Site-specific O-Glycosylation by Polypeptide N-Acetylgalactosaminylltransferase 2 (GalNAc-transferase T2) Co-regulates β₁-Adrenergic Receptor N-terminal Cleavage*§

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The β₁-adrenergic receptor (β₁AR) is a G protein-coupled receptor (GPCR) and the predominant adrenergic receptor subtype in the heart, where it mediates cardiac contractility and the force of contraction. Although it is the most important target for β-adrenergic antagonists, such as β-blockers, relatively little is yet known about its regulation. We have shown previously that β₁AR undergoes constitutive and regulated N-terminal cleavage participating in receptor down-regulation and, moreover, that the receptor is modified by O-glycosylation. Here we demonstrate that the polypeptide GalNAc-transferase 2 (GalNAc-T2) specifically O-glycosylates β₁AR at five residues in the extracellular N terminus, including the Ser-49 residue at the location of the common S49G single-nucleotide polymorphism. Using in vitro O-glycosylation and proteolytic cleavage assays, a cell line deficient in O-glycosylation, GalNAc-T-edited cell line model systems, and a GalNAc-T2 knock-out rat model, we show that GalNAc-T2 co-regulates the metalloproteinase-mediated limited proteolysis of β₁AR. Furthermore, we demonstrate that impaired O-glycosylation and enhanced proteolysis lead to attenuated receptor signaling, because the maximal response elicited by the BAR agonist isoproterenol and its potency in a cAMP accumulation assay were decreased in HEK293 cells lacking GalNAc-T2. Our findings reveal, for the first time, a GPCR as a target for co-regulatory functions of site-specific O-glycosylation mediated by a unique GalNAc-T isoform. The results provide a new level of possibilities for new therapeutic strategies for cardiovascular diseases.

β-Adrenergic receptors (BARs) are G protein-coupled receptors (GPCRs) that activate intracellular signaling pathways mainly via the stimulatory Gs protein after binding of agonists such as adrenaline and noradrenaline (1, 2). The BARs exist as three subtypes, β₁, β₂, and β₃, and the former two are important in the regulation of the excitation-contraction coupling of the myocardium. The β₁AR is the predominant βAR subtype expressed in the heart and the main mediator of the endogenous catecholamine-stimulated positive chronotropy and inotropy (1, 2). Thus, it is the most important target receptor for β-adrenergic antagonists, also called β-blockers, which are widely used in the treatment of cardiac diseases, such as chronic heart failure, coronary artery disease, arrhythmias, and hypertension. However, these therapeutic agents have limited effectiveness in some patients and also exert adverse effects. Consequently, there is a growing need to better understand the underlying mechanisms in cardiac function and disease to develop alternative and more individualized treatment options that can improve clinical outcomes.

During chronic heart failure, a persistent compensatory increase of catecholamines causes β₁AR desensitization and down-regulation. The density of β₁ARs at the plasma membrane is reduced by 50%, whereas that of β₂ARs does not seem to change (3). The resulting partial loss of β₁AR function is believed to be an adaptive mechanism to counteract the cardio-toxicity of chronic adrenergic signaling (4). We have shown previously that β₁AR undergoes limited N-terminal cleavage by metalloproteinases in heterologous expression systems as well as in vivo in rat neonatal cardiomyocytes (5, 6), indicating that limited proteolysis has a regulatory function in receptor down-regulation. We identified two cleavage sites for the human receptor at positions 31R→L²₂ and 35P→L₅₃ in the extracellular

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The abbreviations used are: BAR, β-adrenergic receptor; hβ₁AR, human β₁-adrenergic receptor; ADAM, a disintegrin and metalloproteinase; GalNAc-T, polypeptide GalNAc-transferase; GPCR, G protein-coupled receptor; MMP, matrix metalloproteinase; PAR, proteinase-activated receptors; PC, proprotein convertase; PNGase F, peptide-N-glycosidase F; Endo H, endo-N-acetylglucosaminidase H; hδOR, human δ-opioid receptor.
N terminus and demonstrated that the cleavage at the major site 31R L32 is augmented by agonist-mediated activation of the receptor (5, 6). We also found that β AR undergoes GalNAc-type O-glycosylation (hereafter simply referred to as O-glycosylation) (5), and the NetOGlyc4.0 prediction algorithm predicts the presence of five O-glycan sites in the receptor N-terminal extracellular sequence (7).

O-Glycosylation is found on a majority of proteins passing through the secretory pathway (7). It is a unique type of protein glycosylation because it is differentially regulated by a large family of up to 20 distinct polypeptide GalNAc-transferase isoenzymes (GalNAc-Ts) that catalyze the addition of the first GalNAc residue to Ser and Thr residues (8). These GalNAc-T isoforms have distinct, albeit partly overlapping, acceptor substrate specificities, and they are differentially expressed in cells and tissues, providing a unique scenario for differential and dynamic regulation of site-specific O-glycosylation (8). Whereas tightly spaced O-glycans found in unstructured regions of proteins that serve as linkers between folded domains or as stem regions of membrane proteins may serve to protect these regions from general proteolysis (9–12), it is becoming clear that individual O-glycosites may serve more specific and co-regulatory roles of limited proteolytic processing events with important biological functions. The first example with clear medical relevance was discovered studying the rare disease familial tumoral calcinosis caused by a deficiency in GALNT3, encoding one of the many GalNAc-Ts (13). The loss of GalNAc-T3 was found to eliminate a single O-glycosylation site in fibroblast growth factor 23 adjacent to a proteolytic processing site, thus protecting the central part of the receptor N terminus flanking two previously identified cleavage sites at 31R L32 and 52P L32 (Fig. 1; see also Fig. 3A). The peptides were used for in vitro O-glycosylation assays with a panel of 10 different recombinantly expressed GalNAc-Ts (T1, T2, T3, T4, T5, T11, T12, T13, T14, and T16). This in vitro analysis has been shown previously to be quite predictive of O-glycosylation in vivo (7, 17, 26). Glycosylated peptides were characterized by electrospray ionization–linear ion trap–Fourier transform–MS for site identification. We found that GalNAc-T2 glycosylated both peptides at three positions corresponding to a total of five residues, Thr-28, Ser-37, Ser-41, Ser-47, and Ser-49, in the β AR N terminus (Fig. 1). GalNAc-T3 glycosylated the peptides at three positions (Thr-28, Ser-37, and Ser-41) (Fig. 1) and GalNAc-T1 and GalNAc-T11 at one position each (not determined). However, these latter reactions were slow and never reached completion in an overnight experiment. Thus, the results suggest that GalNAc-T2 is the major isoform controlling O-glycosylation of β AR and initiates O-glycosylation at five Ser/Thr residues in the N-terminal ectodomain of the receptor.

**Site-specific O-Glycosylation by GalNAc-T2 Modulates the in Vitro Proteolytic Cleavage of β AR N-terminal Peptides**

To systematically evaluate the effect of GalNAc O-glycosylation on the metalloproteinase-mediated β AR cleavage, we performed in vitro cleavage assays of peptides and GalNAc glycopeptides using four ADAMs (ADAM8, 10, -12, and -17) and nine matrix metalloproteinases (MMP1, -2, -3, -7, -8, -9, -12, -13, and -14). Of the four ADAMs analyzed, we found that ADAM17 cleaved peptide II at 52P L32 and this was inhibited by GalNAc-T2 glycosylation (Fig. 2A). A comparable effect of glycosylation was observed at 31R L32 of peptide I (Fig. 2A). Furthermore, we found that ADAM10 cleaved peptide I at 30A R31; however, glycosylation did not affect the processing. ADAM8 and -12 did not cleave either of the two peptides (data not shown). Of the panel of nine MMPs tested, several members of this metalloproteinase family cleaved the peptides at 31R L32 and 52P L32 as observed with ADAM17. MMP2 and
GalNAc-T2 Fine-tunes β₂AR Ectodomain Cleavage

FIGURE 1. In vitro analysis of GalNAc-T isoform specificity and a summary of detected glycosylation sites of hβ₂AR. Shown is in vitro O-glycosylation analysis with GalNAc-T1, T2, T3, T4, T5, T11, T12, T13, T14, and T16 of two overlapping peptides (peptides I and II) covering the β₂AR N terminus. Reactions were followed by MALDI-TOF-MS, and spectra are shown for a 24-h incubation time point for T1, T2, T3, and T11. GalNAc-T2 was found to be the most efficient at glycosylating the two peptides, with a total of five sites corresponding to Thr-28, Ser-37, Ser-41, Ser-47, and Ser-49 in the full-length protein. GalNAc-T3 modified three sites at Thr-28, Ser-37, and Ser-41; GalNAc-T1 and GalNAc-T11 modified one position each (not determined). The spectra show relative intensity. The complete hβ₂AR N-terminal sequence and peptide sequences are shown above the spectra. The locations of the attached GalNAcs are shown as gray squares. The results are representative of three independent experiments. TM, transmembrane domain.

FIGURE 2. Site-specific O-glycosylation modulates the in vitro cleavage of hβ₂AR N-terminal peptides. A, in vitro cleavage analysis of β₂AR peptide I and II and respective glycopeptides by ADAM17 monitored by MALDI-TOF. Products formed after 30-min, 1-h, and 4-h incubations are shown. ADAM17 was able to cleave both the naked peptide and the O-glycopeptide at the 31R↓L32 and 52P↓L53 cleavage sites. However, the cleavage of the glycopeptides was less efficient. B, in vitro cleavage analysis of β₂AR peptide I and O-glycopeptide by MMP9. Cleavage was observed at 41S↓L42, and again, the glycopeptide was less efficiently cleaved. C, schematic overview of the total observed cleavage sites by ADAM10, ADAM17, and MMPs, and their relation to the observed glycosylation sites. The boldface font indicates that a protection from cleavage was observed. The spectra show relative intensity. The locations of the attached GalNAcs are shown as gray squares. The results shown are representative of three (A) and two (B) independent experiments.

-3 cleaved peptide I at 31R↓L32 (Fig. 2C) (data not shown), and MMP3, -7, -8, -12, -13, and -14 cleaved peptide II at 52P↓L53 (Fig. 2C) (data not shown). Unexpectedly, we found that MMP2, -7, -8, -9, -12, and -13 cleaved both peptides at a previously uncharacterized cleavage site at 41S↓L42 (Fig. 2C) (data not shown), and this cleavage was also inhibited by O-glycosylation, as exemplified by the MMP9 cleavage (Fig. 2B). Here we analyzed the effect of a simple GalNAc O-glycan; however, it is
GalNac-T2 Fine-tunes β,AR Ectodomain Cleavage

**FIGURE 3.** N-terminal cleavage of hβ3, AR is enhanced in CHO-ldlD cells deficient in GalNAc-type O-glycosylation. A, receptor model highlighting the extracellular N terminus with five O-glycosylation sites (gray squares) and the two cleavage sites and one N-glycosylation site identified previously (5). The two peptides (P1 and PII) used in the in vitro assays and the N- and C-terminal epitope tags are also indicated. B–E, G, and H, the WT and the R31H/L32A cleavage site mutant Myc-hβ3-AR-FLAG and the Myc-hiOR-FLAG were transiently expressed in CHO-K1, CHO-ldlD, or HEK293 cells for 24 h. For ldlD cells, the low serum culture medium (2% FBS (E and G) and 0.5% FBS (H)) was supplemented or not with Gal (20 μM) and/or GalNAc (400 μM). The CHO-K1 cells were cultured at 5% FBS. Expressed receptors were immunoprecipitated from solubilized membranes (B–D) or cellular lysates (E, G, and H) and analyzed by Western blotting. For C and D, the purified receptors were first digested or not with Endo H or PNGase F. The ratios of full-length (black bars) and cleaved (white bars) β,AR species in iDlD cells shown in E are depicted in F. The repeated measures two-way analysis of variance followed by Tukey’s post hoc test was used to compare the ratios in cells supplemented with both Gal and GalNAc with those cultured in the presence of no sugars or only with GalNAc (a). The ratios observed in the two latter culture conditions were also compared (b). ***, p < 0.001. Error bars, S.E. The results are representative of two (B), five (E), four (G), and three (H) independent experiments. Deglycosylation (C and D) was performed once.

likely that extended O-glycans with sialic acids have more pronounced effects on the adjacent proteolysis (14). Taken together, these results indicate that several metalloproteinases in the ADAM and MMP families are able to cleave the β,AR N-terminal peptides at multiple sites.

**O-Glycans Affect β,AR N-terminal Cleavage in Intact Cells—** To further investigate the role of β,AR O-glycosylation, we used the CHO-iDlD cell model system, in which a deficient UDP-Gal/UDP-GalNAc C4-epimerase allows to control O-glycosylation by the addition of exogenous Gal and GalNAc sugars to the cell culture medium (11, 27). Without sugars added to the medium, CHO-iDlD cells do not perform O-glycosylation and galactosylation of N-glycans, whereas with the addition of GalNAc, O-glycosylation is limited to simple GalNAc O-glycans, and by adding both GalNAc and Gal, O- and N-glycosylation are almost fully restored to the level of the parental cell line CHO-K1.

We first expressed β,AR in CHO-K1 cells to confirm that the expressed receptor forms are comparable with those shown previously for HEK293 cells (5, 6). Western blotting using the FLAG M2 antibody that recognizes the C-terminal epitope tag revealed two receptor forms (69 and 54 kDa) in both CHO-K1 and HEK293 cells (Fig. 3B), whereas only the larger 69-kDa form was detected with the c-Myc antibody directed against the N-terminal epitope tag (Fig. 3B). As expected, the larger molecular weight species was digested with peptide-N-glycosidase F (PNGase F) but not with endo-N-acetylglucosaminidase H (Endo H) (Fig. 3C), indicating that it represents the full-length receptor carrying fully processed N-glycans. In contrast, the smaller 54-kDa species was resistant to both enzymes, in line with the expectation that it corresponds to the C-terminal fragment cleaved at the main cleavage site at Asp138 and not to the similar sized receptor precursor (5, 6). In the same conditions, Endo H readily digested the human δ-opioid receptor (δiOR) precursor (Fig. 3D) (19, 28). A 47-kDa β,AR C-terminal fragment cleaved at Lys52 was seen occasionally and only in HEK293 cells (see Fig. 4A).

When expressed in the CHO-iDlD cells without Gal/GalNAc addition, β,AR showed a significant increase in cleavage compared with CHO-K1 cells. The full-length receptor in the iDlD cells migrated at about 57 kDa, and its relative amount was decreased compared with the full-length 69-kDa receptor form seen in CHO-K1 cells (Fig. 3E, lanes 6 and 10, respectively). Concomitantly, there was a significant increase in the relative amount of the corresponding cleaved receptor forms (Fig. 3, E (lanes 1 and 5) and F). Upon the addition of Gal, no clear changes in the relative amount of cleaved receptor forms were detected compared with the corresponding control cells (Fig.
A significant decrease of receptor cleavage (Fig. 3, representative of four (E, lanes 4 and 5) and F). Taken together, these results are fully in line with the in vitro data and show that β1AR O-glycosylation has a protective effect on the proteolysis of the receptor N terminus. Furthermore, the results indicate that the addition of GalNAc alone provides detectable, albeit partial, protection from proteolytic cleavage.

We have shown previously that a mutation of 31R to 31H, flanking the major β1AR cleavage site, inhibits cleavage at this site almost completely when the mutant receptor is expressed in HEK293 cells (5). Thus, we tested the effect of the lack of O-glycosylation on the cleavage of the mutant receptor. As shown in Fig. 3G, the R31H/L32A mutant was cleaved almost to the same extent as the WT receptor when expressed in CHO-ldlD cells without Gal and GalNAc (compare lanes 1 in Fig. 3, G and E). In comparison, GalNAc addition into the culture medium blocked the cleavage almost completely, to the same extent as the full restoration of O-glycosylation by adding both Gal and GalNAc (Fig. 3G, lanes 2 and 3, respectively). These results provide further evidence that the accessibility of metalloproteinases to the receptor cleavage site(s) is significantly modulated by the adjacent O-glycans. Interestingly, for the R31H/L32A mutant (but not for the WT), a smear of smaller molecular mass receptor forms below the 69-kDa full-length receptor was detected when the cells were cultured at lower serum conditions (0.5% FBS instead of 2% FBS used routinely) (Fig. 3H). This is probably because of up-regulated metalloproteinases in the more rigorous culture conditions. This finding could be related to our observation that the tested peptides could be cleaved at alternative sites in the in vitro cleavage assays (Fig. 2). Thus, taking this evidence together, we conclude that the intact β1AR N terminus is a substrate for several metalloproteinases and/or is cleaved at several adjacent sites.

The β1AR N-terminal Cleavage Is Co-regulated by GalNAc-T2 in Human Cell Lines HEK293 and HepG2—The in vitro results pointed to GalNAc-T2 as the major enzyme modifying β1AR but also showed that GalNAc-T3 and to a lesser extent GalNAc-T1 and -T11 were potentially able to glycosylate the β1AR N-terminal peptides. Importantly, although widely expressed, GalNAc-T3 is not found in the heart (8). We therefore used two different isogenic cell line model systems genetically engineered to lack specific GalNAc-Ts to probe isoform specificity in cells with different GalNAc-T expression profiles. HEK293 cells are known to express GalNAc-T1, T2, T3, and T11 (7) and have been used extensively to study the N-terminal cleavage and function of β1AR (5, 6, 29). HepG2 cells express GalNAc-T2, T1, and T11, but not GalNAc-T3 (30), making the GalNAc-T expression profile of HepG2 cells comparable with that described for the heart (8). Thus, we used HEK293 and HepG2 cells with a knock-out of GALN-T2 (ΔT2), HepG2 cells with a knock-out of GALN-T1 (ΔT1) and T11 (ΔT11), and a HepG2 rescue cell line (ΔT2 + T2) with site-directed re-insertion of the GalNAc-T2 coding cDNA into the adeno-associated virus integration site 1 (AAVS1) (30). Furthermore, to confirm the protective effect of truncated O-glycans observed in the CHO-ldlD cells, we used a HEK293 COSMC knock-out cell forms (Fig. 3E, lanes 3 and 8), probably reflecting differentially O-glycosylated receptors. Finally, when both Gal and GalNAc were added, the cleavage was restored to the same level seen in CHO-K1 cells, (Fig. 3, E, lanes 4 and 5) and F). The results shown are fully in line with the in vitro data and show that β1AR O-glycosylation has a protective effect on the proteolysis of the receptor N terminus. Furthermore, the results indicate that the addition of GalNAc alone provides detectable, albeit partial, protection from proteolytic cleavage.

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3E, lanes 1 and 2). However, as expected, the migration of the full-length receptor species was slowed down consistently with the expected restoration of receptor N-glycosylation (Fig. 3E, lanes 2 and 7). Importantly, the addition of GalNAc resulted in a significant decrease of receptor cleavage (Fig. 3, E (lane 3) and F) and in the appearance of a heterogeneous smear of receptor
model, designated HEK293SimpleCells (31). Cosmc is a chaperone for the C1GALT1 core 1 enzyme. Thus, O-glycans are not elongated in SimpleCells, which consequently produce truncated GalNAc O-glycans similar to those produced in CHO-lID cells cultured in the presence of GalNAc alone.

When expressed in HEK293AT2 cells, βAR migrated slightly faster on SDS-PAGE compared with the HEK293WT cells and presented a heterogeneous smear below the major receptor form (Fig. 4A, lanes 2 and 1, respectively, first panel). This suggests a loss of one or more O-glycans in the former cell line. Importantly, there was a significant increase in the relative amount of the two N-terminally cleaved βAR, forms detected with the FLAG M2 antibody (Fig. 4, A (lane 2, second panel) and B). The smaller of these cleaved forms represents a receptor fragment cleaved at 52P↓L33, because it co-migrated with the 47-kDa form observed in the HEK293WT cells (Fig. 4A, lanes 1 and 2, second panel). The larger form probably corresponds to a C-terminal fragment with fewer O-glycans, cleaved at the main cleavage site at 31R↓L32. The smallest receptor form was not seen in all experiments.

We next expressed βAR in HEK293SimpleCells. The observed clear decrease in the apparent molecular mass of the full-length receptor (Fig. 4A, lane 3, first panel) is in line with the expectation that O-glycans are not elongated in these cells (7). In some experiments, the receptor was more extensively cleaved in these cells compared with the HEK293WT cells (Fig. 4A, lanes 3 and 1, respectively, second panel). However, this difference was not consistent and did not reach statistical significance (Fig. 4B). Thus, these results suggest that in HEK293 cells, truncated O-glycans are able to protect the receptor from cleavage, at least partially, in a similar manner as in CHO-lID cells.

To confirm that the enhanced receptor processing observed in the HEK293AT2 cells was mediated by metalloproteinases, we treated the cells with a broad range metalloproteinase inhibitor, marimastat, during transfection. This decreased the amount of the smaller N-terminally cleaved receptor forms with a concomitant increase in the amount of full-length receptors (Fig. 4C, lanes 3 and 4). Similar results were obtained for the HEK293WT cells (Fig. 4C, lanes 1 and 2). This verifies that the smallest receptor forms detected in these cells are the result of metalloproteinase-mediated cleavage.

To investigate the role of GalNAc-T2-mediated O-glycosylation in a cell line without GalNAc-T3, we next expressed βAR in a panel of isogenic HepG2 cells with knock-out or knock-in of specific GalNAc-Ts. We observed a significant increase in receptor cleavage in HepG2ΔT2 cells that was reversed in the rescue HepG2ΔT2 + T2 cell line (Fig. 5, A (lanes 1–3) and B). The cleavage of βAR was consistently more pronounced in the WT HepG2 cells compared with the corresponding HEK293 cells (compare Fig. 4A with Fig. 5A and Fig. 4B with Fig. 5B), which is probably due to partial compensatory glycosylation in HEK293 cells or other cell-dependent differences in O-glycosylation capacities and/or protease activities in the two cell lines.

The cleaved receptor fragments seen in the HepG2ΔT2 cells migrated as a doublet (Fig. 5A, lane 2), most likely representing differentially O-glycosylated and non-modified receptor fragments cleaved at the major cleavage site at 31R↓L32. This was supported by the observation that these forms co-migrated with receptor species truncated at 40R↓L32 (Fig. 5A, compare lanes 2 and 5) and was further verified by enzymatic de-O-glycosylation (Fig. 5D). No clear changes in the pattern or intensity of receptor forms were observed in HepG2ΔT1 or HepG2ΔT11 cell lines (Fig. 5C) (data not shown), although some variability between experiments was found, especially in the case of HepG2ΔT1 cells. Taken together, these results indicate that in the two tested cell lines, HEK293 and HepG2, which express a diverse set of GalNAc-Ts, the major isoform modifying βAR is GalNAc-T2. Other transferases, most likely GalNAc-T3, can,
GalNAc-T2 fine-tunes β1AR ectodomain cleavage

FIGURE 6. Isoproterenol-induced cAMP accumulation is attenuated in HEK293ΔT2/T3 cells. The WT Myc- and FLAG-tagged hβ1AR was transiently transfected into HEK293ΔT2/T3 or HEK293WT cells for 48 h. Cells were stimulated with increasing concentrations of isoproterenol for 60 min, and cAMP was measured using the Cisbio Bioassays cAMP assay. The data represent the mean ± S.E. (error bars) of three independent experiments performed in duplicate. The values were normalized to the maximum signal obtained for the HEK293WT cells, which was set to 100%.

However, partially compensate for GalNAc-T2, possibly involving specific Ser residues in the receptor N terminus.

Impaired O-Glycosylation Attenuates β1AR Signaling—To investigate whether impaired O-glycosylation and the consequent enhanced N-terminal cleavage of β1AR might alter its functional activity, we analyzed the ability of isoproterenol, a β1AR agonist, to induce cAMP production in transfected HEK293ΔT2/T3 and corresponding WT cells in dose-response experiments. As shown in Fig. 6, the EC50 for isoproterenol was 16.8 ± 4.4 nM [H11006], 2.7 ± 0.04 nM [H11005], and 0.8 ± 0.04 nM [H9004] in HEK293WT, HEK293ΔT2/T3, and HEK293ΔT2/T3 KO cells, respectively; the maximum stimulation in HEK293ΔT2/T3 was significantly decreased by 40.4 ± 5.7% (p = 0.009). In comparison, no change in cAMP accumulation was detected in the two cell lines when the endogenously expressed gastric inhibitor peptide receptor (32) was stimulated by an increasing concentration of gastric inhibitor peptide (supplemental Fig. 1). Thus, these data indicate that β1AR O-glycans have a modulatory role in receptor function, having an impact on receptor number as well as on receptor signaling. This occurs most likely via O-glycan-mediated co-regulation of receptor proteolytic processing.

β1AR Proteolytic Processing Is Co-regulated by GalNAc-T2 in Vivo in Rat Hearts—To further investigate the potential co-regulatory function of GalNAc-T2 in the β1AR N-terminal processing and to confirm the in vitro and cell line findings, we compared the cleavage pattern of β1AR in WT and Galnt2−/− rat hearts. Because the N-terminal sequence of the rat β1AR differs slightly from the human sequence, we first confirmed the GalNAc-T2 isomeric-specific glycosylation of peptides corresponding to the rat sequence by in vitro analysis (Fig. 7C). We then analyzed the endogenous β1AR in the rat heart by SDS-PAGE and Western blotting with an antibody directed against the receptor C-terminal domain. This antibody was shown previously to detect both full-length and N-terminally cleaved forms of the rat receptor (6). As shown in Fig. 7, the Galnt2−/− rat hearts exhibited a clear increase in the relative amount of cleaved β1ARs. This was observed when receptors solubilized from isolated membranes were analyzed directly or after immunoprecipitation (Fig. 7, A and B, respectively). The lack of GalNAc-T3 expression in the heart (8) probably explains the extensive receptor processing in the heart muscle comparable with the observations in the HepG2 cell line model system.

**Discussion**

The complex regulation of site-specific O-glycosylation involving up to 20 polypeptide GalNAc-T isoforms offers a compelling system to fine-tune and enhance differential regulation of important proteolytic processing events (15). This has been demonstrated for PC processing regulated by PCs as well as more recently for ectodomain shedding regulated by metalloproteinases of the ADAM and MMP families (15, 17). Here, we expanded the targets for co-regulatory functions of site-specific O-glycosylation to include the cell surface-exposed N-terminal domains of GPCRs using β1AR as an example. We have shown previously that β1AR is O-glycosylated and undergoes limited and regulated processing by metalloproteinases affecting receptor cleavage sites (5, 6) and now demonstrate that O-glycosylation of the receptor is specifically regulated by a single GalNAc-T3 isoform at specific sites close to the metalloproteinase cleavage sites. Furthermore, we confirm *in vitro* and

![Graph showing cAMP accumulation](image-url)
in vivo that the metalloproteinase-mediated processing of $\beta_1$AR is co-regulated by GalNAc-T2 site-specific O-glycosylation and demonstrate that this phenomenon has an effect on receptor function. This is the first example of site-specific O-glycan fine-tuning of GPCR cleavage, and we predict that over 300 GPCRs (30%) could be O-glycosylated and have potential for similar co-regulation by O-glycosylation.

GPCRs are a large group of integral membrane proteins with seven transmembrane segments and a highly variable cell surface-exposed N-terminal sequence. They undergo a variety of post-translational modifications, including N- and O-glycosylation. However, still only a very limited number of GPCRs are known to be O-glycosylated and their actual glycosites determined (7, 18, 19, 22–24, 33). With the introduction of the SimpleCell O-glycoproteomics strategy, our knowledge of O-glycosites has greatly advanced (7), although initially only few glycosites in GPCRs were detected (7, 25). One obstacle is the challenge in detecting juxtamembrane regions, such as GPCR N termini in the bottom-up mass spectrometry that depends on appropriate peptide digestions (34). With the advancing analysis of SimpleCells derived from different organs and improvements in mass spectrometry workflow and instrumentation, we have now identified more than 50 GPCR N termini with one or more O-glycosites, $^5$ supporting the NetOGly4.0 prediction of >300 GPCRs being O-glycosylated (7, 25).

The N-terminal proteolytic cleavage of GPCRs has been demonstrated to participate in various molecular mechanisms. Adhesion GPCRs have a highly conserved GPCR proteolysis site (GPS), which is cleaved by an intramolecular autocatalytic reaction (35). The proteinase-activated receptors (PARs) are a subfamily of GPCRs activated by serine proteases (36), although a metalloproteinase is also involved in the regulated proteolysis of PAR1 (35). For both adhesion receptors and PARs, the proteolytic processing of the receptor N terminus leads to an exposure of a cryptic tethered peptide sequence that acts as an agonist and activates the cognate receptors (35). A growing number of other GPCRs are also reported to undergo N-terminal cleavage mediated by metalloproteinases, including the parathyroid hormone receptor (37), thyrotropin receptor (38), endothelin B receptor (39), C5a receptor (40), GPR37 (41), and GPR37L1 (42) in addition to $\beta_1$AR (5). The functional roles of the N-terminal cleavage of these GPCRs are not fully understood, although several are cleaved in an activation-dependent manner. Interestingly, all of these receptors are predicted to be O-glycosylated in their N termini, and in several cases, the O-glycosites have been determined experimentally. $^5$ Thus, there is mounting evidence to suggest that limited N-terminal proteolysis and O-glycosylation may overlap topologically, thus opening the possibility for a broader functional interplay for GPCRs.

The global discovery of an interplay between O-glycosylation and proteolytic processing has been hampered by limited insight into the location of O-glycosylation and proteolytic processing within the protein sequence or domains. A major challenge is the lack of a clear consensus motif for general GalNAc O-glycosylation or even GalNAc-T isoform-specific glycosylation, although prediction algorithms can provide some guidance (7, 43). Metalloproteinases also lack clear consensus motifs, and a substantial degree of redundancy exists between different isoforms (44, 45). Consequently, peptide libraries and substrate degradomes of knock-out cell lines and animals have been applied to provide information of the preference for sequence features and topology in substrate recognition and used to identify specific substrates (46–50). Due to these challenges, we used here in vitro analyses with peptide substrates derived from the N-terminal sequence of $\beta_1$AR to probe the existence and interplay between site-specific O-glycosylation and metalloproteinase-mediated processing. We previously used this strategy and found a good correlation with in vivo functions (17, 26), and here we could unambiguously validate our findings in cell and animal model systems in vivo. It is important to note that our studies only relate to the direct interplay at the substrate level (i.e. the N terminus of $\beta_1$AR), and we found previously that such interplay of processes occurs within ±3–5 residues (51). Clearly, both players, being the proteolytic processing and O-glycosylation events, have additional layers of complex regulation.

We demonstrated in the in vitro assays that O-glycosylation blocks metalloproteinase-mediated cleavage at $^{52}P_{\rightarrow}L^{53}$ almost completely and inhibits cleavage at $^{31}R_{\rightarrow}L^{32}$ of the $\beta_1$AR N-terminal peptides. We also identified an additional cleavage site at $^{41}S_{\rightarrow}L^{42}$ utilized by a number of MMPs, which was also blocked by O-glycosylation. Thus, our in vitro studies show the potential for multiple cleavage sites in the $\beta_1$AR N terminus regulated by different enzymes. This observation was supported indirectly by results obtained using the cell line models, where mutating the main cleavage site of the receptor at $^{31}R_{\rightarrow}L^{32}$ led to heterogeneity in the expressed receptor forms detected in CHO-ldlD cells. A similar observation was made previously when the mutant receptor was expressed in HEK293 cells (5). In our previous studies, we have demonstrated that the N-terminal cleavage of $\beta_1$AR at $^{31}R_{\rightarrow}L^{32}$ is modulated by agonist-mediated receptor activation (5). Thus, further studies are needed to decipher regulation of $\beta_1$AR processing sites and the ADAMs and MMPs involved in specific situations. A wide range of molecular mechanisms and physiological stimuli are known to regulate ADAM and MMP activities (52), and currently our understanding of the complex regulatory mechanisms is limited.

Similarly, our understanding of the regulation of site-specific O-glycosylation is still incomplete. Although O-glycosylation is widely found on the majority of proteins trafficking the secretory pathway, we predict that only a subset of these are regulated in cells (25). Thus, most O-glycosites are covered by redundancies among the many polypeptide GalNAc-Ts, and a complete loss of a single GalNAc-T isoform does not affect the majority of O-glycosites. However, a subset of glycosites that are often found in isolated sites in proteins are specifically controlled by individual GalNAc-T isoforms and hence can be differentially regulated (25). Here, we found that the N-terminal region of $\beta_1$AR is selectively controlled by the GalNAc-T2 isoform with only minor contributions from a few other isoforms.

$^5$ C. K. Goth and H. Clausen, unpublished data.
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Furthermore, a complete loss of GalNAc-T2 reduced glycosylation and enhanced the metalloprotease-mediated processing of the receptor in vivo. It is therefore likely that the site-specific O-glycosylation of β1AR is controlled through regulation of GalNAc-T2. The GALNT2 gene has been associated with dyslipidemia, and a specific regulatory role of this isoform in O-glycosylation of the lipase inhibitor angiopoietin-3-like 3 has been proposed (25, 30). Importantly, it appears that a liver-specific enhancer element in the intron 1 of the GALNT2 gene selectively regulates this function (54). Furthermore, the murine Gabt2 gene has been shown to be up-regulated in adrenergic-deficient mouse hearts during development, supporting a regulatory link between GalNAc-T2 and β-adrenergic signaling in the heart (55).

Another important aspect, at the substrate level, of the interplay between proteolytic processing and site-specific O-glycosylation is protein sequence variations. Sequence variations in regions of processing and/or O-glycosylation with clinical consequences are often found, but due to the inherent difficulties in predicting processing and O-glycosylation, experimental analysis is required for the evaluation of effects. Here, we mapped the exact locations of O-glycosites in β1AR and identified Ser-49 as an amino acid modified by O-glycosylation. Interestingly, a common single nucleotide polymorphism (S49G) with functional relevance has been identified at this site (56, 57). The less common Gly-49 variant exhibits a higher degree of desensitization and more profound agonist-promoted down-regulation compared with the Ser-49 variant when expressed in cell lines (29, 58). Furthermore, several clinical associations have been suggested for the polymorphism, although the results have been inconsistent (59). The S49G replacement clearly introduces the O-glycosite at this position, but it remains to be determined to what degree this amino acid replacement affects glycosylation of other sites in the region and ultimately proteolytic processing. However, it is compelling to hypothesize that the differences between the variants observed in cells, and the potential clinical associations, are due to the loss of O-glycosylation and to dysregulation of processing. We are currently exploring this possibility.

In summary, our study demonstrates the existence of a regulatory interplay between site-specific O-glycosylation and proteolytic processing in the extracellular N terminus of β1AR. We developed isogenic cell systems and a knock-out rat model, which will be used in future studies to address the physiological consequence of this interplay. Finally, based on the prevalence of O-glycosylation identified and predicted in GPCR N termini, we expect that the phenomenon described here is more widely present in this large family of important drug targets.

Experimental Procedures

Glycosyltransferase Assays—Recombinant glycosyltransferases were expressed as soluble secreted truncated proteins in insect cells (60). In vitro activity assays for GalNAc-T glycosylation of peptides (Schafer-N, NeoBioSci) were performed as described before and monitored with MALDI-TOF (17, 26). Samples were purified for subsequent site determination and cleavage experiments by HPLC using a multistep gradient on a Dionex Ultimate 3000 LC system (Thermo Scientific) with a Kinetex 2.6 μm C18 100A 100 × 4.60-mm column (Phenomenex).

Characterization of O-Glycosites by Electron Transfer Dissociation—MS

Samples were dissolved in methanol/water (1:1) containing 1% formic acid and introduced by direct infusion via a TriVersa NanoMate ESI-Chip interface (Advion Biosystems) at a flow rate of 100 nl/min and 1.4 kV spray voltage. Mass spectra were acquired in positive ion Fourier transform mode using parameters similar to previous studies (17, 61), except at a nominal resolving power of 30,000 or 60,000. Electron transfer dissociation—MS2 spectra were analyzed by comparison with theoretical c and z′ fragment m/z values calculated for all possible combinations of one HexNAc residue distributed on all potential Ser and Thr glycosylation sites in the sequence. Calculations were performed using the web-based Protein Prospector MS-Product software routine. Furthermore, samples were analyzed on a setup composed of an EASY-nanoLC 1000 (Thermo Scientific) connected via a nanoSpray Flex ion source to an LTQ-OrbitrapVelos Pro hybrid mass spectrometer. Glycopeptides were dissolved in 0.1% formic acid and separated on an in-house packed reverse phase column (1.9-μm ReproSil-Pure-AQ C18 particles, Dr. Maisch GmbH). The MS1 precursor ion scan was performed in the Orbitrap using a nominal resolution of 30,000 followed by two MS2 scan events (15,000 resolving power at m/z 400) utilizing HCD and ETD fragmentation modes. Glycopeptide identification and glycosite assignments were accomplished by Proteome Discoverer version 1.4 with final validation through manual inspection of the assigned peaks.

ADAM and MMP Cleavage of Peptides and Glycopeptides—

In vitro metalloprotease cleavage activity was assayed by adding 150–600 nM ADAM8, -10, -12, or -17 (Enzo Life Sciences) or MMP1, -2, -3, -7, -8, -9, -12, -13, or -14 (produced as described previously (46)), using 10 μg of peptide or glycopeptide substrate in a total volume of 25 μl. Reactions were performed in 25 mM Tris-HCl, pH 9 (ADAM17); 25 mM Tris-HCl, pH 9, 2 mM CaCl2, and 0.0005% Brij-35 (ADAM10); 20 mM Tris-HCl, pH 8, 0.0005% Brij-35 (ADAM12); or 20 mM Tris-HCl, pH 8, 25 mM CaCl2, and 0.0005% Brij-35 (ADAM8). Reactions were incubated at 37 °C (ADAM17, -8, -10, and -12) or 30 °C (ADAM8). MMP cleavage assays were performed at 37 °C in 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl2, and 0.05% Brij-35. MMP1, -2, -3, -7, -8, -9, and -13 were activated before the assay by incubation with 10 mM α-methylisoxazole-propionic acid. Product development was evaluated after 0.5, 1, and 4 h by MALDI-TOF-MS.

DNA Constructs and Cell Lines—DNA constructs for the WT hβ1, hAR, the R31H/L32A cleavage site mutant, and the Δ2–31 and Δ2–52 truncation mutants have been described previously (5). The construct for the hδOR Cys-27 variant has been described (28).

Cell Lines—The stably transfected HEK293, cell lines expressing the Myc- and FLAG epitope-tagged hβ1, hAR or hδOR in an inducible manner have been described previously (5, 28). HEK293SimpleCells with a knock-out of COSMC and HEK293 and HepG2 cell lines with a knock-out and knock-in of specific GalNAc-Ts (HEK293ΔT2, HepG2ΔT2, HepG2ΔT2 + T2, and HepG2ΔT1) have been described previously (17, 24). A HepG2...
cell line with a knock-out of GalNAc-T11 (HepG2ΔT11) was produced by ZFN gene-mediated targeting of GALNT11. The ZFN targeting construct for GALNT11 was custom-produced (Sigma-Aldrich) with the following binding sites with the cutting site indicated in parenthesis: 5'-GACCGCTTTGGGCTAC(CACAGA)GATGTGCCAGACACAGG-3'. In short, the HepG2 cells (a kind gift from Novo Nordisk) were transfected with one vial of mRNA (Sigma-Aldrich) using nucleofection on an Amaxa Nucleofector (Lonza). The clones were confirmed to have GALNT11 mutations by PCR and sequencing using the oligonucleotide 5'-TCGCTGACTAACCCTGTTGGA-3' and its reverse complement (62). CHO-K1 cells and the CHO-IdlD cell line were obtained from the ATCC cell culture collection. A HEK293 cell line with a knock-out of both GalNAc-T2 and -T3 was prepared by gene targeting using GFP-tagged CRISPR/Cas9. The 20-nucleotide guide sequences targeting human GALNT2 (5'-GTTAAGCGTTGATTACCCGC-3') and GALNT3 (5'-TATTGGAGTAACCTTACCCG-3') were designed using the online tool. The single-guide RNAs were co-transfected with the Cas9-PBKS plasmid using Lipofectamine 3000 according to the manufacturer’s instructions (Thermo Fisher Scientific). At 24 h after transfection, GFP-positive cells were enriched by FACS and cultured for 1–2 weeks. The sorted cells were then single-sorted again for GFP-negative cells into 96-well plates. Knock-out clones with frameshift mutations were identified by IDAA (indel detection by amplicon analysis) with the following primers: GALNT2, 5'-CATCCCCAGTTGCTAGTCT-3' (forward) and 5'-CTG-TGCTGAGCATCGAGG-3' (reverse); GALNT3, 5'-TCTT-CCTCCAGGTAGTGTTTCT-3' (forward) and 5'-AAAGCAGAACAGTGTGATCATTTCAA-3' (reverse). The mutated sequences of the selected knock-out clone were confirmed by sanger sequencing for each gene, and the loss of the enzyme was characterized by immunocytochemistry with in house monoclonal antibodies to GalNAc-T1 (4D8), GalNAc-T2 (4C4), and GalNAc-T3 (2D10).

All cell lines were cultured in a humidified atmosphere at 37 °C with 5% CO2. HEK293 and HepG2 cells were cultured in DMEM containing 10% FBS, and CHO-K1 and CHO-IdlD cells were cultured in F-12 Ham nutrient mixture with Kighn’s modification containing 5% FBS. The media were supplemented with 100 units/ml penicillin and 0.1 mg/ml streptomycin and with selection antibiotics in the case of the stably transfected HEK293 cells (5, 28). The cell culture reagents were obtained from Sigma-Aldrich, Thermo Fisher Scientific, or Invivogen. For experiments, cells were seeded onto 6- or 10-cm plates and cultured for 1–3 days to 60–80% confluence. For CHO-K1 and CHO-IdlD cells, FBS concentration was lowered to 2 or 0.5%. Cells were transfected with 1–2 μg (WT, R31H/L32A)/3 μg (Δ2–31, Δ2–52) of receptor constructs for 24–48 h using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s instructions or, alternatively, using linear 25-kDa polyethyleneimine (Polysciences) (41). Both reagents were used at a 1:3 DNA/reagent ratio. The metalloproteinase inhibitor marimastat (Tocris Bioscience; Fig. 4C), and the monosaccharides Gal (20 μM) and GalNAc (400 μM) (Sigma-Aldrich; Fig. 3, E, G, and H) were added to the culture medium 4 h after starting the transfection. For some experiments (Fig. 5), cycloheximide (20 μg/ml; Sigma-Aldrich) was added 4 h before cells were harvested to deplete receptor precursors that co-migrate with the main C-terminal fragment on SDS-PAGE (5). Receptor expression in the stably transfected HEK293 cells was induced with 0.5 μg/ml tetracycline for 6 h (Fig. 7B) or 24 h (Fig. 3D). Cells were lifted from plates using warm 2 mM EDTA/PBS and harvested in ice-cold PBS, quick frozen in liquid nitrogen, and stored at −70 °C.

Galnt2−/− Rats—The GalNAc-T2 knock-out rats were prepared by the SAGE Laboratories as described elsewhere (53). Hearts were collected from 12-week-old male homozygote Galnt2−/− and corresponding WT rats, and ventricular samples were quick frozen in liquid nitrogen.

CAMP Accumulation Assay—The assay was performed by applying homogeneous time-resolved FRET using the cAMP cell-based assay kit from Cisbio Bioassays according to recommendations. Briefly, cells were detached from culture plates 48 h post-transfection, resuspended in DMEM with 1 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), counted, and transferred in duplicates to white 384 microplates (Greiner Bio-One) (2,500 cells/well in a total volume of 5 μl). Appropriate concentrations of isoproterenol (Sigma-Aldrich) were then added in a total volume of 5 μl, and the cells were incubated for 60 min at 37 °C. The reaction was terminated by adding 10 μl of the kit’s lysis buffer containing cAMP-d2 (acceptor) and anti-cAMP-Eu3+ Cryptate (donor). The signal was measured after 60 min using the EnSpire multilabel reader (PerkinElmer Life Sciences). The cAMP generated was interpolated from a standard curve generated in parallel for each experiment.

Preparation and Solubilization of Membranes and Whole Cell Extracts—Cellular membranes from transfected cells or from rat heart ventricles were prepared and solubilized, and total cellular lysates were prepared as described previously (6, 28). The detergent n-dodecyl-β-D-maltoside was replaced with Triton X-100 (Sigma-Aldrich).

Immunoprecipitation of Solubilized Receptors—Solubilized receptors from transfected cells and rat heart ventricles were purified by immunoprecipitation using the immobilized mouse monoclonal FLAG M2 antibody (Sigma-Aldrich, A2220) and the polyclonal rabbit V-19 antibody (Santa Cruz Biotechnology, Inc., sc-568), respectively. The latter antibody, which is directed against the receptor C terminus, was cross-linked to magnetic beads (Pierce Protein A/G magnetic beads, Thermo Scientific) according to the manufacturer’s instructions. The one-step immunoprecipitation with the FLAG M2 antibody was performed as described previously (5), and purified receptors were eluted with SDS-sample buffer. Immunoprecipitation with the V-19 antibody was performed using the Pierce Crosslink Magnetic IP/CO-IP kit. The buffers were supplemented with 0.1% Triton X-100, 2 mM EDTA, 0.5 mM PMSF, 2 mM 1,10-phenanthroline 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, and 10 μg/ml benzamidine.

Deglycosylation of Immunoprecipitated Receptors—For deglycosylation, samples were eluted from the FLAG M2 antibody affinity resin with 1% SDS in 50 mM sodium phosphate, pH 5.5, and diluted eluates were digested with Endo H (50 milliunits/ml), PNGase F (50 units/ml), neuraminidase (50 milliunits/ml), and stored at −70 °C.

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and O-glycosidase (50 milliunits/ml) obtained from Roche Applied Science, as described (5).

**SDS-PAGE and Western Blotting**—The analysis of purified receptors was performed by SDS-PAGE and Western blotting as described (6). The blots were probed with FLAG M2 (0.1 μg/ml; Sigma-Aldrich, F3165), FLAG M2-HRP (1:50,000–80,000; Sigma-Aldrich, A8592), c-Myc A14 (1:1,000–3,000; Santa Cruz Biotechnology, sc-789), c-Myc (1:1,000; Sigma-Aldrich, C3956), or V-19 (1:1,000; Santa Cruz Biotechnology) antibodies and, when appropriate, followed by HRP-conjugated donkey anti-mouse F(ab')2/anti-rabbit F(ab')2 antibodies (1:10,000–40,000; GE Healthcare, NA9310 and NA9340, respectively). Pierce ECL or ECL Plus detection reagents (Thermo Scientific) or the Luminata Classico or Crescendo Western HRP substrate (Merck Millipore) were used to reveal the receptor bands. The relative intensities of bands on ECL films were analyzed by densitometric scanning with the Umax PowerLook 1120 color scanner (GE Healthcare) and Image Master 2D Platinum version 6.0 software (GE Healthcare), and quantified using Image version 1.45s, subtracting the local background from each lane.

**Data Analysis**—Data were analyzed using GraphPad Prism version 7.02 (GraphPad Software, La Jolla, CA). Statistical analyses were performed using the paired t test, the one-sample t test, or the two-way analysis of variance followed by Tukey’s multiple-comparison post hoc test. The limit of significance was set at p < 0.05. The data are presented as mean ± S.E.

**Author Contributions**—C. K. G. performed and analyzed the experiments in Figs. 1, 2, 6, and 7C and supplemental Fig. 1 and wrote the first draft of the manuscript. H. E. T. performed and analyzed the experiments in Figs. 3 (B–E, G, and H) and 4C and H. K. performed and analyzed the experiments in Figs. 4A and 5. J. J. L. constructed the receptor model in Fig. 3A and participated in the analysis of results of the cell models. S. W. made the HepG2AT11 cell line, Y. N. made the HEK293TΔT2/T3 cell line, and L. H. H. assisted in performing and analyzing the experiments in Fig. 6 and supplemental Fig. 1. C. M. O. provided the panel of recombinant MMPs. K. T. S. coordinated the preparation of the Galnt2 +/− rats, and U. E. P.-R. performed and analyzed the experiment in Fig. 7 (A and B) and analyzed the data in Figs. 3F, 4B, 5B, and 6 and supplemental Fig. 1. K. T. S., H. C., and U. E. P.-R. conceived and coordinated the study and wrote the final version of the paper. All authors reviewed the results and approved the final version of the manuscript.

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