unnatural amino acids at this position and measured hGOAT octanoylation activity for the modified peptide substrates (Figure 2a,e). Cyclohexylalanine incorporation results in reduced but still detectable substrate reactivity, suggesting that planarity and/or aromaticity plays a role in recognition at this position. Introduction of nitro groups on the phenyl ring reduces reactivity in the context of phenylalanine and does not significantly alter reactivity in the context of tyrosine. Our data supports a strong hGOAT preference for phenylalanine at the F4 position, but the observation of octanoylation activity for the majority of substrates with other side chains at this position suggests that there is potential for chemical diversity at this site in new hGOAT inhibitors.[28,35,36]

Determining the Extent and Context Dependence of Ghrelin Recognition by hGOAT. In its cellular context, hGOAT binds and acylates the 94-residue ghrelin precursor proghrelin. Several studies have shown that the mouse and human isoforms of GOAT accept shorter peptides as substrates,[28,34,37,38] but there have been discrepancies reported in the literature regarding the impact of downstream elements in ghrelin on recognition by GOAT.[28,38] Furthermore, it is uncertain if the selectivity determinants revealed in the studies described above extend to hGOAT recognition of full-length ghrelin and proghrelin.

To investigate these questions, we determined the ability of des-acyl ghrelin peptides to serve as competitive hGOAT inhibitors in $GSSFLC_{AcDan}$ octanoylation reactions, using an

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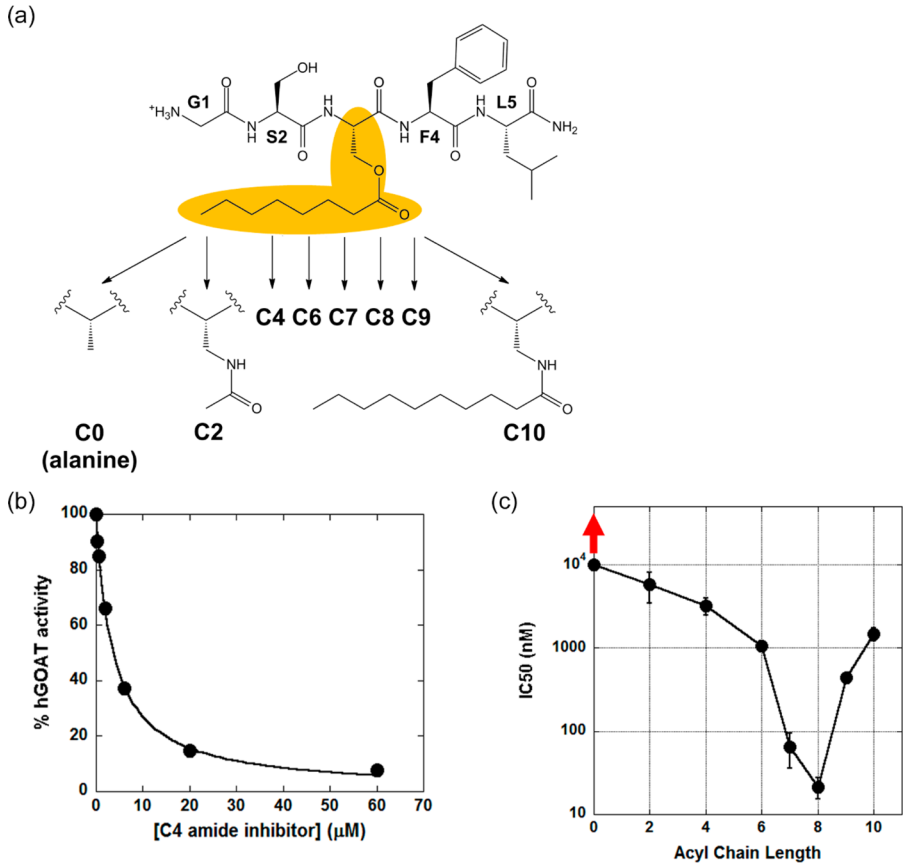

Figure 4. Defining the acyl chain length preference of hGOAT using acylated product inhibitors. (a) Design of the acyl inhibitor series, with replacement of the octanoyl ester of acylated ghrelin with amide side chains of varying length at the S3 position. (b) Inhibition of hGOAT-catalyzed octanoylation of $GSSFLC_{AcDan}$ by C4 amide inhibitor (butyl-[Dap3]-ghrelin (1-5)). Percent activity was calculated as described in the Materials and Methods. (c) Dependence of acyl inhibitor IC_{50} values on acyl chain length. The data point for the GSAFL inhibitor (acyl chain length = 0) indicates a lower limit, as indicated by the red arrow.

assay similar to that reported by Yang and co-workers (Figure 3).[28] As the des-acyl ghrelin peptide is extended from 5 amino acids, representing the minimal recognition motif for GOAT, to the full-length 28-mer ghrelin, the observed IC_{50} values decrease from 130 to 12 μM (Figure 3c). Although the 10-mer peptide does not exhibit inhibitory behavior in the experimentally accessible concentration range, the 18-mer and 28-mer peptides increase in inhibition by approximately 2- and 11-fold, respectively, relative to the behavior of the 5-mer peptide. This indicates that additional binding determinants exist downstream of the GSSF N-terminal sequence of ghrelin, with these interactions most likely occurring with amino acids in the C-terminal half of ghrelin. Inspection of ghrelin reveals the presence of multiple basic amino acids in this region, suggesting that downstream recognition of ghrelin by hGOAT may involve electrostatic interactions; the nature and specific locations of these interactions will be the focus of future study.

To assess the context dependence of hGOAT sequence selectivity, we introduced sequence substitutions into the 28-mer des-acyl ghrelin peptide that were shown to block hGOAT-catalyzed octanoylation of our shorter fluorescent peptide substrate. Removal of the octanoylation site through an alanine substitution at S3 (S3A) reduces but does not eliminate inhibition by the 28-mer peptide, which is consistent with studies of a GSAFL peptide inhibitor by Yang and co-workers.[28] In contrast, substitutions at the S2 and F4 positions that led to loss of octanoylation activity with the 6-mer fluorescent peptide substrate result in loss of inhibition by the 28-mer des-acyl ghrelin peptide (Figures 2 and 3d). The loss of inhibition upon the S2D, S2P, and F4E substitutions is consistent with these amino acid substitutions leading to loss of peptide recognition by hGOAT in the context of both the short $GSSFLC_{AcDan}$ peptide and full-length ghrelin.

Defining the Acyl Chain Length Binding Preference of hGOAT. In addition to proghrelin, hGOAT must also recognize and bind a cosubstrate to catalyze ghrelin acylation: acyl coenzyme A (acyl-CoA). Although ghrelin was originally characterized with an octanoyl (eight-carbon) fatty acid esterified to S3, subsequent studies have shown that the mouse and human forms of GOAT can accept a range of acyl-CoA substrates both in vitro and in vivo.[22,38,46,47] Longer acyl donors, such as myristoyl-CoA or palmitoyl-CoA, are not efficiently accepted by GOAT,[28,38] which is in contrast to other MBOAT family members.[23,24,48] The preference of GOAT for medium-length fatty acid chains is unique among MBOAT family members and provides a potential element for increasing GOAT inhibitor specificity. Toward this end, characterizing the optimal acyl chain length for recognition by hGOAT will provide essential information for the design of inhibitors.

Studies of GOAT-catalyzed ghrelin acylation using acyl-CoA donors of different lengths yield potentially complex relative activity profiles, which is attributable to multiple factors. These factors include the acyl-CoA binding affinity to GOAT, the reactivity of acyl-CoA in GOAT-catalyzed ghrelin acylation, the

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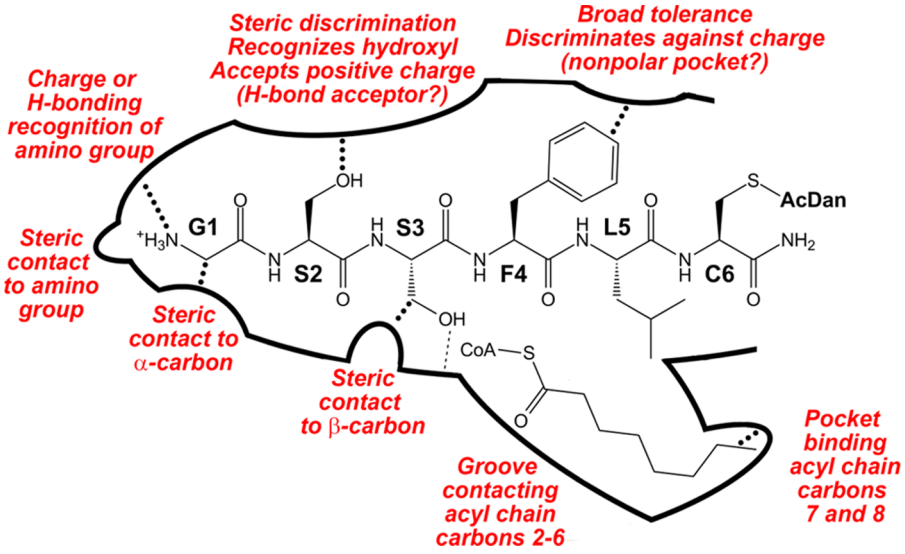

Figure 5. Schematic illustration of substrate-binding-site interactions within the hGOAT active site. The figure incorporates structure−activity data from both this work and previous studies.[28,35,37,38] Proposed interactions between the hGOAT active site and the N-terminal amine, the α carbon of G1, the side-chain hydroxyl of S2, the β carbon of S3, the side chain of F4, and carbons 7 and 8 of the octanoyl chain are labeled and are shown as dotted lines. A proposed contact to the hydroxyl group of S3 is indicated by a dashed line to reflect the potential for a catalytic interaction at this site.

binding affinity of the acylated ghrelin product that acts as a product inhibitor, the hydrolytic stability of the *O*-acylated ghrelin products, and the potential susceptibility of different acyl-CoA donors to contaminating thioesterases.[22,28,34,38] To simplify the analysis of the binding preferences of hGOAT with regard to acyl chain length, we chose to examine the relative binding affinities of acylated product-mimetic inhibitors to hGOAT. Monitoring changes in the IC_{50} values of acylated inhibitors rather than in acyl donor activity allows us to probe changes in binding alone, thus avoiding a potential superposition of the factors described above. To identify the optimal acyl chain length for binding to hGOAT, we synthesized a series of peptidomimetic inhibitors inspired by the octanoyl-[Dap^3]-ghrelin (1-5)-NH_2 inhibitor reported by Yang and co-workers (Figure 4a).[28] This inhibitor mimics acyl ghrelin and replaces the ester group with a more hydrolytically stable amide linkage. Our inhibitor series either eliminates the side-chain amide from the third position by incorporation of alanine (GSAFL) or introduces a variety of amide-linked acyl chains, ranging in length from 2 (acetyl) to 10 (decanoyl) carbons.

With the exception of the GSAFL inhibitor, all of the acylated inhibitors were able to block hGOAT octanoylation of the $GSSFLC_{AcDan}$ substrate (Figure 4b and Table S4). The structure−activity profiles for the acylated inhibitors suggest that the acyl chain binding pocket within hGOAT is composed of two distinct regions, each with different binding energies for individual methylene groups (Figure 4c). No appreciable inhibition was observed with the GSAFL inhibitor within the experimentally accessible concentration range, which provided a lower limit for IC_{50} of >10 μM. The addition of an acetyl group yields a weak inhibitor, with inhibitor binding affinity increasing further (by approximately 5-fold) as the acyl chain is extended from two to six carbons. However, the addition of two more methylene groups leads to much tighter inhibitor binding, with IC_{50} = 22 ± 6 nM for the eight-carbon amide inhibitor. Continued lengthening of the acyl chain beyond eight carbons negatively impacts inhibitor binding affinity for hGOAT. The 9-carbon inhibitor binding affinity is 20-fold weaker than that of the 8-carbon inhibitor, and the 6-carbon and 10-carbon amide inhibitors exhibit roughly equivalent IC_{50} values. The reduced binding affinities for the 9-carbon and 10-carbon inhibitors suggests that the inhibitor acyl chain binds a defined pocket within the hGOAT active site that optimally accommodates an 8-carbon acyl chain.

Evaluating the Potential for hGOAT Substrates Beyond Ghrelin. From the initial discovery of GOAT, ghrelin has been considered the sole substrate for this enzyme because ghrelin is the only known octanoylated protein in animals.[21,22] The reactivity data from this study provides an opportunity to assess the potential for GOAT substrates other than ghrelin within the human proteome. We observed hGOAT-catalyzed octanoylation of peptide substrates bearing substitutions that induce loss of proghrelin acylation in other assays that more closely model the natural ghrelin maturation pathway (e.g., the alanine substitution at F4).[28] These results suggest that the peptide-based assay may be more permissive than previous assays for substrate selectivity. In addition, sequence substitutions in the N-terminal GSSF sequence that block peptide octanoylation by hGOAT also lead to loss of inhibition by unlabeled full-length ghrelin peptides in competitive assays. Therefore, we conclude that loss of activity in the peptide assay will accurately indicate sequence variations that will block proghrelin octanoylation in vivo.

To use the most permissive filter to scan for potential hGOAT substrates, we eliminated only sequences with substitutions at the S2 and F4 positions that were demonstrated to block hGOAT-catalyzed octanoylation in our $GSSFLC_{AcDan}$ substrate and allowed both serine and threonine at the octanoylation site. A PROSITE search with these parameters yielded ~16 000 hits from the ~21 000 human proteins annotated in the Swiss-Prot database (PROSITE search string: <x(0,70)G{DWP}[ST]{EK}, Figure S4).[49] These potential candidates were analyzed using the Signal P algorithm (D-cutoff = 0.34) to determine two factors: (1) if a candidate protein contains a predicted N-terminal signal peptide and (2) if removal of the predicted signal peptide yields the N-terminal glycine required for recognition by hGOAT.[28,37,50] Five human genes are predicted to generate mature proteins with potential hGOAT substrate sequences: B2GP1 (GRTC−), VIPL (GQTF−), SorCS1 (GGSC−),

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C11orf44 (GVTG−), and ghrelin (GSSF−). The first three candidate proteins can be excluded as potential hGOAT substrates on the basis of either previous isolation and characterization of the proposed octanoylation site (B2GP1) or lack of target sequence conservation in the rodent forms of the genes (VIPL and SorCS1).[51−53] Because C11orf44 is a predicted human protein with no reported evidence for protein expression, our analysis supports the conclusion that ghrelin is a unique substrate for GOAT-catalyzed acylation within the human proteome.

DISCUSSION

In these studies, we have applied structure−activity analysis to identify elements within the ghrelin and acyl-donor substrates of hGOAT that are important for enzyme recognition. By examining the effects of both amino acid substitutions and functional group alterations, our work provides the first molecular-level insight into the range of chemical properties that hGOAT recognizes in the ghrelin GSSF target sequence.[28,38] For example, hGOAT requires positive charge and/or hydrogen bond donation character at the N- terminus of its ghrelin substrate and does not tolerate negative charge at any site within the N-terminal sequence of ghrelin. Our work also shows that hGOAT strongly prefers to bind an eight-carbon acyl chain over acyl groups of similar length, which provides an important insight into acyl-donor selection by this enzyme.[22,28,38,46] Our studies also reveal unanticipated aspects of hGOAT substrate recognition, such as the broad tolerance for different side chains at the F4 position. These findings build upon our previous study of hGOAT substrate selectivity at the G1 and S2 positions of the ghrelin GSSF N-terminal sequence.[37] We demonstrated that hGOAT will not tolerate any amino acid side chains at the G1 position, with both alanine and serine substitutions leading to loss of substrate reactivity. Threonine can substitute for serine at the S2 position with no loss of octanoylation activity with hGOAT, whereas larger amino acids at this position lead to loss of peptide reactivity. Taken together, the results from structure−activity analysis of peptide substrate reactivity and acylated inhibitor binding yield a schematic that illustrates the relative arrangement and nature of interactions between the hGOAT active site and its substrates (Figure 5). The array of interactions identified in this study also provides a structure−function context within which to evaluate and design new hGOAT inhibitors.

In addition to the N-terminal GSSF sequence of ghrelin, GOAT isoforms appear to recognize downstream elements of ghrelin. In studies of mouse GOAT, Yang and co-workers demonstrated that both a short 5-mer peptide (GSSFL, IC_{50} = 80 μM) and full-length mouse ghrelin (IC_{50} = 2 μM) can serve as inhibitors of proghrelin octanoylation by GOAT.[28] The lower IC_{50} value for full-length mouse ghrelin in these assays parallels the IC_{50} trend shown for des-acyl ghrelin peptide inhibition of $GSSFL_{AcDan}$ octanoylation in this study and is consistent with downstream elements contributing to binding of ghrelin-derived peptides to GOAT. Our study indicates that the increased binding requires the presence of the C- terminus of ghrelin, with partial binding enhancement occuring in the absence of the last 10 amino acids of ghrelin. These findings provide guidance in defining the specific location of downstream recognition elements within ghrelin and the nature of the interactions leading to increased peptide binding to GOAT. In addition, the possibility that proghrelin sequence elements (removed upon ghrelin maturation) contribute to substrate recognition by hGOAT remains to be explored. However, the ability of point substitutions within the GSSF N-terminal sequence to eliminate peptide inhibition of hGOAT activity provides further support for the hypothesis that the N-terminal sequence of ghrelin serves as the major determinant for recognition by hGOAT.

Several factors must be considered when critically assessing the interactions proposed on the basis of our analysis and their value in predicting hGOAT substrates and designing hGOAT inhibitors. We are using a short peptide to model hGOAT octanoylation of the 94-residue substrate proghrelin. Although this peptide substrate lacks the downstream elements of ghrelin or proghrelin that are recognized by hGOAT, the binding of small-molecule hGOAT inhibitors would be expected to more closely resemble the small peptide than proghrelin. Furthermore, substitutional analysis in the context of full-length ghrelin suggests that hGOAT recognition of the GSSF sequence in the context of the short peptide is similar to that in the context of longer ghrelin peptides. Another important concern when using fluorescently labeled substrates is that the binding and reactivity of the substrate may be dominated by enzyme interactions with the fluorophore. One such example is resveratrol activation of sirtuin-catalyzed lysine deacetylation, which is dependent on the presence of a coumarin fluorophore in the Fluor de Lys substrate.[54] In our studies, both experimental design and observed sequence-dependent substrate reactivity changes minimize the potential for misinterpretation as a result of fluorophore-induced artifacts. By comparing the reactivity of different peptide substrates without altering the location or nature of the fluorophore, the relative reactivity measurement will cancel out effects that arise from fluorophore interactions so long as the reactions are carried out with subsaturating peptide substrate concentrations (as in our assays) to avoid binding artifacts. In addition, the complete loss of peptide reactivity upon small molecular changes (e.g., −NHMe to −OMe at the N-terminus) is consistent with reactivity changes that reflect the effects of amino acid substitutions and functional group modifications rather than the presence of the acrylodan fluorophore.

From its initial discovery, ghrelin has been considered an attractive pharmacological target based on its multiple roles in controlling energy balance and metabolism coupled with its octanoylation posttranslational modification.[1−3,18] Although the unique character ascribed to ghrelin octanoylation was previously based on the lack of detection of other octanoylated proteins,[21] our peptide selectivity data provides a new filter for bioinformatics analysis to identify other potential proteins within the human proteome that are modified by GOAT. This analysis yields the strongest prediction to date in support of ghrelin being the only substrate for GOAT within the human proteome. This finding further supports the ghrelin−GOAT system as a promising target for therapeutic development with a reduced expectation for off-target effects. In addition, studies of Hhat suggest that a small number of human proteins can serve as substrates for palmitoylation by this enzyme,[27] indicating that selecting for a small number of potential substrates among the pool of secreted proteins may be a general property of MBOAT family members that acylate protein targets.

Defining the location and composition of the substrate binding sites and active site within hGOAT presents a key challenge in understanding this enzyme. Given the challenges in obtaining high-resolution structural data for integral membrane proteins such as MBOAT-family enzymes, our structure−function-based approach provides a complementary avenue for investigating the

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hGOAT catalytic apparatus. The interactions and substrate selectivity preferences defined in this work are an array of potential probes for linking groups in the peptides and the acyl-donor substrates to hGOAT residues through functional suppression experiments, thus building the active site from the inside out. For example, scanning mutagenesis can be applied within GOAT to identify sites within the enzyme where the reduction of amino acid volume relieves the strict steric selectivity observed at G1 of the ghrelin substrate.[37] Identifying the residues that compose the hGOAT active site is an essential step toward the mechanistic investigation of hGOAT and the associated development of inhibitors. In light of the growing appreciation for the impact of ghrelin-mediated signaling in human health and disease, developing potent hGOAT inhibitors is essential for exploring the therapeutic potential of the hGOAT−ghrelin pathway.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis procedures for acylated peptide inhibitors, calculated and observed m/z values of hGOAT peptide substrates, quantitation of peptide substrate octanoylation by hGOAT, K_m values for hGOAT octanoylation of peptide substrates, IC_{50} values for inhibition of hGOAT-catalyzed octanoylation by acylated peptide inhibitors, the effect of coincubation of *N*,*N*-dimethylglycine and betaine substrates on hGOAT-catalyzed octanoylation of the $GSSFLC_{AcDan}$ substrate, representative time courses for peptide substrate octanoylation by hGOAT, representative K_m curves for peptide substrate octanoylation by hGOAT, and a flowchart describing filters and output from the hGOAT substrate bioinformatics analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: (315)-443-1134. Fax: (315)443-4070. E-mail: hougland@syr.edu.

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Notes

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■ ABBREVIATIONS

GOAT, ghrelin *O*-acyltransferase; hGOAT, human ghrelin *O*-acyltransferase; AcDan, acrylodan; DMSO, dimethyl sulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; HPLC, high pressure liquid chromatography; MALDI, matrix assisted laser desorption ionization; G1, glycine at the first position of the ghrelin-derived GSSFLC peptide; S2, serine at the second position of the ghrelin-derived GSSFLC peptide; S3, serine at the third position of the ghrelin-derived GSSFLC peptide; F4, phenylalanine at the fourth position of ghrelin; IC_{50}, half maximal inhibitory concentration

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