Role of Glycosylation in Expression and Function of the Human Parathyroid Hormone/Parathyroid Hormone-Related Protein Receptor†

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Received August 21, 1996; Revised Manuscript Received October 17, 1996⊗

ABSTRACT: Parathyroid hormone (PTH) regulates calcium metabolism through a specific G protein-coupled, seven-transmembrane helix-containing receptor. This receptor also binds and is activated by PTH-related protein (PTHrP). The human (h) PTH/PTHrP receptor is a membrane glycoprotein with an apparent molecular weight of approximately 85 000 which contains four putative N-glycosylation sites. To elucidate the functional role of receptor glycosylation, if any, we studied hormone binding and signal transduction in human embryonic kidney cells transfected with hPTH/PTHrP receptor (HEK-293/C-21). These cells stably express 300000−400000 receptors per cell. Inhibition of N-glycosylation with an optimized concentration of tunicamycin yielded completely nonglycosylated hPTH/PTHrP receptor (∼60 kDa). This receptor form is fully functional; it maintains nanomolar binding affinity for PTH- and PTHrP-derived agonists and antagonists. PTH and PTHrP agonists stimulate cyclic AMP accumulation and increases in cytosolic calcium levels. In addition, the highly potent benzophenone (pBz$_2$)-containing PTH-derived radioligand [Nle^{8,18},Lys^{13}(ϵ-pBz$_2$),L-2-Nal^{23},Tyr^{34}(3-^{125}I)]bPTH(1−34)NH$_2$ can photoaffinity cross-link specifically to the nonglycosylated receptor. The molecular weight (∼60 000) of the band representing the photo-cross-linked, nonglycosylated receptor (obtained from the tunicamycin-treated HEK-293/C-21 cells) was similar to that of the deglycosylated photo-cross-linked receptor (obtained by enzymatic treatment with Endoglycosidase-F/*N*-glycosidase-F). Our findings indicate that glycosylation of the hPTH/PTHrP receptor is not essential for its effective expression on the plasma membrane or for the binding of ligands known to interact with the native receptor. The nonglycosylated hPTH/PTHrP receptor remains fully functional with regard to both of its known signal transduction pathways: cAMP−protein kinase A and phospholipase C−cytosolic calcium.

Parathyroid hormone (PTH),[1] the major regulator of calcium homeostasis, and PTH-related protein (PTHrP), an abundantly expressed paracrine growth and differentiation hormone also associated with hypercalcemia of malignancy, bind and activate a specific G protein-coupled seven-transmembrane helix-containing receptor (Chorev & Rosenblatt, 1994; Jüppner, 1994). The PTH/PTHrP receptor is coupled to at least two intracellular signal transduction "second messengers": cyclic AMP and calcium/inositol triphosphate (Jüppner, 1994; Abou-Samra et al., 1992; Jüppner et al., 1991; Civitelli et al., 1989; Pines et al., 1996).

The PTH/PTHrP receptor belongs to a subfamily of G protein-coupled receptors which includes the receptors for calcitonin, growth hormone-releasing hormone, vasoactive intestinal polypeptide, secretin, corticotropin-releasing factor, glucagon, and others (Jüppner, 1994, and references therein). Members of this receptor subfamily share considerable sequence homology, conserved cysteine residues, and at least two conserved putative N-glycosylation sites in the extracellular domain.

Cross-linking studies carried out by different groups (Karpf et al., 1987; Shigeno et al., 1988), employing PTH analogs derivatized with an arylazide group as the photoreactive moiety, demonstrated that the PTH/PTHrP receptor expressed in rat osteosarcoma cells (ROS 17/2.8) (Shigeno et al., 1988) and canine renal cortical membranes (Karpf et al., 1987) is a glycoprotein with an apparent molecular weight (M_r) of approximately 85 000. The PTH/PTHrP receptors appear to contain several complex asparagine-linked (N-linked) oligosaccharide chains which account for almost 30% of the M_r. No O-linked oligosaccharides chains have been identified (Karpf et al., 1987; Shigeno et al., 1988).

The cloning of PTH/PTHrP receptors from four species (rat, opossum, mouse, and human) reveals the presence of four triads (Asn-Xxx-Ser/Thr) which conform with the consensus motif characteristic of potential N-glycosylation sites (Marshall, 1974). These sites are located at the extracellular N-terminal domain of the receptor (positions 151, 161, 166, and 176 in the human receptor) (Schipani et al., 1993). Interestingly, the position of the consensus motif for the four N-glycosylation sites is highly conserved between

† This work was supported, in part, by Grant RO1-DK47940 from the National Institute of Diabetes, Digestive and Kidney Diseases to M.R.

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⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1996.

[1] Abbreviations: b, bovine; C-21, transfected HEK-293, human embryonic kidney cells; b, bovine; PTH, parathyroid hormone; PTHrP, PTH-related protein; C-21, transfected HEK-293 cells stably expressing hPTH/PTHrP receptor; D-MEM, Dulbecco's minimal essential medium; EDTA, ethylenediaminetetraacetic acid; Endo-F, endoglycosidase-F/*N*-glycosidase-F; FBS, fetal bovine serum; h, human; IBMX, 3-isobutyl-1-methylxantine; K13, [Nle^{8,18},Lys^{13}(ϵ-pBz$_2$),L-2-Nal^{23},Tyr^{34}]bPTH(1−34)NH$_2$; PBS, phosphate-buffered saline; pBz$_2$, p-benzoylbenzoyl; PKA, protein kinase A; PLC, phospholipase C.

S0006-2960(96)02111-3 CCC: $12.00

these PTH/PTHrP receptors (Jüppner, 1994; Schipani et al., 1993). In addition, the recently described hPTH2 receptor (Usdin et al., 1995), which displays a stringent ligand specificity for PTH but not for PTHrP, also contains four putative N-glycosylation sites; three of these positions are comparable to those of the PTH/PTHrP receptor. These observations suggest a functional role for the carbohydrate portion of these receptors.

G protein-coupled receptors are often N-glycosylated (Rands et al., 1990; George et al., 1986; Garcia Rodriguez et al., 1995; Chochola et al., 1993; Davis et al., 1995; Liu et al., 1993; van Koppen & Nathanson, 1990; Fukushima et al., 1995). Oligosaccharide chains may play a variety of roles, including involvement in the binding of ligands and signal transduction (George et al., 1986; Chochola et al., 1993), trafficking of the receptor to the cell membrane (Rands et al., 1990; Garcia Rodriguez et al., 1995), and, in general, a contribution to the overall conformation of the receptor (Opdenakker et al., 1993). However, in some cases, such as the histamine H2 and the m2 muscarinic acetylcholine receptors (van Koppen & Nathanson, 1990; Fukushima et al., 1995), it has been shown that nonglycosylated receptors are effectively expressed and fully functional. Inhibition of binding of radioiodinated PTH(1−34) to the canine PTH/PTHrP receptor in the presence of wheat germ agglutinin was interpreted to indicate that terminal sialic acid residues in the extracellular N-terminal domain of the receptor may be important in ligand binding (Karpf et al., 1987). However, to the best of our knowledge, no studies of the role of N-glycosylation on the expression, recognition of ligands, and subsequent signal transduction by the hPTH/PTHrP receptor have been reported.

We used a stably transfected human embryonic kidney cell line (HEK-293/C-21), which expresses 300000−400000 hPTH/PTHrP receptors per cell (Pines et al., 1994), and the cross-linking of a radioactive, benzophenone (pBz$_2$)-containing PTH analog, namely [Nle^{8,18},Lys^{13}(ϵ-pBz$_2$),L-2-Nal^{23},Tyr^{34}(3-^{125}I)]bPTH(1−34)NH$_2$ ([^{125}I]K13) (Nakamoto et al., 1995; Adams et al., 1995), to monitor and optimize expression of the nonglycosylated hPTH/PTHrP receptor in C-21 cells cultured in the presence of tunicamycin (Elbein, 1987). The nonglycosylated hPTH/PTHrP receptor was fully characterized for binding properties using several PTH- and PTHrP-derived peptides, specific inhibition of photoaffinity cross-linking of [^{125}I]K13, hormone-stimulated cAMP accumulation, and increases in cytosolic calcium levels.

MATERIALS AND METHODS

Materials. The analogs [Tyr^{27}]hPTH(27−48)NH$_2$, hPTH(52−84)NH$_2$, hPTH(1−84), and PTHrP(1−34)NH$_2$ were purchased from Bachem (Torrence, CA). B&J brand acetonitrile was obtained from Baxter (McGraw Park, IL). IodoGen was purchased from Pierce Chemical Co. (Rockford, IL). D-MEM, fetal bovine serum (FBS), trypsin, and PBS were obtained from Gibco-BRL (Gaithersburg, MD). Na^{125}I was obtained from Amersham Corp. (Arlington Heights, IL). Fura-2 acetoxymethyl ester (Fura-2/AM) was obtained from Molecular Probes (Eugene, OR). Endoglycosidase-F/*N*-glycosidase-F (Endo-F) was purchased from Boehringer Mannheim (Indianapolis, IN). Tissue culture disposable and plasticware were obtained from Corning (Corning, NY). 3-Isobutyl-1-methylxanthine (IBMX), polylysine, and all other reagents were purchased from Sigma (St. Louis, MO).

Peptide Synthesis and Characterization. [Nle^{8,18},Lys^{13}(ϵ-pBz$_2$),L-2-Nal^{23},Tyr^{34}]bPTH(1−34)NH$_2$ (K13) (Nakamoto et al., 1995), [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$ (Goldman et al., 1988), [Nle^{8,18},D-Trp^{12},Tyr^{34}]bPTH(7−34)NH$_2$ (Chorev et al., 1990), and [Leu^{11},D-Trp^{12}]hPTHrP(7−34)NH$_2$ (Nutt et al., 1990) were synthesized by solid phase methodology and purified to homogeneity by reverse phase high-performance liquid chromatography (RP-HPLC) as previously described (Nakamoto et al., 1995). Chemical characterization included analytical HPLC, amino acid analysis, and fast atom bombardment mass spectrometry (FAB-MS).

Radioiodination. Radioiodination of [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$ and [Nle^{8,18},Lys^{13}(ϵ-pBz$_2$),L-2-Nal^{23},Tyr^{34}]bPTH(1−34)NH$_2$ (K13) was carried out as previously described (Roubini et al., 1992) with the following modifications. The separation employed a linear gradient from 30 to 38% B in A over 30 min at a flow rate of 1.0 mL/min, where eluant A is water containing 0.1% (v/v) trifluoroacetic acid and eluant B is acetonitrile containing 0.1% (v/v) trifluoroacetic acid. The separation was monitored in tandem by UV (220 nm) and a γ-detector. Fractions of 0.5 mL were collected in Nunc tubes (Nunc Inc., Naperville, IL). The fractions containing the radiolabeled PTH agonists [[^{125}I]K13 or [^{125}I][Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$] were stored in the same tubes at −80 °C.

Cell Culture. Parental human embryonic cells (HEK-293) (vector-transfected and derived from a single cell by limiting dilution) and HEK-293 cells stably transfected with recombinant hPTH/PTHrP receptor (clone C-21) (Pines et al., 1994) were maintained in D-MEM supplemented with 10% FBS at 37 °C in a humidified atmosphere of 95% air/5% CO_2. The medium was changed every 2 days before confluency and every day after confluency. The cells were subcultured 1:10 once a week. Treatment with tunicamycin (6.4 μg/mL, unless otherwise indicated) was initiated at confluency and continued for 48 h.

Hormone−PTH/PTHrP Receptor Cross-Linking. Receptor-transfected HEK-293/C-21 cells were subcultured into 12-well tissue culture dishes. Two days after confluency, the cells were washed once with D-MEM and incubated at 37 °C for 30 min in the absence or presence of various concentrations of [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$. RP-HPLC-purified [^{125}I]K13 was then added to all wells (1×10^6 cpm/well, ∼0.2 nM), reaching a total volume of 1.0 mL/well. After an additional 30 min of incubation at room temperature, the plate (without lid) was placed on ice at a distance of 10 cm from six 15 W 365 nm UV lamps, in a Stratalinker 2400 apparatus (Stratagene, La Jolla, CA). Irradiation was carried out for 20 min at 4 °C. Cells were carefully washed three times with cold PBS and harvested with 600 μL of Laemmli reducing sample buffer (Laemmli, 1970).

Endoglycosidase-F/N-Glycosidase-F Treatment. Batches of HEK-293/C-21 cells, not treated with tunicamycin, were cross-linked with [^{125}I]K13 as previously described and incubated in the presence of endoglycosidase-F/*N*-glycosidase-F (Endo-F) at 37 °C for 24 h, according to the manufacturer's procedure.

SDS−PAGE and Autoradiography. Samples of the cross-linked and radiolabeled cells in Laemmli reducing sample buffer (typically 75−100 μL) were analyzed by 7.5% (w/v) SDS−PAGE (16 × 14 cm, 1.5 mm thickness). Gels were

dried, placed between two intensifying screens (Dupont Lightning plus), and exposed at −80 °C to Kodak XAR-5 film for 12−48 h. Molecular mass standards (high molecular mass of 30−220 kDa, Amersham) were also electrophoresed in each gel.

Receptor Binding Assays. Measurement of specific hPTH/PTHrP receptor binding was performed using confluent monolayers of stably transfected HEK-293/C-21 cells in polylysine-coated 24-well tissue culture plates, as described (Pines et al., 1994). A polylysine coating was used in order to improve cell attachment. Bound radioactivity was determined by γ counting (Packard Cobra auto-gamma instrument, Packard Corp., Meriden, CT) and specific binding expressed as a percentage of the total binding of radioligand (Nakamoto et al., 1995; Adams et al., 1995). Curves were fitted by CA-Cricket Graph III (Version 1.0, Computer Association, WI), and Scatchard analysis was performed as described previously using MacLigand (version 1.01) (Pines et al., 1994).

Cyclic AMP Measurements. For cyclic AMP (cAMP) determination, cells in 24-well tissue culture plates were harvested with 0.5 mM EDTA in PBS, washed once with D-MEM, and incubated in suspension with [$Nle^{8,18}$,Tyr^{34}]-bPTH(1−34)NH_2 for 10 min in D-MEM in the presence of 1 mM IBMX. The incubation was terminated by addition of perchloric acid (final concentration of 30%). Samples were neutralized with potassium bicarbonate and acetylated with acetic anhydride and the total cAMP (medium + cells) concentrations determined by radioimmunoassay (RIA) (Pines et al., 1994). Data are presented as cAMP (picomoles) per 100 000 cells, where the cell number was determined with a hematocytometer.

Calcium Measurements. Measurement of $[Ca^{2+}]_i$ was performed using HEK-293/C-21 cells (with or without tunicamycin treatment) as described (Behar et al., 1996).

RESULTS

Confluent HEK-293/C-21 cells were treated with various concentrations of tunicamycin (0, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 μg/mL in D-MEM supplemented with 10% FBS). Tunicamycin treatment blocks N-glycosylation of all expressed proteins. We observed a tunicamycin dose-dependent reduction in adherence of the cell monolayer to the culture plate. In particular, treatment with high doses of tunicamycin (6.4 μg/mL) which completely blocks receptor glycosylation causes the nonglycosylated C-21 cells to "peel off" more extensively than the native cells during the successive washings included in the binding assay protocol. As much as ∼30% of the cells in the confluent monolayer can be lost during the exhaustive washes following competitive binding or photo-cross-linking incubation.

After 48 h of tunicamycin treatment, the cells were photoaffinity cross-linked with [^{125}I]K13 (Figure 1). Untreated cells (Figure 1, lane 1) yielded a broad band at ∼90 kDa, corresponding to the hPTH/PTHrP receptor, which was competed with by an excess of unlabeled PTH(1−34) (Adams et al., 1995). Upon treatment with increasing concentrations of tunicamycin, an array of photolabeled bands appear, with a distribution of molecular masses between 90 and 60 kDa (Figure 1, lanes 2−6). The reduction in the intensity of the 90 kDa radiolabeled band representing the intact ligand−receptor photo-cross-linked conjugate

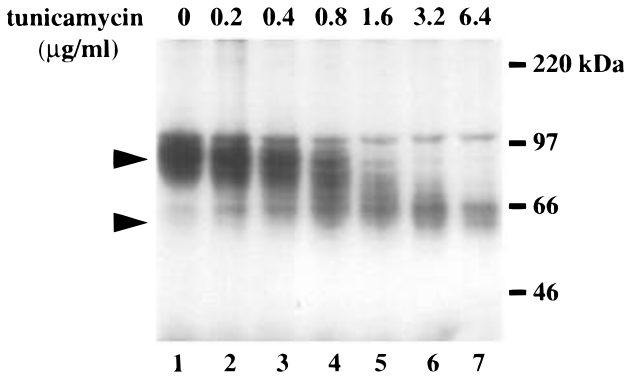

FIGURE 1: Autoradiograph of photolabeled tunicamycin-treated HEK-293/C-21 cells. Cells were treated with various concentrations of tunicamycin (0−6.4 μg/mL) for 48 h and photoaffinity cross-linked in the presence of [^{125}I]K13 (lanes 1−7; see Materials and Methods). Samples were analyzed by 7.5% (w/v) SDS−PAGE and autoradiography. Size markers (kilodaltons) are also shown. The autoradiograph is typical of two additional experiments. Arrows indicate the positions of the predominant hPTH/PTHrP receptor species for each treatment.

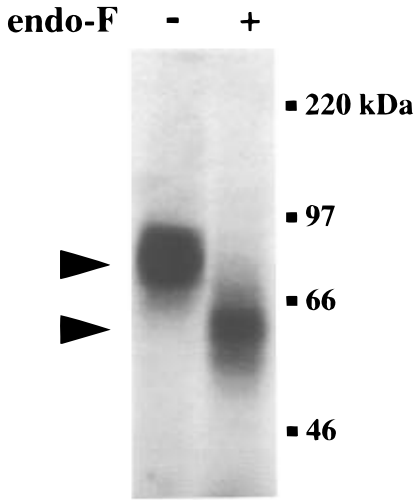

FIGURE 2: Autoradiograph of an Endo-F-treated hormone−receptor photoconjugate obtained from HEK-293/C-21 cells. Photoaffinity-cross-linked cells (−) were treated with Endo-F for 24 h at 37 °C (+). Samples were analyzed by 7.5% (w/v) SDS−PAGE and autoradiography. Size markers (kilodaltons) are also shown. The autoradiograph is typical of two additional experiments. Arrows indicate the position of the native (−) and deglycosylated (+) hormone−receptor photoconjugates.

following tunicamycin treatment, accompanied by the concomitant increase in the intensity of the lower-M_r products, appears to be dose-dependent. At an intermediate tunicamycin concentration (0.8 μg/mL), a full spectrum of bands from 60 to 90 kDa is observed (Figure 1, lane 4). Increasing the tunicamycin concentration to 1.6 and 3.2 μg/mL reduced the average M_r of the cross-linked bands; nevertheless, several radioactive species remain. Upon treatment with the maximum tunicamycin concentration tested (6.4 μg/mL) for 48 h, the heterogeneous mixture collapses into a broad major band at ∼60 kDa. At this concentration of tunicamycin, the native glycosylated PTH/PTHrP receptor (∼90 kDa) is not detected (Figure 1, lane 7).

Endo-F-mediated deglycosylation of the photoaffinity-labeled hPTH/PTHrP receptor yields a band with a M_r of ∼60 000 (Figure 2), similar to that obtained after treatment with 6.4 μg/mL tunicamycin (Figure 1) and in good agreement with the theoretical M_r of the hPTH/PTHrP

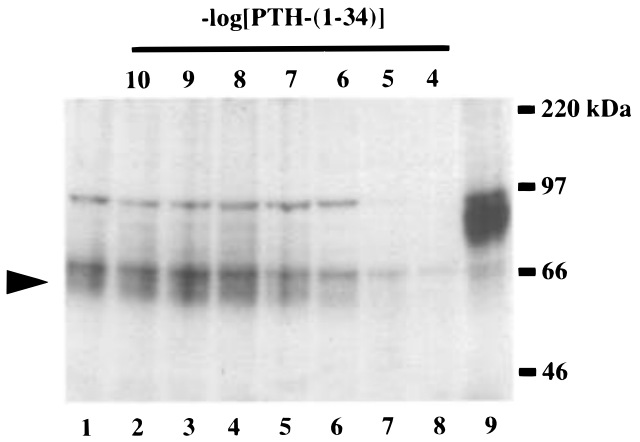

FIGURE 3: Autoradiograph of competitive inhibition of [^{125}I]K13 cross-linking by unlabeled [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$ in tunicamycin-treated HEK-293/C-21 cells. Tunicamycin-treated (6.4 μg/mL for 48 h) cells were preincubated for 15 min with various concentrations (indicated as the log of the molar concentration) of [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$ and photoaffinity cross-linked with [^{125}I]K13. Samples were analyzed by 7.5% (w/v) SDS−PAGE and autoradiography. Cross-linked native hPTH/PTHrP receptor (lane 9) and size markers (kilodaltons) are also shown. The autoradiograph is typical of two additional experiments. An arrow shows the position of the nonglycosylated hPTH/PTHrP receptor (lanes 1−8).

receptor predicted from the translated cDNA sequence. Photoaffinity cross-linking of the nonglycosylated hPTH/PTHrP receptor with [^{125}I]K13 was inhibited dose-dependently by [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$ (Figure 3). In the presence of 10^{-5} M [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$, photoaffinity cross-linking is inhibited completely (Figure 3, lane 7).

Having optimized cell culture conditions for HEK-293/C-21 cells in the presence of tunicamycin in order to obtain expression of completely nonglycosylated hPTH/PTHrP receptor and having demonstrated that this form of receptor is equivalent in size to the product obtained by Endo-F-mediated deglycosylation of the (photo-cross-linked) native receptor, we next undertook the functional characterization of the nonglycosylated hPTH/PTHrP receptor.

Figure 4 shows the competitive binding of the radioligand [^{125}I][Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$ in the presence of 10^{-5} M concentrations of various PTH- and PTHrP-derived peptides in both nontreated and tunicamycin-treated HEK-293/C-21 cells. Although some small differences are apparent, the absence of glycosylation of the hPTH/PTHrP receptor did not significantly affect the binding of a series of previously characterized ligands (Figure 4) (Pines et al., 1994). Moreover, [Tyr^{27}]hPTH(27−48)NH$_2$ (Figure 4, lane H) and hPTH(52−84)NH$_2$ (Figure 4, lane G), the midregion and C-terminal PTH fragments, respectively, which lack detectable avidity for the PTH/PTHrP receptor (Pines et al., 1994; Adams et al., 1995), do not compete for [^{125}I]bPTH binding to the nonglycosylated receptor (Figure 4).

In both tunicamycin-treated and nontreated HEK-293/C-21 cells, [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$ binds to the receptor with a very similar affinity, displaying a K_b of approximately 20 nM (Figure 5A). In multiple similar experiments, total and specific binding were almost equal for both tunicamycin-treated and nontreated cells. Scatchard analysis of the [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$ binding data of tunicamycin-treated and untreated C-21 cells yielded the same receptor

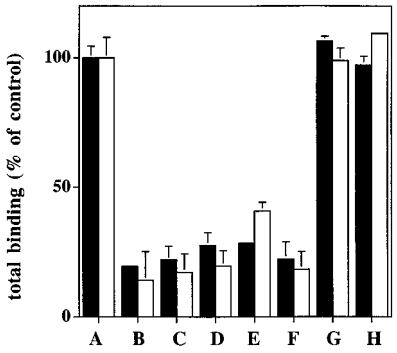

FIGURE 4: Competition for binding of [^{125}I][Nle^{8,18},Tyr^{34}]bPTH-(1−34)NH$_2$ by various unlabeled PTH- and PTHrP-derived peptides (10^{-5} M) in HEK-293/C-21 cells without (open bars) and with tunicamycin treatment (6.4 μg/mL for 48 h) (solid bars). Bars represent total binding of [^{125}I][Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$ alone (A) and in the presence of 10^{-5} M [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$ (B), [Nle^{8,18},D-Trp^{12},Tyr^{34}]bPTH(7−34)NH$_2$ (C), hPTHrP(1−34)-NH$_2$ (D), [Leu^{11},D-Trp^{12}]hPTHrP(7−34)NH$_2$ (E), hPTH(1−84) (F), [Tyr^{27}]hPTH(27−48)NH$_2$ (G), and hPTH(52−84)NH$_2$ (H). Similar results were obtained in two additional experiments.

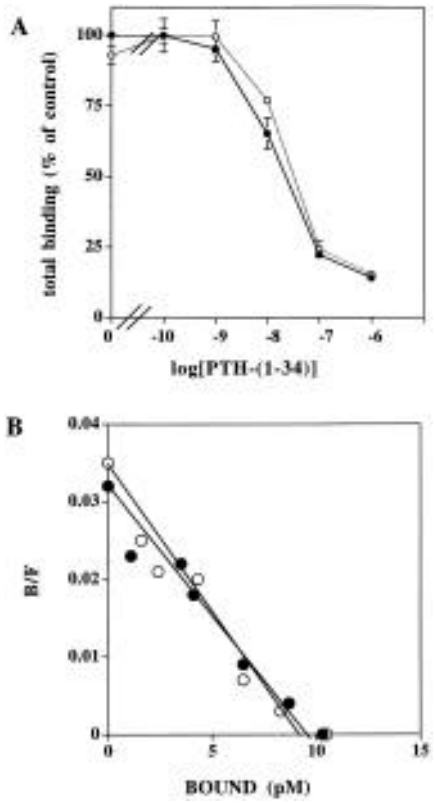

FIGURE 5: Competition for binding of [^{125}I][Nle^{8,18},Tyr^{34}]bPTH-(1−34)NH$_2$ by [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$ in HEK-293/C-21 cells treated (●) and untreated (○) with tunicamycin (6.4 μg/mL for 48 h) (panel A). Scatchard analysis of binding data (panel B). Representative curves are presented, and the apparent dissociation constants and receptor numbers were determined as indicated from three separate experiments.

number per cell (300 000 ± 30 000 receptors/cell) (Figure 5B). The binding affinity for [Nle^{8,18},Tyr^{34}]bPTH(1−34)-NH$_2$ in C-21 cells ($\sim 2 \times 10^{-8}$ M) correlates well with the concentration needed to inhibit photoaffinity cross-linking (Figure 3).

The functionality of the nonglycosylated hPTH/PTHrP receptor expressed by the tunicamycin-treated HEK-293/C-21 cells was examined by characterizing agonist-stimulated increases of intracellular calcium and cAMP accumulation.

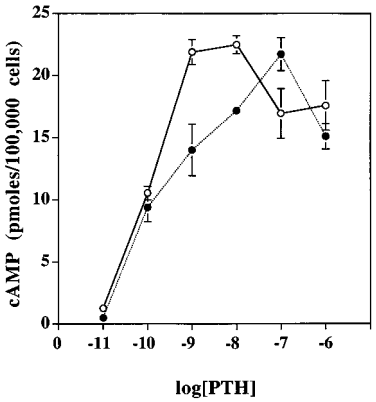

FIGURE 6: Dose-dependent stimulation of cAMP accumulation by [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$ in HEK-293/C-21 cells treated (●) and untreated (○) with tunicamycin (6.4 μg/mL for 48 h). Similar results were obtained in two additional experiments.

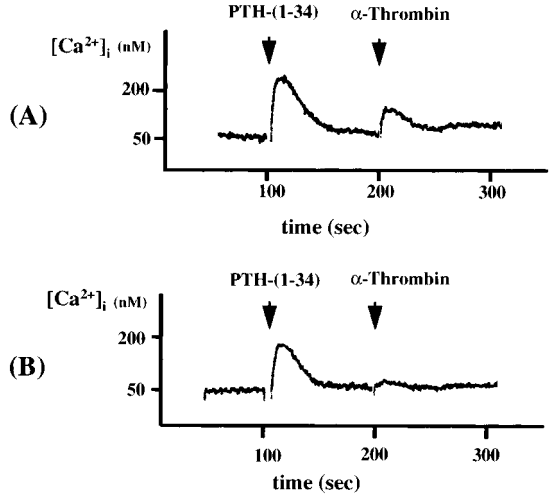

FIGURE 7: Stimulation of intracellular calcium release by [Nle^{8,18},-Tyr^{34}]bPTH(1−34)NH$_2$ (10^{-7} M) in native HEK-293/C-21 cells (A) and tunicamycin-treated (B) (6.4 μg/mL for 48 h). Similar results were obtained in two additional, independent, experiments.

The EC_{50} values for [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$-induced stimulation of cAMP accumulation for both the native and nonglycosylated HEK-293/C-21 receptor forms are similar (0.2 and 0.7 nM, respectively) (Figure 6). The impaired adherence of tunicamycin-treated cells to the culture dishes directed us to perform the cAMP assay on cells in suspension. This was a technically demanding effort which somewhat compromised the quality of the results obtained (Figure 6). Nevertheless, a similar responsiveness for agonist-induced stimulation of intracellular calcium release, carried out with cells in suspension, was observed for tunicamycin-treated and nontreated HEK-293/C-21 cells (Figure 7). The nonglycosylated hPTH/PTHrP receptor responds to [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$ in a manner similar to that of the glycosylated form (Figure 7); in both cases, a rapid increase in intracellular calcium levels (up to ∼200 nM, approximately 4 times above basal) is observed in the presence of PTH agonists (10^{-7} M) (Figure 7). Conversely, tunicamycin treatment eliminates the increase in cytosolic calcium stimulated by treatment with α-thrombin (Figure 7B) as described (Tordai et al., 1995). Comparable results were also obtained with the related agonist, hPTHrP-(1−34)NH$_2$ (data not shown).

DISCUSSION

The high abundance of N-glycosylation in G protein-coupled membrane-bound receptors, in general, and the high degree of conservation of N-glycosylation sites among PTH/PTHrP receptors from different species, in particular, suggest a potential role for the decorating oligosaccharide chains in the correct expression, presentation, and/or function of these receptors. Due to the lack of antibodies to the hPTH/PTHrP receptor, a receptor binding-based approach remains the only method for detecting and quantifying either native or deglycosylated receptors. The availability of [^{125}I]K13, a potent radioactive and photoreactive PTH agonist, allowed us to directly demonstrate both the expression and functionality of nonglycosylated PTH/PTHrP receptors in HEK-293/C-21 cells (Figures 1−3). Tunicamycin-treated C-21 cells both bind and are photo-cross-linked by [^{125}I]K13 to produce an array of radiolabeled PTH/PTHrP receptors with molecular masses ranging from ∼90 to ∼60 kDa. The extremes of this size range represent the native, fully glycosylated and the "naked" nonglycosylated receptor. The heterogeneity of radiolabeled receptors observed is tunicamycin dose-dependent. SDS−PAGE analysis of tunicamycin-treated photo-cross-linked C-21 cells provides a straightforward means for optimizing tunicamycin treatment in order to achieve the expression of uniformly nonglycosylated hPTH/PTHrP receptors. The correspondence of the ∼60 kDa radiolabeled band to the naked nonglycosylated photo-cross-linked PTH/PTHrP receptor (calculated from the combined molecular weight of the hPTH/PTHrP receptor protein, predicted by the cDNA sequence, plus that of [^{125}I]K13) was further validated by an independent approach in which photo-cross-linked C-21 cells were deglycosylated by Endo-F treatment.

Our studies suggest that N-glycosylation of hPTH/PTHrP receptors in C-21 cells does not play an essential role in the expression and functionality of this receptor. Receptor binding studies employing our standard radioligand, [^{125}I]-[Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$, revealed that tunicamycin-treated and nontreated C-21 cells (cultured under identical conditions) express a similar number of receptors (Figure 5). In addition, the avidity of the nonglycosylated receptor for the PTH agonist, [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$, remains unaltered compared to that of the native hPTH/PTHrP receptor in C-21 cells (Figure 5). Furthermore, the nonglycosylated hPTH/PTHrP receptor maintains the same avidity and specificity as the native form (Figure 4). The receptor recognizes full-length PTH and the N-terminal PTHrP-derived agonist PTHrP(1−34)NH$_2$ as well as the N-terminal truncated antagonists, [Nle^{8,18},D-Trp^{12},Tyr^{34}]bPTH-(7−34)NH$_2$ and [Leu^{11},D-Trp^{12}]hPTHrP(7−34)NH$_2$ (Figure 4). However, like the native PTH/PTHrP receptor, the nonglycosylated form does not recognize midregion and C-terminal fragments of PTH [[Tyr^{27}]hPTH(27−48)NH$_2$ and hPTH(52−84)NH$_2$, respectively] (Figure 4).

Evidently, the naked nonglycosylated receptor maintains the native signaling pathways and efficacy displayed by the PTH/PTHrP receptor in C-21 cells. The nonglycosylated receptor is coupled to both the cAMP−PKA and Ca^{2+}−PLC intracellular signal transduction pathways and induces cAMP accumulation and increases in cytosolic Ca^{2+} in response to PTH agonists. Apparently, N-glycosylation of the hPTH/PTHrP receptor does not play a significant role in receptor expression and function in cells of human origin.

Karpf et al. (1987) reported that treatment of canine renal cortical membranes with wheat germ agglutinin inhibited binding of [^{125}I]PTH(1−34). They concluded that the oligosaccharide chains linked to the N-glycosylated PTH/PTHrP receptor might be involved in ligand recognition and binding. In light of our results, the disruption of binding of the radioligand in the presence of lectins ($M_r \sim 36\,000$) may result from the close spatial proximity of the lectin binding site(s) to the PTH/PTHrP ligand binding site(s) within the receptor rather than from direct involvement of the oligosaccharide moieties in ligand binding; the spatial steric hindrance created by the receptor N-linked oligosaccharide−lectin complex may prevent either access or the correct alignment of a PTH-derived radioligand to its binding site(s).

Taken together, these observations indicate that inhibition of glycosylation at a very early stage of receptor biosynthesis does not affect the trafficking or the correct insertion of the hPTH/PTHrP receptor into the cell membrane. Furthermore, the nonglycosylated form of the receptor retains functionality, as assessed by either hormone binding, ligand specificity, or stimulation of intracellular signal transduction pathways. Similar conclusions were reached by analyzing the role of N-glycosylation in the histamine H2 and m2 muscarinic acetylcholine receptors (Davis et al., 1995; Liu et al., 1993). However, this observation concerning G protein-coupled receptors cannot be generalized; deglycosylation of the follicle-stimulating hormone (FSH) receptor, platelet-activating factor (PAF) receptor, and lutropin/choriogonadotropin (LH) receptor interferes with the trafficking of the receptors to the membrane, although ligand binding affinities are unaffected (Rands et al., 1990; Garcia Rodriguez et al., 1995; Chochola et al., 1993). Inhibition of N-glycosylation of the β-adrenergic receptor results in the selective alteration of the response to prostaglandin E1 but not of that to isoproterenol (George et al., 1986).

Therefore, despite the evolutionary conservation of the putative N-glycosylation sites of PTH/PTHrP receptors, the role(s) of the carbohydrate portion of the receptor in ligand binding and signaling does not appear to be essential. The physiological function of the N-glycosylation of the PTH/PTHrP receptor, therefore, remains to be elucidated.

ACKNOWLEDGMENT

We thank Dr. Mark Pines (Institute of Animal Science, The Volcani Center, Israel) for the stimulating discussions during his sabbatical visit in our laboratories.

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BI962111+