Megalin Antagonizes Activation of the Parathyroid Hormone Receptor*

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Parathyroid hormone (PTH) is predominantly cleared from the circulation by glomerular filtration and degradation in the renal proximal tubules. Here, we demonstrate that megalin, a multifunctional endocytic receptor in the proximal tubular epithelium, mediates the uptake and degradation of PTH. Megalin was purified from kidney membranes as the major PTH-binding protein and shown in BIACore analysis to specifically bind full-length PTH and amino-terminal PTH fragments (Kd 0.5 μM). Absence of the receptor in megalin knockout mice resulted in 4-fold increased levels of amino-terminal PTH fragments in the urine. In F9 cells expressing both megalin and the PTH/PTH-related peptide receptor (PTH/PTHrP receptor), uptake and lysosomal degradation of the hormone was mediated through megalin. Blocking megalin-mediated clearance of PTH resulted in 3-fold increased stimulation of the PTH/PTHrP receptor. These data provide evidence that megalin is involved in the renal catabolism of PTH and potentially antagonizes PTH/PTHrP receptor activity in the proximal tubular epithelium.

Calcium concentrations in plasma and extracellular body fluids have to be kept at approximately 2.5 mM to assure normal body functions. Mammalian organisms therefore have developed an elaborate endocrine system to regulate the systemic calcium homeostasis. The parathyroid hormone (PTH)1 is the principal fast acting regulator of the calcium balance. It is produced in the parathyroid glands and released into circulation within minutes after the decline of extracellular calcium concentrations. There, PTH acts on several target tissues to mobilize calcium and to counterregulate hypocalcemia: 1) it induces osteoclastic breakdown of bone; 2) it increases tubular re-absorption of calcium in the kidney; and 3) it stimulates renal conversion of 25-OH vitamin D₃ to 1,25-(OH)₂ vitamin D₃, which in turn increases intestinal absorption of dietary calcium (reviewed in Refs. 1 and 2).

In the circulation, some PTH molecules are proteolytically cleaved between amino acids 33 and 39 to generate amino-terminal and carboxyl-terminal fragments of the hormone. Whereas the carboxyl-terminal fragments are functionally inactive, the amino-terminal fragments exhibit biological properties similar to the full-length hormone (reviewed in Ref. 3). Both PTH 1-84 and amino-terminal fragments bind to a heptahelical receptor expressed in PTH target organs, the PTH/PTH-related peptide receptor (PTH/PTHrP receptor). This 60-kDa protein was cloned initially from proximal tubular cells and osteoblasts. It belongs to a novel family of G protein-coupled receptors characterized by seven transmembrane domains and at least two conserved N-linked glycosylation motifs (4, 5). Upon binding of PTH, the PTH/PTHrP receptor transduces its signal through activation of adenylate cyclase and phospholipase C.

The kidney not only is the prime target organ of PTH activity but it also plays an active role in the removal of the hormone from the circulation. This is suggested by several lines of experimental evidence. First, in patients with renal failure the plasma half-life of the hormone is significantly increased (6, 7). Furthermore, in organ perfusion studies, PTH was shown to be lost from the plasma mainly by glomerular filtration followed by re-absorption and degradation in the proximal tubules (8, 9). Finally, cellular uptake and degradation of PTH was demonstrated directly in cultured proximal tubular cells. In these cells, the PTH/PTHrP receptor was suggested to mediate the uptake and degradation of the hormone (10, 11). Whether this receptor is also responsible for systemic clearance of PTH or whether alternative uptake mechanisms exist in vivo remained unclear.

Recently, a major endocytic receptor pathway for uptake of ligands from the glomerular filtrate has been elucidated in the proximal tubules. Megalin is a multifunctional clearance receptor expressed on the apical surface of the proximal tubular epithelium (12). Cloning of the rat cDNA revealed that megalin is a member of the low density lipoprotein (LDL) receptor gene family (13). Like other members of this gene family, megalin exhibits a broad ligand specificity and mediates the uptake and lysosomal degradation of numerous macromolecules. Ligands internalized by megalin in the proximal tubules include albumin and trans-cobalamin/vitamin B₁₂ complexes (14, 15). The significance of megalin for tubular function is underscored by the finding that the receptor resors aminoglycosides from the glomerular filtrate and is therefore responsible for the nephrotoxicity associated with these compounds (16). The central role of megalin in the tubular resorption processes was further confirmed in mice genetically deficient for the receptor. Most megalin-deficient newborns die perinatally from holoprosen-
cerephaly, a developmental defect of the forebrain (17). The causes underlying this defect have not been elucidated with certainty, but the severity of the phenotype varies among individual animals and 1 in 50 of the megalin/−/− mice survives to adulthood. Absence of the receptor in the kidneys of these mice results in tubular resorption deficiency and excretion of low molecular weight plasma proteins in the urine.

The current study was conducted to investigate a potential role of megalin in the renal catabolism of PTH. We show that megalin mediates the cellular uptake and degradation of PTH and that receptor-deficient mice exhibit impaired renal catabolism of the hormone. Furthermore, we demonstrate that megalin-mediated degradation of PTH antagonizes activation of the PTH/PTHrP receptor.

**EXPERIMENTAL PROCEDURES**

**Materials and General Methods—** Mature bovine PTH as well as bovine and human PTH fragments were obtained from Sigma (Deisenhofen). Rabbit anti-human PTH antibody was purchased from Accurate Chemicals (Westbury, NY). Sheep anti-rat megalin antibody was a gift from P. Verroust (INSERM, Paris). Polyclonal antibodies directed against rat PTH/PTHrP receptor were obtained from BAbCO (Richmond, CA) or kindly provided by A. Abou-Samra (MGH, Boston). Proteins were radiolabeled with 125I using the IODO-GEN method (19).

**Cloning and Purification of Mature Bovine Parathyroid Hormone—** The coding region of amino acids 1 to 84 of mature PTH present on exon 3 of the bovine PTH gene (20) was amplified by polymerase chain reaction from genomic calf liver DNA using primers: 5'-GATCCTCAT-CATATGGCTGTGAGTGAAATACAGTTT-3' and 5'-GGTGGGATCC-TATACGGGGTTAGCTTTAATTAATA-3'. Ndel and BamHI restriction sites in the polymerase chain reaction primers were used to clone the amplified PTH gene fragment into vector pET-14B (Novagen, Madison, WI). Expression of this vector results in a recombinant PTH molecule carrying an amino-terminal extension of 6 histidine residues and a thrombin cleavage site (recPTH). Integrity of the PTH gene sequence was confirmed by DNA sequence analysis. For purification, the expression plasmid was introduced into *Escherichia coli* strain BL-21pLys (Novagen). RecPTH was recovered from bacterial lysate by standard nickel-affinity chromatography (Qiagen, Hilden).

**Purification of PTH-binding Proteins by PTH Affinity Chromatography—** 5 mg of purified recPTH was coupled to 2 ml of CNBr-activated-Sepharose 4B resin (Pharmacia, Uppsala) according to manufacturer's recommendations. Crude rat kidney membrane extracts were prepared as described previously (17). 80 mg of membrane extract were diluted in 100 ml Tris-buffered saline, 0.5% Triton X-100, and circulated over the PTH column for 16 h at 4 °C. The column was washed with 100 ml Tris-buffered saline, 0.5% CHAPS, and bound proteins were eluted in 100 mM glycine, 0.5% CHAPS, pH 2.8.

**Biosensor Measurements—** Binding of PTH fragments to megalin was quantified by BIAcore (Bioensor, Sweden). Briefly, rabbit megalin immobilized on CM5 sensor chip (34 fmol/receptor/mm²) was incubated with PTH peptides (10–50 μg/ml) in 10 mM HEPES, 150 mM NaCl, 1.5 mM CaCl₂, 1 mM EGTA, 0.005% surfactant P20, pH 7.4, and the relative increase in response between megalin and control flow channels was determined. Kinetic parameters were determined by using the BIA evaluation 3.0 software. Number of ligands bound per milligram of total cell protein.

**RESULTS**

In initial experiments, we intended to identify proteins in the kidney that interact with PTH and potentially play a role in the renal catabolism of the hormone. To do so, a fusion protein of mature bovine PTH with an amino-terminal hexahistidine epitope (recPTH) was produced in bacteria and coupled to Sepharose resin. Total membrane extracts from rat kidneys were applied to this recPTH affinity column. The column was washed extensively and bound proteins were eluted in low pH buffer. Re producibly, two major protein bands were identified that bound specifically to the PTH column (Fig. 1, lane 2). No binding was observed in Sepharose columns lacking PTH (data not shown). The 60-kDa protein eluted from the PTH column was identified as the renal PTH/PTHrP receptor using antibodies against rat PTH/PTHrP receptor (lane 3). A second protein of high molecular weight also bound to the PTH column (lane 2). This protein was identified as megalin by Western blot analysis (lane 4).

The ability of megalin to interact with PTH was investigated in more detail using plasmon surface resonance (BIAcore) analysis. As shown in Fig. 2A, megalin immobilized on the BIA sensor chip bound recPTH reversibly. Ligand binding was dependent on calcium and abolished by the addition of EDTA, a characteristic feature of ligand binding to megalin. The intensity of the binding response signal indicated two PTH binding sites per receptor molecule. To further map the receptor binding epitope on PTH, binding of PTH fragments to megalin was analyzed (Fig. 2B). PTH 1–34 bound specifically to the receptor, eliciting a response signal equal to half that of the full-length hormone (300 versus 600 response units). Because PTH 1–34 exhibits half the molecular weight of recPTH, this result indicated that the same number of receptor binding sites were occupied by PTH 1–34 and recPTH. In the same assay, no binding was observed for PTH 39–84. The exact binding affin-
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### Table I

**Affinities of binding of PTH and PTH fragments to megalin**

Binding to immobilized rabbit megalin of purified bovine PTH, recPTH, and synthetic PTH fragments was tested by BIAcore analysis as described under "Experimental Procedures." Each value represents the mean of three to six individual measurements. No binding was detected for PTH fragments 39–84 and 28–48.

| Ligand          | $K_d$ (±S.E.) |
|-----------------|--------------|
| recPTH (1-84)   | 0.9 μM ± 0.46|
| recPTH*         | 0.5 μM ± 0.24|
| bovine PTH      | 0.4 μM ± 0.31|
| PTH 1-44        | 3.2 μM ± 0.7 |
| PTH 1-34        | 26.0 μM ± 4.7|
| PTH 39-84       | ND*          |
| PTH 28-48       | ND*          |

*recPTH*, recombinant PTH cleaved with thrombin to remove the His$_6$ epitope.

*ND*, no binding detectable.

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**Fig. 2.** BIAcore analysis of binding of PTH and PTH fragments to immobilized megalin. RecPTH (amino acids 1-84) was incubated with immobilized megalin in the absence or presence of 20 mM EDTA (A). Binding to the receptor was detected by surface plasmon resonance signal (BIAcore, see Experimental Procedures) and is indicated in response units. Binding of recPTH (1-84), amino-terminal bovine PTH fragment (1-34), and carboxyl-terminal human PTH fragment (39-84) to immobilized megalin (B).

**Fig. 3.** Degradation of $^{125}$I-recPTH by induced and noninduced F9 cells. F9 cells were cultured in the absence (0; noninduced) or presence (+) of 1 μM dibutyryl cAMP and 1 μM trans-retinoic acid for 7 days. Subsequently, the medium was changed to 1 ml of DMEM (without glutamine) containing 0.2% (w/v) bovine serum albumin and 32 ng/ml $^{125}$I-recPTH (3,220 cpm/ng). After incubation at 37 °C for the indicated periods of time, the amount of $^{125}$I-labeled recPTH degradation products (trichloroacetic acid-soluble material) released into the culture medium was determined (23). Where no error bar (mean ± S.E.) is shown, the size of the bar is smaller than the respective symbol. Inset, analysis of receptor expression in F9 cells. Cell extracts from noninduced (0) and induced (+) F9 cells were subjected to 4–15% SDS-PAGE and immunoblot analysis using antiserum against rat megalin, rat LDL receptor-related protein (LRP), bovine LDL receptor (LDL-R), and rat PTH/PTHrP receptor (PTH-R). Bound IgG was visualized by ECL.

It is well established that ligands bound to megalin are subjected to cellular uptake and lysosomal degradation. To test whether the receptor also mediates the degradation of recPTH, we investigated the catabolism of the hormone in F9 mouse embryonic carcinoma cells. Upon stimulation with *trans*-retinoic acid and dibutyryl cAMP, these cells express high levels of megalin and the PTH/PTHrP receptor (Fig. 3, inset) (21, 22). Expression of other receptors of the LDL receptor gene family is either not increased (LDL receptor) or even reduced (LDL receptor-related protein) upon stimulation (Fig. 3, inset). Induction of megalin and PTH/PTHrP receptor expression in F9 cells correlated with a 3-fold increase in $^{125}$I-recPTH degradation as compared with noninduced control cells (Fig 3; $p < 0.009$). To dissect the contribution of different pathways for PTH degradation, we investigated the cellular catabolism of $^{125}$I-recPTH in the presence of various inhibitors (Fig. 4). The receptor-associated protein (RAP) is a cellular chaperone which blocks binding of all known ligands to megalin (25). These antagonistic properties of RAP can be exploited to specifically interfere with megalin-mediated ligand uptake into cells (16, 26). When purified glutathione S-transferase (GST)-RAP fusion protein was added to the culture medium of F9 cells, the amount of PTH degraded was reduced by 50% as compared with control cells that received GST only (Fig. 4A, columns 1 and 2, $p < 0.006$). PTH degradation was blocked to the same extent by chloroquine, an inhibitor of lysosomal degradation (Fig. 4A, column 3, $p < 0.001$). A combination of GST-RAP and chloroquine had no additive inhibitory effect (Fig. 4A, column 4), suggesting that lysosomal degradation of PTH is mediated through a RAP-sensitive receptor. Because only 50% of PTH degradation was mediated through lysosomal pathways, we reasoned that extracellular proteolytic activity might be responsible for the residual catabolism of the hormone. In particular, extracellular processing of $^{125}$I-recPTH into amino-terminal and carboxyl-terminal fragments would result in the generation of labeled PTH peptides that are trichloroacetic acid-soluble material.
Acid-soluble and indistinguishable from lysosomal degradation products in our assay. This hypothesis was confirmed by application of chymostatin, a protease inhibitor that blocks extracellular processing of PTH (27). Addition of chymostatin reduced the amount of 125I-degradation products by 50% (Fig. 4A, column 5, p < 0.001), and the combined application of chymostatin and RAP inhibited the degradation of the hormone almost completely (Fig. 4A, column 6, p < 0.0009). This indicated that a RAP-binding receptor was primarily responsible for lysosomal degradation of full-length PTH in these cells. To test whether the PTH/PTHrP receptor might be affected by RAP, we tested binding of 125I-RAP to F9 cell membrane preparations separated by SDS-PAGE and transferred to nitrocellulose filters. In this ligand binding assay, 125I-RAP bound strongly to a high molecular weight protein co-migrating with megalin (Fig. 4B). No significant binding to other membrane proteins including the PTH/PTHrP receptor was observed (Fig. 4B).

Previously, we have generated mice genetically deficient for megalin (17). These animals were used to evaluate the consequence of megalin deficiency for the renal catabolism of PTH. Consistent with a role of the receptor in the tubular uptake of biologically active PTH peptides, levels of amino-terminal PTH fragments in urine of knockout mice were increased 4-fold as compared with control litter mates (374 ± 48 versus 87.5 ± 29 pmol/liter; p < 0.002). In contrast, levels of carboxyl-terminal PTH fragments were unchanged (354.6 ± 76 versus 345.4 ± 45.2 pmol/liter). At the same time, the megalin gene defect did not affect the total amount or subcellular localization of the PTH/PTHrP receptor in proximal tubular cells as shown by immunohistochemistry (Fig. 5) and Western blot analysis (data not shown). Finally, the significance of megalin for tubular catabolism of PTH was confirmed by microinfusion of 125I-labeled recPTH into rat proximal tubules in situ (with method described in Ref. 16). In these micropuncture experiments, 50.6 ± 0.9% of labeled recPTH was taken up into the tubules. The uptake of 125I-recPTH was significantly impaired when RAP was co-infused into the tubules (41.1 ± 2.8%; p < 0.025). Similar effects have been observed for other receptor ligands previously (16).

The results obtained so far have established a dual receptor system in the renal proximal tubules that recognizes full-length and amino-terminal PTH fragments. This receptor system consists of the PTH/PTHrP receptor that transduces PTH signals and of the endocytic receptor megalin that mediates the catabolism of the hormone. It was of particular interest to learn about the significance of this dual receptor system for PTH activity. So far, the limited number (n = 8) of megalin−/− animals available and their complex phenotype confounded the detailed analysis of their PTH metabolism. We therefore focused on PTH-mediated signal transduction in F9 cells. First, the ability of recPTH to stimulate the PTH/PTHrP receptor was tested (Fig. 6A). When recPTH was added to F9 cells,
cAMP levels did not rise above the level in untreated control cells (columns 1 and 2). However, when recPTH was applied together with an inhibitor of phosphodiesterase activity (isobutylmethylxanthine) a significant increase in cAMP levels was observed (columns 3 and 4, *p < 0.014). To study the influence of megalin on PTH/PTHrP receptor activity, we next determined PTH-induced signal transduction in the presence of the megalin inhibitor RAP (Fig. 6B). As already shown in Fig. 6A, recPTH alone did not increase steady state cAMP levels (column 3). In contrast, addition of recPTH and RAP caused a 3-fold elevation of cellular cAMP concentrations (column 4) even in the absence of isobutylmethylxanthine (*p < 0.012). GST or GST-RAP had no direct effect on cAMP levels (columns 1 and 2). These results demonstrated that megalin-mediated degradation of PTH antagonizes activation of the PTH/PTHrP receptor.

**DISCUSSION**

We have identified megalin as an endocytic receptor for the cellular uptake and degradation of PTH. The receptor was purified from kidney membranes as a major PTH-binding protein and shown in BIAcore analysis to bind full-length PTH and amino-terminal PTH fragments. In F9 cells and in microinjected proximal tubules, uptake and lysosomal degradation of the hormone was mediated through megalin. Expression of the receptor in the proximal tubular epithelium and enhanced excretion of amino-terminal but not carboxy-terminal PTH fragments in urine of megalin−/− mice suggests that the receptor also plays an important role in the renal catabolism of the hormone in vivo.

A renal endocytic pathway for uptake and degradation of PTH has been anticipated by findings that the kidney is largely responsible for removal of the hormone from the circulation. In isolated perfused kidneys, PTH and PTH fragments are mainly cleared through glomerular filtration and re-absorption in the proximal tubules (8, 9). In both patients and laboratory animals, plasma PTH levels are directly correlated with glomerular filtration rates and clearance of PTH is blocked in nonfiltering kidneys (6, 28). Previously, two types of PTH binding sites have been identified in renal membrane preparations that could be involved in the tubular catabolism of the hormone: high affinity (Kd = 10 nM) and low affinity binding sites (Kd = 1 μM) (29, 30). The high affinity binding sites are coupled to G proteins and most likely represent the PTH/PTHrP receptor (31). In contrast, the low affinity binding sites are not linked to G proteins (30) and potentially represent the endocytic receptor megalin. This is indicated by the fact that megalin is a major PTH-binding protein in the kidney (Fig. 1) and exhibits PTH binding affinities in the micromolar range (Table I). Although megalin binds recPTH in vitro with an affinity significantly below the circulating levels of the hormone, this does not exclude a role of the receptor in the renal uptake of PTH in vivo. Megalin is one of the most abundant membrane proteins on the brush border surface of the proximal tubules, and its abundance could compensate for low affinity in ligand uptake. For example, polybasic drugs such as aminoglycosides, aprotinin, and polymyxin B are efficiently internalized by megalin in the proximal tubules. The affinity of the receptor for these compounds is similar to the one for PTH (16).

The degradation of PTH was investigated in a number of established proximal tubular cell lines. In opossum kidney cells most PTH was subjected to endocytosis and lysosomal degradation, whereas a minor fraction of the hormone was metabolized by extracellular chymotrypsin-like endopeptidases (10, 11, 27). It has been suggested that in opossum kidney cells the PTH/PTHrP receptor is responsible for the endocytic uptake of the hormone. However, no specific antagonist other than PTH peptides have been available to specifically block the PTH/PTHrP receptor and to investigate the potential contribution of various receptor pathways to PTH clearance. We have analyzed PTH degradation in F9 embryonic carcinoma cells where expression of megalin and the PTH/PTHrP receptor can be co-induced by trans-retinoic acid and dibutyryl cAMP. Although not of tubular origin, high levels of PTH/PTHrP receptor and megalin expression mimics well the situation in the proximal tubules in vivo. Similar to opossum kidney cells, F9 cells metabolized PTH both by extracellular proteolysis and by lysosomal degradation (Fig. 4A). The latter pathway most likely involves megalin, because lysosomal degradation was blocked by RAP. This inhibitor does not bind to the PTH/PTHrP receptor (Fig. 4B) or affect its function directly (Fig. 6B). In addition, we have investigated the degradation of PTH in porcine proximal tubular cells (LLC-PK1). These cells express megalin but not the PTH/PTHrP receptor (32). Again, more than 80% of PTH degradation in these cells was blocked by RAP (data not shown). Finally, we analyzed the PTH catabolism in mice genetically deficient for megalin. These animals specifically excreted increased levels of amino-terminal but not carboxy-terminal PTH fragments.

Endocytic mechanisms are known to modulate signal transduction pathways. Upon binding of signaling molecules, a number of signaling receptors undergo rapid sequestration and

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\[^{5}\] J. Hilpert, A. Nykjaer, C. Jacobsen, G. Wallukat, R. Nielsen, S. K. Moestrup, H. Haller, F. C. Luft, E. I. Christensen, and T. E. Willnow, unpublished observations.
internalization. Consequently, the total number of receptor molecules on the cell surface is reduced, and target cells are protected from overstimulation by excess ligands (down-regulation). The PTH/PTHrP receptor also has been shown to undergo endocytosis after PTH binding (33, 34). Both positive and negative regulatory elements controlling endocytosis have been identified in the carboxyl-terminal tail of this receptor (35).

An alternative endocytic mechanism regulating signal transduction has been identified in the insulin-like growth factor II (IGF-II) signaling pathway. IGF-II is a growth factor that controls mitogenic processes during embryonic development via binding to the IGF-I receptor. Loss of IGF-I receptor or IGF-II expression in knockout mice results in a growth-deficiency phenotype (36, 37). IGF-II also binds to the IGF-II/cation-independent mannose 6-phosphate receptor, which mediates internalization and lysosomal degradation of the growth factor. Gene inactivation of cation-independent mannose 6-phosphate receptor results in increased circulating levels of IGF-II and, as a consequence, in overgrowth of embryonic tissues. These findings demonstrate that cation-independent mannose 6-phosphate receptor is essential to reduce circulating levels of IGF-II and to protect the IGF-I receptor from overstimulation (18, 38). On the basis of our findings, it is intriguing to speculate that megalin might play a similar role in regulation of the PTH/PTHrP receptor activity. According to this model, endocytosis of the PTH/PTHrP receptor predominantly regulates the availability of signaling receptors on the cell surface, whereas megalin removes excessive ligands from the extracellular space. In F9 cells, such an antagonistic property of megalin was demonstrated (Fig. 6B). Additional support for this hypothesis comes from the fact that megalin only recognizes full-length and amino-terminal PTH fragments that are able to stimulate the PTH/PTHrP receptor. Biologically inactive fragments of the carboxyl-terminal region of the hormone are not bound (Fig. 2 and Table I). Furthermore, megalin and the PTH/PTHrP receptor are co-localized on the brush border surface of the proximal tubular epithelium (Fig. 5) where megalin could regulate PTH concentrations locally.

At present, the limited number of megalin knockout animals available and their complex phenotype makes it difficult to test this concept in vivo. The generation of mouse models with kidney-specific megalin gene defect will be helpful in answering this important question.

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