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The Cytoplasmic Tail of the G-protein-coupled Receptor for Parathyroid Hormone and Parathyroid Hormone-related Protein Contains Positive and Negative Signals for Endocytosis*

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Zhengmin Huang, Ying Chen, and Robert A. Nissenson‡

From the Endocrine Research Unit, Veterans Administration Medical Center and the Departments of Medicine and Physiology, University of California, San Francisco, California 94121

The present studies were done to evaluate the role of the cytoplasmic tail of the G-protein-coupled receptor for parathyroid hormone (PTH) and PTH-related protein (PTHrP) in the endocytosis of agonist-occupied receptors. PTH/PTHrP receptor mutants progressively truncated from the C terminus were expressed in COS-7 cells, and their ability to internalize ^{125}I-PTHrP(1–34)amide was determined. Most of the C-terminal tail (91 of 127 residues) could be deleted without affecting internalization. However, further truncation removing residues 475–494 resulted in a 50–60% decrease in ligand internalization. A mutant with an internal deletion of these 20 amino acids showed a similar reduction in internalization, confirming the presence of a positive endocytic signal. No additional positive signals were found in the membrane-proximal region of the tail. However, alanine mutagenesis of the membrane-proximal residues 459–461 (EVQ → AAA) resulted in a mutant PTH/PTHrP receptor displaying a 40% increase in ligand endocytosis, indicating that EVQ functions as a negative signal. Treatment of COS-7 cells with hypertonic sucrose (to disrupt clathrin lattices) markedly suppressed (by >80%) PTH/PTHrP receptor internalization. These results demonstrate the presence of both positive and negative endocytic signals in the membrane-proximal cytoplasmic tail of the PTH/PTHrP receptor and suggest that these signals regulate the ability of the receptor to accumulate in clathrin-coated pits.

Endocytosis of cell surface receptors is a ubiquitous process that serves multiple functions (1–3). Constitutively recycling receptors, such as those for transferrin and low density lipoprotein, are shuttles that deliver their ligands to the cell interior and then return to the plasma membrane. Signaling receptors, on the other hand, are generally not endocytosed efficiently until agonist is bound and transmembrane signaling has occurred. At this point, endocytosis may allow receptor resensitization (*e.g.* by targeting the receptor to an acidic endosomal compartment that facilitates ligand dissociation, followed by receptor recycling) or may serve as the initial step in the ultimate lysosomal degradation of the receptor (down-regulation) (4).

For many receptors, efficient endocytosis requires discrete cytoplasmic receptor determinants that serve as signals for interaction with the endocytic machinery of the cell. Positive internalization signals have been identified in constitutively recycling receptors such as transferrin receptors, low density lipoprotein receptors, cation-independent mannose 6-phosphate receptor, and asialoglycoprotein receptors (5, 6). They are composed of a short linear stretch of cytoplasmic amino acid residues which lack an obvious consensus sequence, but share certain common features. These motifs generally consist of ≤6 residues that are 20–50 amino acid residues from the plasma membrane and frequently contain an essential tyrosine residue (7). Such sequences are thought to be directly involved in the association of the receptor with the HA-2 adapter proteins of the membrane clathrin lattice, a process that leads to efficient endocytosis via clathrin-coated pits (8).

Relatively little is known concerning the molecular basis of endocytosis of heptahelical G-protein-coupled receptors (GPCRs).[1] Clathrin-coated pits/vesicles have been implicated as mediating internalization of some (9), but not other (10, 11), GPCRs. The sequence NP*X*Y, similar to the NP*X*Y internalization motif in the low density lipoprotein receptor (5), is required for efficient agonist-mediated sequestration of the β-adrenergic receptor away from the cell surface (12). This sequence is highly conserved in the seventh membrane-spanning segment of many GPCRs and might serve as a common endocytic signal. Serine/threonine-rich sequences in the third cytoplasmic loop or cytoplasmic tail contribute to efficient internalization of the GPCRs for thyrotropin-releasing hormone (13), gastrin-releasing peptide (14), muscarinic agonists (15), and yeast α-factor (16).

The receptor for parathyroid hormone (PTH) and PTH-related protein (PTHrP) (17) is a member of a newly recognized, structurally distinct heptahelical GPCR subfamily that also includes receptors for secretin, glucagon and related peptides, calcitonin, and several others (18). Members of this subfamily lack the conserved NP*X*Y sequence; nonetheless, they undergo efficient ligand-mediated endocytosis. The sequence elements in these receptors required for this process have not been identified. Previous studies of the PTH/PTHrP receptor suggest that endocytosis leading to down-regulation of the receptor occurs in bone and kidney cells *in vitro* (19, 20) and may serve to regulate target cell responsiveness *in vivo* (21).

In the present study, we have used a mutagenesis strategy to identify receptor sequences in the cytoplasmic tail of the PTH/PTHrP receptor that influence receptor-mediated endocytosis. The results indicate the presence of two novel determinants. One is within residues 475–494 and is required for efficient

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‡ Research Career Scientist of the Department of Veterans Affairs. To whom correspondence and reprint requests should be addressed. Tel.: 415-750-2089; Fax: 415-750-6929.

[1] The abbreviations used are: GPCR, G-protein-coupled receptor; PTH, parathyroid hormone; bPTH, bovine PTH; PTHrP, parathyroid hormone-related peptide; PCR, polymerase chain reaction; OK, opossum kidney.

receptor internalization; the second is within residues 459–461, which are highly conserved in the PTH/secretin receptor subfamily, and serves to suppress internalization. These studies illustrate that at least one member of the PTH/secretin receptor subfamily of GPCRs is endocytosed by a signal-dependent mechanism and that such signals can suppress or enhance internalization.

EXPERIMENTAL PROCEDURES

Materials—Synthetic bovine PTH(1–34) and human PTHrP(1–34) were purchased from Bachem, Inc. Restriction enzymes and *Taq* polymerase were purchased from Life Technologies, Inc. Sequenase 2.0 sequencing kit was obtained from U. S. Biochemical Corp. DEAE-dextran and chloroquine were from Sigma. Cell culture media and reagents were obtained from the UCSF cell culture facility. All oligonucleotides used were synthesized and purified at UCSF Biomolecular Resource Facility.

Mutagenesis by Overlap Extension Polymerase Chain Reaction (PCR)—Truncation mutant receptor constructs were generated by PCR using the opossum kidney (OK) PTH/PTHrP receptor cDNA in pcDNA1/AMP (OKO2) as a template (a gift from Harold Jüppner). Downstream primers were designed such that a stop codon was introduced at desired positions in the receptor coding region, which was followed by an endonuclease restriction site *Xba*I. An upstream primer was located at about 50 base pairs upstream of an internal restriction site *Nsi*I. PCR fragments were digested with *Xba*I and *Nsi*I, separated by agarose gel electrophoresis and extracted by QIAEX (QIAGEN Corp.) before ligation into OKO2 predigested with *Nsi*I and *Xba*I followed by transformation into *Escherichia coli* strain Top10F′ (Invitrogen Corp.).

Internal deletion and tandem alanine mutation constructs were generated by overlap extension PCR, as described by Pease and co-workers (22). For each receptor construct, four oligonucleotide primers were utilized, two internal primers bearing desired mutation sequences encoding tandem alanines or single alanine and two external primers bearing the 5′ end sequences of the final PCR fragment. Primers were designed such that their melting temperatures are between 55 °C and 65 °C. Two overlapping fragments gel-purified from two PCR reactions with paired external and internal primers were mixed for a second PCR reaction. At a low but significant frequency, two overlapping strands were annealed and extended by the DNA polymerase to generate a full-length template which was then amplified by two external primers. The external primer sites are chosen such that the PCR fragment includes a *Nsi*I and *Sph*I sites to facilitate subcloning into the plasmid of interest. The PCR fragments were gel-purified, digested with *Sph*I and *Nsi*I, and ligated with the predigested OKO2 vector. All receptor constructs derived from pcDNA1/AMP (Invitrogen Corp.) were transformed into, maintained, and amplified in *E. coli* strain, Top10F′. Plasmid DNAs were purified using QIAGEN columns according to the manufacturer's recommended procedure (QIAGEN Corp.). The PCR portions of the final constructs were sequenced to confirm the identity of the PCR product.

Cell Culture and Transfection—COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For transient transfection of COS-7 cells, we used a modification of the DEAE-dextran/chloroquine method (23). In brief, COS-7 cells cultured in T75 flasks were incubated for 3 h with a mixture of 5 μg of plasmid DNA, 400 μg/ml DEAE-dextran, and 0.1 mM chloroquine, followed by a 2-min 10% dimethyl sulfoxide shock. The following day, cells were subcultured into 6-well cluster plates for functional studies, which were carried out 72-h post-transfection.

PTH/PTHrP Receptor Binding and Internalization—PTH/PTHrP receptor binding and internalization studies were carried out essentially as described (19, 24). In brief, COS-7 cells were incubated in 1 ml of media containing 20 mM HEPES, 0.1% bovine serum albumin, 50,000 cpm of ^{125}I-hPTHrP(1–34)amide, plus varying concentrations of unlabeled bPTH(1–34). Previous studies have demonstrated that bPTH(1–34) and hPTHrP(1–34) bind equipotently to the OK PTH/PTHrP receptor (17). Under these conditions, the concentration of hPTHrP(1–34) added as radioligand was approximately 0.1 nM. After a 1-h incubation at room temperature, the cells were washed, collected in 1.5 ml of 0.8 N NaOH, and bound ^{125}I-hPTHrP(1–34)amide was assessed. For internalization studies, after a 1-h incubation at room temperature with ^{125}I-hPTHrP(1–34)amide, cells were washed twice with ice-cold phosphate-buffered saline. Surface-bound ligand was then extracted by two 5-min incubations on ice with 50 mM glycine buffer (pH 3.0) containing 0.1 M NaCl. After acid extraction, the remaining cell-associated radioligand was extracted by exposing cells to 0.8 N NaOH. Receptor internalization is expressed as the percent of cell-associated radioligand remaining after acid washing.

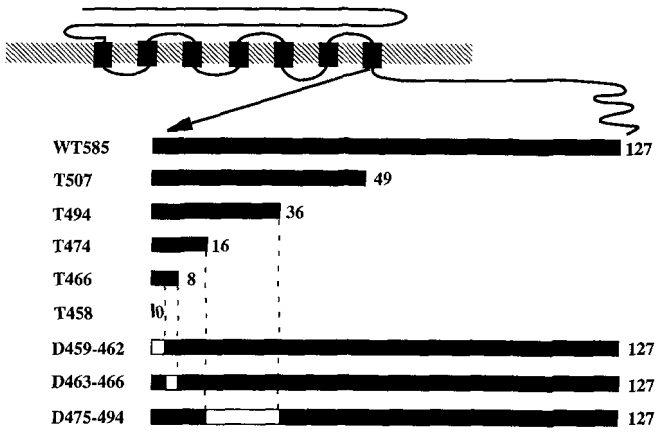

FIG. 1. **A schematic diagram of wild type and mutant PTH/PTHrP receptors evaluated in the present study.** Progressive truncation at the cytoplasmic tail resulted in mutant PTH/PTHrP receptors (T507, T494, T474, T466, and T458) containing 49, 36, 16, 8, and 0 amino acid residue tails, respectively. In mutant receptors D459–462, D463–466, and D475–494, cytoplasmic tail residues (1–4, 5–8, and 17–36, respectively) were replaced by 4 alanines.

cAMP Levels—cAMP was measured by a modification of our published method (25). In brief, 72 h after transfection, cells were incubated for 10 min in medium containing 0.4 mM isobutylmethylxanthine, followed by 10 min in medium containing appropriate concentrations of bPTH(1–34). Cells were washed and cyclic AMP was extracted with 95% ethanol and assayed by radioimmunoassay.

RESULTS

Fig. 1 presents a diagrammatic representation of the mutant OK PTH/PTHrP receptors encoded by the constructs used in this study. These include a series of C-terminal truncation mutants, designated by "T" followed by the residue number of the last amino acid. Also shown are 3 mutants ("D") in which the residues shown were deleted and replaced by a set of 4 alanine residues.

Functional expression of wild-type OK PTH/PTHrP receptors was assessed in COS-7 cells transiently transfected with expression vector OKO2 (Fig. 2). These cells have no detectable endogenous PTH/PTHrP receptors and are thus a suitable model system for these studies. Competitive binding studies (Fig. 2*A*) demonstrated the presence of PTH/PTHrP receptors that display an apparent affinity (IC_{50}) of 4.5 nM, similar to the reported value in an opossum kidney cell line (26). These receptors were found to be functionally coupled to adenylyl cyclase (Fig. 2*B*), conferring PTH-dependent increases in cAMP levels, with an EC_{50} for bPTH(1–34) of 0.21 nM.

Fig. 3 compares the kinetics of ligand binding and of receptor internalization in COS-7 cells expressing either wild-type PTH/PTHrP receptors or receptors lacking all but 16 residues in the cytoplasmic tail (T474). These receptors displayed similar ligand binding kinetics (Fig. 3*A*) and binding affinity (data not shown), but differed markedly in their internalization properties (Fig. 3*B*). Internalization of the wild-type receptor was rapid (half-maximal within 5 min) and reached a maximal level of 50–60% of cell-associated ligand within 20–30 min. Truncation mutant T474 displayed a similar internalization time course, but only about 25% of cell-associated ligand was internalized after 60 min. To define in more detail the determinants in the cytoplasmic tail required for efficient receptor endocytosis, we evaluated a series of PTH/PTHrP receptor mutants progressively truncated at the C terminus of the cytoplasmic tail (Fig. 4). Functional expression of these mutant receptors, assessed by radioligand binding (Fig. 4*A*), was variably reduced

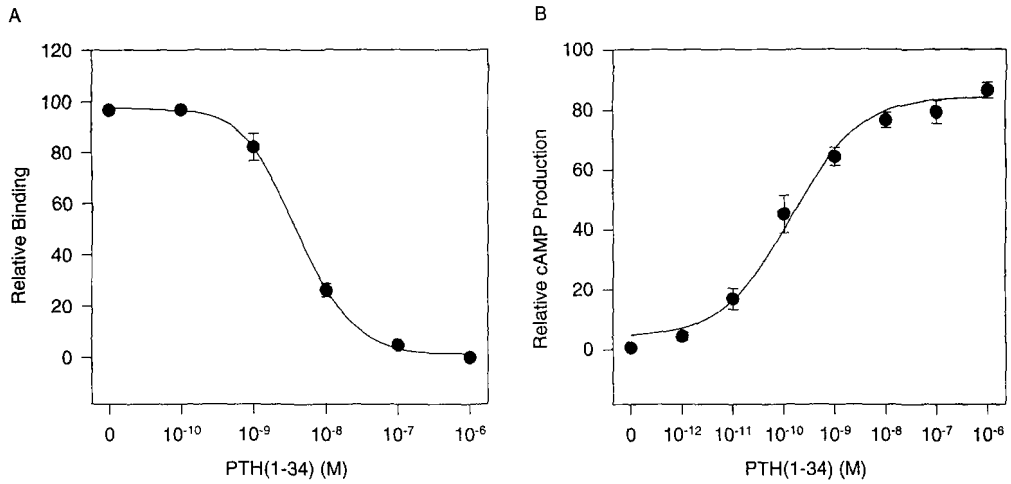

FIG. 2. **^{125}I-labeled PTHrP(1–34)amide binding (*A*) and bPTH(1–34)-stimulated cAMP production (*B*) in COS-7 cells transiently expressing the wild-type OK PTH/PTHrP receptor.** Binding and cAMP data represent the mean ± S.E. of 8 experiments. Total specific binding was 27 ± 3% of added tracer, and the IC_{50} for bPTH(1–34) was 4.5 ± 0.7 nM. Basal and maximal bPTH(1–34)-stimulated cAMP levels were 8 ± 2 and 75 ± 14 (pmol/30-mm well), and the EC_{50} for bPTH(1–34) was 0.21 ± 0.03 nM.

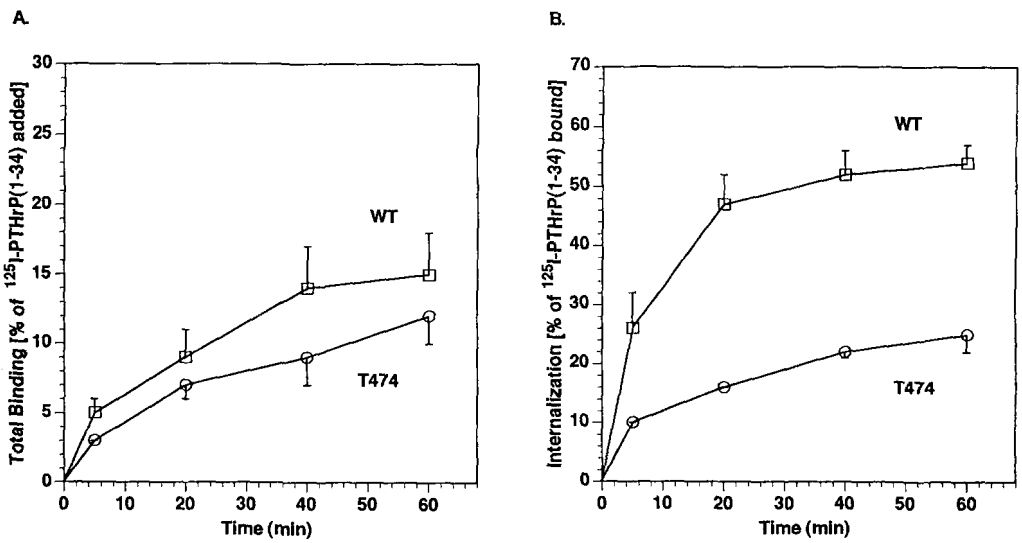

FIG. 3. **Time course of PTH/PTHrP receptor internalization in COS-7 cells.** The wild-type receptor and mutant T474 were transiently expressed in COS-7 cells, and ^{125}I-PTHrP(1–34)amide binding (*A*) and internalization (*B*) were assessed, as described under "Experimental Procedures." Similar results were obtained in two experiments, each with triplicate data points.

compared to wild-type for all mutants except T474. No significant differences in binding affinity were seen (data not shown). Results of internalization assays (Fig. 4*B*) revealed that T507 and T494 were internalized indistinguishably from wild-type, whereas both T474 (as shown above) and T466 were internalized with a 50–60% reduced efficiency.

These results with truncation mutants suggest the presence within amino acid residues 475–494 of a determinant of efficient PTH/PTHrP receptor endocytosis. However, it is also possible that reduced internalization was due to local effects such as the change in the relative position of the C terminus in T474 and T466. Moreover, as redundant endocytic codes/regions have been identified in a number of receptors (13, 27), it is also possible that reduced internalization required the deletion of more C-terminal sequence as well as residues 475–494. To investigate these possibilities, an internal deletion mutant (D475–494) in which a sequence region between amino acid residues 475 and 494 was replaced by 4 alanine residues was generated and expressed in COS-7 cells. As shown in Fig. 4*B*, internalization of this mutant receptor was reduced by 50%, similar to the defect seen with truncation mutant T474. These results indicate that residues 475–494 contain a signal that is required for efficient PTH/PTHrP receptor internalization, and that this signal is functional in the context of the full-length PTH/PTHrP receptor.

Because the truncation mutant T458 was not expressed on the cell surface, it was unclear whether juxtamembrane residues 459–466 contributed to receptor internalization. To investigate this, we generated two tandem alanine mutations targeting this region. The two mutated receptors (D459–462 and D463–466) were expressed at levels somewhat lower than wild-type, based on their ligand binding properties (Fig. 5*A*). Strikingly, D459–462 displayed a 40% increase in internalization relative to wild-type. D463–466 did not differ from wild-type. These results suggest that the juxtamembrane region of the cytoplasmic tail contains a second endocytic signal that serves to constrain receptor internalization.

Exposure of cells to hypertonic medium has been shown to remove membrane-associated clathrin lattices, resulting in a loss of clathrin-coated pits and vesicles (28). When COS-7 cells transiently expressing the wild-type OK PTH/PTHrP receptor were exposed to hypertonic solution (0.45 M sucrose) 1 h before

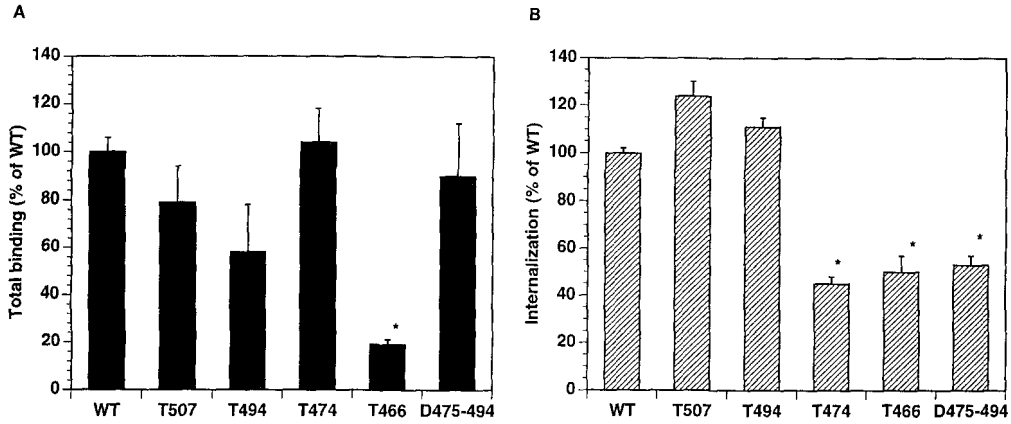

FIG. 4. **Effects of C-terminal truncation and internal deletion on PTH/PTHrP receptor binding (*A*) and endocytosis (*B*).** Mutant receptors are as described in Fig. 1. Values are normalized to those of the wild-type receptor which was assigned a value of 100%. Data represent the mean ± S.E. of three to five experiments with triplicate in each. Values with *asterisks* are significantly different from wild-type ($p < 0.005$, Student's t test).

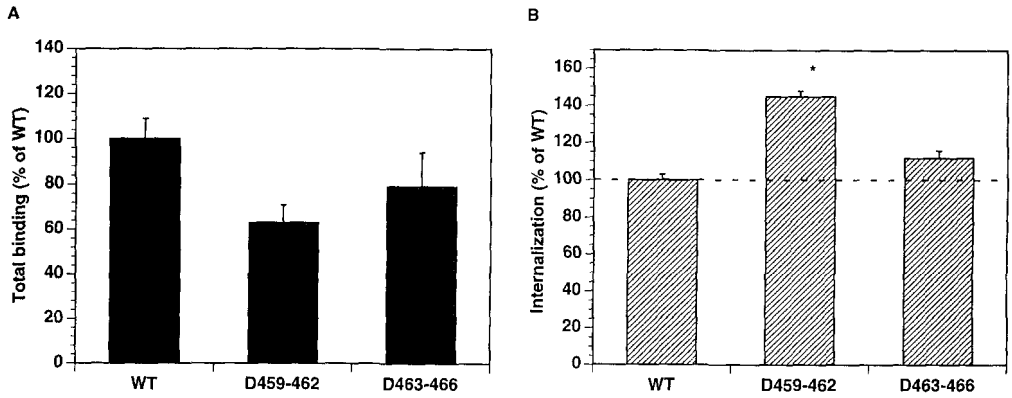

FIG. 5. **Mutations in the juxtamembrane region of the cytoplasmic tail of the PTH/PTHrP receptor: effects on receptor binding (*A*) and endocytosis (*B*).** Mutant receptors are as described in Fig. 1. Data represent the mean ± S.E. of three experiments with triplicate samples in each. Values with *asterisks* are significantly different from wild-type ($p < 0.005$, Student's t test)

the addition of ^{125}I-PTHrP(1–34)amide, there was an 82% reduction in receptor internalization with no effect on receptor binding *per se* (Fig. 6). This effect was fully reversible upon restoration of conditions that allow clathrin reassembly (removal of the sucrose). These results are consistent with the notion that ligand-induced PTH/PTHrP receptor internalization occurs via clathrin-coated pits. The internalization of the mutant T474, which is deficient under standard incubation conditions, is further reduced by hypertonic sucrose, indicating that clathrin pit-mediated internalization of the PTH/PTHrP receptor is not solely dependent upon the positive endocytic signal in the tail.

DISCUSSION

Previous studies have suggested that homologous down-regulation of the PTH/PTHrP receptor may limit target cell responsiveness under conditions of ligand excess *in vivo* (21), and that this process is associated with receptor internalization (19, 20, 29). Molecular cloning of the PTH/PTHrP receptor (17) has now allowed us to address the molecular basis of receptor-mediated endocytosis. The results of the present study indicate that the 90 C-terminal amino acid residues of the cytoplasmic tail can be removed without affecting PTH/PTHrP receptor-mediated endocytosis. However, the proximal region of the tail contains two discrete sequence elements that influence internalization, one a positive endocytic signal between residues 17 and 36 of the tail, and the other a negative signal comprising 3 residues at the interface between membrane and cytoplasm.

Two approaches were used to identify the positive endocytic signal. Expression of C-terminal truncation mutants revealed that wild-type levels of receptor-mediated endocytosis were obtained with PTH/PTHrP receptors possessing only a 36-amino acid tail. However, further truncation deleting residues LDFKRKARSGSSTYSYGPMV resulted in markedly (50–60%) reduced receptor internalization. Therefore, in the context of a severely truncated PTH/PTHrP receptor, this sequence is sufficient to signal enhanced endocytic efficiency. Moreover, this sequence is required for efficient endocytosis since a nontruncated receptor in which this sequence was specifically deleted displayed comparably reduced endocytosis.

The relationship between the signal contained within this sequence and previously identified positive endocytic signals is unclear. The sequence does not contain a heretofore identified internalization signal, but resembles known signals in certain respects. It is rich in serine/threonine residues as are internalization sequences in the cytoplasmic tails of the thyrotropin-releasing hormone (13), gastrin-releasing peptide (14), and yeast α-factor (16) receptors. Recently, we have found that the cytoplasmic tail of the PTH/PTHrP receptor is rapidly phosphorylated upon agonist binding,[2] a modification that could promote receptor internalization. Also present are 2 tyrosine residues in a stretch of sequence likely to have β-turn structure by Chou-Fasman analysis (30). A tyrosine residue in the context of a β-turn is a characteristic feature of internalization signals in several constitutively recycling receptors (7) and could play a

[2] E. Blind and R. A. Nissenson, submitted for publication.

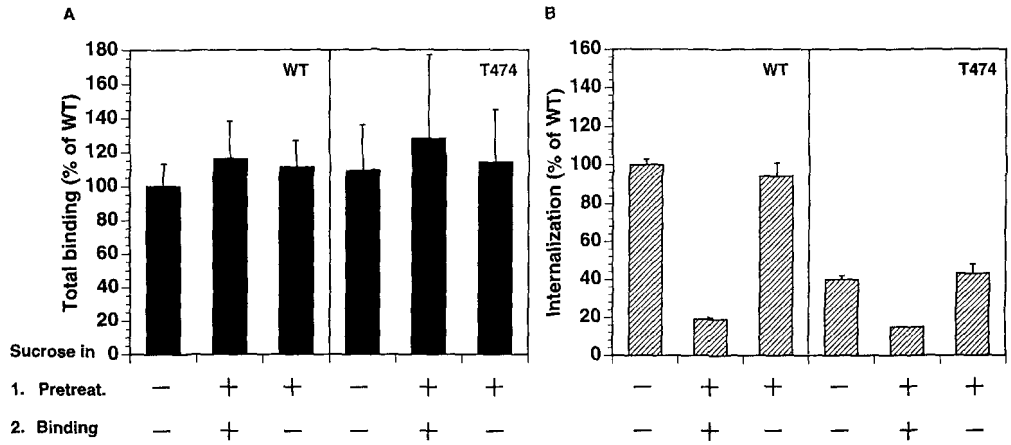

FIG. 6. **Effect of hypertonic sucrose treatment on PTH/PTHrP receptor binding (*A*) and endocytosis (*B*).** COS-7 cells transiently expressing the wild-type or the T474 mutant PTH/PTHrP receptor were preincubated ± hypertonic media (0.45 M sucrose) for 1 h, followed by a 1-h incubation with ^{125}I-PTHrP(1–34) ± 0.45 M sucrose, as indicated. Receptor binding and internalization were then assessed as described under "Experimental Procedures." Values are normalized to those of the wild-type receptor which was assigned a value of 100%. Data represent the mean of two to three independent experiments with triplicate samples in each.

similar role in signaling receptors. In this regard, preliminary mutagenesis studies indicate that the positive signal is contained within the sequence TYSYGPMV.[3] This stretch contains 2 tyrosines included in the sequence TYSYG which has a high probability of forming a turn (30). Unlike most of the cytoplasmic tail, this sequence is highly conserved among PTH/PTHrP receptors from opossum to human (18), suggesting a functional role. Further mutagenesis should serve to clarify the role of serine/threonine phosphorylation and/or tyrosine residues in the endocytic function of this domain.

Mutated PTH/PTHrP receptors lacking the positive internalization signal (*e.g.* T474) are endocytosed, albeit to a reduced extent. This is not due entirely to bulk flow internalization, as disruption of clathrin lattices by treatment with hypertonic sucrose further suppressed internalization. The present signal may act in concert with one or more additional positive endocytic signals located outside of the receptor's cytoplasmic tail. It will be important to establish whether this signal can function autonomously to promote endocytosis, *e.g.* when spliced onto an internalization-defective transferrin receptor.

An important issue for signaling receptors is the relationship between signal transduction and receptor internalization. In the case of epidermal growth factor and insulin receptors, mutations that abolish intrinsic tyrosine kinase activity markedly reduce receptor-mediated endocytosis (31, 32). In the case of the epidermal growth factor receptor, tyrosine kinase activity, but not autophosphorylation, is required, indicating that signal transduction and heterologous protein phosphorylation is a prerequisite for efficient internalization (32, 33). For GPCRs, endocytic signals may be found in cytoplasmic domains that are either independent of (34) or overlap with (35) regions that are required for agonist-stimulated signal transduction. In the present study, truncation mutants lacking the positive endocytic signal appear to function similarly to wild-type with respect to adenylyl cyclase activation,[3] indicating that internalization can be disrupted in mutants that retain the ability to signal adenylyl cyclase. We do not yet know whether the results of transmembrane signaling (*e.g.* activation of protein kinase A or protein kinase C) are involved in regulating PTH/PTHrP receptor endocytosis.

Tandem alanine mutagenesis of the proximal region of the cytoplasmic tail revealed a second domain that influences PTH/PTHrP receptor-mediated endocytosis. Mutation D459–461, in which residues EVQ at the membrane-cytoplasmic interface were mutated to AAA, displayed a marked, 40% increase in internalization. This result suggests that the EVQ sequence serves to limit the entry of receptors into endocytosis-competent structures, either by facilitating interaction of the receptor with poorly endocytosed membrane components or by directly inhibiting the function of a positive endocytic signal(s). It is noteworthy that this EVQ sequence is highly conserved across members of the PTH/secretin receptor subfamily (but not in other GPCRs) and may serve as a suppressor of endocytosis in other subfamily members. Although the vast majority of endocytic signals previously described are positive signals, a juxtamembrane cytoplasmic domain that inhibits coated-pit localization and endocytosis has been identified in an isoform of the Fc receptor (36). In that case, the minimum sequence required for inhibition is not yet clear. Rodriguez *et al.* (37) found that truncation of the distal portion of the cytoplasmic tail of the luteinizing hormone/human choriogonadotropin receptor resulted in enhanced receptor-mediated hormone internalization, suggesting the removal of a negative endocytic signal. Thus, these results and ours indicate that receptor-mediated endocytosis is a highly complex and regulated process, with multiple receptor domains playing facilitory as well as inhibitory roles.

Both clathrin-coated and noncoated endocytic pathways have been suggested for GPCRs. For example, β-adrenergic receptors have been reported to be internalized through noncoated vesicles (11). However, a recent report showed that β-adrenergic receptors co-localized in endosomes with transferrin, which is known to be internalized in clathrin-coated pits/vesicles (38). It is not clear whether this discrepancy is due to differences in the cell lines used or whether noncoated vesicles are able to fuse with clathrin-coated vesicles. The marked inhibition of PTH/PTHrP receptor internalization produced by hypertonic sucrose, a treatment known to disrupt clathrin lattices (28), suggests that PTH/PTHrP receptor endocytosis occurs at least in part through a clathrin-coated pit mechanism. This result is consistent with the previous report of Silve *et al.* (39) who used EM autoradiography to demonstrate the presence of iodinated PTH in clathrin-coated pits on the surface of cells in calvarial bone.

In summary, specific positive and negative signals in the cytoplasmic tail of a GPCR, the PTH/PTHrP receptor, serve to regulate agonist-stimulated endocytosis via clathrin-coated pits/vesicles. These signals are distinct from those previously

[3] Z. Huang and R. A. Nissenson, unpublished data.

identified in both constitutively recycling and signaling receptors. Further delineation of the critical features of these signals and identification of their targets should provide new insights into the molecular basis of endocytosis of the PTH/PTHrP receptor and possibly other GPCRs.

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REFERENCES

1. Trowbridge, I. S., Collawn, J. F., and Hopkins, C. R. (1993) *Annu. Rev. Cell Biol.* **9,** 129–161
2. Pastan, I. H., and Willingham, M. C. (1981) *Annu. Rev. Physiol.* **43,** 239–250
3. Steinman, R. M., Mellman, I. S., Muller, W. A., and Cohn, Z. A. (1983) *J. Cell Biol.* **96,** 1–27
4. Smythe, E., and Warren, G. (1991) *Eur. J. Biochem.* **202,** 689–699
5. Chen, W. J., Goldstein, J. L., and Brown, M. S. (1990) *J. Biol. Chem.* **265,** 3116–3123
6. Jing, S. Q., Spencer, T., Miller, K., Hopkins, C., and Trowbridge, I. S. (1990) *J. Cell Biol.* **110,** 283–294
7. Vaux, D. (1992) *Trends Cell Biol.* **2,** 189–192
8. Pearse, B. M., and Robinson, M. S. (1990) *Annu. Rev. Cell Biol.* **6,** 151–171
9. Ghinea, N., Vu, H. M., Groyer, P. M., Houllier, A., Schoevaert, D., and Milgrom, E. (1992) *J. Cell Biol.* **118,** 1347–1358
10. Raposo, G., Dunia, I., Marullo, S., Andre, C., Guillet, J. G., Strosberg, A. D., Benedetti, E. L., and Hoebeke, J. (1987) *Biol. Cell* **60,** 117–123
11. Raposo, G., Dunia, I., Delavier, K. C., Kaveri, S., Strosberg, A. D., and Benedetti, E. L. (1989) *Eur. J. Cell Biol.* **50,** 340–352
12. Barak, L. S., Tiberi, M., Freedman, N. J., Kwatra, M. M., Lefkowitz, R. J., and Caron, M. G. (1994) *J. Biol. Chem.* **269,** 2790–2795
13. Nussenzveig, D. R., Heinflink, M., and Gershengorn, M. C. (1993) *J. Biol. Chem.* **268,** 2389–2392
14. Benya, R. V., Fathi, Z., Battey, J. F., and Jensen, R. T. (1993) *J. Biol. Chem.* **268,** 20285–20290
15. Lameh, J., Philip, M., Sharma, Y. K., Moro, O., Ramachandran, J., and Sadee, W. (1992) *J. Biol. Chem.* **267,** 13406–13412
16. Roher, J., Benedetti, H., Zanolari, B., and Riezman, H. (1993) *Mol. Biol. Cell* **4,** 511–521
17. Jüppner, H., Abou, S. A., Freeman, M., Kong, X. F., Schipani, E., Richards, J., Kolakowski, L. J., Hock, J., Potts, J. J., Kronenberg, H. M., and Segre, G. V. (1991) *Science* **254,** 1024–1026
18. Segre, G. V., and Goldring, S. R. (1993) *Trends Endocrinol. Metab.* **4,** 309–314
19. Teitelbaum, A. P., Silve, C. M., Nyiredy, K. O., and Arnaud, C. D. (1986) *Endocrinology* **118,** 595–602
20. Pernalete, N., Garcia, J. C., Betts, C. R., and Martin, K. J. (1990) *Endocrinology* **126,** 407–413
21. Mahoney, C. A., and Nissenson, R. A. (1983) *J. Clin. Invest.* **72,** 411–421
22. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene (Amst.)* **77,** 51–59
23. Lopata, M. A., Cleveland, D. W., and Sollner-Webb, B. (1984) *Nucleic Acids Res.* **12,** 5707
24. Nissenson, R. A., Diep, D., and Strewler, G. J. (1988) *J. Biol. Chem.* **263,** 12866–12871
25. Nissenson, R. A., Strewler, G. J., Williams, R. D., and Leung, S. C. (1985) *Cancer Res.* **45,** 5358–5363
26. Teitelbaum, A. P., and Strewler, G. J. (1984) *Endocrinology* **114,** 980–985
27. Chang, C. P., Lazar, C. S., Walsh, B. J., Komuro, M., Collawn, J. F., Kuhn, L. A., Tainer, J. A., Trowbridge, I. S., Farquhar, M. G., Rosenfeld, M. G., Wiley, H. S., and Gill, G. N. (1993) *J. Biol. Chem.* **268,** 19312–19320
28. Hansen, S. H., Sandvig, K., and van Deurs, B. (1993) *J. Cell Biol.* **121,** 61–72
29. Brown, R. C., Silver, A. C., and Woodhead, J. S. (1991) *Am. J. Physiol.* **260,** E544–E552
30. Chou, P. Y., and Fasman, G. D. (1974) *Biochemistry* **13,** 211–222
31. Carpentier, J. L., Paccaud, J. P., Gorden, P., Rutter, W. J., and Orci, L. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 162–166
32. Sorkin, A., Waters, C., Overholser, K. A., and Carpenter, G. (1991) *J. Biol. Chem.* **266,** 8355–8362
33. Sorkin, A., Helin, K., Waters, C. M., Carpenter, G., and Beguinot, L. (1992) *J. Biol. Chem.* **267,** 8672–8678
34. Zanolari, B., Rath, S., Singer-Kruger, B., and Riezman, H. (1992) *Cell* **71,** 755–763
35. Moro, O., Shockley, M. S., Lameh, J., and Sadee, W. (1994) *J. Biol. Chem.* **269,** 6651–6655
36. Miettinen, H. M., Matter, K., Hunziker, W., Rose, J. K., and Mellman, I. (1992) *J. Cell Biol.* **116,** 875–888
37. Rodriguez, M. C., Xie, Y. B., Wang, H., Collison, K., and Segaloff, D. L. (1992) *Mol. Endocrinol.* **6,** 327–336
38. von Zastrow, M., and Kobilka, B. K. (1992) *J. Biol. Chem.* **267,** 3530–3538
39. Silve, C. M., Hradek, G. T., Jones, A. L., and Arnaud, C. D. (1982) *J. Cell Biol.* **94,** 379–386