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Glycosylation of β_1-adrenergic receptors regulates receptor surface expression and dimerization☆

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Abstract

The β_1-adrenergic receptor (β_1AR) has one predicted site of N-linked glycosylation on its extracellular amino-terminus, but the glycosylation and potential functional importance of this site have not yet been examined. We show here that the β_1AR is glycosylated in various cell types and that mutation of the single predicted site of N-linked glycosylation (N15A) results in the formation of receptors that are not N-glycosylated. The β_1AR N15A mutant exhibited significantly decreased basal surface expression relative to the wild-type receptor but had no detectable deficits in ligand binding or agonist-promoted internalization. Co-immunoprecipitation experiments using Flag-tagged and HA-tagged receptors demonstrated that the β_1AR-N15A mutant receptor exhibits a markedly reduced capacity for dimerization relative to wild-type β_1AR. These data reveal that the β_1AR is glycosylated on Asn15 and that this glycosylation plays a role in regulating β_1AR surface expression and dimerization.

Keywords: Regulation; Trafficking; Oligomerization; β-adrenergic receptor; G protein-coupled

β-Adrenergic receptors (βARs) are heptahelical G protein-coupled receptors (GPCRs) that mediate many of the physiological effects of epinephrine and norepinephrine. There are three subtypes of β-adrenergic receptor: β_1AR, β_2AR, and β_3AR. All of these subtypes couple to G_s and therefore increase cellular cyclic AMP levels when stimulated with agonist. β_1AR is found in a variety of tissues but is particularly highly expressed in both the heart, where it mediates the bulk of the effects of epinephrine on cardiac function [1], and the brain, where it plays a key role in regulating synaptic plasticity [2] and memory formation [3]. β_1AR-selective antagonists are very commonly prescribed therapeutic drugs in the treatment of cardiac disease and hypertension [4], and the cellular regulation of β_1AR function is therefore a subject of keen research interest.

β_1AR function is known to be regulated post-translationally via phosphorylation [5] and via association with cytoplasmic proteins. For example, it has been shown that the β_1AR can directly associate in cells with PSD-95 [6] and MAGI-2 [7], two related PDZ domain-containing scaffold proteins. PSD-95 and MAGI-2 bind to the same motif on the β_1AR carboxyl-terminus but exhibit differential regulation of agonist-promoted β_1AR internalization [7]. β_1AR internalization has also been shown to be regulated via receptor association with arrestins [8] and the endophilin family of proteins, which directly associate with the β_1AR third intracellular loop [9].

In addition to phosphorylation and association with cytoplasmic proteins, one additional post-translational modification that many GPCRs are known to undergo is glycosylation. While most GPCRs are known to be N-glycosylated, the functional effects of glycosylation vary significantly from receptor to receptor [10]. The β_2AR is

☆ *Abbreviations:* βAR, β-adrenergic receptor; cAMP, cyclic AMP; ConA, concanavalin A; DHA, dihydroalprenolol; EDTA, ethylenediamine tetraacetic acid; GPCR, G protein-coupled receptor; HA, hemagglutinin; HEK, human embryonic kidney; HRP, horseradish peroxidase; MAGI, membrane-associated guanylate kinase-like protein with an inverted domain structure; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PDZ, PSD-95/Discs-large/ZO-1 homology; PSD-95, post-synaptic density protein of 95 kDa; SDS, sodium dodecyl sulfate; WT, wild-type.

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known to be glycosylated on two residues, Asn6 and Asn15 [11]. Blockade of glycosylation at these sites by either mutation or treatment with tunicamycin results in a significant reduction in β_2AR surface expression [11]. The β_1AR and β_2AR are similar but not identical in their extracellular amino-terminal regions: Asn15 is conserved in the β_1AR while Asn6 is not. Asn15 has been predicted to be a site of β_1AR glycosylation [12], but this prediction has never been explicitly tested. Moreover, nothing is known about the potential functional importance of β_1AR N-glycosylation. It was recently shown by Liggett and colleagues that a polymorphism in the human β_1AR gene that alters a single amino acid in the receptor's amino-terminus also alters the extent of β_1AR glycosylation [13]. Thus, a basic understanding of the functional significance of β_1AR N-glycosylation is critical for achieving a clinical understanding of adrenergic receptor variation between patients.

To examine the physiological importance of β_1AR N-glycosylation, we have prepared an Asn15 β_1AR mutant (N15A) and examined the functional properties of this mutated receptor. Specifically, we have examined the effects of receptor N-glycosylation on β_1AR surface expression, ligand binding, agonist-promoted internalization, cyclic AMP generation, and dimerization. We have found that the β_1AR-N15A mutant is functional but is deficient in surface expression and dimerization relative to the wild-type receptor.

Experimental procedures

Plasmids. Flag-β_1AR has been described previously [7]. HA-β_1AR was provided by Hitoshi Kurose (University of Tokyo). The N15A mutant β_1AR was prepared via polymerase chain reaction amplification from the native human β_1AR cDNA using a mutant sequence oligonucleotide (CTG GGC GCC TCC GAG CCC GGT GCC CTG TCG TCG GCC GCA CCG CTC). The point mutation was confirmed by ABI sequencing.

Cell culture and transfection. All tissue culture media and related reagents were purchased from Gibco/Life Technologies. HEK-293 and COS-7 cells were maintained in complete medium (Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 1% penicillin/streptomycin) in a 37 °C/5%CO_2 incubator. For heterologous expression of receptors, 2 μg DNA was mixed with Lipofectamine (15 μl) and Plus reagent (20 μl) (from Invitrogen) and added to 5 ml complete medium in 10 cm tissue culture plates containing cells at approximately 50–80% confluency. Following a 4 h incubation, the medium was removed and 10 ml fresh complete medium was added. After another 12–16 h, the medium was changed again and the cells were harvested 24 h later.

Western blotting. Samples (5 μg per lane) were run on 4–20% SDS/PAGE gels (Invitrogen) for 1 h at 150 V and then transferred to nitrocellulose. The blots were blocked in "blot buffer" (2% non-fat dried milk, 0.1% Tween 20, 50 mM NaCl, and 10 mM HEPES, pH 7.4) for at least 30 min and then incubated with primary antibody in blot buffer for 1 h at room temperature. The blots were then washed three times with 10 ml blot buffer and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech) in blot buffer. Finally, the blots were washed three more times with 10 ml blot buffer and visualized via enzyme-linked chemiluminescence using the ECL kit from Amersham Pharmacia Biotech.

Immunoprecipitation. Cells were harvested and lysed in 500 μl ice-cold lysis buffer (10 mM HEPES, 50 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, and the protease inhibitor cocktail from Roche). The lysate was solubilized via end-over-end rotation at 4 °C for 30 min and clarified via centrifugation at 14,000 rpm for 15 min. A small fraction of the supernatant was taken at this point and incubated with SDS–PAGE sample buffer to examine expression of proteins in the whole cell extract. The remaining supernatant was incubated with 30 μl beads covalently linked to anti-Flag antibodies (Sigma) for 2 h with end-over-end rotation at 4 °C. After five washes with 1.0 ml lysis buffer, the immunoprecipitated proteins were eluted from the beads with 1× SDS–PAGE sample buffer, resolved by SDS–PAGE, and subjected to Western blot analyses.

Enzymatic deglycosylation. Immunoprecipitates were separated from beads by boiling for 10 min in a denaturing buffer (0.5% SDS containing 1% β-mercaptoethanol). After cooling, NP-40 was added to the supernatants to a final concentration of 1% and Na_2HPO4/NaH_2PO4 buffer (pH 7.5) was added to lysates to a final concentration of 50 mM. PNGase F (1500 U; New England Biolabs) was added to a 30 μl reaction volume and the sample was incubated for 1 h at 37 °C.

Cyclic AMP assay. Intracellular cAMP was measured by using a non-acetylation cAMP enzyme immunoassay kit (Amersham Pharmacia Biotech). Briefly, cultured cells were transfected with either wild-type Flag-β_1AR or the Flag-β_1AR N15A mutant. After 24 h, cells were split into 6-well culture dishes with fresh medium. After another 48 h, cells were treated with varying concentrations of isoproterenol for 10 min and harvested with cell harvest buffer (50 mM Tris, pH 7.4, 250 μM Ro 20-1724 [Tocris, Ellisville, NJ], 5 mM $MgCl_2$, 1 mM ATP, and 1 M GTP). Cell lysates were sonicated, transferred to a 96-well assay plate coated with anti-rabbit IgG, and incubated with an anti-cAMP antibody at 4 °C for 2 h along with a series of cAMP standards. A cAMP-peroxidase conjugate was then added to the microtitre plate and incubated at 4 °C for 1 h. The plate was then washed four times with 400 μl wash buffer and the wells were incubated with 150 μl enzyme substrate at room temperature for 1 h. When the samples were within the linear range of the standards, the reaction was stopped by adding 100 μl of 1.0 M sulfuric acid. Optical density was determined in a plate reader at 450 nm and cAMP levels were determined using standard curves.

Surface expression assay. Transfected cells were grown in 35 mm dishes and incubated in the absence and presence of agonist. The cells were then rinsed in PBS and fixed with 4% paraformaldehyde in PBS for 30 min, then rinsed three times in PBS, and blocked with blocking buffer (2% non-fat dried milk in PBS, pH 7.4) for 30 min. The fixed cells were then incubated with primary antibody in blocking buffer for 1 h at room temperature. The dishes were subsequently washed three times with 2 ml block buffer and incubated for 1 h at room temperature with horseradish-peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech) in blocking buffer. Finally, the dishes were washed three times with 2 ml blocking buffer and one time with 2 ml PBS and then incubated with 2 ml ECL reagent (Pierce) for exactly 15 s. The luminescence, which corresponds to the amount of receptor on the cell surface, was determined by placing the plate inside a TD 20/20 luminometer (Turner Designs). Surface expression values were normalized to total receptor expression by performing Western blots on matching plates of transfected cells for each set of plates examined in the luminometer assay. Total cellular expression of the wild-type and N15A receptors was generally comparable, and any within-experiment differences were accounted for by quantitation of the Western blot data and normalization of the surface expression data.

Ligand binding assays. For preparation of membranes to be used in ligand binding assays, transfected cells grown on 100-mm dishes were rinsed twice with 10 ml PBS and then scraped into 1 ml ice-cold binding buffer (10 mM HEPES, pH 7.4). Cells were then sonicated for 10 s and

the fresh membrane suspension was used in radioligand binding assays. Membranes were incubated with [^{3}H]DHA in binding buffer in the absence or presence of isoproterenol for 30 min at 37 °C. Nonspecific binding was defined as [^{3}H]DHA binding in the presence of 1 mM isoproterenol and represented less than 10% of total binding in all experiments. Incubations were terminated via filtration through GF/C filter paper using a Brandel cell harvester. Filters were rapidly washed three times with ice-cold wash buffer (10 mM HEPES) and radioactive ligand retained by the filters was quantified via liquid scintillation counting.

Results

The β_1-adrenergic receptor has previously been reported to be glycosylated in native tissues [14]. We examined the potential glycosylation of Flag-tagged β_1AR heterologously expressed in HEK-293 cells, which do not express endogenous β_1AR. Following transfection and Western blotting with an anti-Flag antibody, two primary immunoreactive bands were detected at 71 and 58 kDa. Treatment with tunicamycin resulted in a decrease in the size of both of these bands to 65 and 54 kDa, respectively, presumably reflecting a decrease in the apparent size of the receptor due to blockade of N-linked glycosylation (Fig. 1A, lanes 1 and 2). Immunoreactive bands at 71 and 58 kDa were still evident in the tunicamycin samples, however, and these bands probably represent receptors that were synthesized before the addition of the tunicamycin. Similar results were obtained when Flag-β_1AR was transfected into COS-7 cells and examined in the absence and presence of tunicamycin treatment (data not shown). These findings reveal that β_1-adrenergic receptors heterologously expressed in HEK-293 and COS-7 cells are glycosylated like endogenous β_1-adrenergic receptors in native tissues.

We next identified the site of β_1AR N-glycosylation. The primary sequence of the β_1AR reveals only a single asparagine residue conforming to the N-x-S/T consensus site for N-linked glycosylation [12]. We mutated this residue, Asn15, to alanine, creating a Flag-tagged N15A mutant receptor. When the N15A mutant was expressed in HEK-293 cells and then examined via Western blot, it exhibited two primary immunoreactive bands that were decreased in size relative to those observed for wild-type β_1AR (Fig. 1A, lane 3). Moreover, the size of the immunoreactive bands observed in the N15A β_1AR-expressing membranes matched up well with the lower molecular weight immunoreactive bands (at 65 and 54 kDa) seen in the WT β_1AR-expressing membranes following treatment with tunicamycin. These data indicate that the β_1AR is glycosylated at Asn15.

To determine if Asn15 is the only site of β_1AR N-linked glycosylation, we performed enzymatic deglycosylation studies. For immunoprecipitated WT β_1AR, deglycosylation with PNGase F resulted in a decrease in the apparent size of the receptor (Fig. 1B). PNGase F treatment of immunoprecipitated N15A β_1AR, however, did not result in any change in the apparent size of the receptor. These data reveal that Asn15 is the only site on which the β_1AR is N-glycosylated. One additional point that should be noted is that the immunoprecipitated samples exhibited higher molecular weight bands (more than 200 kDa), which may represent β_1AR

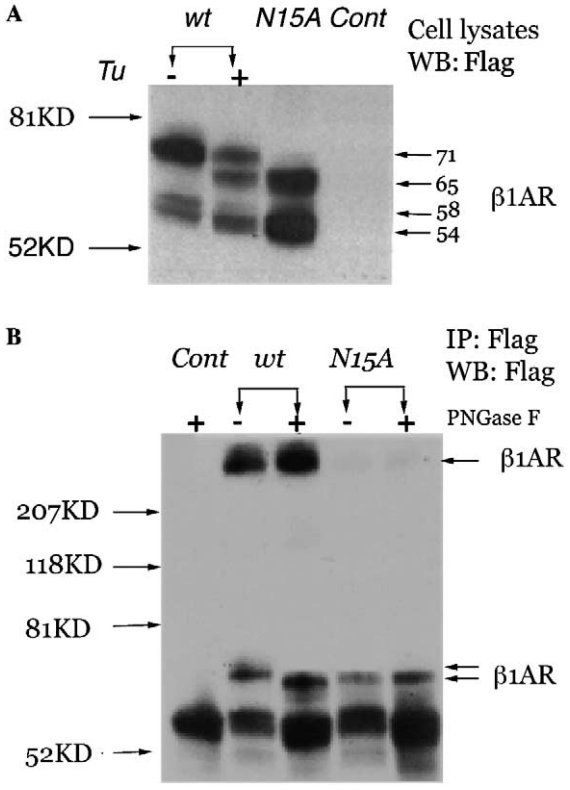

Fig. 1. β_1AR is glycosylated at Asn15. (A) HEK-293 cells transfected with either wild-type Flag-β_1AR ("wt") or Flag-β_1AR carrying a mutation at Asn15 ("N15A") were grown in the absence or presence of 1 μg/ml tunicamycin (Tu) overnight, then harvested, and analyzed via Western blot with an anti-Flag antibody. Both tunicamycin treatment and the N15A mutation significantly reduced the apparent size of the β_1 AR on SDS–PAGE. The final lane of the blot ("Cont") reveals that no anti-Flag immunoreactivity was evident in control cells that were not transfected with Flag-β_1AR. The positions of molecular mass standards (in kDa) are shown on the left side of the panel. The calculated sizes of the various immunoreactive bands are indicated by the arrows and numbers on the right side of the panel. The wild-type receptor (first lane) exhibited two major immunoreactive bands (71 and 58 kDa), while the size of these two bands was reduced (to 65 and 54 kDa, respectively) in the tunicamycin-treated (second lane) and N15A mutant receptor (third lane) samples. (B) Flag-β_1AR in the transfected cell lysates was immunoprecipitated with anti-Flag antibody beads and immunoprecipitated complexes were deglycosylated with PNGase F and analyzed via Western blot with an anti-Flag antibody. The enzymatic deglycosylation reduced the apparent size of wild-type β_1AR but not the N15A mutant, indicating that Asn15 is the sole site of β_1AR N-linked glycosylation. In all lanes of this blot, there is a large non-specific band at approximately 50 kDa, which probably represents the heavy chain of the antibody used for immunoprecipitation. The transfection-specific bands representing Flag-β_1AR are indicated by the arrows. The experiments shown in this figure were repeated three to four times each with identical results.

complexes. These higher molecular weight bands were consistently reduced in intensity for immunoprecipitates of the N15A mutant receptor as compared to the WT β_1AR.

The functional properties of the N15A mutant β_1AR were examined next. Since inhibition of β_2AR N-glycosylation has been shown to result in impaired β_2AR surface expression [11], we studied the surface expression of the N15A mutant β_1AR in COS-7 cells. As shown in Fig. 2, we found that N15A-β_1AR surface expression was reduced by approximately 60% relative to wild-type β_1AR. Similarly, tunicamycin treatment of cells expressing wild-type β_1AR resulted in an approximately 40% decrease in receptor surface expression. Thus, both tunicamycin treatment and mutation of Asn15 reduced β_1AR surface expression, with the latter effect being somewhat more pronounced. This difference may reflect the fact that tunicamycin only partially inhibited β_1AR N-glycosylation while mutation of Asn15 completely blocked N-glycosylation of the β_1AR. It should be noted that the surface expression data were normalized to total receptor expression (as determined by Western blot) for all conditions and that total receptor expression was not significantly altered by tunicamycin treatment or by the N15A mutation.

In contrast to receptor surface expression, agonist-promoted internalization of the β_1AR was unaffected by inhibition of receptor N-glycosylation. Both wild-type β_1AR and the N15A mutant exhibited 20–30% internalization in response to a 30 min treatment with the β-adrenergic agonist isoproterenol (Fig. 3). Treatment with the lectin concanavalin A (Con A) is known to inhibit β_2-adrenergic receptor internalization [15], but Con A regulation of β_1AR internalization has not been studied. We examined internalization induced by stimulation of wild-type β_1AR versus the N15A mutant β_1AR, in the absence and presence of Con A treatment. We found that Con A treatment blocked internalization induced by isoproterenol treatment of wild-type β_1AR (Fig. 3, second bar), consistent with findings for Con A treatment of β_2-adrenergic receptors [15]. Strikingly, however, there was no effect of Con A treatment on internalization of the N15A mutant β_1AR. These data indicate that Con A can block β_1AR internalization and that this effect is dependent upon N-linked glycosylation of the β_1AR at Asn15.

We next examined the effect of receptor N-glycosylation on downstream signaling of the β_1AR. The ability of increasing concentrations of isoproterenol to induce rises in intracellular cyclic AMP was assessed for both

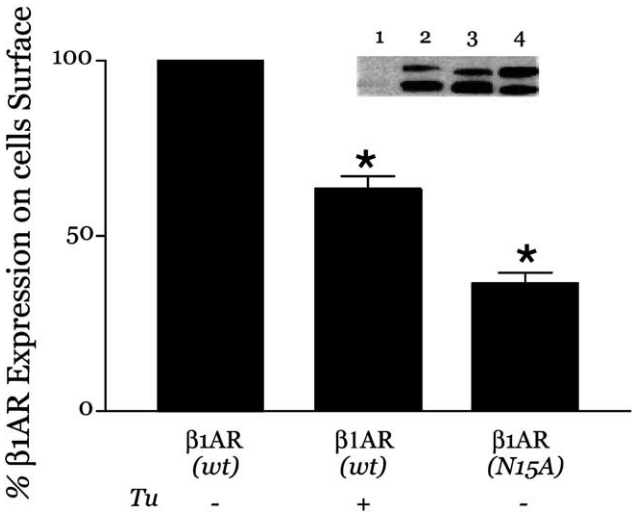

Fig. 2. Blockade of β_1AR N-glycosylation inhibits receptor trafficking to the cell surface. COS-7 cells transfected with either wild-type Flag-β_1AR ("wt") or the Flag-β_1AR N15A mutant ("N15A") were grown overnight in the absence or presence of 1 μg/ml tunicamycin ("Tu"). Receptor expression on the cell surface was quantified via a luminometer-based assay as described under "Experimental procedures." Both tunicamycin treatment and the N15A mutant significantly reduced β_1AR surface expression; data are expressed as percent of wild-type β_1AR surface expression in the absence of tunicamycin. The bars and error bars represent means and standard errors for six independent experiments, and the values for each experiment are normalized to total receptor expression as determined via Western blot. A representative Western blot is shown in the inset (lane 1 = untransfected, lane 2 = WT, lane 3 = tunicamycin-treated, and lane 4 = N15A); all immunoreactive bands between 54 and 71 kDa were scanned and quantitated to normalize for total receptor expression. "*" indicates significantly different from β_1AR wt in the absence of tunicamycin ($p < 0.01$).

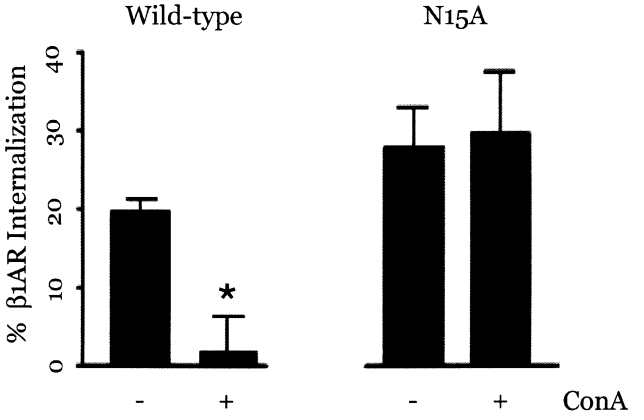

Fig. 3. Concanavalin A blockade of β_1AR internalization is dependent upon receptor N-glycosylation. COS-7 cells were transfected with either wild-type Flag-β_1AR ("wt") or the Flag-β_1AR N15A mutant ("N15A"). Cells were pre-incubated in the absence or presence of 10 μM concanavalin A ("Con A") for 15 min and agonist-induced internalization of Flag-β_1AR was measured by via a luminometer-based assay following a 30 min treatment with 50 μM isoproterenol (Iso) at 37 °C. In the absence of Con A treatment, the internalization of the N15A mutant receptor was not significantly different from that of the wild-type receptor. In the presence of Con A treatment, however, internalization of the wild-type receptor was almost completely blocked while internalization of the N15A mutant receptor was unaffected. The bars and error bars represent means and standard errors for five independent experiments. "*" indicates significantly different from β_1AR wt in the absence of Con A ($p < 0.01$).

HEK-293 cells transfected with wild-type β_1AR or the N15A mutant. As shown in Fig. 4A, the maximum amount of cyclic AMP generated was not significantly different in cells transfected with the wild-type versus mutant receptors. However, the potency of isoproterenol was significantly reduced for the N15A mutant: the EC_{50} for isoproterenol stimulation of cyclic AMP by the wild-type receptor was 0.20 ± 0.05 nM, while the EC_{50} for isoproterenol stimulation of cyclic AMP by the N15A mutant was 0.65 ± 0.17 nM (significantly different from the value for wild-type, $p < 0.01$). These data reveal that the N15A mutant receptor is capable of coupling to G proteins but exhibits a reduced apparent potency for stimulation by isoproterenol.

Ligand binding studies were also performed. Specifically, isoproterenol inhibition of binding of the specific β-adrenergic antagonist [^{3}H]DHA was examined in membranes prepared from transfected cells expressing either wild-type β_1AR or the N15A mutant (Fig. 4B). The K_i values for isoproterenol were 1.9 ± 0.7 μM for the wild-type receptor and 1.0 ± 0.5 for the N15A mutant. The B_{max} values for the [^{3}H]DHA binding were 49.3 ± 10.7 pmol/mg for the wild-type receptor and 45.1 ± 9.7 pmol/mg for the N15A mutant. Thus, these data indicate that neither the affinity of the N15A mutant receptor for isoproterenol nor the total expression level of expression of the N15A mutant was significantly different from wild-type β_1AR.

We recently reported that β_1-adrenergic receptors can dimerize, based on co-immunoprecipitation data and on Western blot data revealing higher-order bands of β_1AR immunoreactivity, which appear to correlate with dimers or oligomers [7]. In our initial Western blot analyses of the N15A mutant, described above, we noted that the higher-order bands of β_1AR immunoreactivity were significantly reduced in immunoprecipitates from cells expressing N15A mutant β_1AR versus cells expressing wild-type β_1AR (see Fig. 1B). These data suggested that β_1AR N-glycosylation might regulate β_1AR oligomerization. To further investigate this possibility, we co-expressed Flag-tagged β_1AR and HA-tagged β_1AR in HEK-293 cells and performed co-immunoprecipitation experiments to assess the ability of the differentially tagged receptors to dimerize with each other. These studies were performed for both wild-type β_1AR and the N15A mutant receptor. As shown in Fig. 5, the N15A mutant β_1AR exhibited a markedly decreased capacity for dimerization, with the efficiency of N15A dimerization being reduced by more than 60% relative to wild-type β_1AR. These data reveal that blocking β_1AR N-linked glycosylation impairs β_1AR dimerization.

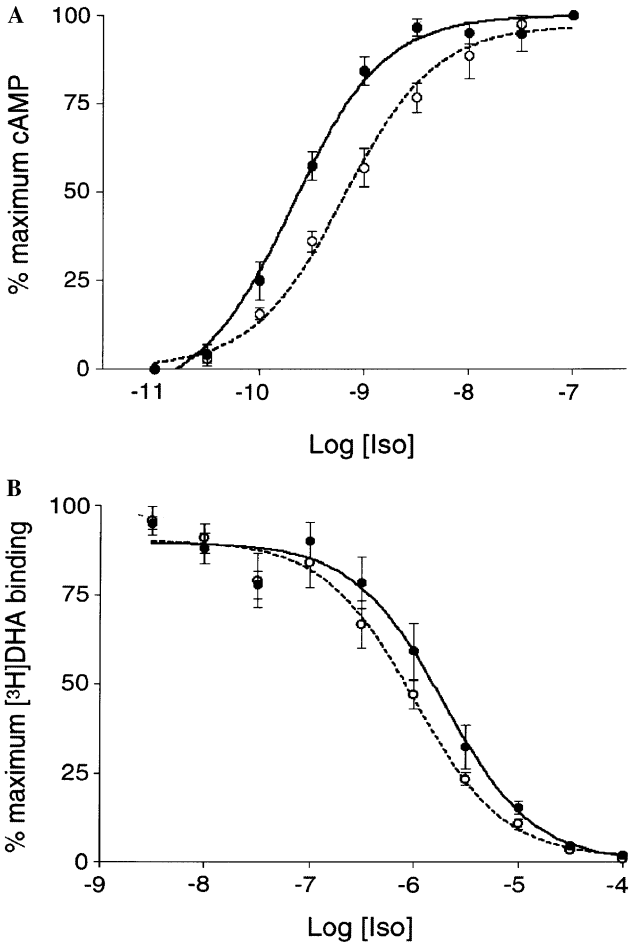

Fig. 4. Blockade of β_1AR N-glycosylation decreases the potency of isoproterenol in cyclic AMP generation assays but not the affinity of isoproterenol in ligand binding assays. (A) HEK-293 cells were transfected with either wild-type Flag-β_1AR (closed circles, solid line) or the Flag-β_1AR N15A mutant (open circles, dashed line). The cells were treated for 10 min at room temperature with increasing concentrations of isoproterenol ("Iso"). Cyclic AMP accumulation was measured and expressed as percentage of cAMP levels when cells were stimulated with a maximal concentration of isoproterenol (10^{-7} M). Mock-transfected cell exhibited only negligible increases in cAMP in response to Iso at the concentrations examined, and thus, the increase in cAMP shown on the graphs is due to activation of the transfected receptors. The maximum amount of cyclic AMP produced in cells transfected with the N15A mutant was not significantly different from that of cyclic AMP produced in cells transfected with wild-type β_1AR (N15A = 112 ± 15% of wild-type). The points and error bars represent means and standard errors for five independent experiments. (B) HEK-293 cells were transfected with either wild-type Flag-β_1AR (closed circles, solid line) or the Flag-β_1AR N15A mutant (open circles, dashed line). The cells were harvested, membranes were prepared, and the binding of 2 nM [^{3}H]DHA in the absence and presence of increasing concentrations of isoproterenol ("Iso") was examined. Non-specific binding was defined as binding in the presence of 1 mM isoproterenol. Mock-transfected cell exhibited only negligible [^{3}H]DHA binding, and thus, the specific binding that was studied represents binding to the transfected receptors. The points and error bars represent means and standard errors for three independent experiments.

Discussion

The functional effects of N-glycosylation on GPCRs are highly variable. For some receptors, such as the M2

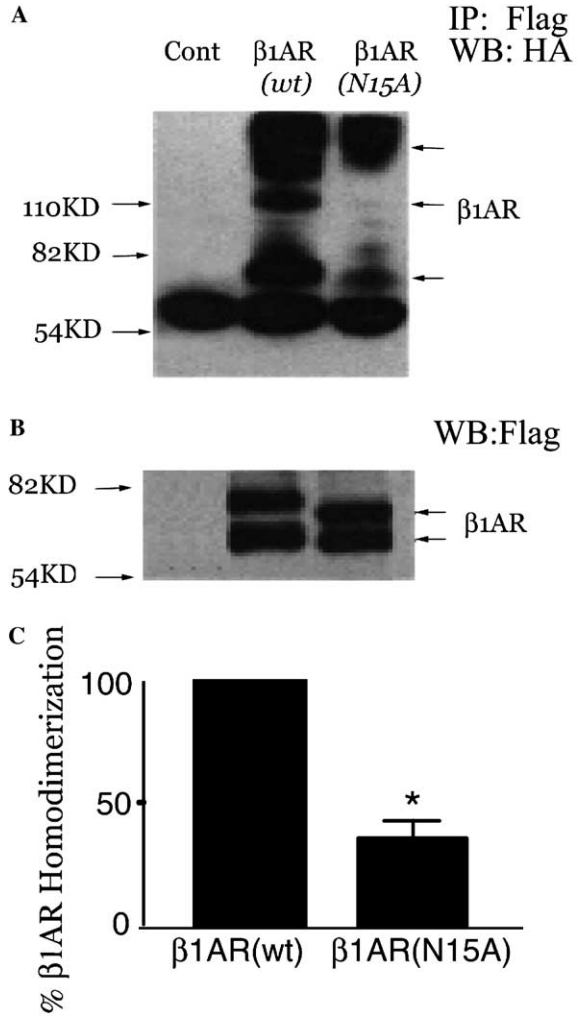

Fig. 5. β_1AR N-glycosylation regulates receptor dimerization. HEK-293 cells were co-transfected with either wild-type Flag-β_1AR (wt)/HA-β_1AR (wt) or Flag-β_1AR (N15A)/HA-β_1AR (N15A). Cells were harvested, solubilized, and subjected to immunoprecipitation with anti-Flag antibody beads. Analysis of immunoprecipitates by Western blotting with an anti-HA antibody (A) revealed significantly more co-immunoprecipitation of wild-type HA-β_1AR than HA-β_1AR-N15A with their Flag-tagged counterparts. Cell lysates were also analyzed by Western blotting with both anti-HA and anti-Flag antibodies to examine total expression levels of the various tagged receptors; the levels of expressed wild-type vs. N15A β_1AR were comparable in all experiments. In all lanes of the blot shown in (A), there is a large non-specific band at approximately 50 kDa, which probably represents the heavy chain of the antibody used for immunoprecipitation. The positions of the transfection-specific bands corresponding to the β_1AR are indicated by the arrows on the right side of the figure, while the positions of molecular mass standards (in kDa) are shown on the left side of the figure. Total expression levels of the wild-type vs. N15A mutant β_1AR were equivalent in the solubilized cell lysates, and the two receptors were recognized equally by the anti-Flag antibody, as shown in (B). The amount of immunoprecipitated HA-β_1AR (N15A) was quantitated and expressed as a percentage of immunoprecipitated HA-β_1AR (wt) within matched sets of immunoprecipitates. These data are shown in bar graph form in (C). Only immunoreactive bands in the range of 65–71 kDa were analyzed for quantitation. Bars and error bars represent means ± SEM ($n = 4$). "*" indicates significantly different from β_1AR wild-type ($p < 0.01$).

muscarinic acetylcholine receptor [16], H2 histamine receptor [17], AT2 angiotensin receptor [18], oxytocin receptor [19], and D1 dopamine receptor [20], there seem to be no detectable deficits in receptor function if N-glycosylation is blocked. For other receptors, such as the β_2-adrenergic receptor [11], D5 dopamine receptor [20], V1a vasopressin receptor [21], and gastrin-releasing peptide receptor [22], the primary effect of disrupting receptor N-glycosylation is to reduce receptor surface expression. For yet other receptors, such as the thyrotropin receptor [23], SSTR3 somatostatin receptor [24], and rhodopsin [25], perturbation of N-glycosylation results in the creation of either non-functional receptors or receptors with severe functional deficits such as disrupted ligand binding. In the present study, we have found that the β_1-adrenergic receptor is N-glycosylated on Asn15. We have furthermore found that N15A mutant β_1-adrenergic receptors are clearly functional, but exhibit reduced surface expression and reduced dimerization relative to wild-type receptors.

The functional deficits observed for the N15A mutant β_1AR may be related to one another. For example, it is possible that reduced surface expression might result in reduced efficiency of dimerization, since a lower density of receptors in the plasma membrane could conceivably reduce the proportion of receptors forming dimers. The causal relationship here is difficult to assert unequivocally, however, since it could also plausibly be the case that the reduced efficiency of dimerization of the N15A mutant leads to its reduced surface expression. GPCR dimerization is known to play a key role in regulating the surface expression of other GPCRs, with $GABA_B$ receptors being perhaps the most striking example of this phenomenon [26]. GPCR dimerization is a relatively recently described phenomenon [27] and the possibility that N-glycosylation might regulate receptor dimerization has not yet been explored for other GPCRs. For growth factor receptors, such as the epidermal growth factor receptor (EGFR), it is known that N-glycosylation strongly regulates receptor dimerization, as EGFR mutants lacking certain glycosylation sites are severely impaired in their ability to dimerize and autophosphorylate [28]. In any case, it will be of significant interest in the future to see if the dimerization of other GPCRs can be regulated by their state of N-glycosylation.

There may also be a relationship between the decreased surface expression of the β_1AR-N15A mutant receptor and the functional deficit observed in the cyclic AMP generation experiments. Isoproterenol was significantly less potent at activating the β_1AR-N15A mutant than the wild-type receptor in the cyclic AMP generation studies, but yet exhibited an equivalent affinity for the wild-type and mutant receptors in ligand binding assays. The most likely interpretation of these data is that the reduced surface expression of the N15A mutant receptor resulted in less receptor reserve and a

concomitant decrease in the apparent potency of isoproterenol. Decreases in spare receptors are known to correlate with decreases in the apparent potency of agonists [29], especially when the receptors are heterologously expressed, as in our experiments.

One additional way that N15A mutant β_1-adrenergic receptors are different from wild-type receptors is that they are not susceptible to regulation by concanavalin A. Con A is a lectin that is known to associate with a number of cell surface receptors. Treatment of cells with Con A blocks internalization of many GPCRs, including the β_2-adrenergic receptor [15], α2A-adrenergic receptor [30], and dopamine D1 receptor [31]. Con A is known to induce a number of diverse cellular effects [32] that might indirectly alter rates of receptor internalization. Our data indicate, however, that the action of Con A on blocking β_1AR internalization is a direct effect mediated via association of Con A with the carbohydrate portion of the receptor, since the N15A mutation that blocks β_1AR N-linked glycosylation also results in the creation of a receptor that is no longer regulated by Con A.

We have previously reported that β_1AR association with the PDZ proteins PSD-95 and MAGI-2 regulates agonist-promoted internalization but not receptor surface expression [7]. In contrast, we have shown here that β_1AR N-glycosylation regulates receptor surface expression but has no effect on agonist-promoted internalization. Thus, distinct aspects of β_1AR cellular trafficking are regulated differentially by N-glycosylation versus association with PDZ proteins. Glycosylation and association with PDZ proteins occur at different times and places during the course of β_1AR post-translational processing, and these spatiotemporal differences may account for the differential effects of N-glycosylation and PDZ association on β_1AR cellular trafficking. Glycosylation occurs in the endoplasmic reticulum and is known to facilitate the proper folding and processing of many proteins [33], perhaps explaining why perturbation of β_1AR N-glycosylation decreases the total amount of β_1AR making it to the cell surface. Association with PDZ proteins, in contrast, probably occurs at a much later time point in β_1AR processing, since PSD-95 [34] and MAGI-2 [35] are typically found to be associated with the plasma membrane. It might be expected that the β_1AR would not encounter these proteins until after it is already at the cell surface, perhaps explaining why these proteins have little effect on β_1AR surface expression. The rate of β_1AR internalization is known to be influenced by the association of β_1AR with a number of membrane-associated proteins, including not only PSD-95 and MAGI-2 but also arrestins [8] and endophilins [9].

Glycosylation of cell surface receptors does not occur uniformly across all cell types, but rather can be quite variable. For example, glycosylation of some receptors is known to vary significantly across different tissues [36,37]. In the specific case of the human β_1AR, the extent of N-glycosylation is known to be markedly affected by a polymorphism in the β_1AR gene [13]. Our findings reveal that alterations in β_1AR N-glycosylation can influence β_1AR surface expression and dimerization. Thus, the data reported here demonstrate that β_1-adrenergic receptor function may be differentially regulated, both between tissues and between individuals, via differences in the extent of receptor N-glycosylation.

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