Human Bradykinin B2 Receptor Sialylation and N-Glycosylation Participate with Disulfide Bonding in Surface Receptor Dimerization†

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ABSTRACT: G-Protein-coupled receptors (GPCRs) act on the cell surface where they recognize and convert external stimuli to modulate cellular activity and are regulated by agonist and various partner molecules. We here studied the cell surface post-translationally modified forms of a GPCR, the human bradykinin B2 receptor. This was by means of detailed molecular analysis of the cell surface forms of N-glycosylation site mutant and wild-type receptors that were treated with glycosidases, neuraminidase, and/or the reducing agent dithiothreitol or not treated before Western blotting. We found that the receptor undergoes similar glycosylation processes and similar cell surface organization in CHO-K1 and HEK 293 cells, used for stable and transient receptor expression, respectively. The receptor is present as dimers and monomers on the cell surface. The dimers result from heterologous association of differently glycosylated mature receptor molecules. Importantly, receptor sialylation and N-glycosylation participate with disulfide bonding in the stabilization of the cell surface human B2 receptor dimers.

Kinin receptors are heptahelical G-protein-coupled receptors (GPCRs).[1] There are two subtypes of kinin receptors (B1 and B2) classified according to their relative affinities for des-Arg9-bradykinin and bradykinin (BK) (*1*). Most of the effects of BK described so far, such as vasodilation and control of vascular tone, ion transfer in epithelia, and pain (*2*), are mediated by the B2 receptor subtype via activation of phospholipases C and A2, and stimulation of the mitogenic activated protein (MAP) kinase pathway. Like many GPCRs, the B2 receptor is functionally regulated by post-translational modifications, such as phosphorylation, acylation, and glycosylation. Phosphorylation of the B2 receptor is dependent on agonist stimulation and is required for sequestration and desensitization processes (*3*, *4*). Palmitoylation influences the internalization process and the ability of the B2 receptor to couple and uncouple to G-proteins (*5*). Finally, N-linked glycosylation has recently been reported to be required for the efficient expression of the receptor on the cell surface and for efficient coupling (*6*).

It has become clear over the past decade that GPCRs form homo- or hetero-oligomers and that agonist-induced conformational changes within interacting receptors modify their pharmacology, signaling, and/or trafficking (*7*). In the case of the rat B2 receptor, its homodimerization has been shown to play a role in the agonist-induced desensitization process (*8*). The B2 receptor also physically associates with the angiotensin II AT1 receptor and the B1 receptor subtype. B2−AT1 heterodimerization increases the responsiveness to angiotensin II (*9*), whereas the recently reported B2−B1 heterodimerization has been found to enhance B1 receptor signaling (*10*).

Receptor−receptor interactions could be mediated by covalent [disulfide (*11*−*15*)] and/or noncovalent interactions; these could be association of the extracellular domains (*8*, *16*), transmembrane domains (*17*, *18*), or the C-terminal tail (*19*, *20*). The extracellular N-terminal domain is apparently involved in the rat B2 receptor dimerization (*8*). Since this domain contains two of the three N-glycosylation sites of the receptor (*6*), we asked whether the carbohydrate chains linked to the N-glycosylation sites could play a role in the association of human B2 receptor molecules. For growth factor receptors, such as the epidermal growth factor receptor, N-glycosylation strongly regulates receptor dimerization and autophosphorylation (*21*). As far as we are aware, the involvement of N-glycosylation in the dimerization of GPCRs has only been described for β1-adrenergic receptor homodimerization (*22*) and for α2A- and β1-adrenergic receptor heterodimerization (*23*).

We here, therefore, examined the role of N-glycosylation in the dimerization of the human B2 receptor. For this purpose, four forms of the receptor having zero, one, two, or its three N-glycosylation sites were stably or transiently expressed in CHO-K1 or HEK 293 cells, respectively, and compared via Western blot analysis. It should be emphasized that our study has focused on only the receptor molecules located on the plasma membrane, i.e., those that recognize external stimuli, convert these stimuli to modulate cellular activity, and are regulated upon agonist action. We have found that the wild-type receptor is expressed as dimers and

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[1] Abbreviations: GPCR, G-protein-coupled receptor; BK, bradykinin; B2 receptor, bradykinin receptor B2 subtype; B1 receptor, bradykinin receptor B1 subtype; PNGase F, peptide-N-glycosidase F.

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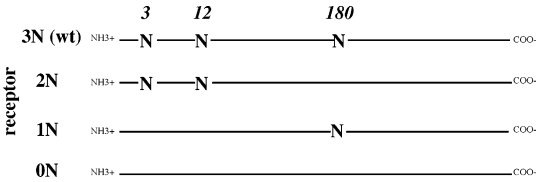

FIGURE 1: Schematic representation of the human B2 receptor with the potential N-glycosylation site(s) in the 0N, 1N, 2N, and 3N (wt) receptors. When absent, asparagine (N) at positions 3, 12, or 180 was replaced with glycine [numbering according to Hess et al. (*24*)].

monomers on the plasma membrane and that not only N-glycosylation but also sialylation and disulfide bonding participate in the stabilization of the dimers.

EXPERIMENTAL PROCEDURES

Materials. Lipofectamine 2000, pcDNA4/myc-HIS vector, anti-myc antibody, and anti-V5 antibody were from Invitrogen (Leek, The Netherlands). Fetal calf serum (lot no. S01190S0180) was from Biowest (ABCYS-Paris, France). The antibiotic cocktail for cell culture, igepal, protease inhibitor cocktail, bovine serum albumin (A-4378), iodoacetamide, trypsin, soybean trypsin inhibitor, and dithiothreitol were from Sigma (Saint Quentin Fallavier, France). The ECL kit was from Amersham Biosciences (Buckinghamshire, U.K.). Protein G magnetic beads were from Dynal (Oslo, Norway). Endoglycosidase H, peptide-N-glycosidase F, and protein markers were from New England BioLabs-Ozyme (Saint-Quentin en Yvelines, France). Neuraminidase and *O*-glycosidase were from Roche Molecular Biochemicals (Meylan, France). Polyacrylamide was from Interchim (Montluçon, France). Peroxidase-conjugated secondary antibody was a goat anti-mouse IgG from Jackson ImmunoResearch Laboratories (West Grove, PA). All other reagents were of analytical grade from Merck (Darmstadt, Germany) or from other suppliers.

cDNA Constructs. The expression vectors for the used V5-tagged wild-type and mutant B2 receptors were constructed as previously described (*6*). They consisted of human B2 receptor cDNAs in which codons for extracellular potential N-glycosylation sites at positions 3, 12, and 180 in the amino acid sequence [numbering according to Hess et al. (*24*)] were mutated into codons for glycine. The mutations involved the three positions for the receptor without N-glycosylation site (0N receptor), positions 3 and 12 for the receptor with one N-glycosylation site (1N receptor), position 180 for the receptor with two N-glycosylation sites (2N receptor), and neither position for the wild-type receptor with three N-glycosylation sites (3N receptor or wt receptor). Each of these cDNAs contained an exogenous V5 epitope coding sequence at the amino-terminal extremity of the receptor, just after the methionine start codon, so that the receptor could be immunoprecipitated with an anti-V5 antibody.

A schematic representation of the receptor with the potential N-glycosylation site(s) in the 0N, 1N, 2N, and wild-type receptors is given in Figure 1.

We also used a C-terminally myc-tagged wild-type B2 receptor cDNA. It was constructed using the pcDNA4-myc/HIS vector. By this way, the myc-tagged receptor amino acid sequence is 37 amino acids longer than that of the V5-tagged receptor. The myc-tagged receptor was used, together with the V5-tagged receptor, in experiments designed to examine whether the receptor molecules undergo self-association during the solubilization of the cellular proteins and during the precipitation.

Cell Culture and Receptor Expression. For characterization of the cell surface receptors, HEK 293 cells were used for transient expression, whereas stable expression was realized in CHO-K1 cells. Cells were transfected using Lipofectamine 2000 reagent according to the manufacturer's instructions. Cells were grown on a 60 mm Petri dish in modified Eagle's medium (HEK 293 cells) and in Ham's F12 medium (CHO-K1 cells) in a humid atmosphere of 95% air and 5% CO_2; both media were supplemented with 10% (v/v) fetal calf serum, antibiotics (0.2 unit/mL penicillin, 20 pg/mL streptomycin, and 0.5 mg/mL amphotericin B), and 1 mM (HEK 293 cells) or 0.5 mM (CHO-K1 cells) glutamine. Experiments were performed in HEK 293 cells, 72 h after transfection with 2 μg (1N, 2N, and 3N receptors) or 4 μg (0N receptor) of vector to produce similar cell surface expression of the various receptors. CHO-K1 cells were used at confluence, i.e., 48−72 h after cell passage.

To examine whether receptor molecules self-associate during the isolation process described below, we used HEK 293 cells transiently transfected with myc- and V5-tagged wild-type receptor cDNAs.

Immunoprecipitation of Cell Surface Receptors. The technique was that of Hilairet et al. (*25*) with slight modifications. It was entirely conducted at 4 °C in the presence of 10 mM iodoacetamide except when indicated. Cells expressing the V5-tagged receptors were washed three times with PBS and incubated for 1 h with 3 mL of blocking buffer [PBS containing 0.2% (w/v) BSA and 10 mM iodoacetamide] and then for 2 h with anti-V5 antibody (1:500 dilution) in 500 μL of blocking buffer. After two washes in blocking buffer and two washes in PBS, the cells were lysed in 500 μL of lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% (v/v) igepal, 0.1% (w/v) SDS, 0.5% (w/v) deoxycholate, protease inhibitor cocktail (1:500 dilution), and 10 mM iodoacetamide], rocked for 45 min, and centrifuged at 15000*g* for 20 min. Cell surface receptor−anti-V5 antibody complexes were then precipitated by rocking the supernatant for 2 h after addition of 50 μL of a protein G magnetic bead suspension. The immunoprecipitate was washed three times with PBS, resuspended in 50 μL of H_2O, and treated as described below before electrophoresis.

In some experiments, the cells were treated with trypsin before the 1 h incubation with blocking buffer. This treatment consisted of incubating cells grown on a Petri dish with 200 μL of 1× trypsin for 15 min at 37 °C, after the PBS wash. They were then transferred into an Eppendorf tube containing 200 μL of 1× soybean trypsin inhibitor and washed three times with PBS before the incubation with the blocking buffer.

Examination of Receptor in Vitro Self-Association. To test whether receptor self-association occurs during the solubilization of the cellular proteins and during the precipitation, HEK 293 cells expressing the V5-tagged wild-type receptor incubated with anti-V5 antibody (see above) were mixed with HEK 293 cells expressing the myc-tagged wild-type receptor, after the cells had received the lysis buffer. After protein solubilization, precipitation of receptor−anti-V5 antibody complexes, PBS washing, and Western blotting (see below),

the presence of the V5-tagged receptor-associated myc-tagged receptor was tested with anti-myc antibody.

Deglycosylation by Endoglycosidase H and Peptide-N-glycosidase F. Immunoprecipitate was incubated (25 μL total incubation volume) for 1 h at 37 °C with 2 mM NaCl, 0.2 mM Na_2EDTA, 1 mM iodoacetamide, 1 mM Tris-HCl, 5 mM sodium citrate buffer (pH 5.5), and 50 IUB milliunits of endoglycosidase H. Deglycosylation via peptide-N-glycosydase F was carried out at the same temperature for 15 min with 3 mM NaCl, 0.3 mM Na_2EDTA, 3% (v/v) glycerol, 1% (v/v) NP-40, 1 mM Tris-HCl, 5 mM sodium phosphate buffer (pH 7.5), and 10 IUB milliunits or varying amounts (from 0.1 to 10 IUB milliunits) of enzyme. The control consisted of the same amount of the same immunoprecipitate incubated without enzyme. At the end of the incubation, each sample was supplemented with 25 μL of nonreducing 2× sample buffer [solution A: 125 mM Tris-HCl (pH 6.8), 25% (v/v) glycerol, 5% (w/v) SDS, and 0.002% (w/v) bromophenol blue] and was heated at 100 °C for 5 min and kept frozen at −20 °C until it was used.

Neuraminidase and O-Glycosidase Treatments. Immunoprecipitate was incubated (25 μL total incubation volume) with 0.5% (w/v) SDS, 4 mM $CaCl_2$, 10 mM sodium phosphate buffer (pH 7), protease inhibitor cocktail (1:250 dilution), 1 mM iodoacetamide, and 50 milliunits or varying amounts (from 0.05 to 50 milliunits) of neuraminidase for 2 h at 37 °C. Neuraminidase action was also studied on the immunoprecipitate digested as described above by 10 IUB milliunits of PNGase F. In this case, neuraminidase was contained in 25 μL of a solution containing SDS, $CaCl_2$, sodium phosphate buffer (pH 7), and protease inhibitor cocktail so that the final concentrations of these compounds were those used for digestion with neuraminidase alone. For both experiments, the control was immunoprecipitate incubated without neuraminidase.

When *O*-glycosidase action was studied, 25 μL of neuraminidase-treated samples (see above) was incubated overnight at 37 °C in a total volume of 50 μL with 2 milliunits of *O*-glycosidase added with 1% (v/v) igepal and 100 mM sodium phosphate buffer (pH 7) (final concentrations). Controls were samples incubated without *O*-glycosidase.

For all experiments, the same amount of immunoprecipitate was used for the treated samples and the corresponding controls. At the end of the incubation, the samples were supplemented with 25 μL (neuraminidase alone) or 50 μL (PNGase F with neuraminidase or neuraminidase and *O*-glycosidase) of nonreducing 2× sample buffer and heated at 100 °C for 5 min and kept frozen at −20 °C until they were used.

Reduction with Dithiothreitol. To study the role of disulfide bonding in receptor dimerization, 25 μL of nonreducing 2× sample buffer or reducing 2× sample buffer (solution A containing 100 mM dithiothreitol) was added to 25 μL of the immunoprecipitate before it was heated at 100 °C and subjected to SDS−PAGE.

Western Blot. Samples were heated at 100 °C for 5 min. Then, proteins contained in 10 μL samples were resolved by 10% (w/v) SDS−PAGE for 50 min, transferred to a nitrocellulose membrane, and subjected to immunoblotting using a monoclonal anti-V5 antibody (1:5000 dilution) or an anti-myc antibody (1:5000 dilution) and a goat anti-mouse peroxidase-conjugated secondary antibody (1:5000 dilution) as previously described (*6*). Finally, proteins were visualized via chemiluminescence reactions using the ECL kit. The molecular weights of observed immunoreactive products were determined using protein markers as standards. Values given in the text are means ± the standard error of the mean of *n* determinations from CHO-K1 cells.

RESULTS

We here studied the human bradykinin B2 receptor cell surface organization by means of a detailed molecular analysis of the cell surface forms of the wild-type (wt) and N-glycosylation site mutant receptors (Figure 1) that we treated with glycosidases [endoglycosidase H, peptide-N-glycosidase F (PNGase F), and *O*-glycosidase], neuraminidase, or the disulfide bond disrupting agent dithiothreitol or did not treat. Endoglycosidase H is known to deglycosylate immature forms of glycoproteins containing high-mannose carbohydrates; on the other hand, PNGase F removes N-linked carbohydrates from both immature and mature glycoproteins modified with complex, fully processed carbohydrates (*26*) and *O*-glycosidase releases the disaccharide Galβ(1−3)-GalNAc bound to serine or threonine. With regard to neuraminidase, this enzyme releases acetylneuraminic acids N- or O-linked to glycoproteins (*27*).

Validation of the Technique Used To Study the Cell Surface Human B2 Receptor Organization. The technique consists of binding an antibody to surface-located receptor molecules of intact cells and then precipitating the complexes with protein G after an extensive washout of nonbound antibody [see also Hilairet et al. (*25*)]. Because no antibody directed to extracellular receptor domains is available commercially, we tagged the human B2 receptor at its N-terminal extracellular extremity with the V5 epitope. The tag does not influence receptor properties, except that it reduces the level of receptor expression (*6*).

Tested on CHO-K1 cells expressing the wild-type receptor (3N receptor) or the mutant lacking N-glycosylation sites (0N receptor), the technique allows immunoprecipitation of extracellularly but not of intracellularly located receptor molecules, since the immunoreactive products observed in the Western blot were absent when the cells were pretreated with trypsin (Figure 2A). Indeed, trypsin digests only extracellular proteins, and particularly the N-terminal extracellular receptor domain.

The observation, in Figure 2B, of low-molecular mass species of 35.4 ± 0.5 and 40.5 ± 0.5 kDa for the 0N receptor ($n = 18$) and 53−60 ± 1 and 65 ± 1 to 77 ± 2 kDa for the 3N receptor ($n = 18$) in the presence but not in the absence of iodoacetamide means that the alkylating agent prevented in vitro association through disulfide bonding of those species to form the high-molecular mass species of 73 ± 1 to 96 ± 3 kDa for the 0N receptor ($n = 18$) and 145−222 ± 5 kDa for the 3N receptor ($n = 18$). Therefore, the alkylating agent was included in all of the solutions used during the receptor isolation.

To definitively validate our technique, we checked that no receptor self-association occurred during the solubilization of the cellular proteins and during the precipitation. For this purpose, HEK 293 cells expressing the V5-tagged 3N

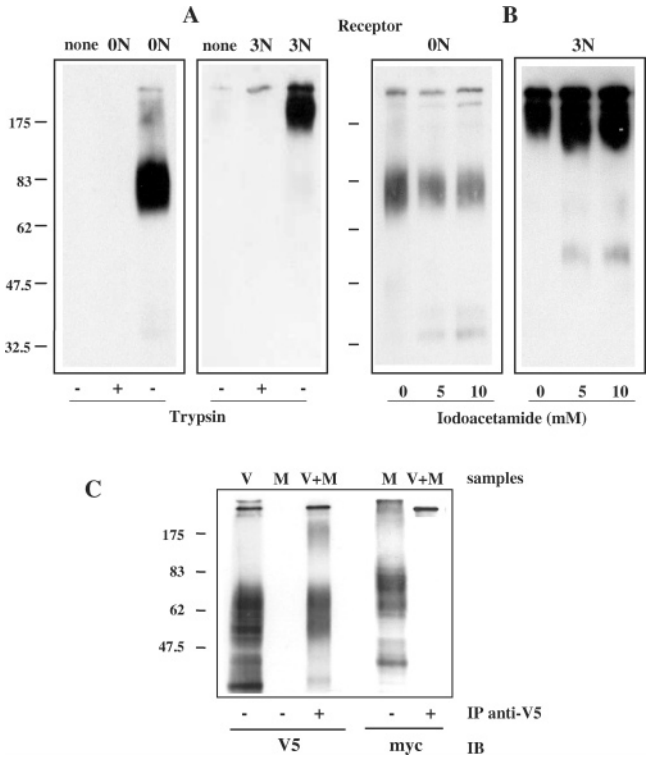

FIGURE 2: Effects of trypsin and iodoacetamide on cell surface wild-type and mutant B2 receptors and demonstration of the absence of in vitro receptor self-association. (A) Nontransfected CHO-K1 cells (none) and CHO-K1 cells expressing the V5-tagged wt receptor (3N) or the mutant lacking all N-glycosylation sites (0N) were treated with trypsin (+) or not treated (−) before receptor immunoprecipitation as described in Experimental Procedures. Immunoprecipitation was carried out using iodoacetamide-free solutions. (B) Immunoprecipitation of 3N and 0N receptors expressed in CHO-K1 cells was performed in the absence or presence of 5 or 10 mM iodoacetamide as described in Experimental Procedures. (C) All solutions contained 10 mM iodoacetamide. HEK 293 cells expressing the V5-tagged wild-type receptor incubated with the anti-V5 antibody were mixed with HEK 293 cells expressing myc-tagged wild-type receptor (V+M) or not mixed (V), just after the cells had received the lysis buffer. After the protein solubilization step, protein G magnetic beads were then added (+) to precipitate (IP anti-V5) the anti-V5 antibody−V5-tagged receptor complexes or not added (−). The immunoprecipitates or the lysate samples of V and M were then treated with nonreducing sample buffer for 5 min at 100 °C, before being run on 10% (w/v) SDS−PAGE and analysis via Western blotting. In panels A and B, an immunoblot (IB) was performed with the anti-V5 antibody, and in panel C, an immunoblot was performed with the anti-V5 antibody (V5) or anti-myc antibody (myc), as indicated. Molecular standard mass (in kilodaltons) positions are indicated at the left of each panel. The results are representative of experiments repeated twice.

receptor were incubated with the anti-V5 antibody in the presence of iodoacetamide. These cells were then mixed with HEK 293 cells expressing the myc-tagged 3N receptor, before cell protein solubilization and precipitation of the anti-V5 antibody−V5-tagged receptor complex. Clearly, no association of the two differentially tagged receptors had occurred, as the immunoblotting with the anti-myc antibody gave no signal (Figure 2C).

N-Glycosylation Site Mutant and wt B2 Receptors Undergo Similar Processes in CHO-K1 and HEK Cells. Similar migration patterns were obtained when the V5-tagged 0N, 1N, 2N, and 3N receptors were transiently or stably expressed in HEK 293 cells or CHO-K1 cells, respectively (Figure 3).

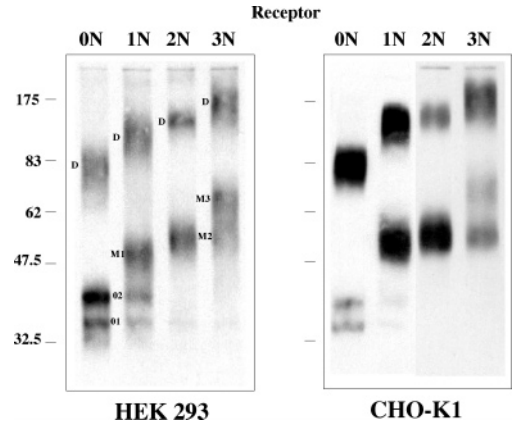

FIGURE 3: Molecular forms of cell surface N-glycosylation site mutant and wild-type B2 receptors in transfected CHO-K1 and HEK 293 cells. Cell surface V5-tagged receptor molecules from transfected HEK 293 cells (left panel) or CHO-K1 cell lines (right panel) expressing the wild-type receptor (3N) or receptors having zero (0N), one (1N), or two (2N) of the three potential N-glycosylation sites of the human B2 receptor were immunoprecipitated using solutions containing 10 mM iodoacetamide as described in Experimental Procedures. After being heated for 5 min in nonreducing 1× sample buffer, they were run on 10% (w/v) SDS−PAGE and analyzed via Western blotting with the anti-V5 antibody. D corresponds to dimers; M1−M3 correspond to glycosylated monomers specific for 1N−3N receptors, respectively, and 01 and 02 to non-N-glycosylated monomers found for 0N and 1N receptors, respectively. Molecular standard mass (in kilodaltons) positions are indicated at the left of each panel. The results are representative of experiments repeated three times.

This indicates that they undergo similar processes and cell surface organization in both cell types.

The receptor lacking N-glycosylation sites (0N receptor) gave during Western blotting two bands of 35.4 ± 0.5 and 40.5 ± 0.5 kDa (n = 18) and a diffuse band of double molecular sizes [73 ± 1 to 96 ± 3 kDa (n = 18)] which would be dimers (D) of the formers (called monomers 01 and 02, respectively). For the other receptors, the dimer bands were also diffuse but of higher molecular masses [105 ± 2 to 168 ± 11 kDa for the 1N receptor (n = 6), 108−174 ± 3 kDa for the 2N receptor (n = 9), and 145−222 ± 5 kDa for the 3N receptor (n = 18)], as a consequence of N-glycosylation (see below). The fact that monomer 01 and some species resulting from receptor deglycosylation or desialylation (see below) migrated at apparent molecular masses lower than the human B2 receptor molecular mass of 41 kDa predicted from its cDNA sequence (*24*) is very likely related to the hydrophobic character of the receptor, as demonstrated for other GPCRs (*28*).

The size overlap among the various dimers together with their diffuse migration pattern suggests that 1N, 2N, and 3N receptors have each at least two kinds of dimers that differ in their glycosylation status. The fact that the 0N receptor dimers lacking N-glycosylation migrated in a diffuse fashion is very likely related to heterologous association of the two non-N-glycosylated monomers 01 and 02, whose sizes match those of the 0N receptor dimers as stated above.

Like the 0N receptor, the 1N receptor displayed monomers 01 and 02. However, the 1N receptor differs from the 0N receptor in monomers M1 that migrated in a diffuse fashion at sizes [50 ± 2 to 57 ± 1 kDa (n = 6)] greater than those of monomers 01 and 02, indicating that monomers M1 are very likely glycosylated forms of monomers 01 and 02, on

Table 1: Molecular Masses and Corresponding Post-Translational Modifications of Cell Surface B2 Receptors[a]

	form	post-translational modification	name	molecular mass (kDa)	receptor
cell surface	dimers	−	−	145−222	3N
		−	−	108−174	2N
		−	−	105−168	1N
		−	−	73−96	0N
cell surface	monomers	N_3/S/O	M3	65−77	3N
		N_2/S/O	M2	52−60	2N and 3N
		N/S/O	M1	50−57	1N
		S/O	02	40.5	0N and 1N
		S/O	01	35.4	0N and 1N
with enzyme	monomers	S/O	01′	34.0	2N and 3N
		O	00	32.6	all
		none	−	31.0	all

[a] The table summarizes the typical forms (dimers or monomers) of the B2 receptor with the corresponding post-translational modifications (N_3, N_2, and N correspond to tri-, di-, and mono-N-glycosylation, respectively; S corresponds to sialylation and O to O-glycosylation), names given in the text, molecular weights (MW), and the receptors with which they were observed [3N, 2N, 1N, and 0N correspond to receptors having three, two, one, and zero potential N-glycosylation sites, respectively]. These forms resulted from Western blot analysis of cell surface receptor immunoprecipitates that were treated with glycosidases and/or neuraminidase (+enzyme) or not treated (cell surface).

the unique N-glycosylation site [asparagine at position 180 (N180)] of the 1N receptor. The observation of both glycosylated (M1) and nonglycosylated (01 and 02) monomers indicates that N180 is not systematically glycosylated.

As opposed to the 1N receptor, the 2N receptor did not show nonglycosylated monomers 01 and 02 and its monomers (monomers M2) migrated in a diffuse fashion at sizes [51−59 ± 1 kDa (n = 9)] only slightly greater than those of monomers M1, suggesting that monomers M2 are diglycosylated forms (on N3 and N12) of monomer(s) lighter than monomers 01 and 02. This indeed is the case (see below the species called monomer 01′ resulting from N-deglycosylation of 2N or 3N receptors).

Finally, the 3N (wt) receptor did not display monomers 01 and 02, like the 2N receptor. It showed two distinct heterogeneous monomer populations, one similar in mass [53−60 ± 1 kDa (n = 18)] to the 2N receptor monomers M2 and another (monomers M3) having higher masses [65 ± 1 to 77 ± 2 kDa (n = 18)] that would be triglycosylated monomers (on N3, N12, and N180). The coexistence of monomers M2 and M3 confirms the fact that N180 is not systematically glycosylated.

Note from Table 1, summarizing the forms and apparent molecular sizes corresponding to the various post-translational modifications of the receptor, that all of the molecular sizes of the observed dimers match those of the corresponding monomers, excluding thus the presence of accessory proteins.

The Human B2 Receptor Is a Mature N-Linked Oligosaccharide-, O-Linked Oligosaccharide-, and Sialic Acid-Modified Protein. As shown in Figure 4A, endoglycosidase H digestion had no effect on any receptor, whereas PNGase F resulted in modifications of the migration profiles of the N-glycosylation site-containing receptors (1N, 2N, and 3N receptors) but not of the 0N receptor (Figure 4B); the overall molecular species (dimers and monomers) had their size reduced by neuraminidase (Figure 4C). These observations

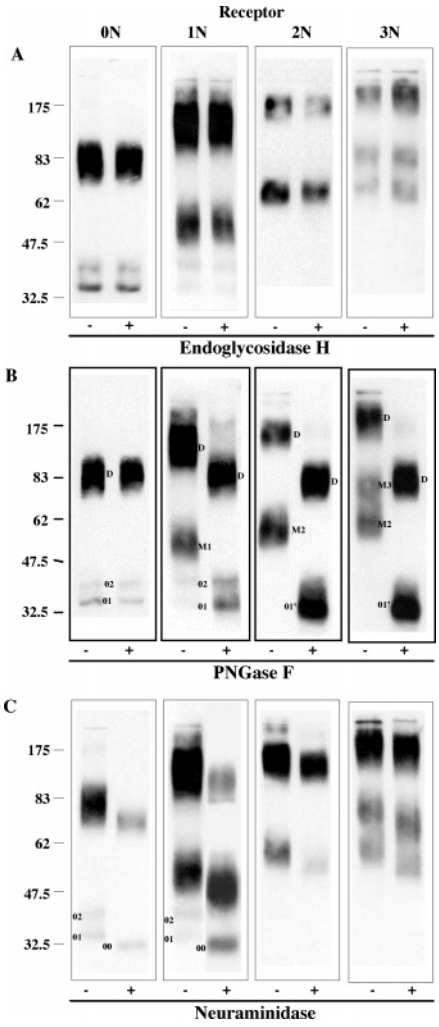

FIGURE 4: Effects of endoglycosidase H, peptide-N-glycosidase F, and neuraminidase on immunopurified cell surface N-glycosylation site mutant and wild-type B2 receptors from CHO-K1 cell lines. The cell surface wild-type receptor (3N) or receptors having zero (0N), one (1N), or two (2N) of the three potential glycosylation sites of the human B2 receptor were immunopurified in the presence of 10 mM iodoacetamide. Before being run on 10% (w/v) SDS−PAGE and Western blotting, immunoprecipitates were incubated (total incubation volume of 25 μL) at 37 °C without (−) or with (+) 50 IUB milliunits of endoglycosidase H for 1 h (panel A), 10 IUB milliunits of peptide-N-glycosydase F for 15 min (panel B), or 50 milliunits of neuraminidase for 2 h (panel C), were supplemented with 25 μL of nonreducing 2× sample buffer, and were heated for 5 min at 100 °C, as described in Experimental Procedures. D corresponds to dimers; M1−M3 correspond to glycosylated monomers specific for 1N−3N receptors, respectively, and 01 and 02 to non-N-glycosylated sialylated monomers specific for 0N and 1N receptors, respectively. 01′ corresponds to the non-N-glycosylated sialylated monomer specific for 2N and 3N and 00 to the non-N-glycosylated sialic acid-free monomer. Molecular standard mass (in kilodaltons) positions are indicated at the left of each panel. The results are representative of experiments repeated two (A) or three times (B and C).

demonstrate that all of the cell surface receptors contain sialic acid and that only mature carbohydrate chains (*26*) are involved in their N-glycosylation.

More specifically, the total amount of 2N and 3N receptor dimers was reduced in size upon PNGase F digestion, demonstrating that all of the receptor molecules of those dimers are N-glycosylated. By contrast, 1N receptor dimers also contain nonglycosylated receptor molecules, since

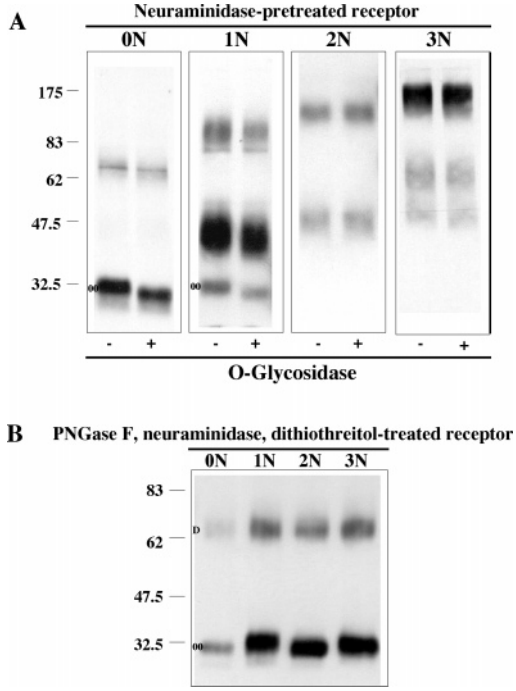

FIGURE 5: Effects of a combination of *O*-glycosidase with neuraminidase and of peptide-N-glycosidase F with neuraminidase on immunopurified cell surface N-glycosylation site mutant and wild-type B2 receptors from CHO-K1 cell lines. The cell surface wild-type receptor (3N) or receptors having zero (0N), one (1N), or two (2N) of the three potential glycosylation sites of the human B2 receptor were immunopurified in the presence of 10 mM iodoacetamide as described in Experimental Procedures. Immunoprecipitates were treated at 37 °C with 50 milliunits of neuraminidase for 2 h and then without (−) or with (+) 2 milliunits of *O*-glycosidase overnight (A), or with 10 IUB milliunits of PNGase F for 15 min and then with 50 milliunits of neuraminidase for 2 h (B), as described in Experimental Procedures. Samples were supplemented with an equal volume of nonreducing 2× sample buffer (A) or of 2× sample buffer containing 100 mM dithiothreitol (B), before being heated for 5 min at 100 °C and run on 10% (w/v) SDS−PAGE. D corresponds to dimer and 00 to the non-N-glycosylated sialic acid-free monomer. Molecular standard mass (in kilodaltons) positions are indicated at the left of the panel. The results are representative of experiments repeated two (A) or three times (B).

PNGase F digested only the dimers that exceeded in size the 0N receptor dimers.

With regard to monomers, all of the heterogeneous monomer M1, M2, and M3 populations disappeared with PNGase F, indicating their N-glycosylated nature. Monomers 01 and 02, observed with 0N and 1N receptors, are clearly not N-glycosylated, but they are sialylated on O-linked oligosaccharide. Indeed, they were endoglycosidase H- and PNGase F-resistant (Figure 4A,B) but disappeared upon neuraminidase treatment (Figure 4C) in favor of a lighter monomer (called monomer 00) of 32.6 ± 0.6 kDa ($n = 6$) that decreased in mass to 31.0 ± 0.5 kDa ($n = 4$) upon combination of *O*-glycosidase with neuraminidase (see the 0N and 1N receptors in Figure 5A).

Unexpectedly, N-deglycosylation of 2N and 3N receptors resulted in the appearance of a monomer called monomer 01′ [34.0 ± 0.4 kDa ($n = 12$)], which was lighter than monomers 01 and 02 (Figure 4C). Contrary to monomers 01 and 02, monomer 01′ is absent in nontreated receptors. This means that monomer 01′ is totally engaged in the N-glycosylation process and does not participate as such in dimer formation. However, it was reduced to monomer 00 upon cotreatment with PNGase F and neuraminidase, like monomers 01 and 02 (Figure 5B). Note that, upon cotreatment with PNGase F and neuraminidase, the various receptors displayed identical migration profiles, showing a monomer migrating as monomer 00 and a dimer migrating at a size twice that of monomer 00.

We therefore conclude that the 31 kDa species becomes O-glycosylated to give monomer 00 which undergoes a process that adds different neuraminidase-sensitive residues (sialic acid) to give either monomers 01 and 02 for 0N and 1N receptors or monomer 01′ for 2N and 3N receptors, the resulting species being N-glycosylated by mature oligosaccharide depending on their N-glycosylation site content (see Table 1).

N-Glycosylation, Sialylation, and Disulfide Bonding Participate in Cell Surface B2 Receptor Dimerization. Before presenting the data, we should emphasize that enzyme or dithiothreitol treatment may modify the epitope reactivity with the antibody due to receptor structural changes. The results, therefore, have to be analyzed in terms of changes in the dimer/monomer ratio rather than on the basis of the absolute Western blot monomer and dimer signals. The comparison of Figures 4A and 5B shows that the dimer/monomer ratio was in favor of monomer for receptors treated with a combination of PNGase F, neuraminidase, and the disulfide bond-disrupting agent dithiothreitol, compared to nontreated receptors. This, therefore, suggests that N-linked glycosylation, sialylation, and/or disulfide bonding might be involved in receptor dimerization.

The role of disulfide bonding is demonstrated in Figure 6A which shows that, for both 0N and 3N receptors, dithiothreitol alone reduced the dimer/monomer ratio.

The role of O-linked oligosaccharide-attached sialic acid is seen in Figure 4C in the case of 0N and 1N receptors (compare receptors treated and not treated with neuraminidase). It was studied further in Figure 6B (left panel) by treating the 0N receptor with increasing amounts of neuraminidase. Dose-dependent desialylation of the 0N receptor resulted in a progressive decrease in the size of both the monomer and dimer, and a decrease in the dimer/monomer ratio. Note that neuraminidase had no apparent effect on the dimer/monomer ratio in the case of 2N and 3N (wt) receptors (see Figure 4C), or when the 3N receptor was treated with this enzyme following N-deglycosylation with PNGase F (Figure 6B, right panel). Together, the observations given above might suggest that, for those heavily N-glycosylated receptors, N-glycosylation would have stabilized a sialylation-dependent process involved in receptor dimerization.

The fact that N-glycosylation could by itself contribute to receptor dimerization was assessed on the 3N (wt) receptor by varying the PNGase F concentration (Figure 6C). Progressive receptor deglycosylation resulted in the parallel progressive disappearance of heavy species and the appearance of lighter species among the dimers. Among monomers, deglycosylation led first to the disappearance of tri- and diglycosylated monomers. This was followed by the appearance (at 0.1 IUB milliunits of PNGase F) of monoglycosylated monomers that progressively disappeared at higher enzyme concentrations, in favor of nonglycosylated monomers. We conclude that receptor dimerization involves N-glycosylation, because deglycosylation of the observed

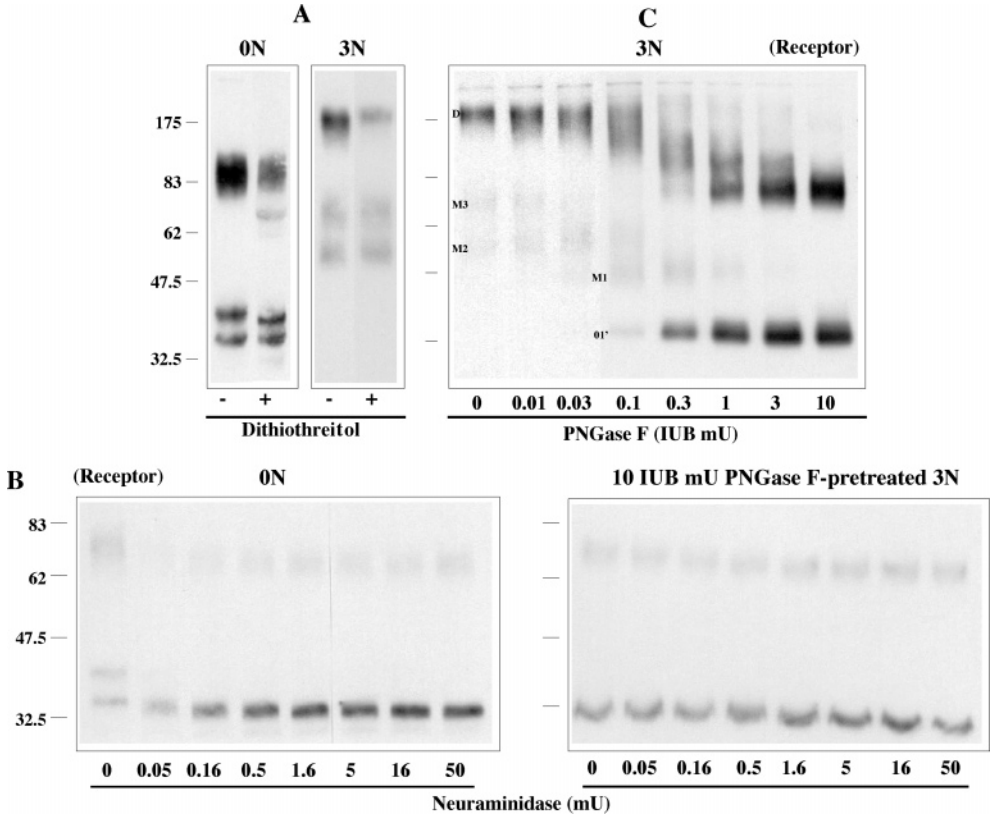

FIGURE 6: Role of disulfide bonding, sialylation, and N-glycosylation in cell surface B2 receptor dimerization. CHO-K1 cell lines were used, and receptor immunopurification was conducted in the presence of 10 mM iodoacetamide as described in Experimental Procedures. (A) Before being run on 10% (w/v) SDS−PAGE, the immunopurified wild-type receptor (3N receptor) or the mutant lacking all N-glycosylation sites (0N receptor) was heated for 5 min at 100 °C with 1× sample buffer with (+) or without (−) 50 mM dithiothreitol. An equal amount of immunoprecipitate was used for dithiothreitol-treated samples and nontreated corresponding controls. (B) The same pool of immunoprecipitates of the mutant (0N receptor) lacking all N-glycosylation sites (left) or of the 3N receptor treated with 10 IUB milliunits of PNGase F for 15 min at 37 °C (right) was divided into eight equal samples that were then incubated for 2 h at 37 °C with the indicated concentrations of neuraminidase, before being heated with nonreducing sample buffer and subjected SDS−PAGE. (C) The same pool of immunoprecipitates of the 3N receptor was divided into eight equal samples that were incubated for 15 min at 37 °C with the indicated concentrations of PNGase F, before being heated with nonreducing sample buffer and subjected to SDS−PAGE. Molecular standard mass (in kilodaltons) positions are indicated at the left. The results are representative of experiments performed three times.

glycosylated monomers hardly accounts for the increase in the level of deglycosylated monomers.

DISCUSSION

On the surface of resting CHO-K1 and HEK 293 cells, used here as expression systems, the human B2 receptor is expressed as N-linked complex-type fully processed oligosaccharide- and sialic acid-containing molecules that are partly arranged in dimers. In dimers, the receptor is present as two forms that assemble in a heterologous manner, thus leading to the existence of a variety of cell surface B2 receptor complexes. These two forms correspond to a receptor species with sialic acid attached to the O-linked oligosaccharide, like the vasopressin V2 receptor (*29*), which is glycosylated on either two (N3 and N12) or three (N3, N12, and N180) potential N-glycosylation sites of the receptor. This differs from the case of the purified rat uterus B2 receptor which appears to be a non-O-glycosylated protein with sialic acid attached to N-linked oligosaccharide chains (*30*). The multiplicity of cell surface oligomers very likely prevails for other GPCRs because many of them (*31*−*38*) display several immunoreactive bands or a diffuse migration pattern in Western blot analysis. But, to our knowledge, this is the first study characterizing the participating receptor molecules.

This study shows that, among the potential N-glycosylation sites of the human B2 receptor, two of them (N3 and N12 on the N-terminal domain) are systematically glycosylated, whereas N180 located on the second extracellular loop is not systematically glycosylated. As N-glycosylation is a cotranslational process and continues throughout protein folding and transport to the *trans*-Golgi (*39*), this difference could be due to a three-dimensional structure in the vicinity of the N180 site that is less permissive with respect to N-glycosylation. The systematical glycosylation of N3 and N12 is functionally important because we have previously observed that the glycosylation of those sites is required for efficient coupling of the receptor (*6*).

Another feature of the human B2 receptor, revealed in this study, is the multiplicity of processes [sialylation of O-linked oligosaccharide, N-glycosylation, disulfide bonding, and yet unknown process(es)] involved in its dimerization. Such a multiplicity of regulatory processes is probably to protect the receptor dimers from dissociation. With regard to other GPCRs, oligomerization has been found to involve disulfide bonding (*11*−*16*) or interactions of transmembrane domains (*17*, *18*) or of the C-terminal tail (*19*, *20*). Several studies have suggested that a combination of interactions between multiple domains occurs during GPCR dimerization (*11*, *17*, *40*), but whether these interactions intervene independently

is not known. For the human B2 receptor, all of the involved processes positively intervene to stabilize its cell surface dimers, and N-glycosylation appears to stabilize a yet unknown O-linked oligosaccharide sialylation-dependent process involved in receptor dimerization. This phenomenon asks for further investigation.

As far as we know, sialylation regulation of GPCR oligomerization has been ignored to date, and there are only two precedents for participation of N-linked oligosaccharides. N-Glycosylation has been found to be a positive and negative regulator for β1-adrenergic receptor homodimerization (*22*) and for α2A- and β1-adrenergic receptor heterodimerization (*23*), respectively.

Finally, unlike that of other glycoproteins such as mucin, follicle-stimulating hormone, or the insulin receptor (*41*−*43*), N-linked glycosylation of the human B2 receptor is not required for dimer formation by disulfide bonds. For those proteins, a conformational effect (*44*, *45*) via hydrophobic contacts between *N*-glycans and amino acids is believed to be responsible for the influence of N-glycosylation on disulfide-dependent dimer formation.

In conclusion, the human B2 receptor possesses several structural properties that allow it to behave as highly stable complex dimers on the cell membrane where the receptor recognizes ligands, converts ligand binding to modulate cellular activity, and is regulated. This would underlie an important functional significance that should be investigated after a means of blocking the receptor dimerization is found.

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