Binding of filamin to the C-terminal tail of the calcitonin receptor controls recycling

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Abbreviation list

aa, amino acid; sCT, salmon calcitonin; CTR, calcitonin receptor; erk, extracellular regulated kinase; FLIP, fluorescence loss in photobleaching; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; MAPK, mitogen-activated protein kinase; PE, phycoerythrin;
Summary

Many G protein-coupled receptors undergo endocytosis, but the mechanisms involved in endocytic sorting and recycling remain to be fully elucidated. We found that the G protein-coupled calcitonin receptor (CTR) undergoes tonic internalization and accumulates within the cell. Using a ‘fluorescence loss in photobleaching’ (FLIP) assay, we classified these vesicles functionally as recycling vesicles. In a two-hybrid screening, we found that the actin binding protein filamin interacted with the C-terminal tail of the CTR. The degradation of the receptor was profoundly increased in the absence of filamin or the CTR-filamin interaction. The absence of filamin was also associated with a marked decrease in recycling of the receptor from the endosomes to the cell surface. In contrast, CT-induced inhibition of spontaneous filamin proteolysis was associated with increased recycling of the receptor to the cell surface and decreased degradation of the CTR, suggesting an important role for filamin in the endocytic sorting and recycling of the internalized CTR.
Introduction

The calcitonin receptor (CTR) belongs to subclass B of the G protein-coupled receptors (GPCRs). It binds calcitonin (CT), which acts on bone and kidney to maintain calcium homeostasis. It also acts on the central nervous system, where it has anorexic and analgesic effects (1). GPCRs comprise the largest known superfamily of cell receptors. They mediate responses to the majority of known hormones, neurotransmitters, and neuromodulators, and also function as sensors for extracellular ions, light, and pheromones. These facts make GPCRs in general extremely interesting targets for therapeutic drugs. GPCRs mediate signal transduction by serving as ligand-regulated guanine nucleotide exchange factors for heterotrimeric GTP-binding proteins, which in turn regulate several downstream effectors. Moreover, several recent reports demonstrate the existence of G protein-independent signaling pathways used by GPCRs (2).

Endocytosis is a common response to ligand stimulation that sequesters the receptors by redistributing them from the cell membrane to intracellular membranes (3). The internalized receptor can then either recycle back to the cell surface or be targeted to lysosomes and degraded, a process inducing downregulation of the GPCR. There is a body of evidence showing receptor- and cell-specific differences in the mechanisms of GPCR endocytosis (3). A common mechanism involves ligand binding and activation of the receptor followed by phosphorylation of the GPCR by GPCR kinases (GRKs), which leads to binding of β-arrestins to the receptor and thereby to the uncoupling from the heterotrimeric G protein (4-6).

In addition to the well-characterized ligand-induced endocytosis, there are reports of GPCRs undergoing tonic internalization (7-14). Mechanisms underlying tonic endocytosis include agonist-independent β–arrestin binding or tyrosine-based endocytic motifs (12, 13). While the reasons for tonic internalization of GPCRs are not understood, it is clear that tonic receptor internalization must be compensated by constant recycling of the receptor back to the cell surface.
if surface expression of the receptor is to be maintained. However, much less is known about endosomal sorting and recycling processes of GPCRs than about the molecular events leading to GPCR endocytosis.

Here we show that the CTR undergoes tonic internalization and recycling to the cell surface. Efficient recycling was found to be dependent on the interaction of the C-terminal tail of the CTR with the actin binding protein filamin. The absence of filamin or disruption of the association between filamin and the C-terminal tail of the receptor resulted in reduced CTR recycling back to the cell surface and the rapid degradation of the CTR, whereas CT-induced inhibition of filamin proteolysis increased recycling to the cell surface and decreased degradation of the CTR.
Experimental Procedures

Reagents and Antibodies – Salmon calcitonin (sCT) was purchased from Peninsula Laboratories, Inc. (Belmont, CA). MG-132 was from Sigma (Saint Louis, MI). The monoclonal anti-HA antibody (F-7) was from Santa Cruz Biotechnology (Santa Cruz, CA), the monoclonal anti-GFP antibody from Clontech (Palo Alto, CA), the monoclonal anti-FLAG antibody (M2) from Sigma, the monoclonal anti-filamin antibody from Chemicon (Temecula, CA), and the monoclonal anti-xpress® antibody from Invitrogen (Carlsbad, CA). Enhanced chemiluminescence solutions and nitrocellulose membranes were from Amersham and Schleicher&Schuell (Keene, NH), respectively.

Cell culture and transient transfections - Minimal essential medium (MEM), Dulbecco’s modified Eagle’s medium (DMEM), methionine-free DMEM, fetal bovine serum (FBS), and newborn calf serum (NCS) were purchased from Gibco-Invitrogen (Carlsbad, CA). All media were supplemented with 100 µg/ml streptomycin and 100 U/ml penicillin. HEK 293 cells were cultured as described before (15). A7 and M2 cells were a generous gift from Dr. John Hartwig (Harvard University). Both cell lines were cultured in MEM with 8% NCS and 2% FBS. The medium of A7 cells contained 500 µg/ml G418 (Life Technologies). For transient transfections, cells were grown to 60-70% confluence and then transfected with Fugene 6 (Roche, Molecular Biochemicals) according to the manufacturer’s protocol.

DNA-constructs – A cDNA encoding the rabbit CTR was generated by PCR and cloned in the Kpn I/ Hind III sites of the p3XFLAG-CMV-13 vector (Sigma) to obtain a CTR with a C-terminal three-fold FLAG tag. The same PCR product was cloned in the Kpn I/ Hind III site of pEGFP-N1 (Clontech) to obtain a C-terminal GFP-tagged CTR. Both constructs contained a HA tag in the extracellular N-terminus of the receptor (after aa 29 of the original sequence). A construct expressing the CTR with three tandem myc epitope tags at the C-terminus was
generated as described before (16). All CTR constructs were compared with the wildtype CTR with respect to ligand binding, phosphorylation after ligand stimulation, and cAMP generation and were found to be indistinguishable. A fragment spanning the seventh transmembrane domain and the C-terminal tail of the receptor (aa 373-474) was cloned between the Hind III and Kpn I sites of the p3XFLAG-CMV-13 vector after generation by PCR. The same was done for the C-terminal tail without the seventh transmembrane domain (aa 397-474). A filamin fragment spanning the immunoglobulin-like repeats 20-23, obtained from a construct encoding the human filamin cDNA (gift of Dr. John Hartwig, Harvard University) was cloned in the Xba I/ BamH I sites of the pcDNA4HisMaxA vector (Invitrogen). All PCR-derived constructs were sequenced by the Yale Keck Sequencing facility. The HA-tagged β2-adrenergic receptor was a gift of Dr. Brian Kobilka (Stamford University).

Yeast two-hybrid screening – A cDNA encoding the C-terminal tail of the rabbit CTR (aa 397-474) was cloned in the bait vector pBTM116 and used to screen a mouse osteoclast-like library in pASV4, as described before (17). The DNA of the colonies that were positive for both reporter assays was extracted and transformed in *E.coli*, strain DH5α. The library plasmid was isolated, amplified and retransformed in L40 cells already containing the bait plasmid to confirm the positive result.

Co-immunoprecipitation and Western blotting – Cells were lysed in mRIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% IGE PAL, 1% sodium deoxycholate, 10 mM NaF, 1 µg/ml pepstatin and 1 mM PMSF) and incubated at 4°C for 30 min. Lysates were then centrifuged for 30 min at 4°C, 16,000 g, the protein concentrations were measured with the BCA protein assay kit (Pierce, Rockford, IL) and equal amounts of protein were used for immunoprecipitation. 30 µl of protein-G agarose slurry and typically 5 µg of antibody were suspended in 500 µl PBS and incubated for one hour at 4°C. The beads were washed three times in mRIPA buffer, then 500 µg
of lysate protein and BSA (0.2% w/v) were added and the mix was incubated for 2 h at 4°C. The immune complexes on the beads were washed four times with washing buffer containing 500 mM NaCl and 0.1% Triton X-100, and once with PBS. Beads were boiled in 2×SDS-PAGE buffer and samples were electrophoresed on precast 10% SDS-PAGE gels (Invitrogen). Proteins were transferred onto nitrocellulose. Non-specific binding was blocked by incubating the membranes in 5% non-fat milk in TBST buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h. Membranes were incubated in the primary antibody for 2 h, washed 3 times for 15 min in TBST and incubated for 1 h in 1:10,000 diluted horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibody (Promega). Blots were developed using the enhanced chemiluminescence system from Amersham.

**Measurement of receptor cell surface expression and endocytosis using FACS** - The surface expression of the CTR was measured by fluorescence activated cell sorter (FACS) analysis. Cells in 6 well plates were trypsinized and the trypsin activity was neutralized by addition of growth medium containing 10% dialysed FBS. Cells were collected by centrifugation at 800g for 3 min. The cell pellet was resuspended in 100 µl ice-cold PBS. Usually about 3×10^5 cells were used for each experiment. Normal goat IgG was added to a final concentration of 200 µg/ml. After 10 min incubation, the anti-HA antibody was added to final concentration of 10 µg/ml.

To measure cell surface expression, a 30 min incubation step was followed by resuspending cells in 100 µl PBS containing 50 µg/ml phycoerythrin (PE)-conjugated goat anti-mouse IgG antibody (Molecular Probes). After a final washing step, cells were resuspended in PBS containing 2% formaldehyde to fix the sample. Bound antibody was analysed by fluorescence flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ). Win MDI software version 2.8 was used for data analysis.
To measure receptor endocytosis, a 30 min incubation step at 4°C was followed by two washes in 100 µl ice-cold PBS. The washed cells were resuspended in ice-cold DMEM, with or without 10 nM sCT. Cells were then quickly warmed to 37°C in a water bath to allow endocytosis of the receptor for different time periods. Cells were then cooled to 4°C, centrifuged and resuspended in ice-cold PBS containing 50 µg/ml phycoerythrin (PE)-conjugated goat anti-mouse IgG antibody (Molecular Probes). After a final washing step, cells were resuspended in PBS containing 2% formaldehyde to fix the sample, and bound antibody was analyzed by flow cytometry.

**Analysis of receptor recycling using FACS** – Cells were prepared as described for the internalization assay, except that cells were incubated for 30 min at 37°C in the presence of the anti-HA antibody to allow endocytosis of the CTR with the bound antibody. After cooling to 4°C, cells were incubated with an acid strip solution (0.2 M acetic acid, 0.5 M NaCl, pH 3) for 3 min to remove the surface-bound antibody. The acid strip reduced the amount of PE-conjugated secondary antibody binding to levels similar to controls without anti-HA antibody. Cells were then warmed to 37°C for different time periods to allow recycling of the CTR. PE-staining and FACS analysis were performed as described above.

**Analysis of receptor endocytosis using cleavable biotin** – CTR-transfected cells in 10 cm dishes were washed twice with ice-cold PBS and incubated with 0.5 mg/ml sulfo-NHS-S-S-biotin (Pierce) in PBS for 30 minutes at 4°C to biotinylate cell surface proteins. Excess biotin was quenched by incubating in Tris-HCl, pH 7.4, (final concentration 50 mM) for 10 min at 4°C. Cells were transferred to medium prewarmed to 37°C with or without 10 nM sCT and incubated at 37°C for various times to allow endocytosis of the receptor, then chilled on ice to stop endocytosis. Biotin was cleaved from proteins on the cell surface by washing cells twice at 4°C with 50 mM glutathione, 75 mM NaCl, 75 mM NaOH, 10% FBS. The cells were then washed twice for 10 minutes at 4°C in iodoacetamide buffer (50 mM iodoacetamide, 1% BSA in PBS) to
quench residual glutathione. Cells were harvested in mRIPA supplemented with 1 mg/ml iodoacetamide. The FLAG-tagged CTR was immunoprecipitated, electrophoresed on 10% SDS-PAGE without reducing agent and transferred electrophoretically to nitrocellulose. The endocytosed, biotinylated receptor was then detected using horseradish peroxidase-conjugated avidin D (Vector Laboratories, Berlingname, CA, USA).

Metabolic labeling, pulse-chase experiments - CTR-FLAG-transfected cells were washed twice with PBS and preincubated in methionine-free labeling DMEM medium, containing 10% dialysed FBS (Gibco, Invitrogen) for 30 min. [35S]-Methionine (Amersham) was added to a final activity of 150 μCi/ml. Cells were incubated for 90 minutes, then washed twice with chase medium (DMEM with 10% FBS, 2 mM methionine), and incubated in chase medium for the indicated time periods. After the chase, cells were washed twice with ice-cold PBS and lysed in mRIPA buffer. The CTR was immunoprecipitated and electrophoresed on 10% SDS-PAGE gel. The gel was dried in a gel dryer at 80°C for 2 hours and exposed to Kodak MR X-ray film for at least 24 h. The X-ray film was scanned and the intensity of the corresponding bands was quantified using the NIH Scion Image 1.62c software, checking that the bands of interest were in the linear range of the X-ray film.

Fluorescence Loss in Photobleaching (FLIP) experiments - Cells were seeded in 35 mm glassbottom dishes (MatTek Corp., Ashland, USA) and 24 hours later transfected with the GFP-tagged CTR constructs as described above. To acquire the images, the dish was placed in a heated stage (DH-35, Warner Instruments, Hamden, USA) in a Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany) to maintain a temperature of 37°C. The culture medium was replaced by 37°C Na⁺-HEPES-buffer (135mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.3, 290 mOsmol/liter). All photobleaching experiments were carried out in the presence of 25 μM cycloheximide added 30 min before the start of the
recordings to rule out contribution from new protein synthesis. A 63 × 1.25 numerical aperture water immersion objective was used. GFP was excited at 488 nm by an argon laser. Emission was measured in the green channel from 505-530 nm. Photobleaching was obtained by scanning the region of interest (a hand-drawn box, the contours of which surrounded the area to bleach) at 75% power and 100% transmission. Imaging was done by scanning the whole cell with attenuated transmission (1%). The fluorescence intensity in membrane areas of the bleached cell distant from the bleached spot and as a control, the fluorescence intensity of the membrane of a neighboring, unbleached cell were measured every 6 seconds for 10 minutes after the bleaching. This procedure was repeated four times. Data were transferred to Excel and for each data point the fluorescence intensity was expressed as percentage of the initial mean fluorescence intensity before bleaching.

Statistical analysis – For analysis of the statistical significance of the observed differences, data from all independent experiments were pooled, mean values and standard errors of mean calculated and a student’s t-test performed. A significant difference was assumed when P was <0.05.
Results

The calcitonin receptor undergoes tonic endocytosis and recycling back to the cell surface

To study the ligand-independent and ligand-induced endocytosis of the CTR, we transfected HEK-293 cells with the CTR-FLAG construct and surface-labeled the cells with a cleavable biotin compound. Internalization of the receptor was allowed to proceed for different time periods, and the biotin remaining on the cell surface was stripped. The cells were lysed and the FLAG-tagged CTR were immunoprecipitated and blotted with avidin (Figure 1A) to detect the endocytosed CTR. We found that a substantial fraction of the CTR was internalized in the absence of ligand, whereas the presence of CT caused only a slightly higher amount of CTR internalization. To confirm this result with a more quantitative method, we performed a flow cytometry-based internalization assay. HEK 293 cells were transfected with C-terminally GFP-tagged, N-terminally HA-tagged CTR. After serum-starving for at least 12 h, cells were incubated at 4°C in the presence of an anti-HA antibody to label the extracellular HA-tag. After this binding step, cells were quickly warmed to 37°C and incubated in the presence or absence of 10 nM sCT for different time periods to allow endocytosis. The remaining anti-HA antibody bound to the CTR at the surface was then labeled with the PE-conjugated secondary antibody, and the cell-associated PE quantified by FACS. This method also showed that a substantial fraction of the CTR on the surface was internalized in the absence of ligand, although more receptor was internalized in the presence of the ligand, with the difference statistically significant at 30 and 60 minutes (Figure 1B). Co-expression of the dominant-negative dynamin I-K44A mutant, which blocks endocytosis via clathrin-coated pits (18), failed to reduce tonic or ligand-induced endocytosis of the CTR (data not shown).
To determine if the internalized CTR is recycled back to the cell surface, we allowed endocytosis of the CTR to proceed in the presence of the anti-HA antibody, then stripped surface-bound HA-antibody with an acid wash. The cells were then re-warmed for different time periods. While the amount of antibody reappearing on the surface was clearly less than what had been endocytosed, consistent with the assumption by other investigators that non-covalently bound antibodies would be lost as the internalized receptor passes through acidified internal compartments (14), we observed a clear increase in the amount of antibody on the cell surface after re-warming, indicating that at least some of the internalized CTR-antibody complex survived the passage through the endocytic vesicles and was recycled to the cell surface. The amount of antibody-decorated CTR on the cell surface reached a steady state by about 20 minutes. When CT was present during both internalization and recycling periods, significantly more of the CTR was recycled at 40 min (Figure 1C). These data, together with the result shown in Figure 1B, suggest that ligand binding induces both more internalization and more recycling of the CTR.

We then determined the intracellular localization of the tonically internalized CTR, using a CTR fused at the C-terminal to green fluorescent protein (GFP). Confocal microscopy showed substantial accumulation of the CTR-GFP within the cell (Figure 2A, left panel). The transferrin receptor, which is constitutively internalized and recycled back to the cell surface, colocalized with the CTR at the cell surface and in a vesicle-rich area in the center of the cell (data not shown). To determine if the CTR was recycling to the cell surface from this region, we used the “fluorescence loss in photobleaching” (FLIP) method in cells expressing the CTR-GFP by repeated photobleaching of the intracellular CTR-GFP accumulations. Figure 2 shows a typical experiment. The intracellular accumulation of CTR-GFP was photobleached in one cell while an adjacent unbleached cell served as a control. The fluorescence intensity at the surface of the
bleached cell began to decrease about 10 min after the initial bleaching and eventually decreased to about 50% of the initial level. The fluorescence of the neighboring unbleached cell was unchanged. This result strongly suggests that the constitutively endocytosed CTR is recycled back to the cell surface via the vesicle-rich area in the center of the cell.

*The C-terminal tail of the calcitonin receptor constitutively interacts with the actin binding protein filamin*

We suspected that an association of the C-terminal tail of the CTR with a cellular protein is necessary for targeting the CTR to the recycling compartment. In order to identify proteins that associate with the C-terminal tail of the CTR and might thereby promote the recycling of the receptor, we screened an osteoclast-like cell yeast two-hybrid library (17) with the CTR C-terminal tail (aa 398-474). Screening of $10^6$ independent clones yielded five positive clones, one of which encoded the C-terminal region of filamin A, analogous to aa 2129-2647 of human filamin A, which contains the 19th to 24th immunglobulin-like repeats and the second hinge region (19) (Figure 3A).

Truncation mutants were used to map the region that binds to the cytoplasmic tail of the CTR. The fragments spanning aa 2129-2458 or aa 2129-2330 were active in the yeast two-hybrid assay, suggesting that the primary interacting region is between aa 2129-2330, corresponding to the immunglobulin-like repeats 20 and 21. However, the activation was weaker than that of the original filamin clone, suggesting either that the smaller fragment folds differently or that sequences elsewhere in the original clone stabilized the binding to the CTR.

The location of the filamin binding site on the CTR C-terminal domain was further refined using the yeast two-hybrid system (Figure 3B). The strong activation in the reporter assays achieved by the complete C-terminal tail (aa 397-474) was seen only with a fragment spanning aa
422-474. The smaller fragment spanning aa 447-474 was also positive in both reporter assays, although less so than the full length tail. This region contains no sequences that correspond to known protein-binding domains such as PDZ, SH2, or SH3 domains. The CTR fragments more proximal to the seventh transmembrane domain (aa 397-447 and aa 397-422) also showed a consistent but much smaller activation in the reporter assays. The results suggest that aa 447-474 span the primary binding site for filamin, but that other more proximal regions could be also contributing to stabilizing the filamin-CTR association.

To show that the interaction of the CTR and filamin occurs in mammalian cells, we immunoprecipitated C-terminally FLAG-tagged CTR from HEK 293 cells and blotted for filamin (Figure 3C) or immunoprecipitated endogenous filamin and blotted for FLAG (data not shown). Both proteins were present in the immune complexes, regardless of the antibody used in the isolation, confirming an interaction of filamin and the CTR in mammalian cells. Treating the cells with sCT had no detectable effect on the amount of filamin that co-immunoprecipitated with the CTR (Figure 3C), indicating that the association between the CTR and filamin is not regulated by CTR-induced signaling effectors.

To further confirm the CTR-filamin interaction, we examined the effect of overexpressing xpress©-tagged filamin repeats 20 to 23, the FLAG-tagged CTR cytoplasmic tail, or the FLAG-tagged CTR cytoplasmic tail plus 7th transmembrane domain on the interaction between the full length CTR and filamin. Each of the fragments strongly reduced the co-immunoprecipitation of endogenous filamin with the myc-tagged CTR (Figure 3D).

The interaction of the calcitonin receptor with filamin reduces the degradation of the receptor

To investigate the functional relevance of the association between filamin and the CTR we used M2 cells, which express no filamin A, and A7 cells, which are M2 cells stably transfected
with filamin A (20). We transfected these cell lines with the CTR and analyzed CT-induced signal transduction by the CTR in the absence of filamin. We found no significant differences in the ligand-induced generation of cAMP, increase in intracellular calcium concentrations, or Erk phosphorylation in M2 and A7 cells (data not shown), suggesting that the association with filamin is not required for these signaling pathways of the CTR.

However, Western blots indicated that there was less CTR in the M2 cells than in the A7 cells (Figure 4A) and both confocal microscopy (Figure 4B) and FACS analysis (Figure 4C) indicated that there was less surface expression of the CTR in M2 cells than in the filamin-containing A7 cells. The lower expression of the CTR in the absence of filamin could result from lower de novo synthesis or from faster degradation of the CTR. To distinguish between these possibilities, we performed a pulse-chase analysis of CTR degradation in M2 and A7 cells (Figure 4D). The synthesis of the CTR was similar in both cell lines (data not shown), but the extent of degradation of the CTR in the M2 cells was about twice that in A7 cells (Figure 4D, left panel). Half of the labeled receptor in the M2 cells was degraded by 3 hours, while more than 60% of the labeled receptor was still present in the A7 cells at 6 hours (Figure 4D, left panel). To rule out the possibility that the accelerated degradation was not specific for the CTR, we expressed another GPCR, the \( \beta_2 \)-adrenergic receptor (\( \beta_2 \)-AR), in M2 and A7 cells and examined its degradation by pulse-chase analysis. In contrast to the results obtained with the CTR, we found essentially no difference in the degradation of the \( \beta_2 \)-AR in the two cell lines (Figure 4D, right panel).

We next asked whether disrupting the filamin/CTR association also increased the degradation of the CTR. Filamin repeats 20-23 were co-expressed with the CTR in A7 and M2 cells and the CTR degradation measured in a pulse-chase assay (Figure 4E). The co-expression of the filamin fragment significantly increased the amount of degradation of the CTR in A7 cells. In contrast
and as expected, the expression of this fragment had little effect on the degradation of the CTR in M2 cells.

We investigated the pathways involved in the degradation of the CTR by treating pulse-labeled M2 and A7 cells with chloroquine or NH₄Cl, both of which inhibit lysosomal degradation, or with MG132, a proteasome inhibitor. MG132 significantly inhibited the degradation of the receptor in M2 cells. Chloroquine and NH₄Cl also slightly inhibited the degradation of the CTR in both cell lines (Figure 4E), although the results failed to reach statistical significance, suggesting that the lysosomal pathway might also be involved in the degradation of the CTR in these cells.

**Filamin is required for efficient recycling of the calcitonin receptor**

The increased degradation of the CTR in the absence of its association with filamin could result from an increased rate of tonic endocytosis of the CTR. Flow cytometric analysis (Figure 5A) clearly showed similar ligand-independent internalization in both M2 and A7 cells. We next examined the CTR recycling in the M2 and A7 cells and found significantly less of the CTR recycled to the surface in M2 cells than in A7 cells (Figure 5B).

We next did a FLIP analysis of CTR-GFP trafficking in M2 and A7 cells to examine the recycling of the CTR from the perinuclear vesicle compartment in the absence of filamin. In A7 cells, the fluorescence intensity of the cell membrane decreased after photobleaching of the intracellular CTR-GFP-containing endosomes (Figure 6, upper panel), suggesting that the CTR recycles back to the cell surface from the photobleached endosomes in A7 cells as it does in the HEK293 cells. In contrast, photobleaching the intensely fluorescent vesicles in M2 cells caused a much smaller decrease in the fluorescence intensity of the cell membrane (Figure 6, lower panel), indicating that fewer of the CTR in the photobleached endosomes recycle to the surface in the
absence of filamin. The more rapid degradation of the CTR in the absence of filamin may therefore be related due to a reduced efficiency of recycling from the perinuclear vesicles.

**Calcitonin affects the degradation of its receptor by inhibiting filamin proteolysis**

Our initial results (Figure 1C) suggested that CT promoted the recycling of the internalized CTR to the cell surface. We considered the possibility that CTR signaling specifically promotes the filamin-dependent recycling of the internalized CTR, thereby protecting it from degradation. We therefore determined how CT treatment affected the degradation of pulse-labeled CTRs in A7 and M2 cells. Exposing the cells to 10 nM CT resulted in about 30% decrease in the amount of degradation in the filamin-containing A7 cells, but had no significant effect on the degradation in the filamin-free M2 cells (Figure 7A).

Cleavage of filamin between the actin binding site and the CTR-binding site by calpain would result in uncoupling of the CTR from the actin cytoskeleton (21). Given that Marzia et al. have demonstrated that CT inhibited calpain activity and induced a transient decrease in the constitutive production of a 190 kDa fragment in osteoclasts (22), we determined whether CT affected filamin fragmentation in HEK 293 cells in a manner similar to what has been observed in osteoclasts. In untreated HEK 293 cells, as in osteoclasts, the 190 kDa proteolytic fragment was clearly detected. Brief exposure to CT (5 min) caused the amount of the fragment to decrease (about 55% of baseline level), suggesting that CT is inhibiting calpain activity. The amount of the fragment slowly increased, approaching basal levels between 45 and 60 min (Figure 7B).

The calpain inhibitor calpeptin also reduced the rate of degradation of the pulse-labeled CTR in the A7 cells, but not in M2 cells (Figure 7A). Together these results suggest that constitutive calpain-catalyzed cleavage of filamin occurs at a low rate, and that inhibiting that cleavage reduces CTR degradation in a filamin-dependent manner.
Discussion

While numerous studies have provided detailed information on the molecular mechanisms of endocytosis of GPCRs (23), much less is known about the endosomal sorting and recycling of these receptors. The mechanisms involved in tonic receptor internalization and recycling, which is observed for a growing number of GPCRs (7-14), are of particular interest because efficient sorting and recycling of the internalized receptor is required in order to avoid extensive degradation of the receptor and to maintain appropriate numbers of receptors at the cell surface. The sorting of tonically endocytosed receptors also provides a mechanism by which cells regulate the level of surface expression of these receptors.

In this paper we show that the CTR is constitutively internalized and recycles to the cell surface. Our results offer insight into several aspects of CTR internalization and recycling. CTR recycling is decreased and its degradation is increased when the CTR cannot bind filamin. In filamin-containing cells, the CTR appears to be recycled through a population of perinuclear vesicles that also stain for the transferrin receptor.

Several different experiments suggest that the transit time for internalized receptors to return to the surface is about 10 min. Both the loss of antibody-decorated CTR from the cell surface and the reappearance of antibody-decorated CTR after acid wash approach a steady state at this time, and the decrease in surface membrane fluorescence in the FLIP experiment, which reflects the insertion of bleached receptors at the surface, begins 8 to 10 min after the initial photobleaching.

In addition, the steady state levels of antibody-decorated CTR in the internalization experiments (40-60% of initial levels, Figure 1B) suggest that on average, about half the receptors that are actively recycling are present on the surface at any one time. The steady state level of antibody-decorated CTR that reappears on the cell surface following the acid strip is necessarily lower, since only a fraction of the initially labeled receptors are internalized and thus
protected from the stripping, and they will become progressively diluted with undecorated receptors as recycling and internalization progresses. Moreover, some of the antibodies may dissociate from the receptors during the internalization and recycling.

The role of filamin in CTR recycling appears to involve the regulation of the transition from the intracellular compartment to the surface. The rates of internalization in the M2 and A7 cells, reflected in the initial slope of the internalization curves (Figure 5A) are indistinguishable, while the return of the CTR to the surface in the filamin-deficient M2 cells is less than half the normal level in both the antibody-tagging and the FLIP experiments. The amount of CTR degradation also changes by about 2-fold in the absence of filamin, about doubling in the M2 cells. The increased degradation could be a consequence of the presumed accumulation of the receptor in an intracellular compartment as a consequence of the impaired recycling. However, we cannot exclude that the accelerated degradation and decrease in recycling via the perinuclear compartment is due to an increased active sorting of the CTR to degradative compartments when it is not bound to filamin. Our data do not allow us to distinguish between these possibilities.

Other proteins that regulate GPCR recycling and degradation have been described. Cong et al. found that the N-ethylmaleimide sensitive factor (NSF) binds to the C-terminal tail of the β2-AR and showed that binding of this protein, known to be involved in membrane fusion processes, is required for recycling of the β2-AR (24). Whistler et al. (25) recently described a protein (GPCR-associated sorting protein, GASP) that targets several GPCR, including the β2-AR and δ opioid receptor, to lysosomal degradation. The same group (26) has identified another protein, EBP-50, that links the β2-AR to F-actin. This linkage, which is required for recycling of the β2-AR to the cell surface, may be regulated by ligand-induced phosphorylation of the β2-AR.

Although we did not find a CT-dependent change in the CTR-filamin interaction, we did observe a CT-induced decrease in CTR degradation. The CT-induced increase in the amount of
antibody-tagged CTR that recycled to the surface following an acid strip might be a consequence of less degradation. On the other hand, the steady-state level of antibody-tagged CTR was somewhat lower in CT-treated cells, possibly reflecting an overall net retention of CTR in intracellular compartments under this condition. Further experiments will be necessary to answer these questions.

We also observed a transient CT-induced decrease in the cleavage of filamin. Cleavage of filamin by calpain separates the CTR-binding site from the N-terminal actin binding domain of filamin, thereby disrupting the connection between the internalized CTR and actin. Inhibition of calpain-induced filamin cleavage could thus increase CTR recycling and consequently inhibit the degradation of the receptor, as illustrated in Figure 8. The inhibition of CTR degradation by calpeptin provides further support for this hypothesis. The inhibition of filamin proteolysis by CT might involve an inhibition of m-calpain due to phosphorylation by PKA (27) or phosphorylation of filamin itself (28).

The dopamine D2 and D3 receptors (29,30), and the calcium sensing receptor (31,32) have also been reported to interact with filamin. Interestingly, the functional consequences of the association seem to be different for the individual GPCRs. For the Gi-coupled D2 receptor, there was less inhibition of adenylyl cyclase reported in the absence of filamin (29), possibly because the receptor was inefficiently expressed on the cell surface of filamin-free cells (30). In the case of the calcium sensing receptor, the absence of filamin or the disruption of the interaction with the receptor abolishes Erk phosphorylation. We failed to find a difference in the CTR-induced Erk phosphorylation in M2 and A7 cells. Differences in the MAPK-activation pathways for the two receptors might underlie these different responses.

In conclusion, the CTR is tonically endocytosed and recycled to the cell surface via a mechanism that depends in part on an interaction between the CTR and filamin. Since receptor
trafficking is shown to be an important regulator of GPCR function (23), it is likely that the mechanism shown in this study, with regulation of CTR degradation and recycling via the association with intact filamin, contributes significantly to the specific biological response induced by the CTR and possibly also other GPCRs.
References


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Figure legends

Figure 1. The calcitonin receptor undergoes tonic endocytosis. (A) CTR-FLAG-transfected HEK 293 cells were labeled with the membrane impermeable sulfo-NHS-S-S-biotin. Cells were then transferred to medium prewarmed to 37°C with or without 10 nM sCT and incubated for 5 to 60 minutes to allow endocytosis of the receptor. The biotin attached to proteins still remaining on the cell surface was cleaved by washing cells twice at 4°C with a glutathione strip solution. The FLAG-tagged CTR receptor was immunoprecipitated and biotinylated receptors were detected using horseradish peroxidase-conjugated avidin D (top). The membrane was then stripped and reprobed with anti-FLAG antibody (bottom). Identical results were obtained from three independent experiments. (B) Serum-starved HEK 293 cells transfected with the CTR-GFP construct were incubated with a monoclonal anti-HA antibody at 4°C, to label the extracellular HA-tag in the receptor construct. After washing, cells were incubated with (■) or without (○) 10 nM sCT. Cells were then quickly warmed to 37°C allow endocytosis of the receptor for different time periods. Measurement of the HA-antibody bound to the cell surface was performed as described under “Experimental Procedures”. The data represent the means of four independent experiments, the error bars represent the standard error of mean of the different experiments. (*, P<0.05). (C) Serum-starved HEK293 cells transfected with the CTR-GFP construct were incubated with anti-HA antibody to internalize antibody-decorated CTR as described in “Experimental Procedures”. After the endocytosis of the antibody-labeled CTR, the cell were cooled to 4°C, the antibody bound to the CTR at the cell surface was removed by an acid strip, and the cells were then warmed to 37°C for different time periods to allow recycling of the CTR incubated with (■) or without (○) 10 nM sCT, then processed for FACS analysis as described in “Experimental Procedures”. The fluorescence intensity at each time-point was normalized to the fluorescence prior to the acid strip. The data represent the means of five independent
experiments, the error bars represent the standard error of mean of the different experiments. (*, P<0.05).

Figure 2. The constitutively endocytosed calcitonin receptor accumulates intracellularly and recycles back to the cell surface. (A) HEK 293 cells transfected with the GFP-tagged CTR construct were placed in a heated stage in a confocal microscope to maintain the temperature at 37°C. The accumulation of CTR-containing vesicles was photobleached as described in “Experimental Procedures”. The fluorescence intensity of the bleached area, the surface membrane of the bleached cell distant from the bleached area and, as a control, the fluorescence intensity of the membrane of a neighboring unbleached cell were measured every 6 seconds for 8 minutes after the bleaching. This procedure was repeated four times. The micrographs show the same cells at the beginning (left panel) and end (right panel) of the experiment. (B) The time course of the change in fluorescence intensity in the different regions. The results are typical of five independent experiments.

Figure 3. Filamin interacts with the C-terminal tail of the CTR. (A) Schematic diagram of the fragments of filamin encoded by the cDNA initially cloned by the yeast two-hybrid screening and truncations of this fragment used to map the filamin binding site for the C-terminal tail of the CTR. ABD, actin binding domain; H1 and H2, hinge regions 1 and 2. The numbers within boxes represent the number of the immunoglobulin-like repeats of human filamin (19), the numbers at the beginning and end of the lines refer to aa positions in the human full-length filamin sequence (33). Both CTR and filamin constructs were co-transformed in yeast strain L40 and assayed for growth on medium lacking histidine and for β-galactosidase activity. ++ indicates a strong interaction based on the assay parameters. + represents also growing on selective medium and
activation in the β–galactosidase assay, but less activity compared to ++. (+) represents a very weak activation of the β–galactosidase activity. – indicates failure of growth and the absence of a β–galactosidase activity. (B) Schematic diagram of the fragments of the CTR that interacted with filamin 2129-2642 in the two-hybrid screening. The numbers refer to residues in the rabbit CTR (34). (C) HEK 293 cells were transfected with the FLAG-tagged CTR construct or with empty vector. Cells were stimulated with 10 nM sCT for the indicated time periods, lysed and the CTR immunoprecipitated. The immune complexes were immunoblottedted with the anti-filamin antibody (upper panel). The membrane was stripped and re-blotted with the M2 anti-FLAG antibody (middle panel). Total cell lysate (TCL) were immunoblottedted with the anti-filamin antibody (lower panel). Identical results were obtained from four independent experiments. (D) HEK 293 cells were co-transfected with a C-terminally myc-tagged CTR construct in combination with a vector encoding the immunoglobulin-like repeats 20-23 of filamin with a xpress® tag (lane 3), a vector encoding the 7th transmembrane domain and the C-terminal tail of the CTR with a FLAG-tag (lane 4), or a vector encoding the C-terminal tail of the CTR with a FLAG-tag (lane 5). The cells were lysed and the CTR immunoprecipitatedated with the anti-myc antibody. The immune complexes were immunoblottedted with the anti-filamin antibody (upper panel), the blot was stripped and re-blotted for myc to check the immunoprecipitation (second panel). The lysates used for the immunoprecipitations were blotted for filamin (third panel) and with the anti-xpress® antibody (lane 3) and M2 anti-FLAG antibody (fourth panel). Identical results were obtained from three independent experiments.

Figure 4. **Expression and degradation of the calcitonin receptor is dependent on the interaction with filamin.** (A) M2 and A7 cells were transfected with the CTR-FLAG construct, cells were lysed and proteins were separated by SDS-PAGE and transferred to nitrocellulose. The
membrane was immunoblotted with the anti-FLAG antibody (upper panel), then stripped and reblootted for actin (lower panel). Identical results were obtained from three independent experiments. (B) A7 (upper panel) and M2 (lower panel) cells were seeded on glass cover slips and transfected with the GFP-tagged CTR construct. 36-48 h later, cells were analyzed by confocal microscopy. The bars represent 10 µm. (C) For quantification of the CTR cell surface expression, M2 cells (right panel) and A7 cells (left panel) were transfected with the GFP-tagged CTR construct and the surface expression was measured by FACS using the antibody against the extracellular HA tag at the N-terminal of the CTR construct as described in “Experimental Procedures”. The mean fluorescence of PE, reflecting the amount of the HA antibody binding, was used as a measure of the receptor cell surface expression. The mean values of four independent samples in M2 and A7 cells are shown (*, P<0.05). (D) A7 (■) and M2 (○) cells were labeled with $[^{35}\text{S}]$-Methionine. The cells were lysed and the CTR immunoprecipitated and subjected to SDS-PAGE. The dried gel was autoradiographed and the labeled CTR quantified by densitometry (left panel). The slopes of the linear regressions in M2 and A7 cells were significantly different (P<0.05). The degradation of the β2-AR was analyzed in a similar manner (right panel). The data represent the means of three independent experiments, the error bars represent the standard error of mean of the different experiments. (E) Pulse-chase analysis was performed as described with proteolysis inhibitors (40 µM MG132, 150 µM chloroquine, 10 mM NH₄Cl) present during a three hour chase period. In addition, the filamin 20-23 fragment, which inhibits the binding of the CTR to filamin, was co-expressed and its effect on CTR degradation examined in the same way. The data represent the means of five independent experiments, the error bars represent the standard error of mean of the different experiments. All data in the different experiments were normalized to the amount of degraded CTR in the untreated control in
the same cell line. In untreated A7 cells, 32% ± 9% was degraded compared to 55% ± 12% in untreated M2 cells. (*, P<0.05).

Figure 5. **Tonic internalization of the calcitonin receptor is unchanged in the presence or absence of filamin, but recycling in the absence of filamin is altered.** (A) Internalization of GFP-tagged CTR in unstimulated A7 (■) or M2 (○) cells was measured by FACS as described in “Experimental Procedures”. The data represent the means of five independent experiments, the error bars represent the standard error of mean of the different experiments. (B) A7 and M2 cells were incubated for 30 min at 37°C in the presence of the anti-HA antibody to allow endocytosis of the CTR with the bound antibody. After cooling to 4°C, surface-bound antibody was removed with an acid wash as described in “Experimental Procedures”. Cells were then warmed to 37°C for different time periods to allow recycling of the internalized CTR to the cell surface. PE-staining and FACS analysis was done as described in “Experimental Procedures”. The fluorescence intensity at each time-point was normalized to the starting fluorescence prior to the acid wash. The data represent the means of five independent experiments, the error bars represent the standard error of the mean of the different experiments. (*, P<0.05).

Figure 6. **Recycling of the calcitonin receptor to the cell surface is markedly reduced in the absence of filamin.** A7 cells (upper panel) or M2 cells (lower panel) were transfected with the GFP-tagged CTR construct. The FLIP experiment was done as described in “Experimental Procedures”. The panels show the time course of the fluorescence intensity in the regions of the surface membrane and the intracellular bleached area. The fluorescence intensities are normalized to the fluorescence intensity at the beginning of the experiment. The figure shows the
result of a typical experiment out of five separate experiments. Identical results were obtained from five independent experiments.

Figure 7. **CT inhibits CTR degradation and filamin proteolysis.** (A) To test the effect of CT on CTR degradation, A7 and M2 cells transfected with the CTR-FLAG were pulse-labeled with $[^{35}S]$-methionine as described in “Experimental Procedures” and the amount of labeled CTR determined 90 min after the pulse labeling as described for Figure 4. The data represent the means of three independent experiments, the error bars represent the standard error of the mean of the different experiments. A t-test was used to compare the undegraded fraction in the treated cells with control cells (* P<0.05). (B) HEK 293 cells transfected with the CTR-FLAG construct were treated with 10 nM sCT for 5 and 45 min. Cell lysates were prepared for immunoblotting as described in “Experimental Procedures”. The membrane was immunoblotted with the anti-filamin antibody (lower panel). The 190 kD filamin fragment was quantified by gel densitometry. The numbers below the panel represent the percentage of the 190 kD filamin fragment compared to the untreated control. Identical results were obtained from three independent experiments. The mean values for the densitometry for all three experiments were 59% after 5 min and 83% after 45 min.

Figure 8. **Model for the role of filamin in regulating the cellular trafficking of the calcitonin receptor.** The CTR undergoes constitutive endocytosis and is targeted to recycling endosomes. The association of the C-terminal tail of the receptor to filamin is necessary for efficient recycling of the CTR to the cell surface.
A

![Bar graph showing the undegraded fraction of A7 and M2 cells treated with control, sCT, and calpeptin.](image)

B

- **sCT (min)**: 0, 5, 45
- **IB: anti-filamin**
  - 100%, 55%, 87%