Large Putative PEST-like Sequence Motif at the Carboxyl Tail of Human Calcium Receptor Directs Lysosomal Degradation and Regulates Cell Surface Receptor Level*

Received for publication, June 13, 2011, and in revised form, November 21, 2011 Published, JBC Papers in Press, December 12, 2011, DOI 10.1074/jbc.M111.271528

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Background: Regulation of cell surface expression and signaling activity of the calcium-sensing receptor plays an important role in human diseases.

Results: A PEST-like degradation motif participates in routing of the receptor to the lysosomal pathway.

Conclusion: This degradation motif regulates cell surface receptor abundance.

Significance: This might be a novel mechanism that connects lysosomal degradation and regulation of cell surface receptor expression.

A deletion between amino acid residues Ser895 and Val1075 in the carboxyl terminus of the human calcium receptor (hCaR), which causes autosomal dominant hypocalcemia, showed enhanced signaling activity and increased cell surface expression in HEK293 cells (Lienhardt, A., Garabédian, M. G., Bai, M., Sinding, C., Zhang, Z., Lagarde, J. P., Boulesteix, J., Rigaud, M., Brown, E. M., and Kottler, M. L. (2000) J. Clin. Endocrinol. Metab. 85, 1695–1702). To identify the underlying mechanism(s) for these increases, we investigated the effects of carboxyl tail truncation and deletion in hCaR mutants using a combination of biochemical and cell imaging approaches to define motifs that participate in regulating cell surface numbers of this G protein-coupled receptor. Our data indicate a rapid constitutive receptor internalization of the cell surface hCaR, accumulating in early (Rab7 positive) and late endosomal (LAMP1 positive) sorting compartments, before targeting to lysosomes for degradation. Recycling of hCaR back to the cell surface was also evident. Truncation and deletion mapping defined a 51-amino acid sequence between residues 920 and 970 that is required for targeting to lysosomes and degradation but not for internalization or recycling of the receptor. No singular sequence motif was identified, instead the required sequence elements seem to distribute throughout this entire interval. This interval includes a high proportion of acidic and hydroxylated amino acid residues, suggesting a similarity to PEST-like degradation motifs (PESTfind score of +10) and several glutamine repeats. The results define a novel large PEST-like sequence that participates in the sorting of internalized hCaR routed to the lysosomal/deg-

radation pathway that regulates cell surface receptor numbers.

The extracellular cation-sensing calcium receptor belongs to the family C/3 G protein-coupled receptor (GPCR)4 gene family consisting of eight metabotropic glutamate (mGluR1–8) receptors, two heterodimeric γ-aminobutyric acid (GABAγ) receptors, the calcium-sensing receptor (CaR), three taste (T1R1–3) receptors, and a promiscuous L-α-amino acid receptor (GPRC6A). This family of GPCRs is characterized by large extracellular amino-terminal domains that bind the endogenous orthosteric agonists (1). The hCaR plays an essential role in maintaining systemic calcium homeostasis and is continuously exposed to extracellular calcium in serum yet remains highly sensitive to small changes in extracellular calcium necessary for the normal physiological regulation of parathyroid hormone secretion (2). The hCaR primarily regulates extracellular systemic calcium by controlling the rate of parathyroid hormone secretion but also regulates the rate of calcium re-absorption by the kidney. In a host of other tissues, including skin, brain, intestine, and breast, the receptor also regulates cellular processes such as secretion, differentiation, and gene expression (2–3). Human calcium homeostasis disorders are primarily associated with inactivating mutations of the hCaR for autosomal dominant loss-of-function disorders like familial hypocalciuric hypercalcaemia and neonatal severe hyperparathyroidism; in contrast, activating mutations are responsible for the gain-of-function disorder autosomal dominant hypocalcemia. On the structural level, functional hCaR requires homodimerization and the cell surface expression is controlled by multiple checkpoints along the secretory pathway to ensure only properly folded and post-translationally modified receptor access the plasma membrane (4–6).

* This work was supported, in whole or in part, by the National Institutes of Health Intramural Research Program of the NIDCD, National Institutes of Health. Present address: Molecular Virology Section, Bldg. 5, Rm. 306, 9000 Rockville Pike, NIDDK, Bethesda, MD 20892.

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3 The abbreviations used are: GPCR, G protein-coupled receptor; hCaR, human Ca2+-sensing or calcium receptor; PI, phosphoinositide hydrolysis; GRK, G protein-coupled receptor kinase; CaM, calmodulin; Endo H, endoglycosidase H; CaR, calcium-sensing receptor.
There is limited information regarding the mechanisms of agonist-induced desensitization and internalization of cell surface-expressed functional hCaR. A common feature among the GPCR signaling system is desensitization, i.e. a loss of responsiveness of a receptor when continuously exposed to the agonist. G protein-coupled receptor kinases (GRKs) and arrestins are important regulators of GPCR desensitization (7). Upon agonist treatment, many GPCRs are rapidly phosphorylated by a GRK, resulting in binding of arrestin, which uncouples the receptor from G proteins and initiates GPCR endocytosis. Once internalized, some GPCRs are dephosphorylated and subsequently recycled back to the cell surface where they can again respond to agonists. The processes of hCaR desensitization and internalization are currently poorly understood. The hCaR is phosphorylated by protein kinase C (PKC) as well as GRK2 and GRK4 and has been shown to bind β-arrestin (8). Interestingly, hCaR undergoes only a minor agonist-dependent internalization and β-arrestin binding seems to be PKC-dependent not GRK-dependent (9). Interestingly, hCaR shows constitutive endocytosis and recycling to the cell surface by a Rab11a-dependent mechanism (10), suggesting that this constitutive receptor internalization requires different endocytic machinery than that commonly found for other GPCRs. This hCaR endocytosis is also essential for the transactivation of epidermal growth factor receptor that leads to the MAP kinase signaling cascade and links receptor signaling to parathyroid hormone-related peptide secretion via a Rab11a-dependent and associated molecule with the SH3 domain of STAM (AMSH)-sensitive mechanism (10, 11). These data suggest internalization and down-regulation might be important regulatory mechanisms for rapid and efficient control of hCaR cell surface expression and for its signaling activities.

The principal mechanism underlying down-regulation of GPCR degradation is a multistep process often involving endocytosis and subsequent delivery of the receptor to lysosomes for degradation (12). Little is known about the molecular mechanisms involved in sorting GPCRs to lysosomes. Once internalized, receptors are often targeted to specialized endosomal compartments, dephosphorylated, and recycled back to the cell surface or targeted to lysosomes for degradation (12, 13). In addition to the lysosomes, intracellular degradation of receptor proteins is also accompanied by proteosomal degradation. The hCaR and other GPCRs including human opioid receptor subtypes, rhodopsin, and follicle-stimulating hormone receptor have been shown to bind ubiquitin and undergo ubiquitin-targeted proteosomal degradation (12, 14). The cytoskeletal actin-binding protein filamin A facilitates the hCaR-mediated MAP kinase signaling pathway and increases the total cellular hCaR level by preventing proteosomal degradation (15). Also, hCaR ubiquitination and degradation are linked to the activity of E3 ubiquitin ligase, also known as dorfin (14). However, it is unclear whether ubiquitination and proteosomal degradation have a direct role in hCaR internalization, or whether they serve as a quality control during the synthesis of the receptor in the endoplasmic reticulum.

A majority of the 215 carboxyl-terminal residues (Lys863 to Ser1077) of the hCaR can be truncated without perturbing the G-protein signaling response in heterologous cell expression systems (16–18). Disease causing mutations in the hCaR carboxyl tail are relatively rare (19). Nevertheless, the proximal carboxyl tail possesses multiple important determinants that regulate functional response of the receptor. PKC-mediated phosphorylation of Thr888 inhibits extracellular calcium-induced release of intracellular Ca2+ stores. High extracellular Ca2+ also induces stoichiometric binding of calmodulin (CaM) to the carboxyl tail of hCaR (residues 871–898) that may interfere with PKC phosphorylation of Thr888 and thus stabilize cell surface expression by reduced internalization of the receptor (17, 21). Truncations at the C terminus can cause either gain-of-function or loss-of-function of the hCaR. A large in-frame naturally occurring deletion in the hCaR carboxyl tail, Ser989–Val1077, leads to increased cell surface expression and gain-of-function in subjects with autosomal dominant hypocalcemia, causing a left-shift in the plasma Ca2+ set point and leading to hypocalcaemia and hypercalcioria (22). Mutations of a proximal putative endoplasmic reticulum retention signal region (Arg980 through Arg988) increased cell surface expression of the receptor (23). A truncation of hCaR, at Ala977 likewise exhibited an increased level of cell surface expression (16, 17). The truncations at residues 874 and 876 of hCaR and point mutations at Arg886 lead to familial hypocalciuric hypercalcemia/neonatal severe hyperparathyroidism by modulating cell surface receptor expression and also inhibiting responsiveness to extracellular calcium (16–17, 24–27). Three common hCaR polymorphisms (A986S, R990G, and Q1011E) are found in the carboxyl tail of the hCaR associate with altered serum calcium levels (28). Given these observations, it is apparent that the hCaR carboxyl tail contains multiple determinants for regulating the cell surface level, desensitization, internalization, and down-regulation of the receptor. We report here a cellular sorting signal encoded by 51 amino acid residues in the hCaR carboxyl tail containing a PEST-like motif and several polyglutamate (QQQ) repeats that specify an endosomal-lysosomal degradation pathway required for down-regulation of the hCaR protein contributing importantly to the regulation of cell surface expression level of the receptor.

EXPERIMENTAL PROCEDURES

Plasmid Constructs and Antibodies—The wild type (WT) hCaR in pCR3.1 and the G903X receptor carboxyl tail truncation mutant (previously named T903) modified to contain a rhodopsin epitope tag (Met-Asn-Gly-Thr-Glu-Gly-Pro-Asn-Phe-Tyr-Val-Pro-Phe-Ser-Asn-Lys-Thr-Gly-Val) at the amino terminus after the signal peptide have been previously described (29). All other carboxyl tail-truncated mutants containing the N-terminal rhodopsin tag (S895X, I914X, W930X, R961X, S970X, I900X, I9040X), deletion mutants 920–927Δ, 920–937Δ, 920–960Δ, 920–970Δ, CaMβD3E3/920–970Δ (mutation at positions F881E, T888E, and V894E, shown in Fig. 1A), XRPM/920–970Δ (mutation at positions R896A, K897A, and R898A, shown in Fig. 1A), PKCμ/920–970Δ (mutation at positions T888A, S895A, and S915A, shown in Fig. 1A), 3K/A (mutation at positions K931A, K963A, K965A in full-length receptor, shown in Fig. 3A), and multi-Q/A (all Gln at positions 926, 930, 932–934, 940, 941, 943–946, 952–954, 957–959, and 964 mutated to Ala in full-length receptor, shown
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in Fig. 3A) were constructed using the QuikChange mutagenesis kit from Stratagene Inc. as described previously (16). All mutant constructs were verified by DNA sequencing using a Taq DyeDeoxy terminator cycle sequencing kit and ABI Prism 377 DNA sequencer. Rab7–RFP and LAMP1–RFP plasmid constructs were obtained from OriGene Technologies Inc. The anti-hCaR-specific monoclonal antibody ADD, and α-actin monoclonal antibody were purchased from Affinity Biosciences and Sigma. A monoclonal antibody (B6–30N) raised against the bovine rhodopsin amino-terminal sequence has been described previously (29) and was kindly provided by Dr. Paul Hargrave, University of Florida.

Cell Culture and Transient Transfection—Human embryonic kidney 293 (HEK293) and HeLa cells were cultured using Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. For transient expressions, plasmid DNA constructs were introduced either in HEK293 or HeLa cells by Lipofectamine transfection (Invitrogen). For Western blots and immunocytochemistry experiments, 6-well plates containing 90% confluent HEK293 or HeLa cells in each well were transfected with a constant amount of plasmid DNA (2 μg). 1 μg of each plasmid DNA was added in cotransfection experiments. For phosphoinositide (PI) hydrolysis experiments, cells were transfected in 80-cm² flasks with DNA adjusted to a total 8 μg and an equal number of cells were re-plated on 12-well plates for assays performed 48 h after transfection. Membrane protein extraction for Western blot and immunocytochemical studies were performed 72 h after transfection.

Immunoblot Analysis with Detergent-solubilized Cell Extracts—Confluent cells in 6-well plates were rinsed with ice-cold PBS and scrapped on ice in solution B containing 20 mM Tris-HCl (pH 6.8), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 1% Triton X-100 with freshly added protease inhibitor mixture (Complete protease inhibitor mixture, Roche Diagnostics) and 10 mM iodoacetamide. Protein samples were loaded and separated on either 6 or 4–12% gradient gels by SDS-PAGE. The proteins from the gel were electrotransferred to nitrocellulose membranes and incubated with primary antibodies after treating with 5% milk as blocking solution. Subsequently, the membranes were incubated with a secondary goat anti-mouse or goat anti-rabbit antibody conjugated to horseradish peroxidase (Kirkegaard and Perry Laboratories). The immunoreactive protein bands were detected using the enhanced chemiluminescence system containing substrate for horseradish peroxidase (Pierce Laboratories). For cleavage with endoglycosidase H (Endo H) (Roche Diagnostics), cell extracts were incubated with 0.5 million units of the enzyme for 1.5 h at room temperature.

Cell Surface Biotinylation—Pierce cell surface protein isolation kit (Thermo Scientific) was used for detecting cell surface-biotinylated hCaR forms. Transiently transfected HeLa cells with WT hCaR and mutant receptors were treated for 2 days with Sulfo-NHS-SS-Biotin for 20 min at 4 °C after transfection. Unreacted biotin was quenched with quenching solution and washed with PBS. Cell lysis and extraction of cell lysates was performed as described in the kit and labeled proteins were isolated using NeutrAvidin beads. Protein loading sample buffer containing DTT was used for protein elution from the avidin column and analyzed by Western blot. Anti-hCaR monoclonal antibody ADD was used to detect cell surface biotin-labeled hCaR protein bands.

Immunofluorescence-based Internalization and Recycling Assays—HeLa cells were plated on coverslips coated with polylsine. They were transfected alone with N-terminal rhodopsin epitope-tagged WT and 920–970Δ receptors or cotransfected with plasmids containing Rab5–RFP or LAMP1–RFP endosomal markers obtained from OriGene Technologies for intracellular colocalization experiments. 72 h after transfection, transfected live cells were incubated with monoclonal antibody B6–30N in Solution A (16) containing 10% normal goat serum for 60 min at 4 °C. For visualizing cell surface receptors, after washing with PBS, nonpermeabilized cells were fixed with 4% paraformaldehyde and stained with Alexa Fluor 488 (green) goat anti-mouse secondary antibody (Invitrogen).

To follow receptor internalization, after washing the primary antibody-labeled live cells with ice-cold complete DMEM, the cells were incubated for 15 to 240 min at 37 °C in a incubator with 5% CO₂. Thereafter, surface-bound antibody was removed by acid wash (25 mM glycine in PBS buffer, pH 2.0) for 15 min on ice, followed by fixation with 4% paraformaldehyde, permeabilized for 5 min with 0.5% Triton X-100 in PBS, followed by staining of internalized receptors with Alexa Fluor 488 (green) goat anti-mouse secondary antibody (1:500 dilution) for 60 min at room temperature. After washing, the cells were embedded in Prolong Gold antifade fluorescence mounting medium with or without DAPI (Invitrogen) and analyzed by confocal laser-scanning microscopy (LSM 510, Zeiss).

For recycling experiments, live HeLa cells expressing cell surface receptors were labeled with antibody B6-30N for 90 min at 37 °C, a condition that permits endocytosis of receptors during the antibody labeling period. Thereafter, surface-bound antibody was removed with two acid washes (25 mM glycine in PBS buffer, pH 2.0) for 15 min on ice. Cells were further incubated at 37 °C for 30 or 60 min for cell surface recycling of internalized receptors. Recycled receptors were stained with a green fluorescent secondary antibody at 4 °C, followed by fixation and permeabilization as described. Internalized receptors were visualized by staining with a red fluorescent secondary antibody for 60 min at room temperature. For co-localization experiments with internalized receptors, the live cell surface-labeled receptors labeled with antibody B6-30N at 4 °C were transferred to the incubator and allowed to internalize for 120 min at 37 °C. Surface receptor-bound antibodies were stripped off by acid washes and followed by fixation with 4% paraformaldehyde. Cells permeabilized with Triton X-100 were further labeled with green secondary antibody. Immunofluorescence was captured using a ×40 objective on a Zeiss LSM 710 confocal laser scanning microscope.

PI Hydrolysis Assay—PI hydrolysis was measured as previously described (16, 29). Briefly, HEK293 cells transfected with test constructs were split into 24-well plates replenished in medium containing 3.0 μCi/ml of myo-[³H]inositol (New England Nuclear) in complete DMEM and cultured overnight. After removal of the medium, attached cells were first washed with Ca²⁺-free phosphate-buffered saline, followed by a
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10-min incubation and wash with PI solution (120 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.1 mM MgCl$_2$, 20 mM LiCl in 25 mM PIPES buffer, pH 7.2) containing no Ca$^{2+}$. After removal of this calcium-free PI solution, cells were treated for 30 min with 0.5–10 mM Ca$^{2+}$ in PI solution. The reactions were terminated by addition of 1 ml of HCl-methanol (1:1000, v/v) per well. Inositol phosphates were separated from phosphoinositides and myoinositol by chromatography on Dowex 1-X8 columns and samples were counted in a Wallace 1219 liquid scintillation spectrometer.

RESULTS

Carboxyl Tail Truncation at Position 895 Leads to Increase in Cell Surface hCaR Expression—An in-frame deletion of 181 amino acids within the carboxyl tail between Ser$^{895}$ and Val$^{1075}$ of hCaR is associated with familial autosomal dominant hypocalcemia (22). This truncated receptor exhibits significantly greater cell surface expression in HEK293 cells as compared with the WT hCaR contributing to a gain-of-function. We sought to determine the mechanisms through which this truncation could produce enhanced signaling activity of the receptor. We created a truncation at position 895 and generated seven other carboxyl tail-truncated receptors (Fig. 1A) to map sequence elements necessary for the higher cell surface receptor expression. S895X mutant receptor expression and its functional activity were first compared with the WT hCaR. Because the hCaR undergoes extensive $N$-linked glycosylation in the endoplasmic reticulum and the Golgi (30), we first monitored maturation and trafficking of the hCaR by an Endo H digestion strategy. Sensitivity to Endo H distinguishes between the fully processed 150-kDa hCaR forms modified with complex carbohydrates (Endo H resistant) and the 130-kDa intermediate high mannosyl-modified hCaR forms (Endo H sensitive) that have not trafficked from the endoplasmic reticulum to the Golgi. As shown in Fig. 1B (WT control lane), the WT hCaR transiently expressed in HeLa cells showed a doublet of immune-reactive bands of ~150 and 130 kDa sizes. The carboxyl tail-truncated S895X mutant showed corresponding changes in the mobility of both immune reactive bands on an immunoblot, and also revealed a significant increase in the intensity of the band representing the fully processed complex carbohydrate-modified mature receptor forms. To confirm that this increase corresponds to the increased cell surface level of the K895X truncation mutant, we performed cell surface biotinylation and immunofluorescence analysis of these mutant receptors transiently expressed in HeLa cells. The cell surface transmembrane proteins were labeled with membrane-impermeant Biotin-7-NHS prior to lysing the cells. The WT hCaR and S895X mutant receptor were precipitated from the cell lysate with streptavidin-agarose beads; then analyzed on immunoblots with anti-hCaR monoclonal antibody ADD. ADD antibody identified only a single band at 150 kDa representing the wild type and the upper 125-kDa band of the S895X-truncated mutant receptor (Fig. 1B). The band intensity of the mature form of this mutant receptor was substantially greater than that of the WT hCaR, suggesting that the S895X mutant receptor is present at a much higher level at the cell surface than the WT hCaR. Immunofluorescence analysis of cells transfected with either WT or the S895X mutant receptors revealed that in nonpermeabilized intact cells, the S895X mutant receptor was expressed at a higher level than WT hCaR at the cell surface (Fig. 1C). Fig. 1D shows typical dose-response curves for the WT and S895X mutant receptors in response to extracellular Ca$^{2+}$ in a PI hydrolysis assay. Compared with the WT hCaR, the S895X mutant receptor exhibited a left-shifted dose-response curve with a reduced EC$_{50}$ for calcium-stimulated changes in phosphoinositide levels (WT EC$_{50}$ 2.9 ± 0.39 versus S895X EC$_{50}$ 1.1 ± 0.25). Taken together, these results indicate that the S895X receptor is expressed at robust levels on the cell surface, and that this increased expression possibly leads to its enhanced signaling activity.

Mapping for Sequence Elements within hCaR Carboxyl Tail Necessary for Higher Expression—Because the S895X truncation removed many residues from the hCaR carboxyl tail, we generated multiple truncation constructs at various loci within this region of the carboxyl tail (Fig. 1A) to map the critical sequence elements involved. Expression of mutants transiently expressed in HEK293 cells was analyzed by immunoblotting. The data showed expression levels of receptors truncated at positions 1040 and 1001 were similar to WT hCaR, whereas truncations at positions 970, 961, 930, 914, 903, and 895 lead to increased expression (Fig. 2A). Quantitative analysis of these deletion constructs showed the highest expression levels for receptors containing truncations at positions 914, 903, and 895, almost 3-fold greater than the WT hCaR. Truncation at position 970 produced a moderate increase (1.5-fold), whereas mutants R961X and Q930X showed intermediate (2- and 2.5-fold) increases in expression. These results suggest that residues between 1001 and 914 may contain sequence elements important for regulating hCaR expression level. We used a web-based algorithm, PESTfind, to identify potential PEST motifs that represent a proteolytic degradation signal found in many rapidly degraded proteins. We found several potential PEST-like regions in the carboxyl tail domain of the hCaR with the PEST scores ranging from low PEST candidates (−7) to a strong PEST candidate (+10). A single stretch of polar amino acids with a PEST score of +10 was identified at residues 917 to 928 of the hCaR carboxyl tail (Fig. 2B). To examine whether this PEST sequence motif and the associated sequences directly alter the expression level of hCaR, various hCaR PEST-disrupting deletion mutants were constructed (Fig. 2B). These were transiently expressed in HEK293 cells for immunoblot analysis (Fig. 2C). The 920–97Δ, 920–937Δ, 920–960Δ, and 920–970Δ receptor mutants all showed doublet band patterns similar to the WT receptor; however, only the 920–960Δ and 920–970Δ mutants showed significant increases in expression level, with expression of the 920–970Δ receptor more than 2-fold higher than that of the WT receptor. These results suggest that deletion of the PEST sequence between residues 920 and 927 had no effect on the expression of the hCaR. In contrast, removal of residues 920–970 led to significantly increased receptor expression.

Sequence alignment of this putative PEST motif and distal sequences between 917 and 970 showed clusters of conserved amino acids for CaR from different mammalian species, particularly serines, threonines, lysines, prolines, and several glut-
mine repeats (Fig. 3A). To measure the cell surface level of the 920–970Δ mutant receptor, we used Endo H digestion, along with immunocytochemical and functional cell-based PI assays to compare receptor expression and activity. Immunoblot studies revealed higher expression of the 920–970Δ receptor protein compared with the WT receptor in transiently transfected
HEK293 as well as HeLa cells in two different plasmid DNA transfection conditions (Fig. 3B). Biotin labeling experiments with immunoblotting confirmed that the upper 150-kDa band was the cell surface forms and confirmed the presence of a significantly higher level of 920–970Δ receptor protein at the cell surface (Fig. 3C). A cell-based PI hydrolysis assay in HEK293 cells also revealed that the 920–970Δ mutant receptor exhibited a gain-of-function with a left-shifted dose-response curve and a reduced EC₅₀ for calcium-stimulated changes in phosphoinositide levels (WT EC₅₀ 2.9 ± 0.39 versus 920–970Δ EC₅₀ 1.9 ± 0.17) (Fig. 3D). Nonpermeabilized HEK293 or HeLa cells transiently expressing WT and 920–970Δ receptors were labeled with B6-30N antibody at 4 °C and fixed, and the amount of cell surface receptors was visualized by confocal microscope. Fig. 3F reveals a higher level of cell surface expression of 920–970Δ receptor compared with WT receptor in both cell lines. Together, these findings provide evidence that deletion of residues between 920 and 970 robustly increases hCaR expression at the cell surface of both HEK293 and HeLa cells and is associated with a gain-of-function receptor activity.

WT hCaR and 920–970Δ Receptors Undergo Constitutive Internalization—To access a potential role of the residues between 920 and 970 in endocytosis and/or down-regulation of the hCaR, we examined the internalization pattern of the WT hCaR and 920–970Δ receptor in transfected HeLa cells. To visualize internalized hCaR, surface receptors of living cells were labeled with monoclonal antibody B6-30N at 4 °C for 60 min, a condition nonpermissive for internalization, and then the cells were incubated at 37 °C for various time intervals and antibody surface-bound antibody was stripped with acid wash as previously described (13). Cells were then fixed, permeabilized, and internalized receptors were labeled with a fluorescent secondary antibody. Shown in Fig. 4, the acid wash procedure completely removed the primary antibody label in intact cells. On incubation at 37 °C, small clusters of internalized antibody were detected after 15 min, and at 60 min a robust increase in accumulation of both WT hCaR and 920–970Δ receptors in large clusters was observed. After 240 min, the clusters were reduced for WT hCaR, which displayed a more perinuclear distribution. Thus, both WT and 920–970Δ receptors undergo rapid constitutive internalization at 37 °C.

Internalized 920–970Δ Receptor Is Recycled Faster than WT hCaR at the Cell Surface—Internalized receptors can be directed to two distinct destinations: to lysosomes for degradation and/or to recycle back to the cell surface. To study the fate of internalized hCaR receptors, we first tested whether they are able to recycle back to the cell surface. Cell surface receptors were labeled with B6-30N antibody for 90 min at 37 °C. Subsequently, antibodies bound to the cell surface hCaR were acid stripped twice. The stripped cells were then further incubated at 37 °C for 30–60 min to test for recycling of receptors to the cell surface. Cell surface receptors were stained with a green fluorescent secondary antibody and internalized receptors were stained, after fixation and permeabilization of the cells, with a red fluorescent secondary antibody. Under these conditions, recycling of the WT hCaR was visible after 30 min, which further increased after 60 min (Fig. 5). The 920–970Δ recycled receptor level seen after 30 min was similar to the WT receptor level at 60 min, indicating a possible faster cell surface recycling rate of 920–970Δ receptor.

Internalized WT hCaR but Not 920–970Δ Receptors Are Targeted to Lysosomes for Degradation—Because internalized receptors can be directed either to lysosomes for degradation or recycled back to the cell surface, we tested this for hCaR and 920–970Δ transiently expressed in HeLa cells. HeLa cells were transiently co-transfected with WT hCaR or 920–970Δ recep-

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**FIGURE 2.** Scanning of expression levels of different hCaR carboxyl tail truncation and deletion mutant receptors. A, HEK293 cells transiently transfected with different carboxyl tail mutant receptors were analyzed by SDS-PAGE on 4–12% gels under reducing conditions. Receptor expressions were detected by anti-hCaR antibody ADD. hCaR bands were quantified by measuring band intensity ratios using a UVP multispectral imaging system and after normalizing against α-actin band and are represented as mean ± S.E. (n = 4–7 independent experiments). B, schematic illustration of PEST-like domain and PEST-included deletions analyzed for expression studies. C, hCaR carboxyl tail deletion mutant receptors transiently expressed in HEK293 were analyzed by SDS-PAGE on 4–12% gels under reducing conditions and band intensities were quantified similarly as stated above.
tor DNA along with plasmid DNA containing Rab5-RFP or LAMP1-RFP reporters. Cell surface receptors were labeled with B6-30N antibody at 4 °C for 60 min, allowed to internalize for another 120 min at 37 °C, surface-bound antibodies were removed by acid wash, and cells were subsequently fixed, permeabilized, and stained for internalized receptors with green fluorescent secondary antibody. Rab5-RFP and LAMP1-RFP cellular localization were detected as red fluorescent signals. Under these conditions internalized WT receptors co-localized in early endosomes with Rab5-RFP and in LAMP1 positive lysosomal compartments (Fig. 6A). Furthermore, blocking lysosomal proteases with 100 μg/mL leupeptin resulted in an increased lysosomal localization of WT hCaR with LAMP1, supporting the notion that endocytosed WT hCaR is degraded in lysosomes. In contrast, internalized 920–970 receptors co-localized with Rab5 positive endosomes, however, no co-localization of 920–970 receptor with lysosomal marker LAMP1 was detected with or without leupeptin treatment (Fig. 6B). Taken together, these results indicate that sequences between residues 920 and 970 contain a sorting signal for targeting of hCaR to LAMP1 positive lysosomal compartments.

Effects of CaM Binding, PKC Phosphorylation, and Arginine-rich RXR Motif Mutations in 920–970Δ Receptor Background—Because an important segment of the hCaR close to the 920–970Δ-deleted region containing the CaM-binding, PKC phosphorylation, and arginine-rich RXR motifs in the proximal hCaR carboxyl tail also contribute to the regulation of plasma membrane receptor abundance and signaling response, we determined whether mutation of these motifs in the 920–970Δ background alters receptor expression or intracellular localization in lysosomal compartments. Selective alanine-scanning mutations of lysines and glutamines within the 920–970 segment were also generated to identify residues important for lysosomal targeting. In the 920–970Δ receptor background, however, the CaMBD3E3, RXRmu, and PKCmu mutants had total expression levels comparable with the 920–970Δ receptor (Fig. 7A), whereas the 3K/A and multi-Q/A mutants had significantly reduced expression similar to that for the WT hCaR (Fig. 7B). Colocalization experiments of internalized 920–970Δ receptors with late endosome/lysosomal marker LAMP1 revealed no co-localization of CaMBD3E3, RXRmu, and PKCmu mutants in the 920–970Δ receptor background with the lysosomal marker LAMP1 (Fig. 7C). In contrast, internalized 3K/A and multi-Q/A mutant receptors were found to accumulate in intracellular compartments with lysosomal marker LAMP1 as patchy clusters supporting the notion that these endocytosed mutants like WT hCaR are degraded in lysosomes (Fig. 7D). We concluded that mutations of this mem-

**FIGURE 3. Deletion of the hCaR carboxyl tail 920–970 sequence interval increases receptor cell surface expression and leads to gain-of-function activity.** A sequence comparison of amino acids 917–970 of the human calcium receptor with those of other mammalian species. The hCaR PEST-like sequence 917–928 is shown and the poly(Q) motifs are boxed. Three potential ubiquitination conserved lysine residues are in bold type. B, WT hCaR and 920–970Δ mutant receptors were transiently transfected with 1 or 2 μg of plasmid DNA in either HEK293 or HeLa cells, and receptor expression was detected by Western blot. C, HeLa cells were transiently transfected with 2 μg of plasmid DNA and the cell surface receptor level was analyzed by cell surface biotinylation and subsequent Western blot. The blot shows total as well as cell surface biotinylated and avidin-precipitated hCaR detected with anti-hCaR antibody ADD. D, calcium dose-responses for PI hydrolysis in HEK293 cells transiently expressing WT hCaR and 920–970Δ receptors were assessed in the presence of the indicated extracellular calcium concentrations from 0.5 to 8 mM. The values shown have been normalized to the control WT values and are the mean ± S.E. of three determinations. Two independent experiments showed similar results. E, the same transfected HEK293 and HeLa cells from the experiment of panel B were stained with an anti-rhodopsin antibody, B6-30N. Cell surface WT hCaR and 920–970Δ receptor expression was detected in fixed nonpermeabilized cells and stained with a secondary green fluorescent antibody using laser confocal microscopy.
brane proximal region of hCaR does not influence sorting of the 920–970 receptor that escapes lysosomal targeting. Localization of both 3K/A and multi-Q/A mutants with the lysosomal marker also points to the fact that these amino acids by themselves are not sorting signals of the hCaR from endosomes to lysosomes.

**DISCUSSION**

In this report, we have characterized a rapid ligand-independent constitutive internalization of the human calcium-sensing receptor and identified a large “PEST-like” sequence interval in the hCaR carboxyl tail that contributes to receptor entry into late endosomes or lysosomal compartments for possible degradation. A series of progressively refined deletion mutants identified a minimal sequence interval between residues 920 and 970 necessary for higher cell surface receptor expression of hCaR. This sequence interval is striking, minimally defined as 51 amino acid residues with high densities of both hydroxylated and acidic amino acids, particularly serine, threonine, proline, and glutamine. Interestingly, in this sequence interval, residues 917–928 resemble a PEST motif and scored highly with the...
In the presence of 100 min at 4 °C, and allowed to internalize at 37 °C for 120 min in the absence or presence of 100 μM leupeptin. Antibody B6-30N for 60 min, therefore, labeling the surface receptors were done with anti-rhodopsin antibody B6-30N for 60 min. Cells then were transfected with a Rab5-RFP plasmid construct, an early endosomal marker, or a LAMP1-RFP plasmid construct, a late endosomal/lysosomal marker. Cell surface receptors were then labeled with anti-rhodopsin antibody B6-30N for 60 min, and then fixed, permeabilized, and receptors were stained with a green fluorescent secondary antibody. Rab5-RFP and LAMP1-RFP localization were visualized by laser scanning microscopy. Panels A and B show the results for the WT hCaR and 920–970Δ receptor. The scale bars are equal to 10 μm.

The results presented here with the hCaR highlight a previously unrecognized complexity in endosomal-lysosomal trafficking of this receptor. The constitutive internalization of WT hCaR in the absence of calcium is unexpected as hCaR was reported to undergo only minor agonist-induced internalization (9). We show this constitutively internalized hCaR can be detected in Rab5 positive early endosomes and internalized receptor recycle back to the cell surface. This futile cycle of hCaR internalization and recycling appears to occur as hCaR also accumulates in the late LAMP1 positive endosomes for trafficking to the lysosomal compartment. The assumption of lysosomal degradation of internalized hCaR is consistent with an increased accumulation of WT hCaR in LAMP1 positive lysosomes on treatment with the lysosomal protease inhibitor leupeptin. This phenomenon is similar to many internalized GPCRs including GABA<sub>γ</sub> receptors, β<sub>2</sub>-adrenergic receptor, angiotensin-1 receptor, and V<sub>2</sub>-vesopressin receptor sorted within the early endosomal compartment to either recycling or lysosomal degradation pathways (12, 13). Both the WT hCaR and 920–970Δ receptor appear to follow a similar internalization and localization pattern in Rab5-positive early endosomes and then into a recycling route, but the 920–970Δ receptor seems to recycle more rapidly. Deletion of the 920–970 sequence interval prevents the mutant 920–970Δ receptor from colocalizing in LAMP1-positive late endosomes for degradation. The results suggest that the 920–970 sequence interval possibly contains sorting signal(s) that mediates sorting of hCaR within the endosomal-lysosomal system. Tyr- and dileucine-based motifs appear to regulate endocytic sorting of several mammalian GPCRs, including β<sub>2</sub>-adrenergic receptor and chemokine receptors CXCR4 and CXCR2 (12). In constitutively internalized PAR1, a distal YKYL motif controls constitutive internalization and the proximal YSIL motif regulates lysosomal sorting (36). No singular tyrosine-based or dileucine-based lysosomal sorting signal was identified in the hCaR 920–970 sequence interval, except for a di-leucine sequence at positions 1012 and 1013 in the distal segment (Fig. 1A). Presence of this putative acidic di-leucine signal in the 920–970Δ receptor background is unable to direct this mutant receptor to the lysosomal pathway. Similarly, absence or presence of this putative di-leucine signal in Q1001X or Q1040X truncation mutant receptors have no significant effect in increasing or decreasing receptor expression levels. This suggests that these distal di-leucine residues may not be important for regulating lysosomal sorting of the hCaR. However, this result does not completely rule out an indirect conformational effect due to deletion of the 920–970 sequence interval extending to the proximal or distal segments of the hCaR carboxyl tail.

**Figure 6.** Fate of endocytosed WT hCaR and 920–970Δ mutant receptors. WT hCaR or the 920–970Δ receptor plasmid DNA construct was co-transfected with a Rab5-RFP plasmid construct, an early endosomal marker, or a LAMP1-RFP plasmid construct, a late endosomal/lysosomal marker. Cell surface receptors were labeled with anti-rhodopsin antibody B6-30N for 60 min at 4 °C, and allowed to internalize at 37 °C for 120 min in the absence or presence of 100 μM leupeptin. After stripping antibody from the remaining background is unable to direct this mutant receptor to the lysosomal compartment. The assumption of lysosomal degradation of internalized hCaR is consistent with an increased accumulation of WT hCaR in LAMP1 positive lysosomes on treatment with the lysosomal protease inhibitor leupeptin. This phenomenon is similar to many internalized GPCRs including GABA<sub>γ</sub> receptors, β<sub>2</sub>-adrenergic receptor, angiotensin-1 receptor, and V<sub>2</sub>-vesopressin receptor sorted within the early endosomal compartment to either recycling or lysosomal degradation pathways (12, 13). Both the WT hCaR and 920–970Δ receptor appear to follow a similar internalization and localization pattern in Rab5-positive early endosomes and then into a recycling route, but the 920–970Δ receptor seems to recycle more rapidly. Deletion of the 920–970 sequence interval prevents the mutant 920–970Δ receptor from colocalizing in LAMP1-positive late endosomes for degradation. The results suggest that the 920–970 sequence interval possibly contains sorting signal(s) that mediates sorting of hCaR within the endosomal-lysosomal system. Tyr- and dileucine-based motifs appear to regulate endocytic sorting of several mammalian GPCRs, including β<sub>2</sub>-adrenergic receptor and chemokine receptors CXCR4 and CXCR2 (12). In constitutively internalized PAR1, a distal YKYL motif controls constitutive internalization and the proximal YSIL motif regulates lysosomal sorting (36). No singular tyrosine-based or dileucine-based lysosomal sorting signal was identified in the hCaR 920–970 sequence interval, except for a di-leucine sequence at positions 1012 and 1013 in the distal segment (Fig. 1A). Presence of this putative acidic di-leucine signal in the 920–970Δ receptor background is unable to direct this mutant receptor to the lysosomal pathway. Similarly, absence or presence of this putative di-leucine signal in Q1001X or Q1040X truncation mutant receptors have no significant effect in increasing or decreasing receptor expression levels. This suggests that these distal di-leucine residues may not be important for regulating lysosomal sorting of the hCaR. However, this result does not completely rule out an indirect conformational effect due to deletion of the 920–970 sequence interval extending to the proximal or distal segments of the hCaR carboxyl tail.

**Algorithm** that has proved to be a powerful indicator of proteins subjected to rapid ubiquitin-dependent proteosomal degradation (31). Deletion of this minimal PEST sequence motif itself, however, had no apparent effect on receptor expression level but the extension of the deletion from residue 920 to 970 resulted in increased receptor expression and enhanced signal-
Because the proximal carboxyl tail of hCaR possesses multiple important determinants that regulate a functional response of the receptor, such as PKC phosphorylation sites, binding of CaM, and an extended arginine-rich (RXR) region regulated by phosphorylation (10, 14, 15), we mutated these proximal residues or motifs in the context of the 920–970Δ receptor background to determine potential effects in expression and late endosomal localization of these mutant receptors. Our results demonstrated that disruption of these proximal residues and motifs have no apparent impact on 920–970Δ receptor expression and these mutations do not re-direct the 920–970Δ receptor for lysosomal sorting and degradation. This data strongly suggests that a sequence element(s) within the 920–970 interval regulates lysosomal degradation of internalized hCaR and thereby contributes to hCaR down-regulation and degradation.

In this report, we have evaluated the fate of cell surface hCaR using immunofluorescent and confocal microscopic experiments and shown constitutive internalization of hCaR. Our data indicate that WT hCaR is subject to continuous internalization and recycling back to the cell surface and in turn undergoes lysosomal degradation in the process, suggesting a possible dynamic equilibrium between receptor intracellular sorting, degradation, and synthesis that maintains a constant hCaR level at the cell surface. Interestingly, hCaR ubiquitination and filamin A interaction are linked as possible mechanisms regulating the receptor level after receptor synthesis via an ERAD-mediated degradation pathway in the endoplasmic reticulum (15). The 920–970 sequence interval overlaps with a high-affinity filamin A binding region with a minimal sequence between 962 and 981 (15, 37). Of the 12 lysines suggested to be polyubiquitylated in the intracellular domains of the hCaR, 3 highly conserved lysines reside within the 920–970 sequence interval (15). We investigated whether ubiquitination of the 3 lysine residues in this sequence interval may serve as sorting signal(s) as shown for other transmembrane proteins (12). However, mutation of the lysines present in the 920–970 interval resulted in minor changes in overall receptor expression as opposed to deletion of the 920–970 sequence interval, and the internalized lysine mutant receptor undergoes similar sorting and co-localization with the lysosomal marker LAMP1 as the WT hCaR. Therefore, if ubiquitination indeed plays a role in lysosomal sorting of hCaR, it appears that ubiquitination of lysines within the 920–970 interval is not sufficient to initiate lysosomal degradation of the receptor. Similarly, the presence of several polyglutamine (QQQ) repeats in this 920–970 sequence interval is intriguing. Limited evidence supports that proteosomes can cleave at multiple sites within a poly(Q) tract and degradation of poly(Q)-expanded proteins both by proteosomes can cleave at multiple sites within a poly(Q) tract and degradation of poly(Q)-expanded proteins both by autophagy aggregates and proteosomes (20, 38). Mutation of these poly(Q) tracts in the full-length hCaR does not increase either the expression level or prevent sorting of the receptor to the late endosome or lysosome. Interestingly, because filamin A...
stabilizes hCaR and is reported to protect against degradation (15), it remains to be determined whether the PEST-like sequence interval identified in this study is involved in lysosomal sorting of hCaR in a filamin A-dependent manner.

In summary, the results presented here extend characterization of the postendocytic trafficking of the hCaR and highlight a previously unrecognized complexity in this process. Although the reasons for the differential fate of the internalized WT hCaR and the 920–970Δ mutant receptor are not fully understood, experiments presented here are important because they document a mechanism that connects an increase in the cell surface expression level and lack of lysosomal accumulation and degradation of the internalized 920–970Δ mutant receptor. It is currently unclear whether this mechanism of hCaR endocytosis and expression detected using heterologously expressed hCaR also applies to the endogenous hCaR expressed in parathyroid or other differentiated cell types. Data obtained for hCaR expressed in HEK293 cells indicate constitutive internalization and recycling of hCaR in a Rab11a-dependent manner (10), which is well in line with the findings of the present study. Clearly, more experiments are needed to determine the endocytosis mechanisms of hCaR in endogenously expressing cells.

Acknowledgments—We thank Dennis Drayna at the Division of Intramural Research, NIDCD, for critical reading of the manuscript and Kaylin Adipietro for help with preparation of some plasmid constructs.

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