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N-glycosylation of Asparagine 130 in the Extracellular Domain of the Human Calcitonin Receptor Significantly Increases Peptide Hormone Affinity

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Abstract

The calcitonin receptor (CTR) is a class B G protein-coupled receptor that is activated by the peptide hormones calcitonin and amylin. Calcitonin regulates bone remodeling through CTR, whereas amylin regulates blood glucose and food intake by activating CTR in complex with receptor activity-modifying proteins (RAMPs). These receptors are targeted clinically for treatment of osteoporosis and diabetes. Here, we define the role of CTR N-glycosylation in hormone binding using purified calcitonin and amylin receptor extracellular domain (ECD) glycoforms and fluorescence polarization/anisotropy and isothermal titration calorimetry peptide-binding assays. N-glycan-free CTR ECD produced in *Escherichia coli* exhibited ~10-fold lower peptide affinity than CTR ECD produced in HEK293T cells, which yield complex N-glycans, or in HEK293S GnTI⁻ cells, which yield core N-glycans (Man₅GlcNAc₂). PNGase F-catalyzed removal of N-glycans at N73, N125, and N130 in the CTR ECD decreased peptide affinity ~10-fold, whereas Endo H-catalyzed trimming of the N-glycans to single GlcNAc residues had no effect on peptide binding. Similar results were observed for an amylin receptor RAMP2-CTR ECD complex. Characterization of peptide-binding affinities of purified N→Q CTR ECD glycan site mutants combined with PNGase F and Endo H treatment strategies and mass spectrometry to define the glycan species indicated that a single GlcNAc residue at CTR N130 was responsible for the peptide affinity enhancement. Molecular modeling suggested that this GlcNAc functions

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Author Contributions SML and AAP conceived and designed experiments and analyzed data. SML performed plasmid construction, mammalian and bacterial cell expression, protein purification, and binding assays. JMB performed plasmid construction and developed the purification methods for bacterially-expressed MBP-CTR ECD and MBP-RAMP2-CTR ECD and purified these proteins. JIG and DLH designed, performed, and analyzed bacterial expression and purification of isolated CTR ECD and the ITC assays. VS performed mass spectrometry and ms data analysis. SML and AAP wrote the manuscript with input from all authors.

CONFLICT OF INTEREST

None.

through an allosteric mechanism rather than by directly contacting the peptide. These results reveal an important role for N-linked glycosylation in the peptide hormone binding of a clinically relevant class B GPCR.

Graphical Abstract

INTRODUCTION

The human calcitonin receptor (CTR) is a class B/Secretin family G protein-coupled receptor (GPCR) that mediates the actions of the endocrine peptide hormones calcitonin (CT) and amylin (Amy)¹. CT activation of CTR on osteoclasts inhibits bone resorption². This action provides the basis for the pharmacologic use of calcitonins to treat hypercalcemia, Paget's disease, and osteoporosis. Salmon CT (sCT) is the most commonly used calcitonin drug because it has higher affinity for CTR than human CT³. Amy activates CTR with lower potency than CT, however, upon association of CTR with any of three receptor activity-modifying proteins (RAMP1-3) Amy affinity is increased such that it becomes equipotent to or of greater potency than CT⁴⁻⁷. The three amylin receptors, designated AMY₁, AMY₂, and AMY₃, for CTR complexes with RAMP1, -2, or -3, respectively, are expressed at sites of Amy action in the brain⁸. Amy reduces food intake and inhibits gastric emptying and glucagon secretion⁹. An Amy analog pramlintide is available as an insulin adjunct therapy for types I and II diabetes and next generation analogs are actively pursued as an obesity therapy^{9, 10}. The physiological and clinical importance of these receptors provides an impetus to define their peptide hormone binding and activation mechanisms.

CTR has a typical class B GPCR two domain architecture comprising an N-terminal extracellular domain (ECD) of approximately 120 amino acid residues followed by a membrane-embedded 7-transmembrane (7-TM) helical bundle domain¹¹. CT and Amy are 32 and 37 amino acid residue C-terminally amidated peptides, respectively. These peptides comprise an N-terminal region with a disulfide bond structure (residues 1-7) followed by a central α -helical region and an unstructured C-terminal region. The bound hormone spans the two CTR domains with the peptide C-terminal region contacting the ECD and the N-terminal region contacting and activating the 7-TM domain¹¹. The RAMPs have a single TM segment and an N-terminal ECD that is similar in size to the CTR ECD. How RAMPs increase CTR affinity for Amy is unclear, but the RAMP ECD is important for altering ligand selectivity¹², possibly by allosterically modulating CTR conformation^{13, 14}.

We showed that the peptide selectivity profiles of the intact CTR, AMY₁, and AMY₂ receptors were reproduced by purified CTR ECD and tethered RAMP1-CTR ECD and RAMP2-CTR ECD proteins¹⁴. Mutagenesis and modeling allowed us to propose how sCT and an Amy analog AC413 occupy a shared binding site on the CTR ECD. Our sCT model was consistent with the recently published crystal structure of the sCT analog-bound CTR ECD, which showed an extended conformation for the C-terminal sCT fragment and a β -turn structure near the C-terminus¹⁵.

Despite the recent progress on understanding how CT and Amy bind to their receptor ECDs, the role of CTR N-linked glycosylation in hormone binding remains as an important unresolved issue. CTR is a glycoprotein with N-linked glycan sites at N28, N73, N125, and N130 in the ECD. The first of these sites is absent in a functional splice variant¹⁶. Several groups have examined how N-glycans affect CTR function, with mixed results. Tunicamycin treatment of the human breast cancer cell line T 47D, which expresses CTR, reduced the cell surface CTR level, but did not seem to alter affinity for sCT¹⁷. Using membranes prepared from BHK cells stably expressing human CTR Quiza *et al.* observed no difference in sCT binding affinity between control membranes and those treated with PNGase F, although the authors were unable to fully deglycosylate the membrane-embedded receptor under the non-denaturing conditions required for subsequent assessment of binding affinity¹⁸. Ho *et al.* provided the first evidence that CTR N-glycans may be important for function¹⁹.

Tunicamycin treatment of COS-1 cells transiently expressing human CTR reduced sCT binding and cAMP signaling potency while having no apparent effect on cell surface expression. In addition, ~10- to 40-fold reductions in sCT cAMP signaling potency, as well as E_{max} reductions, were observed for CTR N125A or N130A glycan site mutants, whereas the N73A mutant retained normal response to sCT. The authors concluded that CTR N-glycans are important for high-affinity binding and potency of sCT¹⁹. However, sCT binding affinity was not measured at the glycan site mutants and their data could also be explained by a role for N-glycans in CTR folding, or by effects of the mutations on receptor expression level and/or receptor structure.

Recent studies with purified CTR ECD have added further confusion regarding the role of the N-glycans in hormone binding. Using a purified N-glycosylated maltose binding protein (MBP)-CTR ECD fusion protein secreted from HEK293T cells and an AlphaLISATM luminescent bead- based proximity assay to assess peptide binding we observed that sCT(8-32) bound the ECD with an IC₅₀ of 2 μ M in a competition assay¹⁴. In contrast, Johansson *et al.* used N-glycan-free CTR ECD produced in *E. coli* for their sCT analog-bound crystal structure and they reported that sCT(8-32) bound it with an IC₅₀ of 50 nM in a similar AlphaScreenTM competition peptide-binding assay¹⁵. One interpretation of these results is that CTR N-glycosylation reduces hormone affinity, however, the two studies used different constructs and binding assay conditions so they are not directly comparable. In addition, the AlphaLISA/Screen technology, although powerful, is ill-suited for the determination of accurate binding constants. Thus, the role of CTR N-glycans in peptide binding remains unclear. Here, we developed a quantitative fluorescence polarization/ anisotropy peptide-binding assay and used it to characterize the hormone binding affinities of purified CTR and AMY₂ receptor ECD expressed in HEK293 and *E. coli* cells. PNGase F and Endo H enzymes were used to alter the glycosylation state of the HEK293 cell-produced

proteins to probe the role of glycans in hormone binding and purified glycan site mutants were characterized to assess the role of glycosylation at each position in the ECD. Our results demonstrate that while N-glycans are not required for hormone binding, the N130 N-glycan enhances hormone affinity ~10-fold and a single GlcNAc residue is sufficient for this effect.

MATERIALS AND METHODS

Reagents and Materials

HEK293T (HCL4517) and HEK293S GnTI⁻ cell lines (CRL-3022) were from Thermo Scientific Open Biosystems and ATCC, respectively. Dulbecco's modified Eagle's medium (DMEM, 12-604Q) containing 4.5g/L of glucose and L-glutamine and fetal bovine serum (FBS, 16000-044) were from Lonza and Life Technologies, respectively. Non-essential amino acid mixture (13-144E) was from Lonza. PNGase F (P0704L) and Endo H (P0702L) enzymes and Gibson Assembly Master Mix (M5510A) were from New England Biolabs (NEB).

Plasmid constructs

pHLsec-based vectors²⁰ for mammalian cell expression of MBP-hCTR.36-151-H₆ (pAP348) and tethered MBP-hRAMP2.55-140-(Gly-Ser)₅-hCTR.36-151-H₆ (pSL002) were previously described¹⁴. The Gibson Assembly method was used for site-directed mutagenesis to introduce the N73Q (pAP349), N125Q (pSL006), N130Q (pSL007), N130A (pSL010), or N73Q/N125Q (pSL012) mutations into MBP-CTR ECD. pETDuet-1-based *E. coli* expression vectors encoding the same wild-type proteins as above were constructed using PCR and restriction-enzyme based cloning methods as previously described (pAP263 for MBP-CTR ECD and pJB041 for MBP-RAMP2-CTR ECD fusion)^{21, 22}. These vectors encode the MBP fusion proteins in the first multiple cloning site and the *E. coli* disulfide isomerase DsbC in the 2nd multiple cloning site. For *E. coli* expression of isolated RAMP2 ECD, a pETDuet-1 vector encoding MBP-TEV protease cleavage site-hRAMP2.36-140-H₆ (pJB004) was constructed using PCR and restriction enzyme-based methods to insert the fragment encoding RAMP2.36-140 into the pETDuet-1 vector with MBP-TEV cleavage site-(His)₆ (pJB003)²¹. Coding regions of the plasmids were confirmed by automated DNA sequencing by the Laboratory for Molecular Biology and Cytometry Research core facility at the University of Oklahoma Health Sciences Center. The primer sequences used for plasmid construction are provided as Supporting Information. Plasmids used for transient transfections of HEK293T or HEK293S GnTI⁻ cells were purified using the Macherey-Nagel Midi kit (740412.50) or Qiagen Giga kit (12191) according to the manufacturer's directions.

Protein expression from HEK293T or HEK293S GnTI⁻ cells and purification

Proteins were expressed in HEK293T cells as described¹⁴. For HEK293S GnTI⁻ cell culture, 1X non-essential amino acid (NEAA) as a final concentration (13-144E, Lonza) was added as a supplement to DMEM with 10% FBS, 50 units/ml penicillin, and 50 µg/ml streptomycin. HEK293T or HEK293S GnTI⁻ cells were cultured in six to eight T175 cell culture flasks (Corning) at 37°C and 5% CO₂. Cells at 80% confluency were transiently

transfected with 50 µg of plasmid DNA and 75 µg of PEI per flask according to standard methods²⁰. After transfection, the cells were incubated for 3 days at 37°C and 5% CO₂ before harvesting the media. ECD proteins other than those noted below were expressed in this manner. For CTR ECD N73Q, HEK293T cells were incubated at 30°C for 5 days after transfection due to its poor expression. For the expression of CTR ECD N73Q/N125Q in HEK293S GnTI⁻ cells, six expanded surface area roller bottles (681072, Greiner Bio-One) were used due to its very poor expression. HEK293S GnTI⁻ cells were cultured in the bottles to 80 % confluency at 0.3 RPM, 37°C, and 5% CO₂. For transient transfection per bottle, 500 µg of DNA plasmid and 750 µg of PEI were mixed in 40 ml of DMEM and incubated at room temperature. After 10 min, the cell culture media was replaced with the DNA and PEI mixture and DMEM with 2% FBS, 1X NEAA, 4.2 mM valproic acid in a total 250 ml volume per bottle. Then, the roller bottles were incubated at 0.3 RPM, 30°C, and 5% CO₂ for 5 days. After incubation, the media (~1.5 L) was centrifuged at 15000g for 20 min and the supernatant was filtered with 0.22 µm filter membrane (Millipore) and concentrated to ~250 ml by tangential flow filtration as described¹⁴. For each protein the harvested media was dialyzed overnight against 4 L of 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM imidazole and then subjected to IMAC and gel filtration chromatography on an AKTA purifier (GE Healthcare) as described¹⁴. All harvesting and purification steps were performed at 4°C. The purified proteins were transferred to storage buffer and stored at -80 °C as described¹⁴ or directly used in assays after diluting the glycerol content to less than 1%.

Bacterial protein expression and purification

Expression of MBP-CTR ECD-H₆, MBP- RAMP2-(GS)₅-CTR ECD-H₆, and MBP-TEV cleavage site-RAMP2 ECD-H₆ in *E. coli* Origami B (DE3) cells was as described^{21, 22}. Purifications were performed at 4°C using an AKTA purifier unless otherwise noted. MBP-RAMP2-(GS)₅-CTR ECD was purified using the method previously described for a tethered fusion of RAMP2 ECD and a different class B GPCR ECD²² using 5 mM GSH and 1 mM GSSG for the *in vitro* disulfide shuffling step. For MBP-CTR ECD, cells from a 6 L culture were harvested, lysed and the clarified lysate supernatant was subjected to IMAC (GE Healthcare) as described²¹. Peak fractions were pooled, dialyzed to *in vitro* disulfide shuffling (DSS) buffer (50 mM Tris-HCl, pH 8.0, 5% (v/v) glycerol, 150 mM NaCl), and subjected to DSS with MBP-CTR ECD at 1 mg/ml in the presence of purified DsbC, purified isolated RAMP2 ECD-H₆ (residues 36-140), and 5 mM GSH and 1 mM GSSG. The molar ratio of DsbC dimer : MBP-CTR ECD monomer was 0.5:1 and that of RAMP2 ECD monomer : MBP-CTR ECD monomer was 1.5:1. RAMP2 ECD was included because it promoted proper folding of CTR ECD. DsbC and RAMP2 ECD were purified as previously described²¹, except that for RAMP2 a new plasmid incorporating a TEV protease cleavage site instead of a thrombin cleavage site was used and TEV protease was used in place of thrombin. The DSS mixture was incubated for ~40 h at 20°C and then subjected to amylose affinity chromatography (NEB). The column was pre-equilibrated in DSS reaction buffer, loaded, and extensively washed with 50 mM Tris-HCl, pH 8.0, 5% (v/v) glycerol, 500 mM NaCl to remove RAMP2 ECD and DsbC. The amylose column was eluted with a linear gradient of 0-10 mM maltose in DSS buffer. Peak fractions were pooled and the protein was concentrated by ammonium sulfate precipitation as described²¹ and applied to a Superdex

S200HR gel filtration column (GE Healthcare). After elution with 50 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 150 mM NaCl, 0.5 mM EDTA, peak fractions were pooled and dialyzed to storage buffer (50 mM Tris-HCl, pH 7.5, 50% (v/v) glycerol, 150 mM NaCl), and stored at -80°C . MBP-free H₆-CTR ECD (residues 32-140) was expressed as inclusion bodies in *E. coli* and purified using the denaturant-based refolding method previously described for a related class B GPCR ECD ²³.

Protein quantification and polyacrylamide gel electrophoresis

The concentrations of purified proteins were measured by UV absorbance at 280 nm using their calculated extinction coefficient from Tyr, Trp, and Cystine residues and are stated in terms of the monomers. SDS gel electrophoresis was according to standard methods and native gel electrophoresis was as previously described ^{21, 22}.

Synthetic peptides

Peptides were custom-synthesized and HPLC-purified by RS synthesis (Louisville, KY). The sequences used are:

FITC-Ahx (aminohexanoic acid)-AC413(6-25) Y25P, FITC-Ahx-ANFLVRLQTYPRTNVGANTP-NH₂; FITC-Ahx-AC413(6-25), FITC-Ahx-ANFLVRLQTYPRTNVGANTY-NH₂; AC413(6-25) Y25P, ANFLVRLQTYPRTNVGANTP-NH₂; sCT(8-32), VLGKLSQELHKLQTYPRTNTGSGTP-NH₂; Biotin-sCT C1A/C7A, Biotin-ASNLSTAVLGKLSQELHKLQTYPRTNTGSGTP-NH₂. Lyophilized peptide powder was dissolved in sterile ultrapure water and aliquots were stored at -80°C . The concentrations of FITC-labeled peptides were calculated by measuring visible absorbance of samples diluted in 5 mM HEPES, pH 7.0 at 495 nm and using a FITC extinction coefficient of $63000\text{ M}^{-1}\text{cm}^{-1}$. The concentrations of unlabeled peptides were measured by UV absorbance at 280 nm with their extinction coefficient calculated from the Tyr residues. sCT and sCT(8-32) for ITC experiments were purchased from American Peptide. Peptide sequence alignment was performed as described ¹⁴.

Fluorescence polarization/anisotropy peptide binding assay

For saturation binding, 10 μl of purified receptor at varying concentrations in reaction buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.5 mg/ml fatty acid-free BSA, 0.5 mM Maltose, 0.5 mM EDTA, and 0.1% (v/v) Tween 20) was placed per well in a black half-area Costar® 96-well plate (3694, Corning). 40 μl of 12.5 nM FITC-labeled AC413(6-25) Y25P or AC413(6-25) in reaction buffer was added to each well with mixing, and the plate was spun down at 1000g for 3 min, and incubated in the dark for 1 h at room temperature to reach equilibrium. Fluorescence polarization/anisotropy was measured with a PolarStar Omega plate reader (BMG Labtech, Germany) equipped with a 485 nm excitation filter, dual 520 nm emission filters, and FP optics. The gain was set with a target mP of 50 and target value of 40% on a reaction containing FITC-labeled peptide alone in reaction buffer. For competition binding, 10 μl of a competitor peptide in reaction buffer at varying concentrations was placed in wells of the plate. Two master mixes were prepared: master mix A included receptor in reaction buffer at 225 nM (MBP-CTR ECD from HEK293T cells and HEK293S GnTI⁻ cells), 3000 nM (MBP-CTR ECD from *E. coli*), 442.5 nM (MBP-CTR

ECD N73Q from HEK293T cells), 530 nM (MBP-CTR ECD N125Q from HEK293T cells) and master mix B included 25 nM FITC-labeled peptide in reaction buffer. A reaction containing 20 μ l master mix B and 30 μ l reaction buffer was used to set the gain as above. Equal volumes of master mix A and B were mixed and 40 μ l of this mixture was added with mixing to the 10 μ l of competitor peptide per well. The plate was spun as above and incubated in the dark for 2 h at room temperature to reach equilibrium before reading as above. For both assay formats reactions containing reaction buffer alone were included for subtraction of background fluorescence. Receptor at the concentrations used did not appreciably alter the background fluorescence.

FP data analysis

Background corrected fluorescence intensity (FI) values for each channel ($\parallel$ and $\perp$) were used to calculate the total FI and anisotropy as follows:

$$\begin{aligned}\text{Total FI} &= I_{\parallel} + 2I_{\perp} \\ \text{Anisotropy} &= (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})\end{aligned}$$

For saturation binding experiments the observed anisotropies were corrected for the total FI quenching observed upon receptor binding (Fig. 2A) as described in Dandliker *et al.*²⁴ using the following equation:

$$\text{Corrected anisotropy} = \{ [(A - A_f) / (A_b - A)] (Q_f / Q_b) (A_b) \} + A_f / \{ 1 + [(A - A_f) / (A_b - A)] (Q_f / Q_b) \}$$

where:

A = measured anisotropy

A_f = anisotropy of free probe

A_b = anisotropy of bound probe

Q_f = total FI of free probe

Q_b = total FI of bound probe

A_b and Q_b were obtained using equations 9 and 10 from Dandliker *et al.*²⁴, respectively, except that anisotropy replaced polarization in equation 9. Equation 9 describes a plot of anisotropy vs. $[(Q_f)(A - A_f)] / [(Q_b)(\text{receptor concentration})]$, from which the y-intercept provides the value A_b, and equation 10 describes a plot of observed total FI (Q) vs. $(Q_f - Q) / (\text{receptor concentration})$, from which the y-intercept provides Q_b. For competition binding assays no correction was applied because determination of the K_I value is insensitive to the minor total FI variations observed (Fig. 2B)²⁵. Nonlinear regression curve fitting of the saturation binding data to equations 6 and 39 and the competition binding data to equations 17 and 39 from Roehrl *et al.*²⁵ was used to determine pK_D and pK_I values, respectively. Q = 1 in equation 39 because we used corrected anisotropies. These equations are formulated in terms of the total receptor, competitor, and probe concentrations. Nonlinear regression was performed in PRISM 5.0 (GraphPad Software, San Diego) with user-defined equations. The equation syntax used for PRISM is provided in Supporting Information.

AlphaLISA competition peptide binding and isothermal titration calorimetry (ITC) assays

These assays were performed as previously described^{14, 23}. Briefly, for the AlphaLISA assay the receptor, biotinylated-sCT, and competitor peptides at the indicated concentrations were incubated with streptavidin-coated donor beads and anti-MBP antibody-coated acceptor beads (15 µg/ml each) [Perkin-Elmer] in a reaction buffer of 50 mM MOPS, pH 7.4, 150 mM NaCl, 7 mg/ml fatty acid-free BSA for 5 h to reach equilibrium and luminescence was read using a Polarstar Omega Plate reader (BMG labtech). The competition AlphaLISA data were fit by nonlinear regression to the competitive binding “One site-Fit logIC50” equation in PRISM 5.0. ITC experiments used a MicroCal ITC instrument. The ITC data were fit to the standard 1:1 binding model in the Origin software provided with the MicroCal instrument to determine K_D , stoichiometry (n), enthalpy, and entropy.

Enzymatic deglycosylation and glycan trimming

Receptor ECD either in storage buffer or in the pool of fractions after the gel filtration column was diluted with 50 mM HEPES pH 7.4, 150 mM NaCl and spin-concentrated using tube concentrators (UFC500396, Amicon Ultra 0.5mL, MWCO 3kDa) according to the manufacturer's directions to reduce the glycerol content to less than 1%. The receptor ECDs were concentrated to ~80 µM to 150 µM. To remove all N-glycans 13 to 40 µg of the ECD proteins (~5 to 16 µM for MBP-CTR ECD and ~13 µM for MBP-RAMP2-CTR ECD fusion) were incubated with 4 µl of PNGase F (500 U/µl) in 1X GlycoBuffer 2 (NEB) at 37°C for 4 h (MBP-CTR ECD) or 5 h (MBP-RAMP2-CTR ECD) in a reaction volume of 44 µl. To trim core N-glycans to a single GlcNAc residue, 12 to 40 µg MBP-CTR ECD or N73Q/N125Q double mutant produced in HEK293S GnT1⁻ cells (~5 to 18 µM) were treated with 4 µl of Endo H (500 U/µl) in 1X GlycoBuffer 3 (NEB) at 22°C for 30 min in a reaction volume of 40 µl. The digests were cooled on ice for 3 min (PNGase F only), spun down in a microcentrifuge, and aliquots were directly used in the FP assay and analyzed by SDS-PAGE to monitor deglycosylation/trimming. Protein samples after deglycosylation reactions were used for serial dilution to vary receptor concentrations. Maximum 34 µl out of 44 µl PNGase F reaction volume and 33 µl out of 40 µl Endo H reaction volume were used to make 10 µl of receptor samples with the highest 3 µM receptor concentration for FP assays. No enzyme control digests indicated as – PNGase F or – Endo H groups in Tables 2 and 3 included 1X enzyme reaction buffer. Heat-inactivated enzyme control digests received PNGase F or Endo H that was pre-incubated at 75°C for 10 min and then cooled prior to addition. To purify Endo H-treated MBP-CTR ECD away from the enzyme and released glycans a scaled-up digestion reaction was subjected to IMAC and gel filtration chromatography as above.

Glycan modeling

The crystal structure of sCT analog bound-N-glycan-free CTR ECD (PDB: 5II0)¹⁵ was submitted to the GlyProt webserver(<http://www.glycosciences.de/modeling/glyprot/php/main.php>) to create a model bearing core Man₃GlcNAc₂ N-glycans. Pymol (Schrodinger) was used for visualization and figure preparation.

MS sample preparation and LC-MS/MS measurement

SDS-PAGE bands corresponding to control *E. coli*-produced MBP-CTR ECD and Endo H-digested MBP-CTR ECD produced in GnTI⁻ cells were cut and subjected to in gel digestion according to standard procedure²⁶. Liquid chromatography tandem mass spectrometry was performed by coupling a nanoAcquity UPLC (Waters Corp., Manchester, UK) to a Q-TOF SYNAPT G2S instrument (Waters Corp., Manchester, UK). Each protein digest (about 100 ng of peptide) was delivered to a trap column (300 μ m $\times$ 50 mm nanoAcquity UPLC NanoEase Column 5 μ m BEH C18, Waters Corp, Manchester, UK) at a flow rate of 2 μ l/min in 99.9% solvent A (10 mM ammonium formate, pH 10, in HPLC grade water). After 3 min of loading and washing, peptides were transferred to another trap column (180 μ m $\times$ 20 nanoAcquity UPLC 2G-V/MTrap 5 μ m Symmetry C18, Waters Corp., Manchester, UK) using a gradient from 1% to 60% solvent B (100% acetonitrile). The peptides were then eluted and separated at a flow rate of 200 nl/min using a gradient from 1% to 40% solvent B (0.1% formic acid in acetonitrile) for 60 min on an analytical column (7.5 μ m $\times$ 150 mm nanoAcquity UPLC 1.8 μ m HSST3, Waters Corp, Manchester, UK). The eluent was sprayed via PicoTip Emitters (Waters Corp., Manchester, UK) at a spray voltage of 3.0 kV and a sampling cone voltage of 30 V and a source offset of 60 V. The source temperature was set to 70 °C. The cone gas flow was turned off, the nano flow gas pressure was set at 0.3 bar and the purge gas flow was set at 750 ml/h. The SYNAPT G2S instrument was operated in data-independent mode with ion mobility (HDMSe). Full scan MS and MS2 spectra (m/z 50 - 2000) were acquired in resolution mode (20,000 resolution FWHM at m/z 400). Tandem mass spectra were generated in the trapping region of the ion mobility cell by using a collisional energy ramp from 20 V (low mass, start/end) to 35 V (high mass, start/end). A variable IMS wave velocity was used. Wave velocity was ramped from 300 m/s to 600 m/s (start to end) and the ramp was applied over the full IMS cycle. A manual release time of 500 μ s was set for the mobility trapping and a trap height of 15 V with an extract height of 0 V. The pusher/ion mobility synchronization for the HDMSe method was performed using MassLynx V4.1 and DriftScope v2.4. LockSpray of Glufibrinopeptide-B (m/z 785.8427) was acquired every 60 s and lock mass correction was applied post acquisition.

Protein Identification and modification localization

Raw MS data were processed by PLGS (ProteinLynx Global Server, Waters Corp., Manchester, UK) for peptide and protein identification. MS/MS spectra were searched against the Uniprot human database (release date September 01, 2016 containing 48,355 sequences and the protein construct of interest) with the following search parameters: full tryptic specificity up to two missed cleavage sites, carbamidomethylation of cysteine residues set as a fixed modification, and N-terminal protein acetylation, methionine oxidation and asparagine bound to a GlcNAc (+203.0794 Da) as variable modifications. The peptides that could contain a GlcNAc modification were further validated by manual sequencing.

Parameter reporting and Statistical analysis

For the FP assay the equilibrium binding constants obtained from nonlinear regression fitting of the binding curves are reported in Tables 1–3 as the negative log forms, pK_D for

saturation binding, or pK_I for competition binding, as is common in the pharmacology field. In the text we occasionally convert these to K_D or K_I values where it helps for comparison to previously published results or the ITC results. The equilibrium binding constants obtained from the ITC experiments are reported as the K_D values generated by the curve fitting in the Origin software. Scatter plots are provided in Supporting Information to show the individual parameter values determined from each of the three independent experiments along with the mean and S.E.M. reported in Tables 1–3. One-way analysis of variance (ANOVA) was used to compare multiple groups with Tukey's post hoc test. For two groups, Student's t test was used. All analyses were performed in PRISM 5.0 (GraphPad software) using the pK_D or pK_I values. The data used for the statistical analysis were from three independent experiments unless otherwise noted in Tables.

RESULTS

Development of a quantitative fluorescence polarization/anisotropy peptide-binding assay

We developed a fluorescence polarization (FP) assay based on binding of a fluorescein isothiocyanate (FITC)-labeled peptide probe to our previously described MBP-CTR ECD fusion protein¹⁴ (Fig. 1A). The CTR ECD comprises residues 36-151 and thus contains the N73, N125, and N130 N-glycan sites common to human CTR splice variants. MBP was originally included to act as a "crystallization module" to promote crystallization of the fusion protein²⁷, but its presence is also advantageous for the FP assay because it increases the size difference between the receptor and peptide probe, thereby enabling a more robust anisotropy signal than would be possible with the ECD alone. We designed a probe based on the AC413 peptide, which is an Amy/CT hybrid (Fig. 1B). FITC was linked via an Ahx spacer to the N-terminus of AC413 (6-25) containing the Y25P substitution. The 6-25 fragment is the minimal ECD-binding region and the Y25P mutation significantly enhances affinity for CTR ECD as well as tethered RAMP1-CTR ECD (AMY₁) and RAMP2-CTR ECD (AMY₂) complexes¹⁴. This probe was thus chosen for its high-affinity.

In saturation binding format a robust anisotropy signal was observed for purified MBP-CTR ECD expressed in HEK293T cells binding to the probe (Fig. 2A). Notably, probe binding decreased its total fluorescence intensity (FI) indicative of quenching upon receptor binding (Fig. 2A). The observed anisotropies were thus corrected for the total FI variation as described in Materials and Methods. The pK_D of FITC-AC413(6-25) Y25P determined from the saturation binding data was 6.94 (K_D 115 nM), whereas the pK_I of unlabeled AC413(6-25) Y25P determined by competition assay was 6.20 (K_I 631 nM) (Fig. 2A, B and Table 1). This suggested that the FITC-Ahx-labeling increased probe affinity for the fusion protein. Using our previously described Alpha-LISA competition peptide binding assay¹⁴, the relative binding of FITC-labeled and unlabeled AC413(6-25) Y25P was compared. This bead-based assay uses different excitation and emission wavelengths (680 nm/615 nm) than the FP assay (485 nm/520 nm) and indeed it was not affected by free fluorescein at 10 μ M (Fig. 2C). The pIC_{50} of FITC-labeled and unlabeled AC413(6-25) Y25P was 7.32 (IC_{50} 47.9 nM) and 6.15 (IC_{50} 708 nM), respectively (Fig. 2C and Table 1). The FP and Alpha-LISA assay results taken together strongly suggested that the FITC-Ahx moiety enhanced probe

affinity, presumably by contacting the fusion protein, which likely caused the quenching of total FI observed in the saturation FP assay.

Production of distinct recombinant glycoforms of MBP-CTR ECD

Three different expression systems were used to produce distinct glycoforms of MBP-CTR ECD: HEK293T cells, HEK293S N-acetylglucosaminyl transferase I negative cells (GnTI⁻)²⁸, and *E. coli*. HEK293T cells produce MBP-CTR ECD bearing complex N-glycans, whereas GnTI⁻ cells yield Endo H-sensitive core N-glycans that have been identified as Man₅GlcNAc₂ for several proteins^{28–30}. Notably, MBP lacks consensus NxS/T sequons so only CTR ECD within the fusion protein bears N-glycans. *E. coli* provides MBP-CTR ECD devoid of N-glycans. MBP-CTR ECD from HEK293T cells was produced and purified as previously described¹⁴ and similar methods were used here to produce the protein from the GnTI⁻ cell line.

Producing N-glycan-free MBP-CTR ECD in *E. coli* is challenging because the ECD has three disulfide bonds that must be properly formed. We used novel methodology similar to that we previously developed for producing various class B GPCR ECDs as MBP-ECD fusions in *E. coli*^{21, 22, 31}. Our denaturant-free method is distinct from the traditional inclusion body/denaturant-based refolding methods employed by Johansson *et al.* in their study of *E. coli*-produced CTR ECD¹⁵. Using our methodology we could not obtain functional MBP-CTR ECD unless isolated RAMP2 ECD was included in the *in vitro* disulfide shuffling (DSS) step (data not shown). Presumably the RAMP2 ECD binding partner promoted proper folding of the CTR ECD. DSS in the presence of RAMP2 ECD, a bacterial disulfide isomerase protein DsbC, and glutathione redox buffer diminished aggregates and mis-folded MBP-CTR ECD and enriched the properly folded species as monitored by native gel electrophoresis as described^{21, 22} (Fig. 3A). Fortunately, because the RAMP2 ECD interaction with MBP-CTR ECD is weak the RAMP2 ECD could be removed after the DSS step using amylose affinity chromatography to immobilize the MBP-CTR ECD and a high-salt buffer to wash away the RAMP2 ECD prior to elution of MBP-CTR ECD in a maltose-containing buffer. Remaining misfolded MBP-CTR ECD was removed by gel filtration chromatography. The gel filtration elution profiles of MBP-CTR ECD from each expression system were similar and consistent with each protein being a monomer (Fig. 3B). Analysis of the final purified samples by SDS and native gel electrophoresis confirmed their different glycosylation states (Fig. 3C).

N-glycosylation of CTR ECD significantly enhances peptide hormone affinity

In the FP assay N-glycan-free MBP-CTR ECD produced in *E. coli* exhibited a dramatic decrease in peptide affinity as compared to MBP-CTR ECD from HEK293T or GnTI⁻ cells (Fig. 4A). pK_D of FITC-AC413(6-25) Y25P was ~7.0 for MBP-CTR ECD from HEK293T or GnTI⁻ cells, while it was 5.94 (> 10-fold change) for MBP-CTR ECD from *E. coli* (Table 2). To confirm that this result was not just an oddity of the FITC-AC413(6-25) Y25P probe, we measured the affinity of the antagonist sCT(8-32) in competition binding assays and observed a similar marked decrease in sCT(8-32) affinity for the *E. coli*-produced protein (Fig. 4B). The pK_I of sCT(8-32) for MBP-CTR ECD from the HEK293 cell lines was ~5.5 (K_I ~3 μM) compared to 4.61 (K_I ~25 μM) for that from *E. coli* (Table 2).

As the measured sCT(8-32) affinity of our *E. coli*-produced MBP-CTR ECD protein was very far from the 50 nM IC₅₀ reported for isolated CTR ECD refolded from *E. coli* inclusion bodies¹⁵, we considered the possibilities that MBP in our fusion protein had an inhibitory effect on peptide binding or that our unique DSS methodology yielded improperly folded CTR ECD. To test these possibilities we bacterially expressed and purified the isolated CTR ECD (residues 32-140) to homogeneity (Fig. 5A) using a traditional inclusion body/denaturant-based refolding method and assessed its affinity for sCT peptides by isothermal titration calorimetry (ITC). The isolated N-glycan-free CTR ECD bound sCT(1-32) agonist with a K_D of 35 μM (Fig. 5B). In a second experiment we determined a K_D of 16 μM for sCT(8-32) antagonist (Fig. 5C). The similar affinities of these two peptides are consistent with previous findings that the 22-32 fragment constitutes the ECD binding region^{14, 15}. Unfortunately, these low affinities make obtaining accurate K_D values difficult because obtaining ideal sigmoid isotherms for the curve fitting would require high μM concentrations of ECD and peptide that are difficult and costly to obtain. Nonetheless, despite some uncertainty in the K_D values these results were in good agreement with those obtained for sCT(8-32) binding to MBP-CTR ECD in the FP assay, which strongly suggested that MBP in our fusion protein did not alter the ligand binding properties of the CTR ECD and that our unique folding methodology did not yield CTR ECD substantially different from that obtained by the traditional folding method.

To rule out the possibility that the decreased peptide affinities of the *E. coli*-produced proteins were the result of slight misfolding, we treated the HEK293T cell-produced MBP-CTR ECD with PNGase F to remove all N-linked glycans and assessed peptide-binding affinity in the FP assay. The PNGase F-treated protein exhibited ~10-fold reduced affinity for FITC-AC413(6-25) Y25P similar to the bacterially-produced proteins (Fig. 6A and Table 2). Complete deglycosylation was confirmed by SDS-PAGE with comparison to MBP-CTR ECD from *E. coli* (Fig. 6A). The decreased peptide binding affinity was a result of PNGase F enzymatic action because heat-inactivated PNGase F did not decrease the peptide affinity (Fig. 6A and Table 2).

Since core-glycosylated MBP-CTR ECD from GnTI⁻ cells had the same affinity as that bearing complex N-glycans we sought to determine the effect of further trimming the core Man₅GlcNAc₂ structures to single GlcNAc residues with Endo H. Endo H-treatment trimmed the core N-glycans as demonstrated by SDS-PAGE, but had no effect on peptide affinity (Fig. 6B and Table 2). The lack of effect was not due to the cleaved Man₅GlcNAc structures remaining in the digestion reaction because Endo H-treated MBP-CTR ECD that was purified away from the released glycans and Endo H retained wild-type peptide binding affinity (Fig. 6B and Table 2). These results strongly suggested that single GlcNAc residues at the three N-glycosylation sites in the CTR ECD are sufficient to enhance its peptide affinity. Additional control experiments indicated that the PNGase F and Endo H enzymes at the concentrations used had no effects on probe FI or anisotropy in the absence of receptor (data not shown).

N-glycans enhance peptide affinity in a heterodimeric amylin receptor ECD complex

To test if N-glycans are important for peptide binding to an AMY receptor ECD complex we used the FP assay to assess the peptide binding affinity of our previously described tethered MBP-RAMP2-CTR ECD fusion protein produced in HEK293T cells¹⁴ (Fig. 7A). This protein has an additional N-glycan site present in the RAMP2 ECD. We also produced purified N-glycan-free MBP-RAMP2-CTR ECD from *E. coli* using our denaturant-free *in vitro* DSS methodology (data not shown). For these AMY₂ receptor ECD complexes we used FITC-AC413(6-25) WT as a probe, which has lower affinity than the Y25P version, but is more similar in sequence to the endogenous Amy ligand of AMY receptors (Fig. 1B). Using proteins produced in HEK293T cells, MBP-RAMP2-CTR ECD showed higher affinity for FITC-AC413(6-25) than MBP-CTR ECD as expected for an AMY receptor and consistent with our previous results using the Alpha-LISA assay¹⁴ (Fig. 7B and Table 3). MBP-RAMP2-CTR ECD produced in *E. coli* showed markedly lower peptide affinity than the equivalent protein produced in HEK293T cells (Fig. 7B and Table 3). pK_D of FITC-AC413(6-25) was 6.03 (K_D 933 nM) for N-glycosylated MBP-RAMP2-CTR ECD versus 5.34 (K_D 4.57 μM) for the N-glycan-free protein (~5-fold decrease in affinity) (Table 3). In addition, PNGase F-catalyzed deglycosylation of HEK293T cell-produced MBP-RAMP2-CTR ECD dramatically decreased its peptide affinity (Fig. 7C). These results indicated that N-glycans also enhance peptide affinity in an AMY receptor.

Identification of the glycan site and sugar moiety responsible for CTR ECD peptide affinity enhancement

The crystal structure of sCT analog-bound N-glycan-free CTR ECD¹⁵ was used to model the N-glycans at N73, N125, and N130 (Fig. 8A). To identify which of these sites was responsible for peptide affinity enhancement, we substituted each Asn residue with Gln in MBP-CTR ECD to prevent glycosylation, expressed the mutants in HEK293T cells, and purified the proteins. The N125Q and N130Q mutants expressed at levels similar to WT. The N73Q mutant expressed poorly, but we were able to purify enough for FP assays. Each site was utilized in the HEK293T cells as evidenced by the mobility shifts of the mutants as compared to WT in SDS-PAGE (Fig. 8B). The N73Q mutation did not change the affinity of FITC-AC413(6-25) Y25P as compared to WT, while the N125Q mutation showed a minor 2-fold decrease in affinity (Fig. 8C and Table 2). The affinity of sCT(8-32) was not changed by either N73Q or N125Q mutations when pK_I was determined from competition binding assays (Table 2). In contrast, the N130Q mutation dramatically diminished probe binding, unexpectedly even to a greater degree than the effect mediated by PNGase F treatment (Fig. 8C), which suggested that the N130Q mutation had negative effects beyond glycosylation inhibition. To test this hypothesis we tried to produce MBP-CTR ECD with an N130A mutation, but this mutant did not express in HEK293T cells (data not shown).

The data presented thus far suggested that a GlcNAc residue at position N130 was sufficient to enhance peptide affinity. To provide further evidence in support of this model, we constructed MBP-CTR ECD with N73Q/N125Q double mutations and expressed and purified it from GnTI⁻ cells. The double mutant expressed very poorly, but by scaling up in roller bottles we were able to purify a quantity sufficient for FP assays. This double mutant with core glycans only at N130 retained essentially WT affinity for FITC-AC413(6-25)

Y25P and Endo H-catalyzed trimming of the glycans to a single GlcNAc had no effect on affinity (Fig. 9A and Table 2). In contrast, PNGase F treatment to completely deglycosylate position N130 significantly decreased the peptide affinity by over 10-fold (Fig. 9B and Table 2).

To confirm that N130 of Endo H-treated MBP-CTR ECD retained only a single GlcNAc residue we subjected control *E. coli*-produced MBP-CTR ECD and Endo H-treated MBP-CTR ECD from GnTI⁻ cells to LC-MS/MS after in-gel tryptic digest of their respective SDS-PAGE gel bands. The automated PLGS search of the samples did not yield any GlcNAc modified peptides. However, sugars are known to be labile and thus, a manual inspection of the peptide fragmentation pattern was warranted. Tryptic fragment 51 (T51) of the recombinant protein (containing N130) elutes at 25.0 min for the *E. coli*-produced protein but the same peptide, that supposedly has the same m/z as the control peptide (m/z 925.40) elutes at 24.1 min in the Endo H-treated sample. The main peak in the spectrum of the glycosylated protein is the mass of the control peptide plus the mass of a GlcNAc (y15²⁺) indicating that indeed, T51 in the Endo H-treated protein is modified with a single GlcNAc (Fig. 10B). We also detected GlcNAc on its own at m/z 204.08, which confirms the loss of GlcNAc upon fragmentation of the peptide. Manual inspection of the fragmentation of both peptides shows that many fragments overlap (y1 to y11) (Fig. 10A and B). However, we see y ions for both glycosylated and loss of glycosylation of the peptide at y13 (insert of Fig. 10B). This indicated that it is indeed N130 that is glycosylated. Together, the glycan site mutant and mass spectrometry results indicated that a single GlcNAc at N130 of CTR ECD is sufficient and responsible for the peptide affinity enhancement mediated by N-glycosylation.

DISCUSSION

Studies over several decades by various groups yielded mixed results with no clear answer to the question of the role of N-glycosylation in the function of the class B GPCR CTR^{14, 15, 17–19}. Defining the role of N-glycans can be challenging because it is often difficult to disentangle their effects on receptor biogenesis from effects on ligand binding and/or signaling, especially for intact receptors in cell-culture systems. Here, we directly addressed the role of N-glycans in ligand binding by characterizing the peptide hormone binding affinities of recombinant ECD proteins produced in three expression systems that each yielded a different glycosylation state: N-glycan-free ECD from *E. coli*, ECD bearing complex N-glycans from HEK293T cells, and ECD bearing Endo H-sensitive core N-glycans (Man₅GlcNAc₂) from HEK293S GnTI⁻ cells. We chose to use the soluble ECDs for this study because they contain all of the N-glycan sites, are a major determinant of hormone binding affinity, and are easier to work with than purified intact receptors.

We developed a quantitative FP assay to enable accurate assessment of the affinities of peptide hormone ligands for the recombinant proteins (Fig. 1-2 and Table 1). Using this assay, we showed that the N-glycan-free forms of the CTR ECD and a tethered RAMP2-CTR ECD complex produced in *E. coli* exhibited ~5- to 10-fold lower peptide hormone affinities than their N-glycosylated counterparts produced in HEK293 cells (Fig. 4, 5, 7 and Table 2-3). We ruled out the possibility that the *E. coli*-produced proteins were simply

misfolded by showing that PNGase F-catalyzed deglycosylation of the HEK293T-produced proteins resulted in similar diminishments of hormone affinities (Fig. 6–7 and Table 2–3). Notably, the PNGase F-treated proteins are not identical to the N-glycan-free *E. coli*-produced proteins because PNGase F action converts the Asn to Asp³². Nonetheless, the good agreement between the affinities measured for the *E. coli*-produced proteins and the PNGase F-treated proteins from HEK293T cells indicated that the absence of the N-glycan moieties was responsible for the decreased peptide affinities. These results define a clear role for N-glycans in enhancing peptide hormone affinity at the CTR and AMY₂ receptors. We did not include the AMY₁ and AMY₃ receptor ECD complexes in this study, but we suspect that CTR N-glycans also enhance hormone affinity in these receptors.

One of the impetuses for developing the FP assay was that our previous use of the Alpha-LISATM luminescent bead-based proximity assay limited us to determination of IC₅₀ values rather than equilibrium constants¹⁴. Determining equilibrium constants with Alpha technology is complicated by multivalency resulting from the two-bead assay format, multiple equilibria (the one of interest and those for each binding partner interacting with the beads), and possible steric effects resulting from immobilization of binding partners on the beads. In some cases IC₅₀ values from AlphaLISA or AlphaScreen competition assays provide reasonable estimates of the equilibrium dissociation constants^{31, 33} and this appears to be the case here as the FP and AlphaLISA assay yielded similar results for AC413 Y25P (Table 1). Moreover, our previously reported IC₅₀ of 2 μ M for sCT(8-32) binding to N-glycosylated MBP-CTR ECD by AlphaLISA assay¹⁴ agrees well with the $\sim$ 3 μ M K_I determined here using the FP assay (Table 2). In contrast, our observations of a K_I value of $\sim$ 25 μ M for sCT(8-32) binding to *E. coli*-produced N-glycan-free MBP-CTR ECD in the FP assay and K_D values of 16–35 μ M for sCT peptides binding *E. coli*-produced CTR ECD in the ITC assay are significantly different than the 50 nM IC₅₀ determined by Johansson *et al.* for binding of sCT(8-32) to their *E. coli*-produced CTR ECD in an AlphaScreen assay¹⁵. The basis for this apparent discrepancy may lie in the different binding assay formats and/or conditions used such as temperature differences (room temperature vs. 4 °C). The μ M sCT affinity we observed here for the CTR ECD is in line with the μ M affinities reported for several other class B GPCR ECDs binding their natural peptide ligands^{31, 33–35}.

Through the use of N→Q glycan site substitutions we identified N130 as the site responsible for peptide hormone affinity enhancement in CTR (Fig. 8–9). We did not determine if this site is also responsible for affinity enhancement in the AMY₂ receptor ECD complex, but we suspect this to be the case. Our data for N73 and N130 are consistent with those of Ho *et al.* who examined N→A glycan site substitutions in intact CTR expressed in COS-1 cells¹⁹. Their N73A mutant exhibited wild-type sCT signaling response and their N130A mutant had $\sim$ 40-fold reduced sCT potency. Their N130A data can now be understood as resulting—at least in part—from decreased sCT binding affinity due to the loss of the N130 N-glycans. In contrast, whereas they observed a 10-fold reduced sCT signaling potency with N125A, we observed no effect of N125Q on sCT(8-32) ECD-binding affinity. One explanation for this discrepancy is that the N125 N-glycan might be important in the intact receptor, but not in the isolated ECD. Alternatively, their result may have been due to an effect of the N125A mutation on CTR biogenesis and/or structure. The N-glycan sites can significantly affect receptor expression even for the ECD. Our N125Q and N130Q mutants expressed at levels

similar to wild-type, but the N73Q and N73Q/N125Q mutants expressed poorly and we were unable to obtain any N130A CTR ECD. The extreme difference in expression behavior of the N130Q and N130A mutants shows how subtle differences at N-glycan sites can significantly affect receptor biogenesis. Also notable was the difference in magnitude of the defect in peptide binding observed for the N130Q mutant (Fig. 8C) vs. the PNGase F-treated proteins (N→D) (Fig. 6, 9), which suggested that N130Q affected ECD folding and/or structure beyond simple loss of the N-glycan. These results emphasize that caution is needed when interpreting results from glycan site mutants expressed in cell culture systems and highlight the value of studies of purified receptor glycoforms and glycan site mutants such as those presented here for defining N-glycan function(s).

While this paper was under review Liang *et al.* reported a cryo-EM structure of full-length CTR in complex with sCT and heterotrimeric G protein using glycosylated CTR expressed in insect cells³⁶. The resolution of this structure was particularly low for the receptor ECD apparently due to interdomain flexibility and the ECD and peptide were not included in the deposited model. Nonetheless, they observed density in the cryo-EM map that they attributed to N-glycans at position N130 of the ECD and they further assessed the role of CTR N-glycosylation in hormone binding and signaling using N→D glycan site mutants in full-length CTR expressed in COS-7 cells. They observed an ~10-fold decrease in sCT binding affinity and a corresponding ~10-fold decrease in cAMP signaling potency for the CTR N130D mutant. The N130D substitution had no effect on $E_{\max}$ in the signaling assay consistent with the idea that the N130 glycan only affects hormone binding affinity and not efficacy. N73D had no effect on binding and signaling and their N125D mutant exhibited only a minor defect in binding and signaling. These results are in excellent agreement with our findings with purified proteins and indicate that the CTR N130 N-glycan enhancement of hormone binding affinity is relevant for downstream signaling. The two studies thus complement each other to define the role of CTR N-glycans in hormone binding and signaling.

Trimming the recombinant CTR ECD N-glycans with Endo H allowed us to determine that a single GlcNAc residue at N130 is the sugar moiety responsible for the peptide hormone affinity enhancement (Fig. 6B, 9A). The presence of only a single GlcNAc residue at N130 was confirmed by LC-MS/MS (Fig. 10). N130 is the closest of the three N-glycan sites to the peptide-binding site (Fig. 8A); however, modeling a GlcNAc at this position suggested that it is likely still too distant to directly contact the bound peptide. We therefore favor a model whereby the N130 GlcNAc alters CTR ECD conformation to enhance hormone affinity, thus functioning through an allosteric mechanism. Although speculative, a putative allosteric mechanism might involve the N130 GlcNAc altering the conformation of the adjacent CTR ECD loop that contacts the peptide hormone β -turn (Fig. 8A). Future studies are needed to test this hypothesis.

Our results and those of Liang *et al.*³⁶ provide the first clear evidence defining an important role for N-glycans in ligand binding by a class B GPCR. The role of N-glycans has been studied for several other class B GPCRs using glycosylation pathway inhibitors, PNGase F treatments, and/or glycan site mutant strategies with the receptors expressed in cell culture. In many cases the N-glycans play an important role in trafficking the receptors to the cell

surface^{37–41}, but this is not true for all receptors^{42,43}. For several receptors there is good evidence that the N-glycans are not required for normal ligand binding and signaling^{38,39,43,44}, whereas for others the data are less clear^{45–47}. It thus remains to be determined if CTR is unique or if there are other class B GPCRs for which their N-glycans modulate ligand binding and/or signaling. Notably, it was recently demonstrated that N-glycans in the class A GPCR PAR1 regulate its G-protein signaling bias⁴⁸. Clearly GPCR N-glycans can modulate their pharmacology and it will be important to determine the structural basis of these effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Principle of the fluorescence polarization/anisotropy peptide-binding assay and sequence alignment of peptides related to this study. A, Format of the fluorescence polarization/anisotropy assay. FITC, fluorescein isothiocyanate; MBP, maltose-binding protein; CTR, calcitonin receptor ECD. B, Alignment of antagonist peptide fragments is shown using ClusterX2.1 with %Equivalent of sequence similarity depiction parameter. Similar residues were shown in black bold character and yellow boxes and identical residues in red boxes.

Figure 2.

FITC-labeling increased AC413(6-25) Y25P affinity to MBP-CTR ECD. A, FP assay in saturation binding format. 10 nM of FITC-labeled AC413(6-25) Y25P and varying concentrations of MBP-CTR produced from HEK293T cells were incubated for 1 h, then parallel and perpendicular fluorescence intensities were measured. B, FP assay in competition binding format with non-labeled AC413(6-25) Y25P. 10 nM of FITC-labeled AC413(6-25) Y25P, 90 nM of MBP-CTR ECD and AC413(6-25) Y25P at varying concentrations were incubated for 2 h before fluorescence intensity measurement. C, Competition Alpha-LISA assay with 150 nM of MBP-CTR ECD produced from HEK293T cells, 150 nM of biotin-labeled sCT, and the indicated competitor peptides concentrations. 10 μ M fluorescein was used as a control for interference of FITC with Alpha-LISA signal. For this and all subsequent figures data shown are a representative experiment from three independent experiments each performed with duplicate samples unless otherwise noted. Error bars represent the S.E.M. of the duplicate samples.

Figure 3.

Production of three distinct glycoforms of recombinant MBP-CTR ECD. A, Comparison of *E. coli*-produced MBP-CTR ECD before and after *in vitro* disulfide shuffling shown in a 12% Tris-borate native gel. B, Gel filtration elution profiles of MBP-CTR ECD produced from three distinct expression systems. Pooled fractions used for the study were light orange-colored. C, Non-reducing SDS-PAGE and Tris-glycine native gel analysis of the purified MBP-CTR ECD samples. Molecular mass markers are in kDa. Gels were coomassie-blue stained.

Figure 4.

N-glycosylation of MBP-CTR ECD significantly enhanced peptide hormone affinity. A, FP assay in saturation binding format. MBP-CTR ECD produced from three expression systems at varying concentrations was incubated with 10 nM of FITC-labeled AC413(6-25) Y25P. B, FP assay in competition binding format. 90 nM of MBP-CTR ECD produced from HEK293T or GnTI⁻ cells or 3000 nM of MBP-CTR ECD produced from *E. coli* were incubated with 10 nM of FITC-labeled AC413(6-25) Y25P and sCT(8-32) competitor peptide at varying concentrations. Anisotropy was expressed as % of Max anisotropy assuming the anisotropy obtained from the reaction mixture without a competitor as 100%. Competition binding curves of sCT(8-32) with three distinct MBP-CTR ECD proteins were superimposed into one figure.

Figure 5.

Purification and function of MBP-free CTR ECD refolded from *E. coli* inclusion bodies. A, SDS-PAGE showing the purified ECD after IMAC and gel filtration chromatography purification steps. Non-adjacent lanes from the same gel are shown. Molecular mass markers are in kDa. The gel was coomassie-blue stained. B, ITC experiment performed at 25 °C with 16.5 μM CTR ECD in the cell and 165 μM sCT(1-32) in the syringe. Fitting to a 1:1 binding model yielded $K_D=35\ \mu\text{M}$, $n=0.89$, $H=-60.8\ \text{kcal/mol}$, and $S=-183\ \text{cal/mol/K}$. C, ITC experiment performed at 25 °C with 28.3 μM CTR ECD in the cell and 283 μM sCT(8-32) in the syringe. Fitting to a 1:1 binding model yielded $K_D=16\ \mu\text{M}$, $n=1.17$, $H=-24.2\ \text{kcal/mol}$, and $S=-59.3\ \text{cal/mol/K}$. Data shown are from single experiments.

Figure 6.

Effects of PNGase F- or Endo H-catalyzed glycan alteration on peptide affinity to MBP-CTR ECD. A, FP assay with HEK293T-produced MBP-CTR ECD treated with PNGase F at 37°C for 4 h. PNGase F heat-inactivated (HI) at 75°C for 10 min was used as a control. Reducing SDS-PAGE with corresponding samples is shown on the right side. MBP-CTR ECD produced from *E. coli* was used as an N-glycan-free control. B, FP assay with GnTI⁻-produced MBP-CTR ECD treated with Endo H at 22°C for 30 min. Endo H heat-inactivated (HI) at 75°C for 10 min was used as a control. Endo-H treated MBP-CTR ECD was purified away from the enzyme and released glycans by IMAC and gel filtration chromatography. Reducing SDS-PAGE with corresponding samples is shown on the right side. MBP-CTR ECD produced from *E. coli* was used as an N-glycan-free control. * indicates either PNGase F (Panel A) or Endo H (Panel B). Conditions for FP assay are the same as in Figure 4A. Molecular mass markers are in kDa. The gels were coomassie-blue stained.

Figure 7.

CTR N-glycans enhanced peptide affinity for a heterodimeric amylin receptor ECD complex. A, Overview of MBP-RAMP2-CTR ECD fusion binding to FITC-labeled peptide. B, FP assay with MBP-CTR ECD produced from HEK293T cells and MBP-RAMP2-CTR ECD fusion produced from either HEK293T cells or *E. coli*. 10 nM of FITC-labeled AC413 WT was incubated with receptor ECDs at varying concentrations. C, FP assay with MBP-RAMP2-CTR ECD fusion deglycosylated with PNGase F at 37°C for 5 h. PNGase F heat-inactivated (HI) at 75°C for 10 min was used as a control. Reducing SDS-PAGE with corresponding samples was shown on the right side. MBP-RAMP2-CTR ECD fusion produced from *E. coli* was used as an N-glycan-free control. * indicates PNGase F. Conditions for FP assay are the same as shown in panel B. Molecular mass markers are in kDa. The gel was coomassie-blue stained.

Figure 8.

N-glycosylation at Asn 130 of MBP-CTR ECD was critical for peptide binding affinity. A, Orthogonal views of the sCT analog-bound CTR ECD crystal structure (PDB: 5II0) with single GlcNAc residues modeled at N73, N125, and N130. The CTR ECD loop above the bound sCT is dark blue-colored, and the interacting counterpart of sCT β-turn residues are red-colored. B, Non-reducing SDS-PAGE with purified MBP-CTR ECD glycan site mutants. MBP-CTR ECD produced from GnTI⁻ cells and from *E. coli* was used as a core glycan-containing control and an N-glycan-free control, respectively. Molecular mass markers are in kDa. The gel was coomassie-blue stained. C, FP assay with MBP-CTR ECD glycan site mutants. Conditions for FP assay were the same as in Figure 4A.

Figure 9.

A single GlcNAc at N130 of MBP-CTR ECD was sufficient for peptide affinity enhancement mediated by N-glycosylation. A, FP assay with MBP-CTR ECD N73Q/N125Q double mutant treated with Endo H at 22°C for 30 min. Reducing SDS-PAGE with corresponding samples is shown on the right side. MBP-CTR ECD produced from *E. coli* was used as an N-glycan-free control. B, FP assay with MBP-CTR ECD N73Q/N125Q double mutant treated with PNGase F at 37°C for 4 h. PNGase F heat-inactivated (HI) at 75°C for 10 min was used as a control. Reducing SDS-PAGE with corresponding samples is shown on the right side. MBP-CTR ECD produced from *E. coli* was used as an N-glycan-free control. * indicates either Endo H (Panel A) or PNGase F (Panel B). Molecular mass markers are in kDa. The gels were coomassie-blue stained. Conditions for FP assays were the same as in Figure 4A.

Figure 10.

Mass spectrometry analysis of the N130-containing tryptic fragment 51 (T51) peptide of MBP-CTR ECD. A, Nano LC-MS/MS spectrum of the T51 peptide of the doubly protonated peptide ion 925.40²⁺ of the control MBP-CTR ECD produced in *E. coli*. B, Nano LC-MS/MS spectrum of the T51 peptide of the doubly protonated peptide ion 1026.95²⁺ of Endo H-treated MBP-CTR ECD produced in GnTII⁻ cells. The arrow indicates the neutral loss of GlcNAc. The b and y ions with a 0 are ions with a GlcNAc neutral loss. The absence of GlcNAc neutral loss until y10 clearly indicates that a GlcNAc is bound to N130. The black circle represents a free GlcNAc (m/z 204.08, 203.08 Da).

Table 1
Effect of FITC-labeling on peptide affinity for MBP-CTR ECD from HEK293T cells

	FITC-AC413(6-25) Y25P			AC413(6-25) Y25P		
	n	Mean	S.E.M.	n	Mean	S.E.M.
pK _D or pK _i (FP assay)	3	6.94 ^a	0.08	3	6.20	0.06
pIC ₅₀ (α-LISA)	3	7.32 ^a	0.06	3	6.15	0.07

^a *p* < 0.05 compared to AC413(6-25) Y25P.
Student's *t* test was used for statistical analysis.

Table 2
Peptide affinities measured by FP assays for MBP-CTR ECD glycoforms and glycan site mutants

ECD protein	Expression system	Treatment	pK _D			pK _I		
			n	Mean	S.E.M.	n	Mean	S.E.M.
CTR WT	HEK293T	None	6	7.04	0.01	3	5.46	0.01
		– PNGase F	3	7.07	0.01			
		+ PNGase F	3	5.92 ^b	0.02			
		+ HI PNGase F	3	7.06	0.01			
		None	3	7.05	0.02	3	5.50	0.04
GnTI [−]	GnTI [−]	– Endo H	3	7.02	0.02			
		+ Endo H	3	6.95	0.03			
		+ HI Endo H	3	7.06	0.05			
		+ Endo H &	3	6.85 ^{c*}	0.01			
		Purified						
CTR N73Q	HEK293T	None	3	5.94 ^a	0.02	3	4.61 ^a	0.03
		None	3	6.98	0.09	3	5.63	0.06
		None	3	6.70 ^a	0.02	3	5.43	0.04
		None	3	< 4.5				
		– PNGase F	3	6.91	0.05			
CTR N125Q	HEK293T	+ PNGase F	3	5.85 ^b	0.10			
		+ HI PNGase F	2	6.97	0.03			
		– Endo H	3	6.93	0.01			
		+ Endo H	3	6.87 ^{c*}	0.02			

^a $p < 0.05$ compared to the CTR ECD WT expressed in HEK293T cells,

^b $p < 0.05$ compared to – PNGase F,

^c $p < 0.05$ compared to – Endo H.

* No more than 1.5-fold change in K_D values when compared to –Endo H.

ANOVA with Tukey's post hoc test or Student's *t* test (for two groups) was used for statistical analysis.

Table 3

Affinities measured for AC413 at CTR and AMY2 ECDs by FP assays

ECD protein	Expression system	Treatment	pK _D		
			n	Mean	S.E.M.
CTR	HEK293T	None	3	5.44	0.03
RAMP2-CTR	HEK293T	None	3	6.03 ^a	0.07
		– PNGase F	3	6.01	0.05
		+ PNGase F	3	< 5.5	
		+ HI PNGase F	3	5.99	0.05
	<i>E.coli</i>	None	3	5.34 ^b	0.06

^a $p < 0.05$ compared to the CTR ECD expressed in HEK293T cells.
^b $p < 0.05$ compared to the RAMP2-CTR ECD fusion expressed in HEK293T cells.
ANOVA with Tukey's post hoc test was used for statistical analysis.