Role of Asparagine-Linked Oligosaccharides in the Function of the Rat PTH/PTHrP Receptor†

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ABSTRACT: The receptor for parathyroid hormone (PTH) and PTH-related peptide (PTHrP) is a G-protein-coupled receptor with four potential sites for N-linked glycosylation. The contribution of the oligosaccharide moieties to cell surface expression, ligand binding, and signal transduction was investigated. Site-directed mutagenesis of the rat PTH/PTHrP receptor cDNA was performed at single or combination of the four potential glycosylation sites to determine the effect of the putative carbohydrate chains on the activities of the receptor. The results revealed that all four potential N-glycosylation sites in the PTH/PTHrP receptor are glycosylated. Receptors missing a single or multiple glycosylation consensus but with at least one intact glycosylation site expressed sufficiently and functioned normally. In contrast, the nonglycosylated receptor, in which all four glycosylation sites were mutated, is deficient in these functions. These data indicate important roles for N-linked glycosylation in PTH/PTHrP receptor functions.

Parathyroid hormone (PTH)[1] is an important regulator of calcium and phosphate homeostasis. PTH-related peptide (PTHrP) causes the syndrome of humoral hypercalcemia of malignancy and has a critical role in fetal development. The amino-terminal fragments of PTH and PTHrP bind to a G-protein-coupled receptor, the PTH/PTHrP receptor, with similar affinities. The PTH/PTHrP receptor belongs to a subfamily of G-protein-coupled receptors (GPCRs), that includes receptors for several peptide hormones such as calcitonin (*1*), corticotropin-releasing factor (*2*), secretin (*3*), and several others (*4*). The PTH/PTHrP receptor, like other GPCRs, has seven membrane-spanning domains and is glycosylated on asparagine residues (*5*−*7*).

Glycosylation is a common posttranslational feature in the GPCR superfamily. The oligosaccharide moieties of the receptors may be involved in a variety of biological activities (*4*, *8*). These activities include maintenance of receptor stability and solubility, folding, and trafficking of the receptor to the cell surface, ligand binding, and stimulation of biological functions (*8*−*10*).

Previous studies have shown that treatment of the rat PTH/PTHrP receptor with endoglycosidase F decreased its apparent molecular weight on SDS−PAGE (*11*, *12*), indicating that the PTH/PTHrP receptor is N-glycosylated. The molecular cloning of the PTH/PTHrP receptor from a variety of species revealed four highly conserved potential N-linked glycosylation sites containing the N−X−S or N−X−T motifs at the amino terminal extracellular domain (N151, N161, N166, and N176).

The region containing the four potential N-linked glycosylation sites, N151−N176, overlaps with a putative ligand binding domain. The importance of this domain for ligand binding was demonstrated by mutagenesis studies (*13*), which showed that replacement of the rat PTH/PTHrP receptor fragment (L171−M189) with the corresponding sequence from the rat secretin receptor abolished PTH binding (*13*). The potential role of this region in ligand binding was also confirmed by a chemical cross-linking mapping approach, which suggested that the F173−M189 region is in a close proximity to the bound ligand (*14*). Interestingly, this region contains one potential glycosylation site, Asn-176; this raises the possibility that this site might participate in ligand interaction.

Inhibition of glycosylation by tunicamycin suggested that the N-linked carbohydrates are not essential for high-affinity ligand binding in HEK293 cells, stably expressing the human PTH/PTHrP receptor (*15*). However, tunicamycin usually disrupts the glycosylation of all the cellular proteins and reduces the viability of the cells. Additionally, the fully glycosylated receptors, which are already inserted in the plasma membrane, might have a long half-life in tunicamycin-treated cells and might not be fully replaced with newly synthesized nonglycosylated receptors.

To assess the role of the oligosaccharides attached to the PTH/PTHrP receptor, site-directed mutagenesis was performed to prevent glycosylation at a single or combined potential N-linked glycosylation sites. The Asn residues within the glycosylation consensus sequence (Asn−X−Ser/Thr) were replaced with Gln residues. This approach allows evaluation of the contribution of each potential N-linked

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[1] Abbreviations: CNBr, cyanogen bromide; DMEM, Dulbecco's modified Eagle's medium; Endo F, endoglycosidase F; FSH, follicle-stimulating hormone; GPCRs, G-protein-coupled receptors; LHR, luteinizing hormone receptor; NlePTH, [$Nle^{8,18}$,Tyr^{34}]bPTH(1−34)NH_2; PTH, parathyroid hormone; PTHrP, PTH-related peptide; SDS−PAGE, SDS polyacrylamide gel electrophoresis; TM, tunicamycin; TXA2R, thromboxane A2 receptor; WT, wild-type.

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glycosylation site to the expression, binding, and signaling properties of the receptor without disturbing the cellular environment. Since there is an absolute requirement for the hydroxyl groups of either Ser or Thr to serve as hydrogen bond donors during glycosylation of the Asn residue (*16*), substitution of the Ser or Thr residues within a glycosylation consensus prevents glycosylation at the corresponding Asn residues (*16*, *17*). Therefore, another set of mutants were constructed to alter the glycosylation consensus sequences by substituting Ser or Thr with Ala. Comparing the properties of the two sets of mutations addresses the issue that the biological properties of the mutant receptors are secondary to lack of glycosylation at these sites rather than secondary to amino acid substitutions.

MATERIALS AND METHODS

Materials. [$Nle^{8,18}$,Tyr^{34}]bPTH(1−34)NH_2 (NlePTH) was synthesized by a solid-phase method on an Applied Biosystem 430A automated peptide synthesizer (Applied Biosystems, Foster City, CA). All chemicals used in the experiments were sequencing grade from Sigma Chemical Co (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). Restriction enzymes were purchased from United States Biochemical Corp. (Cleveland, OH) and Promega (Madison, WI); endoglycosidase F (Endo F) was from New England BioLab (Beverly, MA). Tissue culture media was prepared by the Massachusetts General Hospital Media Facility, fetal bovine serum (FBS) was from Sigma (St. Louis, MO), streptomycin, penicillin, and lipofectin were from Gibco-BRL (Gaithersburg, MD), and tissue culture flask and plate were from Corning (Oneonta, NY). NaI^{125} (2125 Ci/mmol) and chemiluminescence reagents were from NEN Life Science Products (Boston, MA).

Site-Directed Mutagenesis and Expression of the PTH/PTHrP Receptor in COS-7 Cells. Oligonucleotide-mediated site-directed mutagenesis was performed using the method described by Kunkel et al. (*18*). Four oligonucleotides were designed to replace Asn residues at positions N151, N161, N166, and N176 with Gln residues. Additional other four oligonucleotides were constructed to replace Ser153, Thr163, Ser168, and Thr178 residues with Ala residues. Single-site mutations were first constructed. To understand the cumulative effects of lack of glycosylation at multiple sites, three double, N151Q/N166Q, N161Q/N166Q, and N166Q/N176Q, and one triple, N161Q/N166Q/N176Q (3NQ), receptor mutants were constructed. Two nonglycosylated mutants were also constructed, 4NQ and S153A/3NQ. In 4NQ the four potential glycosylated Asn residues were replaced with Gln residues, N151Q/N161Q/N166Q/N176Q. In S153A/3NQ the first potential glycosylation consensus was disrupted by S153A mutation and the three other Asn residues in the consensus were replaced by Gln residues, S153A/N161Q/N166Q/N176Q. The nucleotide sequence of the mutagenized region and adjacent sequences were confirmed by sequencing. A V156M mutant was made for peptide mapping of glycosylation sites using CNBr cleavage and SDS−PAGE analysis.

Cell Cultures and Transient Expression of the Receptor Mutants. The mutated and wild-type PTH/PTHrP receptors were transiently expressed in COS-7 cells, which do not express endogenous PTH/PTHrP receptor. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin, and streptomycin in a humidified atmosphere containing 95% air and 5% CO_2 at 37 °C. The cells were transfected with cDNAs, cloned in the PcDNA1 plasmid using the DEAE−dextran method. Three days after transfection the cells were tested for receptor expression, capacity and affinity of binding, and PTH-stimulated cAMP accumulation.

Lipofectin Transfection and Tunicamycin Treatment. COS-7 and LLCPK-1 cells were trypsinized and replated in a 24-well plate at a density of 1×10^5 cells/well. More cells (2×10^5 cells/well) were plated in tunicamycin (TM) treated wells because almost half of the cells floated during the transfection**.** COS-7 and LLCPK-1 cells were transfected with 2 μg of cDNA and 6 μL of lipofectin reagent per well in serum-free DMEM for 16 h. TM (1 μg/mL) was added into the culture at the moment of adding the plasmid DNA. After 16 h the medium was replaced with DMEM with 10% FBS and 1 μg/mL TM. The receptors expressed were analyzed after 48 h of transfection. For the stably transfected LLCPK-1 cell line TM (7.5 μg/mL) was added 1 h after the cells were plated in 24-well plates. The receptors expressed in the stably transfected cell line were analyzed after 48 h of incubation. Different concentrations of TM were used in the transient transfected cell lines (1 μg/mL) and stably transfected cell line (7.5 μg/mL). These concentrations inhibited glycosylation completely in these cell lines.

Radioiodination of NlePTH. NlePTH peptide was radioiodinated with 1.5 mCi NaI^{125} using chloramine T and purified by HPLC (*12*).

Cell Surface Receptor Expression Assay. Cell surface expression of PTH/PTHrP receptor was quantified by an antibody binding assay on intact cells. Transfected cells were rinsed three times with PBS, pH 7.4, and then incubated for 1 h with an antirat PTH/PTHrP receptor antiserum, G48, which was diluted to 1:5000 in PBS containing 5% FBS (fetal bovine serum). The antiserum G48 was developed against a synthetic 18-amino acid peptide (DKGWTPASTSGKPRKEKA), representing an epitope from the amino terminal extracellular region of the receptor, named E2 epitope. The G48 antiserum was purified using the antigen peptide immobilized onto a CNBr-activated Sepharose 4B column (16). Cells were then rinsed three times with PBS, incubated with a rabbit antisheep IgG (H + L) (Kirkland & Perry Laboratories, Inc., Gaithersburg, MD) for 1 h, rinsed three times with PBS, and then incubated with ^{125}I-labeled goat antirabbit IgG (200,000 cpm/well) for an additional 2 h period. The cells were then rinsed with PBS three times and solublized in 1 N NaOH, and the radioactivity was counted by a γ counter (Micromedic System Inc., model 6/400).

I^{125}-NlePTH Ligand Binding Analysis. Transfected cells in 24-well plates (90% confluent) were washed twice with 0.5 mL binding buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5% heat-inactivated horse serum, and 0.5% heat-inactivated fetal bovine serum, pH 7.7). The cells were then incubated with ^{125}I-NlePTH (200 000 cpm/well) in the presence of different concentrations of unlabeled NlePTH (0, 0.01, 1.0, 3.0, 10, 30, 100, and 1000 nM) at 15 °C for 4 h. The cells were rinsed three times with ice-cold binding buffer and lysed with 0.5 mL of 1 N NaOH. After the lysates were collected each well was rinsed twice with

Table 1: Properties of PTH/PTHrP Receptors of Wild Type and Mutations of N-Linked Glycosylation Sites[a]

			PTH binding			PTH-stimulated cAMP accumulation		
receptor	expression (wt %)	*p*	Bo (wt %)[b]	*p*	k_d (nM)	wt %	*p*	EC50 (nM)
WT	100 ± 8		100 ± 3		12 ± 2	100 ± 5		4.0 ± 1.1
N151Q	89 ± 7		97 ± 5		13 ± 1	137 ± 5		0.6 ± 0.1
N161Q	82 ± 6		81 ± 2		13 ± 2	95 ± 2		1.5 ± 0.2
N166Q	83 ± 2		83 ± 6		13 ± 2	116 ± 8		0.4 ± 0.1
N176Q	98 ± 6		71 ± 2	>0.05	14 ± 3	119 ± 1		1.5 ± 0.3
S153A	95 ± 5		88 ± 2		14 ± 1	80 ± 1	<0.05	0.7 ± 0.2
T163A	85 ± 6		67 ± 3	<0.05	16 ± 2	114 ± 3		0.4 ± 0.1
S168A	83 ± 9		74 ± 2	<0.05	16 ± 1	112 ± 4		0.6 ± 0.1
T178A	81 ± 4		79 ± 4	<0.05	13 ± 3	139 ± 2		1.0 ± 0.2
N151/166Q	82 ± 9		69 ± 4	<0.05	11 ± 3	118 ± 4		2.0 ± 0.2
N161/166Q	85 ± 4		65 ± 5	<0.05	11 ± 2	95 ± 7		0.7 ± 0.1
N166/176Q	97 ± 2		70 ± 2	<0.05	11 ± 2	142 ± 3		0.3 ± 0.1
3NQ	59 ± 1	<0.001	40 ± 4	<0.05	10 ± 4	55 ± 5	<0.01	7.0 ± 0.4
4NQ	38 ± 3	<0.001	9 ± 1	<0.01	ND	36 ± 3	<0.01	28.0 ± 1.9
S153A/3NQ	46 ± 4	<0.001	11 ± 1	<0.01	ND	39 ± 2	<0.05	6.0 ± 2.2
PcDNA	9 ± 1		10 ± 1		ND	not detected		

[a] Cell surface expression and PTH binding were measured by G48 antibody binding and ^{125}I-NlePTH binding to intact cells, respectively. PTH-stimulated cAMP accumulation was determined in cells challenged with increasing concentrations of NlePTH (0.01−1000 nm) in presence of IBMX (2 mM). The data are means ± SD of three experiments. ND: not determined. [b] Total ligand binding (Bo) was measured for each mutant in 24-well plates and reported as percentage of total ligand binding in COS-7 cells transfected with WT PTH/PTHrP receptor cDNA. Nonspecific binding was not substracted.

0.5 mL of 1 N NaOH and the rinses were collected in the same tube. The radioactivity of the lysate was measured by a γ counter.

Cellular cAMP Measurement. Transfected cells were chilled on ice for 30 min, rinsed with ice-cold PBS, and incubated with DMEM containing 3-isobutyl-1-methylxanthine (IBMX) (2 mM), BSA (1 mg/mL), and HEPES (35 mM), pH 7.4. NlePTH was then added to the chilled cells. The 24-well plates were transferred to a 37 °C water bath for 15 min. At the end of stimulation the supernatant was removed and the plates were frozen on dry ice for 10 min. Intracellular cAMP was extracted by thawing the cells in 1 mL of 50 mM HCl. An aliquot of the acid extract was diluted (1:100) in Na acetate buffer (50 mM, pH 5.5), and the cAMP concentration was measured by a radioimmunoassay (*20*).

Western Blot and SDS−PAGE. Transfected cells in 10 cm dish were lysed in 0.5 mL of 1% SDS in Tris/HCl (62.5 mM, pH 6.8). The lysate was forced 5 times through a fine needle (25G) to break the DNA. The samples were centrifuged at 12 000 rpm in a Beckman microcentrifuge at 4 °C for 30 min, and the precipitates were discarded. The 4 μL volumes of the lysates were analyzed on a gradient (5−20%) SDS−PAGE. The SDS−PAGE was performed according to Laemmli (*21*) using a Bio-Rad minigel apparatus. After SDS−PAGE, the separated proteins were electrophoretically transferred onto nitrocellulose sheets and probed with the G48 first antibody (1:5000) followed by a peroxidase-labeled antisheep second antibody (1:200). The protein bands were visualized with a chemiluminescence reagent kit (NEN).

Cyanogen Bromide (CNBr) Cleavage of the Wild-Type and V156M Mutant Receptors. Cell lysates (80 μL**)** were incubated with CNBr (30 mg/mL) in 70% formic acid at room temperature in the dark for 16 h. The CNBr-cleaved proteins were lyophilized for 20 h, and the lyophilized samples were reconstituted with double distilled H_2O.

Endoglycosidase F (Endo F) Treatment of CNBr Derived Fragments. The CNBr-cleaved fragments were incubated at 37 °C with 2500 U PNGase F in 50 mM sodium phosphate buffer containing 1% NP40, 0.5% SDS, and 1% β-mercaptoethanol (pH 7.5). The deglycosylated fragments were probed with the G48 antibody by Western blot.

RESULTS

Cell Surface Expression of PTH/PTHrP Mutant Receptors. Quantification of the PTH/PTHrP receptor immunoreactivity on intact cells showed no significant change in cell surface expression of the single mutants, N151Q, N161Q, N166Q, N176Q, S153A, T163A, S168A, and T178A (Table 1). The double mutant receptors, N151Q/N166Q, N161Q/N166Q, and N166Q/N176Q, were also well expressed (Table 1). Expression of the mutants, in which three or four glycosylation sites were mutated, was dramatically decreased (Table 1). The expression of 3NQ, 4NQ, and S153A/3NQ was decreased by 41%, 62%, and 54%, respectively (Table 1).

Ligand-Binding Properties. Binding of the radioligand ^{125}I-NlePTH in the presence of increasing concentrations of NlePTH was examined in the wild-type and the mutant receptors. Specific binding of the receptor mutants was compared to that of the wild type. The single mutants, N151Q, N161Q, and N166Q, had normal ligand binding (Table 1), whereas N176Q had a significant decrease in its binding activity (29%, $p < 0.05$, Table 1). The binding affinities of the N to Q mutants were comparable to that of the wild type. Significant decreases in the binding activity were also observed in three S/T to A substitution mutants: T163A, S168A, and T178A (22−33% decreases, $p < 0.05$, Table 1). The fourth S to A mutant, S153A, had a normal binding activity (Table 1). The binding affinity of the S/T to A mutants were similar to that of the wild type.

The effects of multiple combined glycosylation site mutations were then investigated. The mutants N151Q/N166Q, N161Q/N166Q, and N166Q/N176Q exhibited about 30−35% reduced specific binding activity ($p < 0.05$, Table 1), whereas their binding affinity was not significantly different from that of the wild type (Table 1). The binding capacity of the triple mutant, 3NQ, was impaired (40% of wild type, $p < 0.05$) with a normal binding affinity (Table

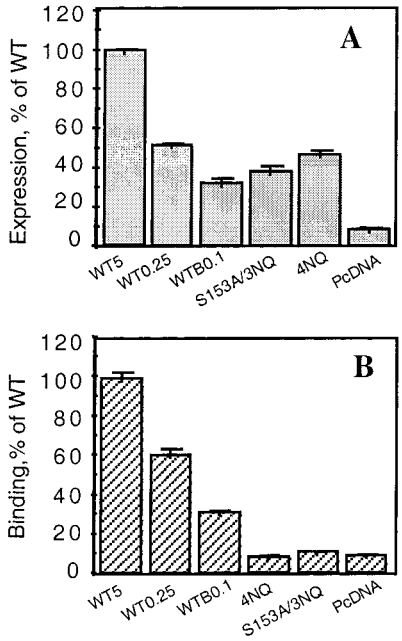

FIGURE 1: Cell surface expression and radioligand binding level of wild-type and nonglycosylated receptors. The amount of plasmid DNA of wild-type receptor used for transfection was 5, 0.25, and 0.1 μg. The amount of plasmid DNA used for transfection of the nonglycosylated mutants 4NQ and S153A/3NQ and pcDNA1 (vector) was 5 μg. Expression (A) and redioligand binding (B) were measured by antibody and I^{125}-NlePTH binding to intact cells, respectively, as described in Materials and Methods. The data are the mean of three experiments.

1). These data suggest that the impaired binding capacity was caused by a lower cell surface expression level of the mutant receptors.

Ligand binding of the nonglycosylated receptor mutant 4NQ was dramatically impaired ($p < 0.01$, Table 1). The low cell surface expression, which was 38% of that of the wild type, may contribute to its low binding. However, ligand binding to the other nonglycosylated mutant, S153A/3NQ, which was better expressed (46% of that of wild type), was also dramatically impaired ($p < 0.01$, Table 1). The ligand-binding affinity could not be estimated because of the nondetectable radioligand binding, however, that these nonglycosylated mutants had about 7-fold decrease in their cAMP stimulation potency suggests that these mutants have decreased ligand-binding affinities.

To examine if the low expression levels were responsible for the low binding of the nonglycosylated receptors we compared ligand binding levels of the wild-type and nonglycosylated receptors expressed at similar levels. To lower the expression level of the wild-type receptor, a smaller amount of plasmid cDNA was used for transfection. Thus transfection of COS-7 cells with 0.25 μg or 0.1 μg of wild-type DNA resulted in an expression level that was similar to that of 4NQ and S153A/3NQ, transfected with 5 μg of cDNA/10 cm plate (Figure 1 A). Ligand binding of the wild-type receptor transfected at 0.1 μg of DNA/dish was 30% of that transfected at 5 μg of DNA/dish (Figure 1B). In contrast, the nonglycosylated receptor mutants, had no detectable radioligand binding (Figure 1 B). These data suggest that the impaired binding of nonglycosylated receptors is not secondary to their low level of expression.

Tunicamycin (TM) treatment decreased receptor expression in both transiently transfected cell lines (LLCPK-1 and COS-7) and stably transfected cell line (LLCPK-1) by about 70% (Table 2). Tunicamycin treatment impaired the binding capacity of the receptors expressed transiently in LLCPK-1 and COS-7 cells; both did not show detectable binding activity (Table 2). In contrast, treatment with tunicamycin did not affect ligand binding in LLCPK-1 cells stably expressing the PTH/PTHrP receptor; both expression and binding decreased proportionally (Table 2). The PTH binding affinities of the stably expressed receptors were 3.7 nM for untreated cells and 3.3 nM for TM treated cells, respectively.

Table 2: Effects of Tunicamycin Treatment on PTH/PTHrP Receptor Expressed in COS-7 and LLCPK Cell Lines[a]

	% of untreated			EC50 (nM)	
cell lines	expression	binding	cAMP	untreated	treated
COS-7 (transient)	30 ± 2	ND	20 ± 2	4.0 ± 0.50	280.0 ± 15.0
LLCPK-1 (transient)	30 ± 1	ND	16 ± 1	5.0 ± 0.70	300.0 ± 20.0
LLCPK-1 (stable)	36 ± 3	30 ± 2	32 ± 3	0.2 ± 0.04	0.30 ± 0.02

[a] The PTH/PTHrP receptor was transiently or stably expressed in COS-7 and LLCPK-1 cell lines. Cells were treated with tunicamycin, 1 μg/mL for transiently transfected COS-7 and LLCPK-1 cells and 7.5 μg/mL for stably transfected LLCPK-1 cells. Cell surface expression, PTH binding, and PTH-stimulation of cAMP accumulation were measured and reported as % of these values in untreated cells. ND: not detected. The data are the means ± SD of three experiments.

Effect of Glycosylation on PTH-Stimulated cAMP Accumulation. The PTH/PTHrP mutant receptors were further characterized for their ability to stimulate cAMP accumulation. Cells expressing wild-type and mutant receptors were challenged with increasing concentrations of NlePTH in the presence of IBMX (2 mM).

All the single and double mutant receptors, including both N to Q and S/T to A mutations, produced similar or slightly higher levels of cAMP in response to NlePTH stimulation, except for the S153A mutant, which had 80% of the response of wild type. N151Q had a higher cAMP response when compared to that of the wild type; this indicates that the 20% lower level of cAMP in S153A is caused by amino acid residue mutation rather than the lack of glycosylation at this site. The EC50 values of the single and double mutants are lower than that of the wild type (Table 1).

The cAMP level measured in the triple mutant 3NQ reached 55% of the response of the wild-type receptor ($p < 0.01$, Table 1), a response that was proportional to its decreased expression. The nonglycosylated mutant, 4NQ, displayed only 36% of the response to NlePTH, compared to that of the WT ($p < 0.01$, Table 1). The EC50 of the 4NQ mutant was more than 7-fold higher than that of the wild type (28 nM versus 4 nM NlePTH for 4NQ and wild type, respectively). The other nonglycosylated mutant, S153A/3NQ, had 39% of the wild-type cAMP response to NlePTH ($p < 0.01$, Table 1) with about 6-fold higher EC50 (26 nM versus 4 nM for S153A/3NQ mutant and wild-type respectively).

Tunicamycin treatment impaired cAMP signal transduction in the transiently transfected cell lines (LLCPK-1 and COS-7); the cAMP simulation levels were only 16−20% of that in the nontreated control (Table 2). The EC50s, in both transiently transfected cell lines (LLCPK-1 and COS-7), increased about 60−70-fold compared to the values in nontreated cells. In the stably transfected LLCPK-1 cell line, tunicamycin treatment decreased cAMP accumulation, ligand

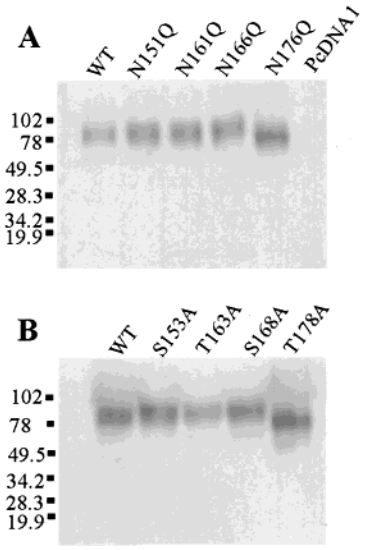

FIGURE 2: Immunoblots of wild-type and glycosylation mutant receptors. The wild-type and the mutant receptors were detergent solubilized, applied on SDS−PAGE, and separated under reduction and denature conditions. Nitrocellulose blots of proteins were probed with G48 antibody and then rabbit antisheep IgG second antibody as described in Materials and Methods. (A) Comparison of wild-type receptor with the receptors from Asn−Gln single mutants. (B) Comparison of wild-type receptor with the receptors from Ser/Thr−Ala single mutants.

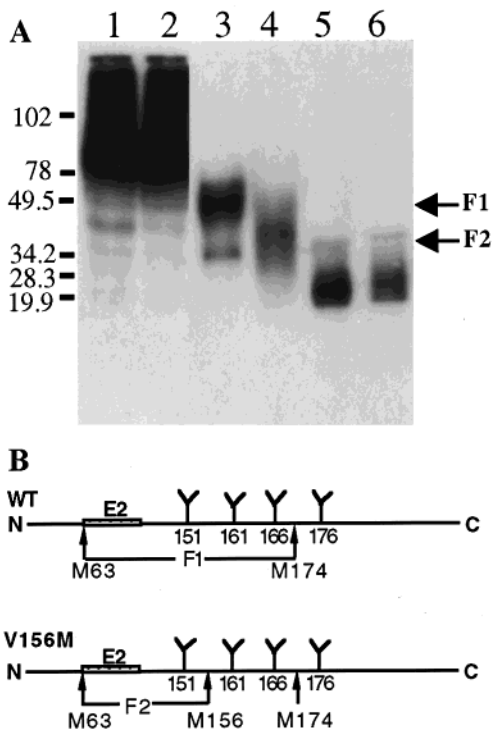

FIGURE 3: Schematic representation of the CNBr cleavage fragments of wild-type and V156M receptors. (A) Cleavage of wild-type and V156M mutant receptors with CNBr and deglycosylation of derived fragments: lane 1, wild-type receptor; lane 2, V156M receptor; lane 3, wild-type receptor fragment cleaved by CNBr; lane 4, V156M receptor fragment cleaved by CNBr; lane 5, deglycosylated fragment F1 from lane 3 with PNGase F; lane 6, deglycosylated fragment F2 from lane 4 with PNGase F. (B) Arrows point to the positions of authentic and mutated methionine. Y represents the potential glycosylation moiety.

binding, and expression proportionally. The EC50 values in the TM-treated and untreated stably transfected LLCPK-1 cells were also comparable (Table 2).

Analysis of Glycosylation Mutants on SDS−PAGE. To identify if any of the four potential glycosylation sites are glycosylated, the mutated receptors were analyzed on SDS−PAGE. No significant change in the apparent molecular mass was observed in the single mutants: N151Q, N161Q, and N166Q (Figure 2A). In contrast, the mutant N176Q had a reduced apparent molecular mass (Figure 2A). A similar SDS−PAGE migration pattern was observed for the Ser/Thr−Ala substitution mutants (Figure 2B); i.e., S153A, T163A, and S168A had a molecular mass similar to that of the wild type while T178A had a reduced molecular weight. Since migration on SDS−PAGE is influenced by the physical and chemical properties of the glycoproteins, these results do not exclude glycosylation at N151, N161, and N166; however, these results indicate that N176 is glycosylated.

Peptide mapping of CNBr-derived fragments was performed to assess if any of the other sites are glycosylated. CNBr cleavage of the wild-type receptor followed by Western blot generated a broad band with an apparent molecular mass of 49 kDa (F1, Figure 3A, lane 3). F1 represents the Gly64−Met174 fragment (Figure 3B), which contains three potential sites for N-linked glycosylation (N151, N161, and N166) and the E2 immunoreactive domain. This fragment is recognized by the G48 antibody, which probes the E2 domain. After CNBr cleavage of the V156M mutant receptor, a 39 kDa broad band was observed (F2, Figure 3A, lane 4). F2 represents the Gly64−Met156 fragment, which contains one potential glycosylation site (N151) and the E2 epitope (Figure 3B). The reduction in molecular weight of F2 after deglycosylation (Figure 3A, lane 6) indicates that N151 is glycosylated. The difference between F1 and F2 is a short peptide Val157−Met174 (18 amino acid residues) and two potential glycosylation sites (N161 and N166). Deglycosylation of F1 and F2 resulted in two fragments with a similar size on SDS−PAGE (Figure 3A, lanes 5 and 6). These data indicate that the glycosylation moieties attached to N161 and/or N166 contribute to most of the difference in molecular weight between F1 and F2. These results suggest that N161 and/or N166 are also glycosylated.

The double mutant N151Q/N166Q had a lower molecular mass compared to that of the WT receptor (Figure 4); this indicates that N166 and/or N151 is/are glycosylated as the single mutant of N151 and N166Q has the same size as that of the WT (Figure 2A). Since CNBr cleavage and peptide mapping established that N151 is glycosylated, the decreased apparent molecular mass of N151Q/N166Q, as compared to the single mutants, indicates that N166 is also glycosylated. The double mutant N161Q/N166Q has a lower molecular mass than the single mutants N161Q and N166Q and the double mutant N151Q/N166Q receptors (Figure 4). These data suggest that N161 is also glycosylated and confirm the results of CNBr cleavage which indicate that N161 and N166 are glycosylated. The triple mutant, 3NQ, has a further reduction in its molecular mass compared to double mutants. The 4NQ mutant has the lowest molecular mass compared to all the other mutants, which is equivalent to the expected mass of the backbone peptide of the PTH/PTHrP receptor, which is about 60 kDa.

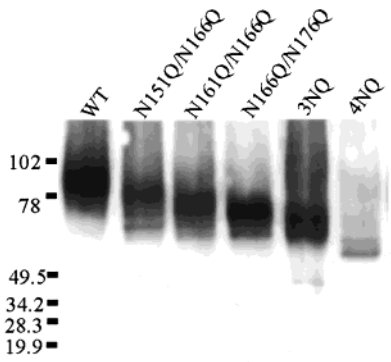

FIGURE 4: Comparison of wild-type receptor with the receptors from double, triple, and nonglycosylated mutants. The wild type and the mutant of double, triple, and nonglycosylated receptors were detergent solubilized, applied on SDS−PAGE, and separated under same conditions as in Figure 2. The Westen blot was also performed under the same condition.

DISCUSSION

This study demonstrates that all four potential glycosylation sites of PTH/PTHrP receptor are glycosylated. It is interesting to note that Western blot has revealed that mutation of the N176Q site markedly decreased the apparent molecular weight whereas single mutations of the other sites had no effect on the apparent molecular weight. It is possible that the other sites are minimally glycosylated or that removal of the glycosylation moiety at these sites does not influence the apparent molecular weight on SDS−PAGE. The progressive reductions of the apparent molecular mass observed with double, triple, and nonglycosylated mutants prove that loss of molecular weight at each of the single mutant is not detectable on SDS−PAGE, except with N176Q and T178A. Peptide mapping of CNBr-cleaved fragments of the wild type and V156M mutant followed by PNGase F digestion confirmed that N151, N161, and N166 are glycosylated.

This study also shows that glycosylation plays important roles in the functions of the receptor. The decreased expressions of the nonglycosylated receptor mutants may be secondary to the decreased stability of the receptor protein and/or missorting of the receptor to the membranes. Therefore, glycosylation may influence the receptor half-life on the cell surface**.** The specific PTH binding levels and binding affinities of the single mutants were comparable to those of the wild-type receptor (Table 1, Figure 2). These results indicate that the carbohydrate moiety at each single glycosylation site is not absolutely required for ligand binding. Even in the double mutants, in which two of the glycosylation sites were disrupted, the binding levels were similar to that of the single mutants. The disruption of three glycosylation sites is, however, less tolerated. A decreased binding of the triple mutant was observed, though with a normal affinity; these data suggest that the decreased binding of the 3NQ mutant is the result of its reduced expression. The nonglycosylated mutants, 4NQ and S153A/3NQ, exhibit no detectable radioligand binding.

These data indicate that glycosylation of at least one site is required for ligand binding. This finding was also observed in other G-protein coupled receptors. For example, follicle-stimulating hormone (FSH) receptor lost its binding activity when both of its glycosylation sites were disrupted, even though the receptor maintained the binding activity with either of the single glycosylation sites intact (*9*). Thromboxane A2 receptor (TXA2R) has also good binding when one glycosylation site was intact, and when both sites were mutated the ligand binding of TXA2R was abolished (*22*). Glycosylation of the PTH/PTHrP receptor may be also important for the signal transduction. The EC50 values for PTH-stimulated cAMP accumulation by the two nonglycosylated receptors increased by 6−7-fold; this indicates that theoligosaccharides at least on one glycosylation site may be required for optimal stimulation of cAMP accumulation.

Normally, decreased ligand binding is secondary to decreased affinity and/or receptor number. However, single and double mutations of some glycosylation sites, such as N176Q, T163A, S168A, T178A, N151/166Q, N161/166Q, and N166/176Q, had decreased ligand binding without decreasing the apparent binding affinity or cell surface expression, as measured by an antibody to the extracellular domain of the receptor. These data raise several possibilities. It is possible that two immunoreactive receptor populations are present on the cell surface, a fully functional receptor population with a normal apparent ligand-binding affinity and a nonfunctional receptor population with no ligand binding capacity. The nonfunctional receptor population may have an impaired receptor conformation secondary to the mutation(s) and/or because of the lack of glycosylation at these sites. Alternatively, ligand−receptor interaction in intact cells may increase the internalization of these mutants more than that of the wild type, or the antiserum binding may delay the rate of internalization of the wild-type receptor as compared to the mutants. Effects of the mutations on receptor internalization and receptor−G-protein coupling may also explain the decreased EC50 of PTH-stimulated cAMP accumulation despite a normal apparent binding K_d. Further studies will be required to distinguish between these alternate hypotheses.

In presence of tunicamycin, the PTH/PTHrP receptor, transiently transfected in COS-7 or LLCPK-1 cells, showed impaired expression, binding activity, and cAMP stimulation. In contrast, tunicamycin did not affect ligand binding and cAMP stimulation of the stably transfected LLCPK-1 cell line. It has been reported that the human PTH/PTHrP receptor, stably expressed in HEK293 cells, maintained good binding activity and signal transduction levels in the presence of tunicamycin (*15*). It is noticed that tunicamycin treatment of transiently expressed LH, TXA_2, and calcitonin receptors resulted in loss of binding (*8*, *22*, *23*). In contrast, tunicamycin treatment did not affect the binding activity of stably transfected LH and PTH/PTHrP receptors (*9*, *15*). These reported findings suggest that the functional assessment of deglycosylated receptors, using tunicamycin treatment, should be interpreted with caution. The possible explanation for this finding is that tunicamycin treatment of stable cell lines may prevent glycosylation of newly synthesized receptor but does not affect the glycosylation of receptors that are already inserted in the membranes.

In conclusion, all of the potential N-glycosylation sites N151, N161, N166 and N176 in PTH/PTHrP receptor are glycosylated. At least one N-linked glycosylation of the receptor is required for its expression, ligand binding, and signal transduction.

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