Articles

Agonist-Dependent Phosphorylation of the Parathyroid Hormone/Parathyroid Hormone-Related Peptide Receptor†

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ABSTRACT: Native PTH/PTHrP receptors in ROS 17/2.8 cells are downregulated after PTH treatment. Since downregulation may involve receptor phosphorylation, we examined PTH/PTHrP receptor phosphorylation in ROS 17/2.8 cells and we mapped the agonist-induced phosphorylation sites using recombinant PTH/PTHrP receptors expressed in LLCPK-1 and COS-7 cells. The data show that the PTH/PTHrP receptor is rapidly phosphorylated in ROS 17/2.8 cells with a maximum occurring at 20 min. The phosphorylation was dose-dependent; it occurred with PTH concentrations that are known to downregulate the PTH/PTHrP receptor in ROS 17/2.8 cells. The time course and the dose requirement for phosphorylation were similar in ROS 17/2.8 cells, which express native PTH/PTHrP receptors, and in LLCPK-1 cells stably transfected with the PTH/PTHrP receptor cDNA. PTH/PTHrP receptor phosphorylation in ROS 17/2.8, COS-7, and LLCPK-1 cells was also stimulated with forskolin and phorbol myristate acetate (PMA). Additionally, in LLCPK-1 cells, which express native clacitonin receptors, PTH/PTHrP receptor phosphorylation was stimulated by calcitonin. These data suggest involvement of second messenger-stimulated kinases in PTH/PTHrP receptor phosphorylation. However, staurosporine, which fully blocked the effects of PMA, forskolin, and clacitonin, partially decreased the effects of PTH on PTH/PTHrP receptor phosphorylation. These data indicate involvement of other kinase(s) in PTH-induced PTH/PTHrP receptor phosphorylation. CNBr cleavage of recombinant receptors expressed in COS-7 cells combined with site-directed mutagenesis revealed that the phosphorylated residues of the PTH/PTHrP receptor map to two regions of the carboxyl-terminal tail located between residues A480 and M499 and residues M499 and M553. These data indicate that the PTH/PTHrP receptor is phosphorylated after PTH stimulation on two regions of the carboxyl-terminal tail and that agonist-dependent phosphorylation involves both staurosporine-sensitive and -insensitive kinases.

Parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP) bind to a common G protein-coupled receptor that activates at least two intracellular second messenger systems (*4*). The molecular cloning of the rat (*4*) and opossum (*20*) PTH/PTHrP receptors revealed significant sequence homology between the PTH/PTHrP receptors and the calcitonin (*23*) and secretin (*16*) receptors which allowed the categorization of these receptors into a new subfamily of the G protein-coupled receptor superfamily (*20*).

Cellular responsiveness to PTH depends upon the number of the PTH receptors on the target cell and upon their G protein-coupled status. The number of PTH binding sites and the responsiveness to PTH has been shown to be dramatically regulated by PTH in many cell lines that express native PTH/PTHrP receptors (*1*−*3*, *5*, *6*, *8*, *11*, *17*, *26*−*28*, *33*). Among these cell lines, ROS 17/2.8 cells, which express several osteoblastic features such as alkaline phosphatase, osteocalcin, collagen type I, and PTH-sensitive adenylate cyclase, represent a model in which to study PTH actions in vitro. Treatment of ROS 17/2.8 cells with PTH causes dramatic downregulation and desensitization of the PTH/PTHrP receptor (*3*, *33*). In contrast, a similar treatment had no effect on the steady-state levels of the PTH/PTHrP receptor mRNA (*30*). These data suggest that downregulation of the PTH/PTHrP receptor in ROS 17/2.8 cells does not involve changes in mRNA levels but may involve covalent modification of the receptor protein.

It has been recently shown that the opossum PTH/PTHrP receptor, stably overexpressed in HEK293 cells, undergoes a rapid phosphorylation after a PTH challenge (*7*). However, it is not known if the native PTH/PTHrP receptors in the osteoblastic cell lines are regulated by phosphorylation. The molecular cloning of the PTH/PTHrP receptor from ROS 17/2.8 cells (*4*) allowed the development of specific antibodies against the rat PTH/PTHrP receptors for studying the molecular mechanisms for receptor regulation (*31*).

In this report, we show that the native PTH/PTHrP receptors in ROS 17/2.8 cells are rapidly phosphorylated after

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PTH stimulation. The agonist-dependent phosphorylation sites have been mapped by cyanogen bromide cleavage and site-directed mutagenesis to two regions in the cytoplasmic tail of the rat PTH/PTHrP receptor.

MATERIALS AND METHODS

Materials. [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH_2 (NlePTH) was synthesized by a solid phase method (Endocrine Unit, Massachusetts General Hospital, Boston, MA). All chemicals were of the highest grade available and were obtained from either Sigma Chemicals Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Na^{125}I (2125 Ci/mmol) and [^{32}P]-orthophosphate (8500−9120 Ci/mmol) and the chemoluminescence kit were purchased from New England Nuclear (Boston, MA). Restriction enzymes were purchased from either United States Biochemical Corp. (Cleveland, OH), New England BioLab (Beverly, MA), Promega (Madison, WI), or Gibco-BRL (Gaithersburg, MD). Fetal bovine serum (FBS), normal sheep serum, and peroxidase-conjugated anti-sheep IgG were from Sigma; streptomycin−penicillin was from Gibco-BRL. Staurosporine and phorbol 12-myristate 13-acetate were from RBI (Natick, MA). CNBr-activated Sepharose 4B was from Pharmacia (Uppsala, Sweden). Tissue culture media were prepared by the Massachusetts General Hospital media facility. Flasks, plates, and other tissue culture supplies were from Corning (Oneonta, NY).

Cell Culture and Cell Transfection. ROS 17/2.8 cells were cultured in Ham F12 medium supplemented with 5% FBS. COS-7 cells and LLCPK-1 cells were cultured in DME medium supplemented with 10% FBS. All media contained 1 μg/mL streptomycin and 100 units/mL penicillin. The cells were incubated in a humidified atmosphere containing 95% air and 5% CO_2 at 37 °C. Media were replaced every other days. When the cells become confluent, they were trypsinized, diluted, and replated in new flasks. LLCPK1 cells stably expressing the rat PTH/PTHrP receptor were developed by cotransfection of the cell lines with psv2-Neo and a plasmid encoding the rat PTH/PTHrP receptor (R15B); the cells were selected in the presence of G418 as previously described (*10*). COS-7 cells were transiently transfected with wild type (R15B) or C-terminally truncated (R480) PTH/PTHrP receptor using the DEAE-dextran/chloroquine method (*25*).

Phosphorylation of the PTH/PTHrP receptor. Confluent cells, in 10 cm tissue culture dishes, were washed once with phosphate- and serum-free DME medium and then incubated with this medium for 40 min in a cell culture incubator. After incubation, the medium was replaced and [^{32}P]orthophosphate (0.5 mCi/3.5 mL) was added for 2 h (labeling period) at 37 °C. The cells were treated with PTH, other stimulators, or kinase inhibitors for variable time periods (0−40 min) during the last 0−40 min of the 2 h labeling period. At the end of the labeling period, the cells were rinsed (three times) with ice-cold PBS and lysed with 0.8 mL/dish of RIPA buffer [140 mM NaCl, 50 mM Tris (pH 8.0), 1% Triton X-100, 0.5% deoxycholic acid (DOC), and 0.1% sodium dodecyl sulfate (SDS)] containing phosphatase inhibitors (300 nM okadaic acid, 10 mM tetrasodium pyrophosphate, 0.1 mM sodium orthovanadate, and 10 mM NaF) and proteinase inhibitors (1 mM PMSF and 20 μg/mL aprotinin). The cell lysate was immunoprecipitated with the PTH/PTHrP receptor antiserum beads.

Immunoprecipitation and Western Blot. For immunoprecipitation and Western blots, we used a previously described anti PTH/PTHrP receptor antiserum, G48, that was raised in sheep against a synthetic rat PTH/PTHrP receptor peptide, which corresponds to residues 88−108 (*31*), and subsequently boosted with eight other synthetic receptor fragments that represent different domains from the extracellular loops, the cytoplasmic loops, and the carboxyl-terminal tail. The crude immunoglobulins were precipitated with saturated ammonium sulfate, dissolved in PBS, dialyzed against coupling buffer [0.5 M NaCl and 0.1 M $NaHCO_3$ (pH 8.3)], and then coupled to CNBr-activated Sepharose 4B beads following the manufacture's recommendations. Normal sheep serum was processed in an identical manner to construct normal IgG−sepharose beads. The cell lysate (0.5 mL) was first incubated with 50 μL of normal sheep IgG beads for 1 h. The supernatant was then collected and further incubated with 30 μL of PTH/PTHrP receptor antiserum beads for 1 h at 4 °C. The beads were rinsed (six times) with 0.8 mL of ice-cold RIPA buffer. The receptor protein was then eluted from the beads by adding 35 μL of SDS sample buffer and incubating for 10 min at room temperature. The eluted receptor was then analyzed on a 5−20% SDS−PAGE gradient gel and autoradiographed for 16−48 h.

For Western blots, the receptor protein which had been resolved on a 5−20% SDS−PAGE gel was electrotransferred onto an Immobilon-P membrane from Millipore (Bedford, MA); the membrane was blocked with 5% nonfat dry milk and 0.2% Tween 20 in PBS and then incubated with the PTH/PTHrP receptor antiserum, G48, at a dilution of 1:2000. A second peroxidase-labeled antiserum (Sigma) was further added, and the bands were developed using a chemoluminescence kit.

CNBr Digestion of the Receptor Protein. The receptor protein, adsorbed on the PTH/PTHrP receptor antiserum beads, was directly subjected to the CNBr cleavage. CNBr (0.5 mL of a 100 mM solution in 70% formic acid) was added to the drained beads at room temperature for 16 h on a rotator. The supernatant was collected, air-dried, dissolved in ddH_2O, and lyophilized. The cleaved receptor fragments were then resolved on 16.5% Tricine-SDS−PAGE. The gel was dried and autoradiographed for 48 h.

Site-Directed Mutagenesis. R15B was used for single-strand plasmid preparation. All the receptor mutations were then created on the R15B backbone by site-directed mutagenesis according to the method of Kunkel et al. (*21*). Mutants were confirmed by sequence analysis.

RESULTS

Effects of PTH on PTH/PTHrP Receptor Phosphorylation in COS-7, LLCPK-1, and ROS 17/2.8 Cells. We have previously described a sheep anti-rat PTH/PTHrP receptor antiserum that is useful for measuring the number of PTH/PTHrP receptors on the surface of intact cells (*31*). Using this antiserum, an immunoprecipitation procedure was developed by coupling the IgG fraction of the antiserum to CNBr-activated Sepharose beads. To assess the specificity of the immunoprecipitation procedure, we examined phosphorylation of recombinant rat PTH/PTHrP receptor stably expressed in LLCPK-1 cells or transiently transfected in COS-7 cells. Untransfected LLCPK-1 cells or COS-7 cells

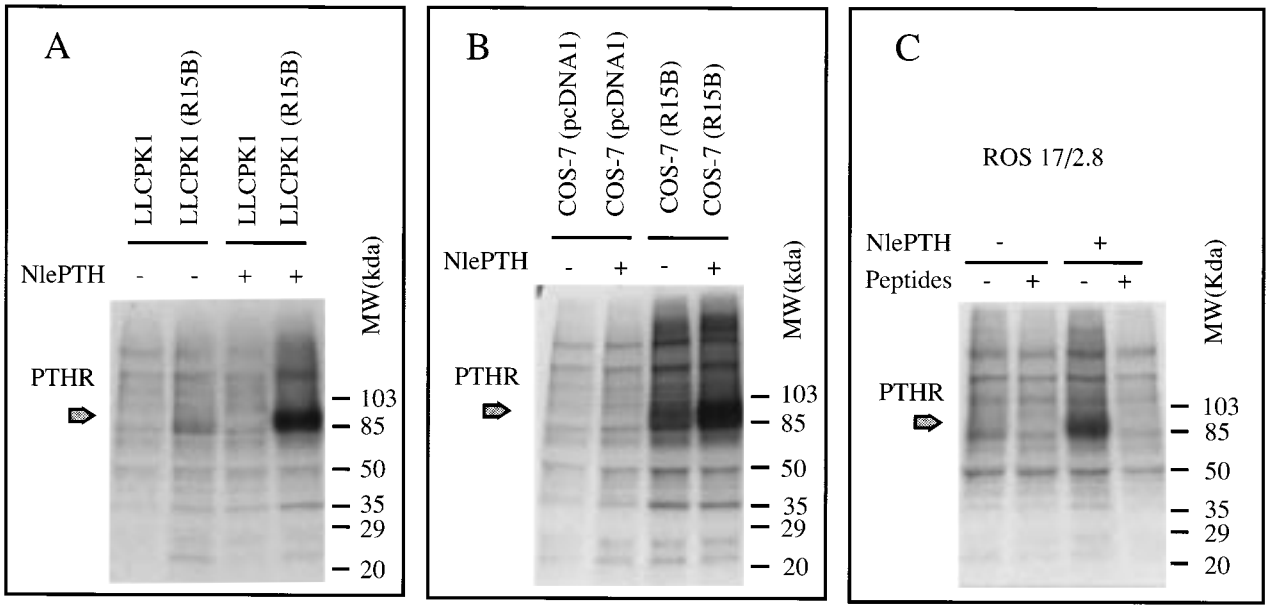

FIGURE 1: Phosphorylation of the PTH/PTHrP receptor in LLCPK-1, COS-7, and ROS 17/2.8 cells. The cells were labeled with ^{32}P for 2 h. NlePTH (200 nM) or vehicle was added during the last 40 min of the labeling period. The cell were lysed with RIPA buffer, and the lysates were immunoprecipitated with the PTH/PTHrP receptor antiserum beads as indicated in Materials and Methods. The immunoprecipitates were analyzed on 5−20% SDS−PAGE. The gels were dried and autoradiographed for 16-48 h. (A) Immunoprecipitation of cell lysate prepared from LLCPK-1 cells transiently transfected with the PTH/PTHrP receptor cDNA (R15B) or from the parent LLCPK-1 cell line. (B) Immunoprecipitation of the cell lysate prepared from COS-7 cells transiently transfected with the PTH/PTHrP receptor cDNA (R15B) or with the empty vector (pcDNA1). (C) Immunoprecipitation of ROS 17/2.8 cell lysates in the presence or absence of an excess of the peptides that were used for immunization (100 ng/mL).

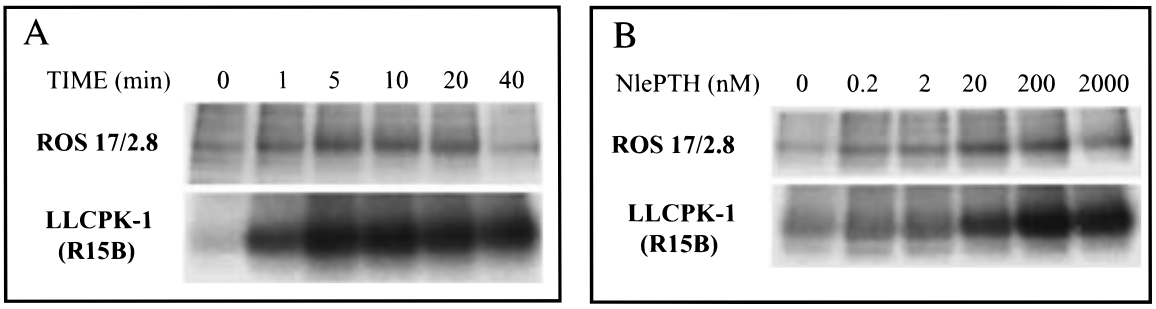

FIGURE 2: Time-dependent and dose-dependent PTH/PTHrP receptor phosphorylation in ROS 17/2.8 cells and LLCPK-1 cells stably expressing the rat PTH/PTHrP receptor. (A) NlePTH (200 nM) was added during the last 1−40 min of the labeling period. (B) NlePTH (0.2−2000 nM) was added during the last 20 min of the labeling period.

transfected with vector only served as a negative control. The PTH/PTHrP receptor expressed in LLCPK-1 and COS-7 cells was phosphorylated at basal levels, and its phosphorylation was dramatically increased after addition of 200 nM PTH (Figure 1A,B). The phosphorylated PTH/PTHrP receptor appears as a 90 kDa broad band, which is consistent with the PTH/PTHrP receptor analyzed by photoaffinity cross-linking experiments (*18*, *19*). The specificity of the antiserum was excellent as shown from the low background phosphorylation in naive LLCPK-1 cells or in COS-7 cells transfected with the pcDNA1 vector (Figure 1A,B). To examine the specificity of the immunoprecipitation procedure in ROS 17/2.8 cells, the cell lysate was immunoprecipitated in the presence and absence of the peptides that were used for immunization (each peptide at 0.1 μg/mL, all added together). A 90 kDa phosphorylated receptor band, which was specifically competed off by these peptides, was detected in ROS 17/2.8 cells (Figure 1C). Receptor phosphorylation was also enhanced after stimulation with PTH (Figure 1C).

We then examined the sensitivity and the time course of PTH-stimulated phosphorylation of the PTH/PTHrP receptor in the native cell line, ROS 17/2.8 cells, and compared them to those observed in LLCPK-1 cells stably expressing the PTH/PTHrP receptor (Figure 2A,B). NlePTH caused time- and dose-dependent phosphorylation of the native PTH/PTHrP receptor in ROS 17/2.8 cells that was parallel to the phosphorylation of recombinant receptors stably expressed in LLCPK-1 cells (Figure 2A,B). Receptor phosphorylation increased to a maximum occurring at 5−20 min and then decreased after incubation for 40 min with 200 nM NlePTH (Figure 2A). The least effective concentration was 0.1 nM, and the maximum effective concentration was 100 nM (Figure 2B).

Heterologous Phosphorylation of the Recombinant PTH/PTHrP Receptor in LLCPK-1 and COS-7 Cells and of the Native PTH/PTHrP Receptor in ROS 17/2.8 Cells. To determine if protein kinase A or C is involved in PTH/PTHrP receptor phosphorylation, a protein kinase A and C inhibitor, staurosporine, was used at increasing concentrations (0.2, 1, and 5 μM, Figure 3). Phorbol myristate acetate (PMA)- and forskolin-stimulated phosphorylation of the PTH/PTHrP receptor, transiently expressed in COS-7 cells, was dose-

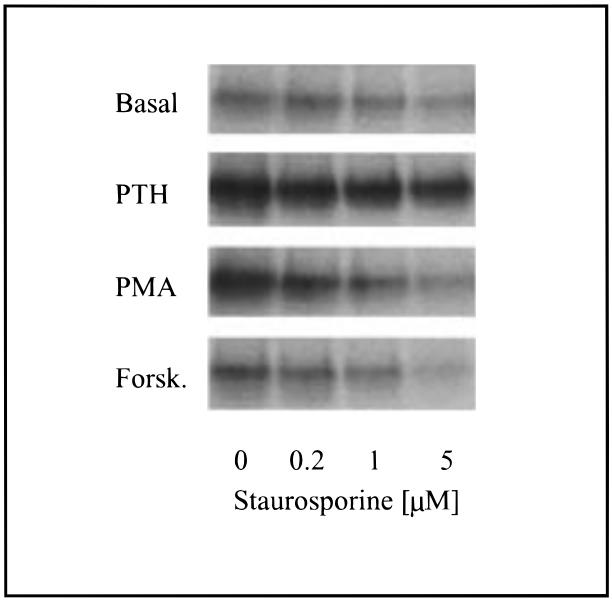

FIGURE 3: Effects of increasing concentrations of staurosporine on PTH/PTHrP receptor phosphorylation stimulated by PTH, PMA, and forskolin. COS-7 cells transiently transfected with the rat PTH/PTHrP receptor cDNA (R15B) were labeled with ^{32}P and then incubated with 0, 0.2, 1, or 5 μM staurosporin for 40 min. During the last 20 min, 200 nM NlePTH, 1 μM PMA, 100 μM forskolin, or vehicle was added.

dependently inhibited by staurosporine (Figure 3). Staurosporine fully blocked the effects of PMA and forskolin at a concentration of 5 μM (Figure 3). In contrast, the effects of PTH were only partially inhibited (Figure 3). These data are consistent with those of Gates et al., who determined that staurosporine (5 μM) fully blocks PKA and PKC but not βARK (*12*). Then 5 μM stauropsorine was used to block phosphorylation of the PTH/PTHrP receptor stably expressed in LLCPK-1 cells or of the native PTH/PTHrP receptor in ROS 17/2.8 cells (Figure 4A−C). Phosphorylation of the PTH/PTHrP receptor was much higher after activation of PKC as compared to that observed after activation of PKA. Addition of staurosporine fully blocked phosphorylation stimulated by PMA and forskolin in all three cell lines; however, it only partially decreased phosphorylation by PTH (Figure 4A−C). Since LLCPK-1 cells express endogenous calcitonin receptor, we examined whether activation of the calcitonin receptor increases the phosphorylation of the PTH/PTHrP receptor in LLCPK-1 cells. Interestingly, addition of salmon calcitonin (sCT) also increased the phosphorylation of the PTH/PTHrP receptor (Figure 4A). The effects of sCT, however, were fully blocked by staurosporine; this suggests that activation of the calcitonin receptors in LLCPK-1 cells causes PTH/PTHrP receptor phosphorylation through activation of PKA and/or PKC. Since inhibition of PKA and PKC fully blocked sCT-, phorbol ester-, and forskolin-induced receptor phosphorylation but only partially decreased the effects of PTH, we conclude that agonist-induced PTH/PTHrP receptor phosphorylation involves another kinase(s) that is distinct from PKA or PKC and that is insensitive to staurosporine.

Phosphorylation of Carboxyl-Terminally Truncated PTH/PTHrP Receptors. To map the phosphorylation sites on the receptor, a carboxyl-terminally truncated receptor mutant, R480, in which the amino acids after position 480 were

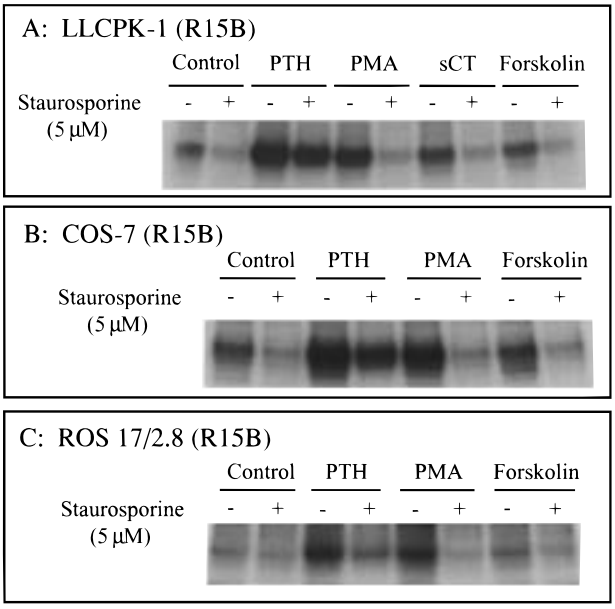

FIGURE 4: Heterologous phosphorylation of the PTH/PTHrP receptor in LLCPK-1 cells stably transfected with the PTH/PTHrP receptor cDNA (A), COS-7 cells transiently transfected with the PTH/PTHrP receptor cDNA (B), and ROS 17/2.8 cells expressing their native PTH/PTHrP receptor (C). The cells were challenged with phorbol myristate acetate (PMA, 1000 nM), forskolin (100 μM), or salmon calcitonin (sCT, 200 nM) in the presence and absence of staurosporine (5 μM). PTH, PMA, forskolin, and sCT were added during the last 20 min of the labeling period, whereas staurosporine was added during the last 40 min.

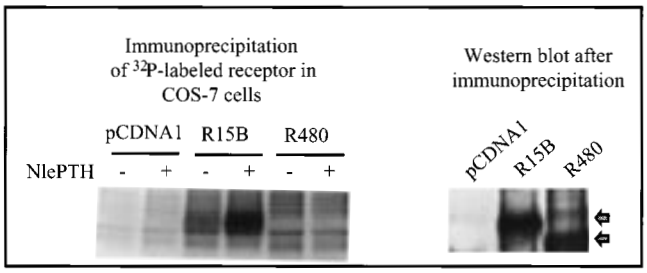

FIGURE 5: Phosphorylation of carboxyl-terminally truncated PTH/PTHrP receptors. COS-7 cells transfected with vector (pcDNA1), R15B, or R480 were labeled with [^{32}P]orthophosphate for 2 h in the presence of NlePTH (200 nM) during the last 20 min of the labeling period. The cell lysates were immunoprecipitated. Aliquots from the immunoprecipitates were analyzed by SDS−PAGE followed by autoradiography or by SDS−PAGE followed by Western blot.

deleted, was expressed in COS-7 cells and examined for PTH-dependent phosphorylation. The functional characteristics of this receptor mutant were previously reported (*15*). Expression of the truncated receptor mutants was similar to that of the wild type receptor, R15B, as shown by Western blot analysis (Figure 5). However, no phosphorylation was detected for R480 (Figure 5). These results suggest that agonist-stimulated phosphorylation sites are located on the carboxyl-terminal tail of the receptor. However, these data can not exclude the possibility that the tail may be required for phosphorylation on other receptor regions, such as the cytoplasmic loops, which also contain several serine and threonine residues.

Mapping of the Phosphorylated Region by CNBr Cleavage. To examine the possibility that the carboxyl-terminal tail of the PTH/PTHrP receptor may not contain the

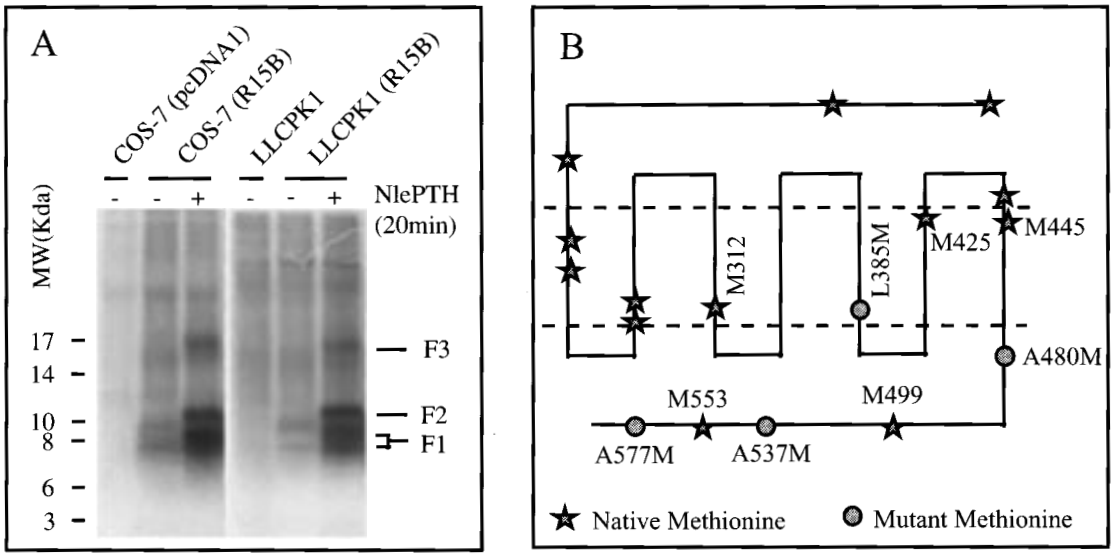

FIGURE 6: Phosphopeptide mapping of the PTH/PTHrP receptor. (A) CNBr cleavage of the immunoprecipitated phosphoreceptor prepared from COS-7 cells transiently transfected with the rat PTH/PTHrP receptor cDNA (R15B) or LLCPK-1 cells stably expressing the rat PTH/PTHrP receptor (R15B). Parent LLCPK-1 cells or COS-7 cells transfected with vector (pcDNA1) were used as a negative control. The cells were labeled for 2 h with [^{32}P]orthophosphate in the presence or absence of NlePTH (200 nM) added during the last 20 min of the labeling period. The CNBr-cleaved fragments were analyzed on tricine SDS−PAGE, and the gels were dried and autoradiographed. (B) Schematic representation of the PTH/PTHrP receptor showing the position of methionine residues: (☆) native methionine residues and (●) methionine residues introduced by site-directed mutagenesis.

phosphorylation sites and/or that the tail may be required for phosphorylation on other receptor regions, we used cyanogen bromide (CNBr) cleavage to map the phosphorylated regions. CNBr cleavage of the wild type receptor generated three phosphorylated receptor fragments (F1, F2, and F3 in Figure 6A) with apparent molecular masses of 8, 10, and 16 kDa, respectively. Phosphorylation of all three fragments was dramatically increased by PTH treatment (Figure 6A). F1 is a broad band which could correspond to several fragments or to one peptide phosphorylated on multiple heterogeneous sites. F2 appears as a sharp band, whereas F3 appears as a diffuse band that is phosphorylated to a very low level (Figure 6A). Since the rat PTH/PTHrP receptor contains several methionine residues, CNBr cleavage can generate several receptor fragments (Figure 6B). Among these fragments, those that are contained between M312 and M425, M445 and M499, M499 and M559, and M559 and the end are rich in serine and threonine residues. There is no potential tyrosine phosphorylation site in the carboxyl-terminal tail or in the cytoplasmic loops of the rat PTH/PTHrP receptor.

To identify the phosphorylated fragments, we introduced L385M, A480M, A537M, or A577M point mutations (Figure 6B). These mutant receptors were tested for expression, ligand binding, and signaling and all shown to express and signal normally (data not shown). They were also shown to be phosphorylated to the same level observed in the wild type receptor (Figure 7A).

Comparison of the phosphopeptide mapping patterns of the CNBr-cleaved fragments of the receptor mutants with those of the wild type receptor revealed that the 8 and 10 kDa peptide bands correspond to receptor regions extending between M445 and M499 and between M499 and M553 respectively (compare Figures 6B and 7B). For example, CNBr cleavage of the receptor carrying the A480M or A537M point mutations resulted in the cleavage of the F1 or F2 bands, respectively, to smaller fragments. No change

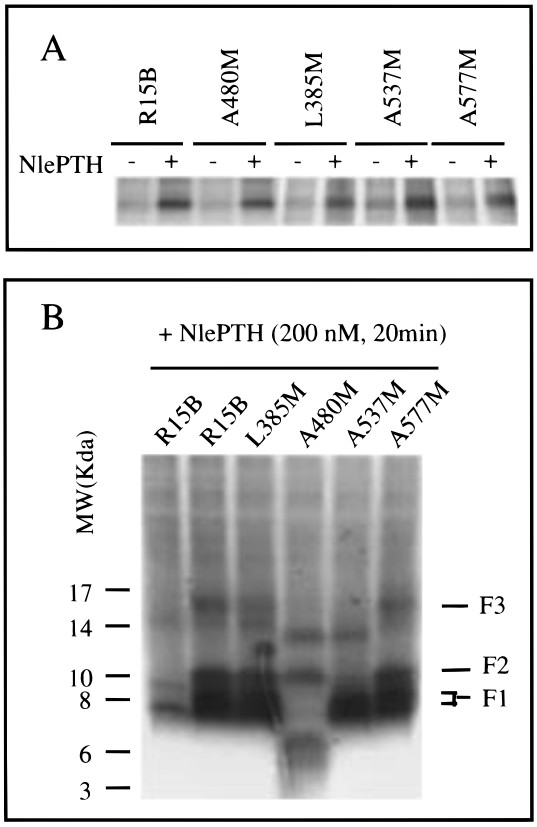

FIGURE 7: CNBr cleavage and methionine point mutations mapping of phosphorylated PTH/PTHrP receptor fragments. (A) Phosphorylation of the wild type PTH/PTHrP receptor (R15B) and mutant PTH/PTHrP receptors carrying the following point mutations: L385M, A480M, A537M, or A577M (schematically depicted in Figure 6B). (B) CNBr cleavage of the phosphorylated wild type (R15B) and mutant PTH/PTHrP receptors. CNBr cleavage of immunoprecipitated phosphoreceptors prepared from COS-7 cells transfected with the wild type R15B or R15B carrying the following point mutations: L385M, A480M, A537M, or A577M (schematically depicted in Figure 6B).

in the phosphorylated peptide fragments was observed with receptors carrying the L385M or A577M point mutations. In contrast, the F3 band decreased in size from 16 to 12 kDa in both the A480M and A537M mutant receptors. These data indicate that the F3 fragment is a partial cleavage product that represents uncleaved F1 and F2 and extends from M445 to M553. These data support the observation that the PTH/PTHrP receptor mutant truncated at position 480 is not phosphorylated. Taken together, we conclude that ligand-activated phosphorylation of the PTH/PTHrP receptor occurs on the cytoplasmic tail on serine and threonine residues that are located between A480 and M553.

DISCUSSION

Homologous downregulation and desensitization of the PTH/PTHrP receptor have been reported in several cell line models for PTH target cells that express their endogenous PTH/PTHrP receptor gene. For example, in the rat osteoblastic osteosarcoma cell line, ROS 17/2.8 cells (*3*, *33*), and in the opossum renal tubular cell line, OK cells (*2*, *29*), the number of PTH/PTHrP receptors is dramatically decreased after PTH treatment. The decrease in the number of the PTH binding sites is associated with a dramatic loss in PTH-stimulated adenylate cyclase (*2*, *3*, *28*, *32*). The dramatic decrease in the receptor number and receptor functions was not associated with any appreciable change in the steady-state mRNA levels (*30*). These data have raised the possibility that homologous regulation of the PTH/PTHrP receptor in these cell lines occurs through post-transcriptional mechanisms.

Phosphorylation of recombinant opossum PTH/PTHrP receptors in HEK293 cells has been recently reported (*7*). The physiological relevance of this phosphorylation, however, is questionable because HEK293 cells do not represent a well-characterized model for a PTH target cell; and, the overexpressed recombinant PTH/PTHrP receptors are not regulated. In this study, we have used a well- characterized cell line model in which the PTH/PTHrP receptor is dramatically regulated by PTH. We now show, for the first time, that the PTH/PTHrP receptor in ROS 17/2.8 cells is phosphorylated at the basal levels and that its phosphorylation is rapidly enhanced by PTH.

Regulation of several G protein-coupled receptors is initiated by receptor phosphorylation through second messenger-activated kinases, such as protein kinases A and C (*9*, *34*), or through receptor specific kinases, such as G protein receptor kinases (*22*). Our data that show that staurosporine fully inhibited PTH/PTHrP receptor phosphorylation by calcitonin, PMA, and forskolin and that staurosporine partially inhibited receptor phosphorylation by PTH suggest involvement of multiple kinases in PTH/PTHrP receptor regulation. Protein kinases A and C appear to play an important role in the heterologous PTH/PTHrP receptor phosphorylation, whereas a different kinase plays the major role in the homologous PTH/PTHrP receptor phosphorylation. It is possible that a receptor kinase from the G protein receptor kinase family is involved in PTH/PTHrP receptor phosphorylation. Alternatively, a PTH/PTHrP receptor specific kinase may be involved. Receptor phosphorylation is an important step for molecular recognition by other cellular proteins, such as β-arrestins (*24*), which may compete with the G protein(s) for binding to the receptor, thereby uncoupling the receptor from the G protein(s), and/or may initiate the internalization pathway through acting as an adapter molecule between the phosphorylated receptor and clathrin (*13*). It is possible that PTH receptor phosphorylation is also followed by some (or all) of these processes.

The molecular domains involved in PTH/PTHrP receptor regulation and phosphorylation have not been fully elucidated. It has been shown that a carboxyl-terminally truncated opossum PTH/PTHrP receptor is not phosphorylated (*7*). This observation is similar to ours where a carboxyl-terminally truncated rat PTH/PTHrP receptor is not phosphorylated although it is well expressed and it stimulates signaling through both adenylate cyclase and phospholipase C (*15*). These data suggest that the carboxyl-terminal tail may be the site for phosphorylation; however, these data could not exclude the possibility that the carboxyl-terminal tail is merely required for phosphorylation of the receptor on other receptor domains. In that regard, the data obtained with CNBr cleavage combined with methionine point mutations, shown in this paper, provided the most conclusive evidence that the PTH/PTHrP receptor is phosphorylated on its carboxyl-terminal tail. There is no phosphorylation detected on the cytoplasmic loops, which contain several serine and threonine residues. Additionally, all the potential phosphorylation sites could be accounted for by phosphorylation of serine and threonine residues contained within the F1 and F2 fragments.

The broadness of the phosphorylated F1 fragment on the Tricine-SDS−PAGE indicates that this fragment is phosphorylated on several residues and that phosphorylation within this fragment is heterogeneous; this suggests the presence of multiple receptor species, each with a different phosphorylation pattern at these residues. In contrast, the phosphorylated F1 fragment appears as a sharp band on the Tricine-SDS−PAGE; this suggests that phosphorylation of the residues contained within this fragment is less heterogeneous than that of F1.

Since the receptor truncated at residue 480 was not phosphorylated after activation, we conclude that the phosphorylation sites contained in F1 must occur after A480 and before M499. This region in F1 (KRKARSGSSSYSYGP) contains a cluster of five serine residues at positions 489, 491, 492, 493, and 495, of which S489 and S491 fit the consensus K/RxxS/T known to be phosphorylated by the cAMP-dependent protein kinase (PKA). Since inhibition of PKA and PKC did not fully block PTH/PTHrP receptor phosphorylation, these sites may be phosphorylated by another kinase(s).

The molecular domains involved in PTH/PTHrP receptor regulation have not been fully established. A complex role for the carboxyl-terminal tail in receptor internalization has been recently suggested. Mutation of an EVQ motif in the opossum PTH/PTHrP receptor to AAA increased internalization, whereas an internal deletion of 20 amino acids (residues 475−494) decreased internalization; these data suggested that both positive and negative domains within the carboxyl-terminal tail are involved in receptor internalization (*14*). These residues, 475−494, in the opossum PTH/PTHrP receptor correspond to a region in the F1 fragment that contains a cluster of five serine residues (SGSSSYS) that we show here to be heavily phosphorylated. Our data and

those of Huang et al. (*14*), taken together, suggest that phosphorylation of these residues may be involed in internalization of the PTH/PTHrP receptor.

In conclusion, our data show that the PTH/PTHrP receptor in native cells is phosphorylated and that phosphorylation is increased by PTH stimulation. Additionally, we have mapped the phosphorylation sites to two regions on the carboxyl-terminal tail of the PTH/PTHrP receptor.

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