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PTH/PTH-related protein receptor interacts directly with Tctex-1 through its COOH terminus☆

Maki Sugai,[a,1] Masaki Saito,[b,1] Izumi Sukegawa,[c] Yuriko Katsushima,[d] Yoshitaka Kinouchi,[a] Norimichi Nakahata,[b] Tooru Shimosegawa,[a] Teruyuki Yanagisawa,[e] and Jun Sukegawa[e,*]

[a] *Department of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan*
[b] *Department of Cellular Signaling, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai 980-8578, Japan*
[c] *Izumi Chu-oh Medical Clinic, Sendai 981-3133, Japan*
[d] *Department of Pediatrics, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan*
[e] *Department of Molecular Pharmacology, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan*

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Abstract

COOH-terminal cytoplasmic domains of G protein-coupled receptors (GPCRs) have been shown to carry determinants that control their cell surface localization, internalization, and recycling. In attempts to seek cellular proteins that mediate these processes of PTH/PTH-related protein receptor (PTHR), one of the class B GPCRs, we have found that Tctex-1, a 14 kDa light chain of cytoplasmic dynein motor complex, interacts with the COOH-terminal tail of the receptor. A 34-amino-acid stretch of the receptor responsible for binding to Tctex-1 has a bipartite structure consisting of a motif previously implicated in binding of some proteins to Tctex-1 and a putative new consensus sequence. Site-directed mutations or a 20-amino-acid deletion in the bipartite consensus binding sequence abolished the association of the PTHR COOH terminus with Tctex-1 in vitro. A GFP-fused mutant PTHR impaired in binding to Tctex-1 expressed in MDCK cells showed a decreased rate of internalization in response to PTH compared to that of the wild type.

Keywords: PTH/PTHrP receptor; Dynein; Tctex-1; Internalization

Molecular mechanisms by which GPCRs transduce extracellular signals to intracellular compartments have been extensively studied. Especially, recent works have indicated that processes of cell surface localization, internalization, and recycling of GPCRs have profound influence on signal transduction efficiencies. It has also been demonstrated that GPCRs carry sorting signals for these processes at their COOH-terminal cytoplasmic domains. Internalization of prototype GPCRs through clathrin-coated pits is initiated by phosphorylation of the COOH-terminal cytoplasmic domain of the receptor by G protein-coupled receptor kinases and/or second messenger-dependent kinases. Then, β-arrestin binds to the phosphorylated GPCR [1,2] and commits the receptor to internalization [3–5]. It has also been reported recently that COOH-terminal domains of a variety of GPCRs interact with PSD95, Disks-large, ZO-1 homologous (PDZ) domain or PDZ-like domain-containing proteins. Proteins with multiple PDZ domains recognize specific cytoplasmic tails of GPCRs, and thereby serve as molecular scaffolds for organizing large multiprotein signaling complexes beneath cortical membrane of the cells [6]. On the other hand, Homer proteins, with their single PDZ-like domains, have been shown to play a role in regulating trafficking of metabotropic glutamate receptors from ER to cell

☆ *Abbreviations:* DIC, dynein intermediate chain; DLC, dynein light chain; GPCR, G protein-coupled receptor; MBP, maltose-binding protein; MesNa, 2-mercaptoethanesulfonic acid sodium salt; PDZ, PSD95, Disks-large, ZO-1 homologous; PTH, parathyroid hormone; PTHR, PTH/PTH-related protein receptor.

* Corresponding author. Fax: +81-22-717-8065.
E-mail address: jsukegaw@mail.tains.tohoku.ac.jp (J. Sukegawa).

[1] Both authors contributed equally to this work.

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surface [7]. Consequently, PDZ or PDZ-like domain-containing proteins target the cognate GPCRs to cell surface. While biological importance of COOH-terminal domains of GPCRs has become increasingly evident, molecular details of the determinants carried by the domain and cellular factors interacting with them have been largely undefined.

PTH is involved in regulation of calcium homeostasis, bone remodeling, and maintaining blood phosphate concentration [8,9]. While the receptor for PTH is expressed abundantly in primary target organs of the hormone, i.e., kidney and bone, the receptor is also expressed in specific areas of central nervous system and has been implicated in some neuronal functions [10,11]. It has been demonstrated that PTHR internalization occurs through a clathrin and β-arrestin2-dependent manner [12]. Also, Huang et al. [13] have suggested that a stretch of amino acids in the cytoplasmic tail of PTHR confers a positive signal for the receptor endocytosis. However, molecular entities that mediate this signal for directing the PTHR-containing membrane vesicles to endocytosis have remained obscure [14].

In order to understand the roles of COOH-terminal cytoplasmic domain of GPCR more precisely, we have screened human brain cDNA library to seek proteins that would interact with the cytoplasmic tail of PTHR and regulate processes of the receptor internalization. Here, we report that the COOH-terminal cytoplasmic tail of PTHR interacts directly with Tctex-1, a 14 kDa light chain of cytoplasmic dynein motor complex, and that the Tctex-1-interaction motif is essential for efficient agonist-induced internalization of the receptor.

Materials and methods

Antibodies and other reagents. Rabbit antiserum against MBP and pMAL-c2 plasmid vector were purchased from New England Biolabs (Beverly, USA). Anti-HA 12CA5 monoclonal antibody (mAb) and FuGENE 6 transfection reagents were obtained from Roche Diagnostics (Basel, Switzerland). Anti-FLAG epitope M2 mAb and 2-mercaptoethanesulfonic acid, sodium salt (MesNa) were from Sigma–Aldrich (St. Louis, USA). Anti-PTH/PTHrP receptor mAb (3D1.1) was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Glutathione–Sepharose 4B, protein A–Sepharose 4 Fast Flow, and pGEX-5X-2 plasmid were purchased from Amersham Bioscience. Human PTH(1–34) was from Peptide Institute (Osaka, Japan). Sulfo-NHS-SS-Biotin was purchased from Pierce Biotechnology (Rockford, USA). ProQuest two-hybrid system, a human brain cDNA library, and pcDNA vectors were obtained from Invitrogen (Carlsbad, USA). Quick Change site-directed mutagenesis kit was from Stratagene (La Jolla, USA). pEGFP-N1 plasmid was obtained from BD Biosciences Clontech (Palo Alto, USA).

Plasmids. The coding sequence for PTHR COOH-terminal cytoplasmic domain (amino acid residues 467–593) was PCR-amplified from human placental DNA and inserted in-frame into pDBLeu bait plasmid. Plasmids encoding GST-fused PTHR-c-end were constructed by transferring inserts of the pDBLeu constructs into pGEX-5X-2 plasmid. A plasmid encoding MBP-Tctex-1 fusion protein was constructed by inserting the Tctex-1 cDNA into pMAL-c2 vector. pcDNA3.1(+)-PTHR was generated by inserting *Avr*II–*Xba*I fragment of pcDNA1-PTHR cDNA [15] into a mammalian expression vector pcDNA3.1(+) plasmid digested with *Nhe*I and *Xba*I. HA-tagged PTHRs were prepared by inserting HA tag sequence into *Bss*SI site of the PTHR cDNA that placed the tag just upstream of the first transmembrane domain of the receptor. pcDNA3-Flag-Tctex-1 plasmid was constructed so that FLAG epitope sequence was located at the amino-terminus of Tctex-1. PTHR-KR, VS, and KRVS mutant plasmids were constructed with the aid of Quick Change site-directed mutagenesis kit to replace desired amino acids with alanines. pcDNA3.1(+)-PTHR-Δ and pGEX-PTHR-Δ mutants coding for the receptors lacking amino acid residues from F483 to H502 were generated by employing PCR to delete the corresponding nucleotide sequence. In order to express PTHR-GFP fusion proteins in mammalian cells, a full-length coding sequence of human PTHR in pcDNA1 vector was PCR-amplified, recloned in pEGFP-N1 plasmid, and designated PTHR-WT-GFP/pEGFP-N1. For constructing PTHR-KRVS-GFP/pEGFP-N1 to express the mutant PTHR-GFP fusion protein, cDNA fragments containing the mutations were PCR amplified and inserted in place of the wild type DNA in PTHR-WT-GFP/pEGFP-N1. Nucleotide sequences of all the plasmid clones and subclones were confirmed by using ABI310 sequencer.

Yeast two-hybrid screening. The screening for histidine prototrophs was done according to the company's instructions. Candidate clones were assessed further for their URA3 and β-galactosidase reporter gene expressions.

In vitro pull-down assay. GST-PTHR, MBP-Tctex-1, and MBP-RP3 fusion proteins were expressed in *Escherichia coli* and the cells were lysed by using a French Pressure Cell in Tris-buffered saline (TBS: 20 mM Tris–HCl, pH 7.4, 150 mM NaCl) supplemented with 1 mM DTT and 1 mM PMSF. Cleared lysates containing one of the GST-PTHR fusion proteins and MBP-Tctex-1 or MBP-RP3 protein were mixed in TBS containing 1 mM DTT and 0.5% NP-40 and incubated for 1 h at 4 °C. The mixture was combined with glutathione–Sepharose 4B and incubated further for 1 h at 4 °C. Proteins associated with the beads were separated on SDS–PAGE and probed with anti-MBP antibody.

Cell culture and transfection. HEK293 cells were obtained from Cell Resource Center for Biomedical Research, Tohoku University, Japan. Transfection of cells was performed by using FuGENE 6 transfection regents according to the manufacturer's instructions. Stable cell lines of MDCK cells were established in the presence of G418 at 400 μg/ml.

Immunoprecipitation. Forty-eight hours after transfection, cells were lysed in IP buffer (TBS containing 1% Triton X-100 and 1 mM EDTA). The cleared lysates were mixed with anti-HA mAb and incubated overnight at 4 °C. After a further incubation with pre-washed protein A–Sepharose beads, the proteins precipitated with the beads were subjected to SDS–PAGE. Separated proteins were transferred onto nitrocellulose membranes and incubated with anti-FLAG M2 mAb.

Cell surface biotinylation and receptor internalization assay. Biotinylation and removal of the biotin molecules from labeled cell surface proteins were performed essentially as described previously [16,17]. Briefly, cells stably expressing PTHR-GFP fusion proteins were seeded on 35 mm culture plates one day before the experiment. The culture plates were placed on ice and the cells were incubated for 20 min twice with 1.4 ml of sulfo-NHS-SS-biotin (0.5 mg/ml) in PBS(+). After the cells were rinsed once and incubated for 15 min on ice with 1.5 ml DME supplemented with 0.5% of BSA (DME/BSA), the media were replaced with pre-warmed DME/BSA supplemented with 100 nM PTH(1–34) and the cells were incubated at 37 °C for 5, 10, or 20 min. The cells were then subjected to three 40-min incubations with 50 mM MesNa in NT buffer (100 mM NaCl, 50 mM Tris–HCl, pH 8.6, 1 mM Mg^{2+}, and 0.1 mM Ca^{2+}) in order to cleave the disulfide bonds of surface-bound biotin molecules. The cells were lysed and PTHR proteins were immunoprecipitated with anti-PTHR mAb. The proteins precipitated with the antibody were separated on SDS–PAGE and

transferred onto nitrocellulose membranes. The blots were probed with streptavidin conjugated with HRP and the signals were quantitated by using Molecular Imager GS-525 (Bio-Rad).

GFP-fused PTHR internalization experiments. MDCK cell lines stably expressing either PTHR-WT-GFP or PTHR-KRVS-GFP were seeded on coverslips placed in 12-well culture plates one day before the experiments. Culture media were replaced with DMEM supplemented with 0.5% BSA with or without PTH(1–34) (100 nM) and incubated in a 5% CO_2 incubator at 37 °C for 30 min. After two rinses with cold PBS, the cells were fixed in 4% paraformaldehyde in PBS, mounted, and observed under Axioplan2 Imaging microscope (Zeiss). Images were captured by Digital Eclipse C1 confocal microscope (Nikon).

Results

Screening for PTHR interacting proteins

In order to identify proteins interacting with the COOH-terminal cytoplasmic domain (amino acid residues from Q467 to M593) of PTHR, we employed yeast two-hybrid system [18]. Screening of 1×10^6 clones derived from a human brain cDNA library yielded 15 positive clones. Nucleotide sequencing of the cDNA clones exhibiting strong interaction signals revealed a clone harboring a full-length coding sequence for cytoplasmic dynein light chain (DLC), Tctex-1.

Mapping of the interacting domain of PTHR COOH terminus

To define amino acid sequences responsible for the interaction with Tctex-1, PTHR c-end was divided into smaller segments and subjected again to yeast two-hybrid assays. Tctex-1 interacted strongly with PTHR CN-11 and very weakly with PTHR CN-10 (Fig. 1A). We also performed in vitro pull-down assays with each part of PTHR and Tctex-1 proteins produced in *E. coli* as GST and maltose-binding protein (MBP) fusion proteins, respectively. Each one of GST-PTHR fusion

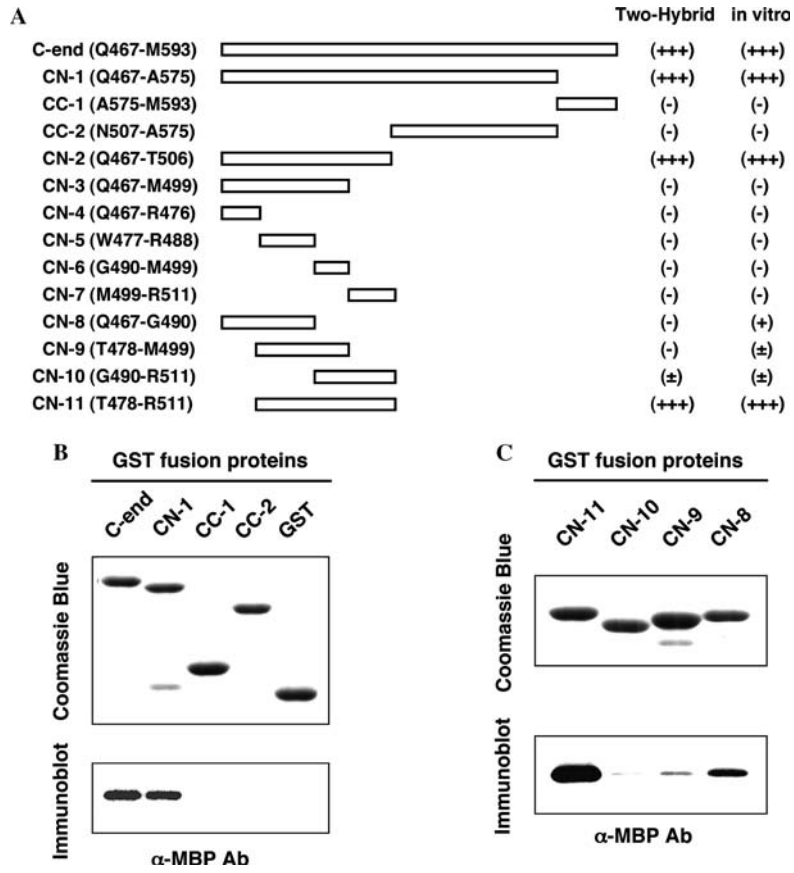

Fig. 1. Mapping of the interacting domain of PTHR cytoplasmic tail with dynein light chain, Tctex-1. Amino acid sequences of PTHR responsible for the interaction with Tctex-1 were determined by yeast two-hybrid system and in vitro binding assays. (A) Schematic diagram of wild type (c-end) and distinct parts of the COOH-terminal domain of PTHR and their activities to interact with Tctex-1. Names of the clones and their amino acid residue numbers are indicated on the left. Interaction activities of the clones with Tctex-1 in two-hybrid system and in vitro binding assays are summarized on the right. (B) and (C) In vitro binding activity of Tctex-1 with various parts of COOH-terminal region of PTHR. Each one of GST-PTHR fusion proteins and MBP-Tctex-1 fusion protein were mixed together with glutathione–Sepharose beads. Proteins bound to the beads were then separated by SDS–PAGE and analyzed by immunoblotting with anti-MBP antibody. Upper panels show Coomassie blue staining of separately prepared gels, indicating that the reaction mixtures contain approximately equal amounts of GST fusion proteins. Lower panels show MBP-fused Tctex-1 protein specifically bound to each GST-PTHR fusion protein. Note that CN-8, CN-9, and CN-10 showed reduced activities to interact with Tctex-1.

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CN-11       478 TLALDFKRKARSGSSSYSYGPMVSHTSVTNVGPR 511
                  * *           * *

Rhodopsin   315 NCMLTTICCGKNPLGDDEASATVSKTETSQVAPA 348(term)
DIC         120 SDSELGRRLHKLG---------VSK--VTQVDFL 142
CD155α      362 GIYFYWSKCSREVLWHCHLC
                        PSSTEHASASANGHVSYSAVSRENSS 407
DOC2α         1 MRGRRGDRMTINIQEHMAINVCPGPIRPIRQ 31
DOC2β         1 MTLRRRGEKATISIQEHMAIDVCPGPIRPIKQ 32
CD5         376 CGPLAYKKLVKKFRQKKQRQWIGPTGMNQNMSFH 409
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Fig. 2. Amino acid sequence alignment of various Tctex-1-binding domains. Amino acid sequences of Tctex-1 binding domains of PTHR CN-11, rhodopsin, DIC, CD155α, DOC2α, and β, and CD5 are aligned. Putative consensus amino acid residues conserved for the binding Tctex-1 are shown in boldface letters. In addition to the consensus, R/K-R/K-X-X-R/K, that was reported previously, another consensus, V-S-K/H-T/S-X-V/T-T/S-N/Q-V, is discernible. Asterisks indicate positions of amino acids that were replaced with alanines in mutant clones. Amino acids deleted in PTHR-Δ mutants are underlined. Amino acids are numbered on both ends. Gaps, shown by dashes are introduced for the best alignment.

proteins and MBP-Tctex-1 fusion protein were incubated together in vitro and proteins bound to glutathione–Sepharose 4B beads were separated by SDS–PAGE and immunoblotted with anti-MBP antibody (Figs. 1B and C). MBP-Tctex-1 was coprecipitated strongly with GST-PTHR CN-11, confirming the result of the two-hybrid assays. GST-PTHR CN-8, -9, and -10 showed weak interactions with MBP-Tctex-1. These results indicate that PTHR binds Tctex-1 via its 34-amino-acid stretch from residue T478 through R511. Previous study of Mok et al. [19] demonstrated that Tctex-1 bound several different proteins through their specific amino acid sequence motif, R/K-R/K-X-X-R/K. We found that amino acid residues from K484 to R488 (KRKAR) of CN-11 conformed to the Tctex-1-binding motif (Fig. 2). Furthermore, it was noticed that Tctex-1-binding proteins, dynein intermediate chain (DIC) and rhodopsin, had another amino acid stretch, V-S-K/H-T/S-X-V/T-T/S-N/Q-V, that was shared with the sequence in PTHR from V500 to V508 (see Fig. 2). CD155α shows a weak homology to this motif. Accordingly, this 34-amino-acid stretch of PTHR COOH terminus appeared to comprise a bipartite motif for binding to Tctex-1. The subclones, CN-8, -9, and -10, that interacted weakly with Tctex-1 in vitro covered only a part of this putative bipartite motif.

PTHR interaction with DLC is Tctex-1 specific

We examined also whether RP3, a close homolog of Tctex-1 with 75% amino acid similarity [20,21], interacts with PTHR COOH terminus. In vitro binding experiments demonstrated that the interaction of the COOH terminus of PTHR is specific to Tctex-1 but not to RP3 (Fig. 3), suggesting that each DLC component of dynein motor complex might interact with specific cognate cargo proteins as indicated previously [22].

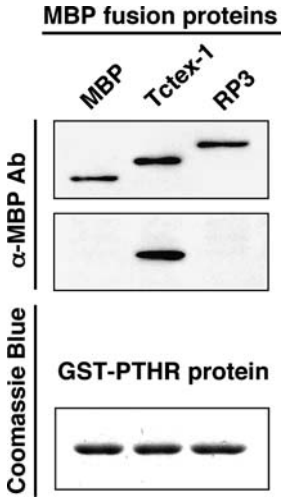

Fig. 3. Specificity of PTHR interaction with Tctex-1. MBP, MBP-fused Tctex-1 or RP3, a homolog of Tctex-1, was incubated with the COOH terminus of PTHR fused with GST. After incubating further with glutathione–Sepharose beads, proteins precipitated with the beads were separated by SDS–PAGE and analyzed by immunoblotting with anti-MBP antibody. Upper panel shows immunoblot of lysates containing MBP, MBP-fused Tctex-1 or RP3 proteins used in the binding reactions. Middle panel shows that MBP-fused Tctex-1, but not RP3, binds to GST-PTHR-c-end fusion protein. Lower panel is the Coomassie blue stain of proteins adsorbed to glutathione beads showing that similar amounts of GST-PTHR fusion protein were added to reaction mixtures.

Tctex-1 does not bind mutated COOH terminus of PTHR in vitro

In order to further specify the amino acid sequence responsible for the interaction with Tctex-1, several mutations were introduced into the COOH terminus of PTHR (see Fig. 2). While the PTHR-KR mutant disrupts the basic amino acid stretch in the proximal part of the putative bipartite consensus sequence, the PTHR-VS mutant destroys a well-conserved motif in the distal part of the sequence. Pull-down assays in vitro revealed that both of the PTHR-KR and PTHR-VS mutants showed significant decreases in their binding efficiency to Tctex-1 compared to the wild type receptor. Moreover, the PTHR-KRVS and PTHR-Δ mutants completely lost the binding activity (Fig. 4). These results suggested that the 20-amino-acid stretch from F483 to H502 of PTHR, deleted in the PTHR-Δ mutant, comprised an essential part for binding to Tctex-1, and that both of the pairs of mutated amino acids (K486, R488, and V500, S501) or at least one of the two amino acids in each pair of the mutations were critically involved in the interaction.

Association of Tctex-1 and PTHR in HEK293 cells

To investigate whether PTHR would interact with Tctex-1 in cells, immunoprecipitation experiments were

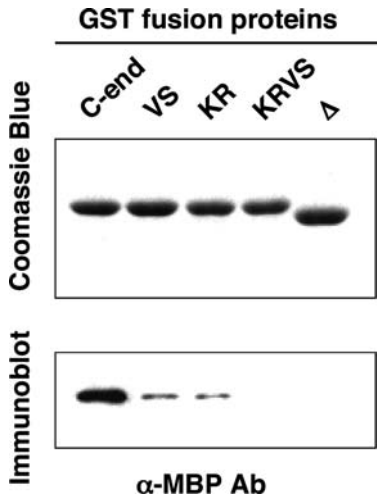

Fig. 4. Tctex-1 binding activities of PTHR mutants in vitro. Wild type GST-PTHR fusion protein or the fusion protein with either KR, VS, KRVS, or Δ mutation was incubated with MBP-Tctex-1. Proteins associated with glutathione–Sepharose beads were separated by SDS–PAGE and stained with Coomassie blue (upper panel) or probed with anti-MBP antibody (lower panel). Coomassie blue stain demonstrates that similar amounts of GST fusion proteins were used in each reaction mixture. While PTHR with the KR or VS mutation exhibited reduced binding to MBP-Tctex-1, both of the KRVS and Δ mutants were severely impaired in their abilities to associate with MBP-Tctex-1.

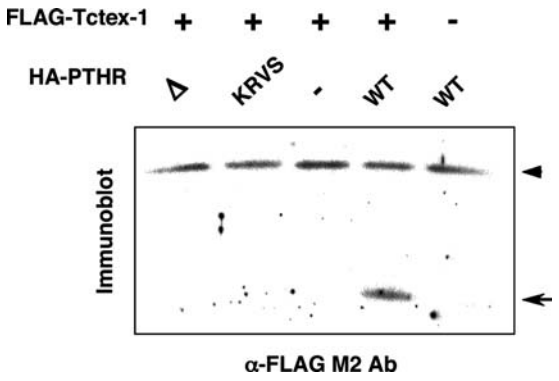

Fig. 5. Co-immunoprecipitation of Tctex-1 with wild type but not with mutant PTHRs. HEK293 cells were co-transfected with one of the plasmids expressing wild type HA-PTHR, HA-PTHR-KRVS, or HA-PTHR-Δ, and with or without the plasmid coding for Flag-Tctex-1. Lysates of the transfected cells were incubated with anti-HA mAb and proteins associated with the antibody were collected by protein A–Sepharose beads. The proteins were separated by SDS–PAGE and analyzed by immunoblotting with anti-FLAG mAb. FLAG-Tctex-1 is marked by an arrow. An arrowhead indicates light chains of anti-HA mAb decorated by the secondary antibody. Note that Tctex-1 co-precipitated with wild type PTHR, but not with mutant PTHRs.

performed. HEK293 cells were transfected with plasmids coding for FLAG-tagged Tctex-1 (FLAG-Tctex-1) and HA-tagged PTHR (HA-PTHR) with or without various mutations. Lysates of the transfected cells were incubated with anti-HA mAb and the antibody was adsorbed to protein A–Sepharose beads. Proteins precipitated with the beads were subjected to SDS–PAGE and analyzed by anti-FLAG mAb. FLAG-Tctex-1 protein precipitated only in the presence of the wild type PTHR, confirming that PTHR could interact with the dynein light chain in cells and that the interaction was impeded by the mutations in the putative Tctex-1-binding consensus sequence (Fig. 5).

Localization of mutant PTHR-GFP protein in cells

Cytoplasmic dynein is a microtubule-based motor protein complex and carries cargo molecules or membrane vesicles along microtubules toward their minus ends. In most cells, minus ends of the microtubules are anchored in the centrosome adjacent to cell nucleus and their plus ends extend outward to cell periphery [23]. Therefore, we reasoned that, during the course of receptor internalization, Tctex-1 would interact with the cytoplasmic domain of PTHR and carry the molecule along the microtubules toward the interior of the cells. In order to investigate whether the mutation that disrupts the interaction of Tctex-1 with the PTHR affects the behavior of the receptor, we constructed plasmids encoding wild type and the KRVS mutant of PTHR fused to GFP at their COOH termini. Wild type PTHR-GFP fusion proteins expressed in MDCK cells are localized primarily at cell surface and, to some extent, in cells' interior around nuclei (Fig. 6, upper left). This observation is in agreement with the report that small fraction of PTHR could be constitutively internalized without agonist stimulations [17] and is also consistent

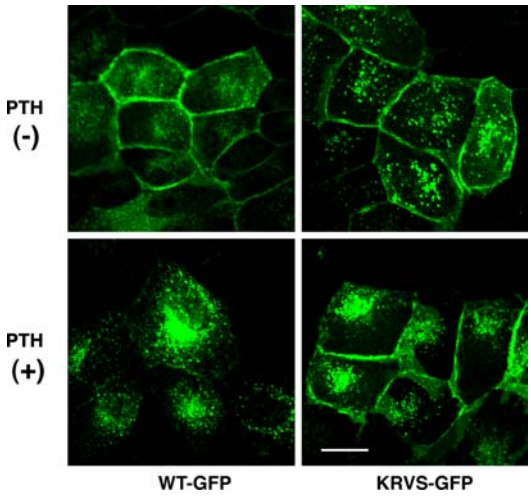

Fig. 6. Agonist-induced internalization of wild type and a mutant PTHR fused with GFP. MDCK cells stably expressing PTHR-WT-GFP (left side) or PTHR-KRVS-GFP (right side) were incubated at 37 °C for 30 min with or without 100 nM PTH(1–34). The cells were then fixed with 4% paraformaldehyde and mounted. Images were obtained with a GFP filter set on Digital Eclipse C1 confocal microscope (Nikon). Upper row shows images without PTH(1–34) treatment. Lower images were taken after treatment of the cells with the agonist. Bar, 20 μm.

with the previous report of the localization of GFP-fused PTHR in cells [24]. On the other hand, many cells that express the KRVS mutant of PTHR-GFP proteins showed some punctate cytoplasmic signals in addition to the cell surface staining (Fig. 6, upper right). These punctate signals of the receptor are reminiscent of the early endosomal localization of ligand-stimulated PTHR in cells treated with the microtubule-disrupting agent, nocodazole [24]. Because the movement of internalized GPCRs from early sorting endosomes to late endosomes has been shown to depend on the structural integrity of microtubules [25], nocodazole treatment blocks the movement of the internalized GPCRs from cell periphery to a perinuclear compartment deep within the cells [24]. We assume that the KRVS mutant of PTHR, with its ability to interact with Tctex-1 impaired, is unable to associate efficiently with microtubule motor system and is blocked at early endosomal compartment in constitutive recycling mechanisms in cells. Upon PTH(1–34) treatment, cell surface signals representing the wild type PTHR-GFP were redistributed into the cells' interior around nuclei that corresponds to endocytic recycling compartment as reported earlier [24] (Fig. 6, lower left). While majority of the mutant PTHR-GFP molecules seem to be internalized normally after 30 min incubation with the agonist, appreciable amount of the mutant molecules stayed at the cell surface as yet in many cells (Fig. 6, lower right). Although the degree of internalization of PTHR seemed to vary depending primarily on cell density seeded on plates, careful adjustment of the density and investigation of several independent cell lines resulted in similar observations.

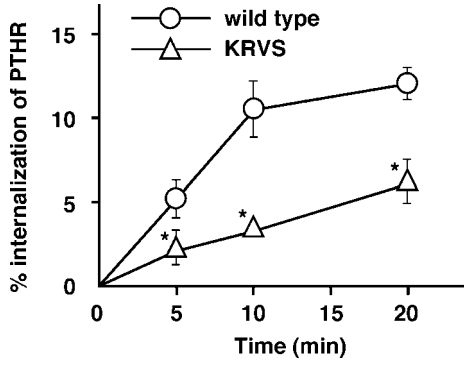

Fig. 7. Effects of KRVS mutation on agonist-induced internalization of PTHR. Cell surface proteins of MDCK cells expressing wild type or KRVS mutant of PTHR-GFP were biotinylated. After treatment with 100 nM PTH(1–34) for indicated time, the cells were exposed to MesNa. PTHR molecules were then immunoprecipitated from cleared cell lysates with anti-PTHR mAb, separated on SDS–PAGE, and blotted onto nitrocellulose membranes. Biotinylated PTHRs protected from MesNa treatment were probed with streptavidin conjugated with HRP. The panel shows the percentage of PTHR internalization at each time point obtained by scanning the blot. Data are means ± SEM of triplicate experiments. $^*P < 0.05$.

Mutant PTHR shows a decreased rate of internalization

In order to measure the rate of receptor internalization, we utilized the cell surface biotinylation technique [16,17]. Cell surface proteins of MDCK cells stably expressing the wild type or the KRVS mutant of PTHR-GFP were labeled with a cell-impermeant biotinylation reagent, sulfo-NHS-SS-Biotin. The cells are then treated with PTH(1–34) and the PTHR internalization was allowed to proceed for the limited time before the cells were treated next with cell-impermeable reducing reagent MesNa. The treatment by the reducing agent releases biotin molecules from non-internalized cell surface proteins. PTHR was immunoprecipitated from the cell lysate by using anti-PTHR mAb, subjected to SDS–PAGE, and blotted. PTHR molecules that are internalized and thereby protected from MesNa treatment were detected by using streptavidin conjugated with HRP. Compared to the wild type receptor, the KRVS mutant showed a decreased rate of internalization (Fig. 7).

Discussion

So far, more than 10 cellular molecules have been reported to interact directly with Tctex-1: rod photoreceptor protein, rhodopsin [26], DIC [19,27], poliovirus receptor, CD155α [28], Doc2, a modulator of Ca^{2+}-dependent neurotransmitter release [29], lymphocyte surface membrane protein, CD5 [30], voltage-dependent anion-selective channel, VDAC [31], FIP-1, a protein that interacts with adenovirus immunoregulatory protein Ad E3-14.7K [32], Trk neurotrophin receptor [33], Fyn protein tyrosine kinase [34,35], and a trophinin binding protein, tastin [36]. In most of these cases, the interactions of the proteins with Tctex-1 have been demonstrated or suggested to be critical in targeting or sorting the proteins through microtubule-based vesicular transport systems within the cells. Our findings that Tctex-1 interacts with PTHR through the specific amino acid motif in the receptor's cytoplasmic tail and that the motif plays an important role in receptor trafficking in cells support the view that Tctex-1 would work as an adapter between cytoplasmic dynein motor complexes and specific vesicular cargos [37,38].

Tctex-1-binding site of PTHR has a bipartite structure. The proximal part of the structure complies well with the previously reported consensus binding motif for Tctex-1, R/K-R/K-X-X-R/K [19]. The distal part of the structure represents the consensus sequence, V-S-K/H-T/S-X-V/T-T/S-N/Q-V, and shares homologies with rhodopsin, DIC, and partly with CD155α. While in vitro experiments indicate that both parts of the structure are involved in binding Tctex-1, it is surprising to note that some Tctex-1-binding proteins lack the distal

portion of the structure. More intriguing, however, is the fact that COOH terminus of rhodopsin, the postulated Tctex-1-binding domain, shares a homology only with the distal part of the structure [26]. This fact clearly suggests that, at least in some cases, either one of the proximal or the distal part of the bipartite structure would be sufficient to bind Tctex-1 in cells and to carry out particular functions assumed by the interaction. It would be interesting to know whether PTHR needs this complete form of the bipartite structure to bind Tctex-1 in cells to accomplish some specific biological processes of the receptor regulation including the receptor internalization. It is also tempting to speculate that each part of the bipartite structure for binding Tctex-1 would be necessary to fulfill specific requirements for distinct pathways of vesicular sorting mechanisms. In this context, it is noteworthy that rhodopsin, one of the class A GPCRs [39], is vectorially transported to stacked membrane disks in rod outer segment by interacting with Tctex-1 [26], showing a marked contrast to PTHR in its sorting pathway.

Previous report by Huang et al. [13] suggested that the 20-amino-acid stretch, LDFKRKARSGSSTYSYGP MV, in the cytoplasmic tail of opossum kidney PTHR carries a positive endocytic signal. This sequence closely matches the core of the bipartite Tctex-1-binding sequence of the human PTHR described in this report. Direct interaction of PTHR with a component of cytoplasmic dynein motor complex would provide mechanical bases for the endocytic movement of PTHR along the microtubule cytoskeleton within cells. It is likely, therefore, that this positive endocytic signal is mediated at least in part by Tctex-1 binding to the sequence during the course of the receptor internalization. The involvement of Tctex-1 in the movement of PTHR in cells is further implicated by our preliminary experiments that showed overexpression of RP3, another 14 kDa DLC, decreased initial rate of PTHR internalization in response to PTH(1–34) (data not shown). Tai et al. [40] reported that overexpression of RP3 displaces endogenous Tctex-1 from the dynein motor complex and alters the binding specificities of the dynein to cargos.

The cytoplasmic tail of PTHR is phosphorylated in an agonist-dependent manner on serine residues at positions 489, 491, 492, 493, 495, 501, and 504 [41,42]. It has also been reported that the phosphorylation on these serine residues is required for stable association of β-arrestin with PTHR and for efficient internalization of the receptor [43]. S501, one of the amino acids mutated in the KRVS mutant, is among the residues phosphorylated in response to PTH stimulation. Therefore, it would be possible that the decreased rate of internalization demonstrated with the KRVS mutant is due partly to reduced phosphorylation of the receptor on agonist stimulation. Because the previous reports showed the reduction of the PTHR internalization only for phosphorylation-null mutants (receptors with all the relevant serine residues mutated) [42,43], further experiments are necessary to determine whether S501A mutation itself would affect the rate of the receptor internalization. Alternatively, it might also be conceivable that the inefficient interaction of the mutant PTHR with dynein motor complex would cause mechanical stress on cells and hinder the normal trafficking mechanisms of the receptor in response to agonist stimulation. In addition, all the serine residues phosphorylated on agonist stimulation are found in the 34-amino-acid sequence of CN-11 that binds Tctex-1. Because this serine-containing sequence would modify the stability of β-arrestin–PTHR complex [43], and at the same time could be the core Tctex-1-binding site, it is presumable that Tctex-1 itself might regulate the interaction between β-arrestin and PTHR.

Abnormalities in regulatory processes of cell surface localization, internalization, recycling, or downregulation of cell surface GPCRs are implicated in various pathological conditions. As for PTHR, developments of secondary hyperparathyroidism and adynamic bone diseases in chronic renal failure have been attributed at least partly to increased downregulation of the receptor [44]. This report on Tctex-1 interaction with PTHR will shed light not only on the basic mechanisms of PTHR internalization but also the general control mechanisms of cell surface expression of GPCRs.

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