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Sar1 -dependent trafficking of the human calcium receptor to the cell surface

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Abstract

The molecular mechanisms underlying the exit from the endoplasmic reticulum (ER) for cell surface trafficking of the human calcium receptor (hCaR) remain poorly understood. We investigated the role of the Sar1 small GTP-binding protein in cell surface transport of the hCaR. Disruptions of endogenous Sar1 function with the constitutively active Sar1H79G mutant or depletion using small interfering RNA, attenuates cell surface expression of the hCaR. Mutation of several putative di-acidic ER export motifs in the carboxyl-tail of the receptor revealed no apparent defect in cell surface expression. Truncated mutants lacking most of the carboxyl-terminal sequences or all intracellular domains also showed no impairment in cell surface expression at steady state. A truncated receptor containing only the large amino-terminal extracellular ligand binding domain (ECD) is secreted into the culture medium and Sar1H79G inhibits this secretion. ECD receptor variants with the cysteines essential for intermolecular disulfide-linked dimerization mutated to serine or four of the asparagine sites for N-glycosylation mutated to alanine also disrupt secretion, indicating proper ECD conformation is critical for forward transport of this receptor.

Keywords

Calcium receptor; family-C/3 G-protein-coupled receptors; Sar1; ER exit

1. Introduction

Calcium receptor belongs in family-C/3 G-protein-coupled receptor (GPCR) gene family and contains a uniquely large extracellular amino-terminal ligand-binding domain (ECD) of 600 amino acids connected to a seven transmembrane helical domain (7TMD), prototypical for all GPCRs responsible for G-protein activation [1]. While the trafficking of rhodopsin-like family-A/1 GPCRs has been the subject of extensive investigation, cellular processing of most family-C/3 GPCRs remains largely unexplored. The hCaR regulates extracellular calcium ion homeostasis by controlling the rate of parathyroid hormone secretion from the parathyroid gland and the rate of calcium re-absorption by the kidney. Inactivating mutations of the hCaR cause familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT), whereas, activating mutations are responsible for a form of autosomal dominant hypocalcemia (ADH). Several missense mutations causing FHH/NSPHT reduce hCaR

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function by impairing cell surface expression of the receptor by mostly remaining trapped intracellularly [2,3].

Little is known about the folding, maturation, and translocation of the hCaR from the endoplasmic reticulum (ER). The large hCaR ECD faces the luminal side of the ER and proper folding may be achieved by disulfide linkages, posttranslational modifications and possibly by other quality control mechanisms [4,5]. ER exit for many transmembrane proteins involve selective concentration of cargos in vesicular or tubular complexes destined for Golgi. This complex formation involve interactions with the ER-derived coat protein complex-II (COPII), COPII-associated proteins, or cargo receptor. Assembly and disassembly of COPII-coated vesicles from ER membranes requires the recruitment of the Sar1 small GTP-binding protein to the ER outer membrane. Sar1 cycles between a GTP-bound form stimulated by the GTP-GDP exchange factor Sec12p, and a GDP-bound form stimulated by GTPase activation by Sec23p. Binding of Sar1-GTP to the ER outer membranes, induces sequential recruitment of coat complexes Sec23p-Sec24p and Sec13p-Sec31p and formation of ER exit sites (reviewed in [6]). While trafficking of many cargo proteins in the secretory pathway utilize conventional COPII-complex in most cell types, non-conventional trafficking has also been reported for some plasma membrane bound proteins [7,8]. ER export of α -adrenergic and angiotensin-II receptors have been found to be differentially regulated by Sar1 [9].

Recognition signals involved in the ER exit and role of carboxyl-terminal sequences in regulating GPCR export have been demonstrated for a relatively small number of GPCRs (reviewed in [10]). No direct evidence is available to suggest that GPCR cargo proteins are recruited to COPII-coat vesicles via interactions with any of these putative export signals. In contrast, for a number of mammalian proteins such as the vesicular stomatitis glycoprotein (VSVG), potassium channels, and cystic fibrosis transmembrane conductance regulator (CFTR), at least two distinct types of ER export signals, a diphenylalanine (FF) hydrophobic motif and/or a diacidic motif (DXE) have been identified. Both these motifs are found in the carboxyl-terminal domains of these proteins that promote cargo enrichment in COPII transport vesicles. Deletion or mutation of these export motifs results in either retention of these proteins in the ER or a delayed exit from the ER resulting in a lowered steady-state level cell surface expression (reviewed in [11]). However, many cargo proteins also require a diversity of signals and may not employ a simple, well-defined motif like DXE or FF. These signals may include hetero- or homo-oligomerization, glycosylation, and interaction with adaptor proteins in the ER lumen [6,11]. In this study, we investigated the intracellular trafficking of hCaR and found that exit from the ER is Sar1-dependent via COPII vesicles. A large portion of the hCaR carboxyl-tail is dispensable and mutations of putative clusters of di-acidic carboxyl-terminal sequences or truncation of the majority of carboxyl-terminus do not interfere with trafficking of the receptor. Instead, we show presence of properly folded ECD is necessary for forward trafficking of the receptor.

2. Materials and Methods

2.1. Constructs and mutagenesis

The wild type hCaR, TM1, T903, twenty amino-terminal sequence tag of rhodopsin at the amino-terminus of T903-Rhoc, hCaR (r-hCaR) and T903 (r-T903) have been described [12]. VSVG epitope-tagged Sar1 and mutant Sar1H79G, were kind gifts from Dr. Victor Rebois. T613, N4 T613 C129, I31 T613, r-AXA-2 mutants were generated by site-directed mutagenesis as described previously [5]. All mutant constructs were verified by automated DNA sequencing using an ABI prism-377 DNA sequencer.

2.2. Cell culture and transient transfection

HEK293 and HeLa cells (ATCC) were cultured as per supplier recommendations. For transient expressions, plasmid DNA constructs were introduced into HEK293 cells by lipofectamine transfection (Invitrogen) [4].

2.3. Immunoblot analysis with detergent solubilized cell extracts

Transfected HEK293 cell extracts were resolved by SDS-PAGE on either 12% or 4–12% gradient gels, transferred to nitrocellulose membrane, probed with either anti-hCaR ADD antibody (Affinity Biosciences), VSVG antibody and α -actin (Sigma Corporation), and detected using enhanced chemiluminescence system containing substrate for horseradish peroxidase (Pierce Laboratories). For cleavage with Endoglycosidase-F (PNGaseF) and Endoglycosidase-H (EndoH) (Roche), cell extracts were incubated with 0.5 milliunits of each enzyme for 1.5 h at room temperature.

2.4. siRNA knockdown

Endogenous Sar1 mRNA in HEK293 cells were targeted with Sar1-specific “Stealth” custom-made siRNA duplex oligonucleotides obtained from Invitrogen. For each transfection, 10 μ l of lipofectamine reagent was mixed with 10 μ l of serum-free DMEM and 80 μ l of 500 nM double-stranded RNA oligonucleotides, incubated for 30 min, and transfection mix was added to the culture dish containing 800 μ l of serum-free DMEM.

2.5. Immunocytochemistry

HeLa cells were grown on gelatin coated coverslips, fixed in 3% paraformaldehyde solution in PBS, blocked with 10% normal goat serum (NGS), then incubated with the primary anti-rhodopsin monoclonal antibody, B6-30N and secondary antibody (Alexa Fluor 488 goat anti-mouse IgG). The cells were next fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. The fixed cells were again exposed to B6-30N and anti-VSVG (Covance Corp.) rabbit polyclonal antibody and then with Alexa Fluor-555 goat anti mouse and Alexa Fluor-660 goat anti rabbit secondary antibodies (Molecular Probes). Immunofluorescence were captured using a 40 $\times$ objective on a Zeiss LSM 710 confocal laser scanning microscope.

2.6. Intact cell enzyme-linked immunoassay (ELISA)

Transiently transfected HEK293 intact cells were incubated with anti-rhodopsin B6-30N antibody, after wash incubated with peroxidase-conjugated goat anti-mouse IgG (Kierkegaard and Perry Laboratories) as described [12]. Peroxidase substrate was added to each sample and the color reaction was stopped by centrifugation and removal of the cells. Absorbance at 580 nm was measured using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech).

2.7. Phosphoinositide (PI) hydrolysis assay

PI hydrolysis was measured as previously described [7]. Briefly, HEK293 cells transfected with test constructs were replenished in medium containing 3.0 μ Ci/ml of 3 H-myoinositol (New England Nuclear) for 16 h. Cells were treated for 30 min with 10 mM Ca^{2+} in PI solution. The reactions were terminated by addition of acid-methanol. Inositol phosphates were separated from phosphoinositides and myoinositols by chromatography on Dowex 1-X8 columns and samples were counted in a Wallace 1219 liquid scintillation spectrometer.

3. Results

3.1. Sar1 regulates cell surface expression of hCaR

To test the role of Sar1 in ER exit of the full length hCaR and several truncated receptor variants (Fig. 1A), we first transiently co-expressed wild type hCaR with a constitutively active GTP-bound mutant Sar1H79G which competes with endogenous Sar1 and inhibits disassembly or uncoating of COPII vesicles, leading to the accumulation of ER to Golgi transport intermediates [6]. Because hCaR undergoes extensive glycosylation both in the ER and the Golgi, we monitored the maturation and trafficking of the hCaR by an EndoH digestion strategy. As seen on Western blot (Fig. 1B, control lane), the core glycosylated high mannose 130 kDa band-B, characteristic of hCaR forms that have not yet trafficked from the ER to the Golgi, showed EndoH sensitivity (band-A). As hCaR traffics through the cis- and medial-Golgi, the N-glycan is processed to a complex oligosaccharide decreasing the receptor mobility to 150 kDa (band-C). The band-C modified with complex carbohydrates of the hCaR exhibits EndoH resistance, identifying them as receptor forms passaged through the ER to the Golgi. Cells expressing hCaR alone (control) or with Sar1 contain a pool of band-C protein resistant to EndoH digestion. This band-C was not detectable for hCaR co-expressed with Sar1H79G with the receptor mostly migrating as the 130 kDa EndoH-sensitive form. Quantitative analysis of EndoH resistant band-C ratios for hCaR exhibited a 75% reduction in Sar1H79G sample compared to control (Fig. 1C). Cell surface expression levels of hCaR were determined using two different experimental strategies: intact cell ELISA for quantitation of cell surface expression and extracellular Ca^{2+} -stimulated phosphoinositide (PI) hydrolysis assay for quantitation of signaling competence of the receptor. For intact cell ELISA, we utilized r-hCaR and anti-rhodopsin antibody B6-30N which detects the native folded amino-terminal ECD of the receptor. Consistent with the EndoH digestion analysis, the cell surface receptor number was reduced 80% in cells expressing Sar1H79G compared to control cells expressing receptor alone (Fig. 1D). To determine the functional activity of the hCaR in Sar1 and Sar1H79G expressing cells, we measured Ca^{2+} -stimulated PI hydrolysis in intact cells. As shown in Fig. 1E, cells expressing mutant Sar1H79G co-expressed with the hCaR reduced the maximal Ca^{2+} response of the receptor to 50% of that for control cells expressing receptor alone.

Because the above experiments relied on overexpression of activated Sar1H79G mutant as a competitor for disruption of endogenous Sar1 function, we also utilized an alternative approach using a siRNA-mediated knockdown of endogenous Sar1 expression to test the requirement for Sar1 expression on hCaR trafficking. To confirm the specificity of the Sar1 siRNA, we co-expressed VSVG epitope tagged Sar1 with Sar1 siRNA and control low GC content siRNA in HEK293 cells and determined the expression of Sar1 by Western blot (Fig. 1F). Sar1 siRNA knocked down the transiently expressed Sar1 but the negative control siRNA did not (upper panel). Coexpression of hCaR with Sar1 siRNA reduced the intensity of fully mature complex carbohydrate-modified band-C of the hCaR by about 60% and 20% of band-B level when compared to cells transfected with negative control siRNA.

We next determined the consequence of Sar1H79G on the subcellular distribution of hCaR. The cell surface expression and intracellular localization of r-hCaR along with co-expressed Sar1 or Sar1H79G in transiently transfected HeLa cells were revealed by confocal microscopic analysis. hCaR was expressed at the cell surface in cells co-expressing Sar1 (Fig. 1G), confirmed by double labeling of the intracellular hCaR and Sar1. hCaR exhibited an even distribution of the receptor in the perinuclear region which co-localized with Sar1. Consistent with the dramatic reduction in cell surface expression as measured by biochemical and functional assays, cell surface expression of the hCaR was absent in cells co-expressing Sar1H79G (Fig. 1H). Subcellular localization of hCaR in these same cells showed highly concentrated receptor density distributed in the perinuclear region which co-localized with Sar1H79G.

3.2. Analysis of putative export motifs in the carboxyl-tail of hCaR

A cluster of potential diacidic signal motifs between positions 1019 and 1052 in carboxyl-tail of calcium receptors are largely conserved among human, mouse, bovine and in rat (Fig. 2A). To test for function of these putative hCaR DXE-like motifs, cell surface expression of r-AXA-2 receptor mutant was examined. Immunoblot analysis showed the band-C expression level of this r-AXA-2 mutant to be similar as the wild type hCaR (Fig. 2B). In intact cell ELISA assay, the r-AXA-2 mutant receptor also displayed comparable cell surface expression level as the wild type receptor (Fig. 2C). Two additional AXA mutants with putative Asp and Glu residues at positions 1020 and 1022 (r-AXA-1 mutant) and 1046 and 1048 (r-AXA-3 mutant) on Western blot/EndoH glycosylation analysis showed similar expression levels (data not shown).

Next, we tested the possibility of other classes of export motifs within this carboxyl-tail of the hCaR. The T903 receptor is expressed at higher level and lacks the majority of carboxyl-tail sequences including the putative DXE-like sequences. Coexpression studies with T903 receptor expressed alone or with Sar1 displayed two bands with similar EndoH resistant band-C intensities (Fig. 2D). Conversely, Sar1H79G significantly decreased the Endo-H resistant band-C intensity level. Quantitative densitometry of EndoH-resistant band-C ratios for T903 receptor confirmed a 60% reduction in Sar1H79G expressing cells (data not shown). Intact cell ELISA confirmed that Sar1H79G mutant inhibited the cell surface expression of the T903 receptor and the surface T903 receptor level exhibited 65% reduction in cells expressing SarH79G compared to cells expressing receptor only, as control (Fig. 2E).

3.3. Secretion of hCaR ECD is Sar1, N-glycosylation and disulfide-linked dimerization dependent

Since carboxyl-tail motifs typical for cytoplasmic recognition are not identified for hCaR ER export, we examined for presence of other structural features within the large amino-terminal ECD. We first tested the ability of cell surface expression of two truncated receptor variants, r-T903 and T903-Rhoc. Quantitative analysis by ELISA shown in Fig. 3A revealed that the r-T903 expression at the cell surface is 4.5 fold higher than T903-Rhoc. The contribution of the ECD structure in forward trafficking was further examined by using TM1 ECD variant. Co-expression of the TM1 mutant receptor with Sar1H79G mutant significantly reduced the level of mature, EndoH resistant 97 kDa TM1 protein band (Fig. 3B). In contrast, cells expressing TM1 alone (control) or with Sar1 contain a distinct pool of mature complex carbohydrate-modified protein forms resistant to EndoH digestion. To refine this test, T613 containing the ECD but lacking all the transmembrane domains was further tested. Fig. 3C shows that T613 is secreted into the culture media as a 95 kDa protein band (upper panel I, left). This band is resistant to EndoH digestion but showed sensitivity to PNGaseF digestion, indicating the N-glycan is processed to a complex oligosaccharide. In contrast, the intracellular T613 forms remained fully sensitive to both EndoH as well as PNGaseF digestion, confirming these ECD forms modified with high mannose oligosaccharides have not yet trafficked from the ER to the Golgi (lower panel I, left). Sar1 dependence of T613 expression was tested by co-expression with Sar1 or the Sar1H79G mutant. The results shown in upper panel-II (middle), confirm the presence of very little or no secreted forms of T613 in the culture medium in cells co-expressing Sar1H79G. Instead, the abundantly expressed T613 protein mostly remains trapped intracellularly in Sar1H79G expressing cells (lower panel II, middle). Lastly, T613 N4 mutant was not secreted into the cell culture medium and secretion of T 613 C129,131S mutant was highly diminished compared to control T613 (panel III, upper right). Both of these mutant T613 receptors were expressed but mostly remained trapped intracellularly. (panel III, lower right).

4. Discussion

The molecular mechanism(s) underlying GPCR export into ER-derived COPII-coated vesicles are largely unexplored. Recent studies suggest differential and selective ER export of α_2 -adrenergic receptor, β_2 -adrenergic receptor and angiotensin-1 receptor is modulated by Sar1 [9]. In the present study, we provide the first characterization of the export trafficking mechanism of a family-C/3 GPCR between the ER to Golgi and to plasma membrane. We addressed this issue by investigating the role of small GTP-binding proteins Sar1, known to play critical roles in the assembly and budding of COPII-coated vesicles in the ER, in regulating hCaR transport to the cell surface. Using both Sar1H79G and siRNA specific for Sar1, we found that disruption of endogenous Sar1 function significantly inhibit cell surface expression of the hCaR. Our data demonstrate that transient expression of Sar1H79G leads to retention of hCaR in the ER mostly as immature high-mannose type receptor. Confocal microscopic analysis revealed that Sar1H79G arrested hCaR in distinct perinuclear compartments. A significant reduction in cell surface expression of the hCaR was also confirmed quantitatively by intact cell surface ELISA and by analysis of hCaR signaling efficacy. In contrast, expression of wild-type Sar1 did not significantly inhibit the cell surface expression of the receptor or its functional activity. Coexpression of Sar1 with hCaR in several experiments augmented the total and cell surface expression of the hCaR. This suggests that this small GTP-binding protein may be limiting for hCaR transport from the ER to the cell surface in our transient expression paradigm. We conclude that ER export of hCaR is likely mediated in ER-derived COPII-coated vesicles.

A number of studies suggest ER export signals DXE and FF motifs play an important role in the COPII-mediated transport of transmembrane proteins [11]. Several other motifs such as (F9X)3F(X)3F, FN(X2)LL(X)3L and F(X)6LL have also been reported to be required for GPCR export trafficking [10]. Unlike the DXE and FF motifs, there is little evidence that these motifs associate with the components of COPII vesicles for GPCR transport from ER to the Golgi. Site-directed mutagenesis and truncation of the carboxyl-tail eliminating putative DXE-like motifs did not reduce cell surface expression of the hCaR mutant receptors at steady state. Similarly, removal of all intracellular loops and the carboxyl-tail of hCaR did not affect cell surface expression and exhibited Sar1-dependent trafficking. These data indicate that DXE or other ER export signals in the carboxyl-tail of the hCaR are not critical for forward trafficking and possibly in binding with components of COPII machinery.

Our present data demonstrated that presence of the autonomously folded large amino-terminal ECD module of hCaR is necessary for forward trafficking of the receptor. A receptor variant devoid of this ECD poorly express at the cell surface, suggesting that the ECD plays an important role in plasma membrane transport activity. Presence of ECD structure in hCaR variants improved cell surface transport of the receptor. The secretion of ECD variant is inhibited greatly by mutating cysteines responsible for disulfide-linked dimerization and elimination of four N-glycosylation sites, indicating that disulfide-linked dimerization and addition of carbohydrates on the ECD in the ER lumen are critical for ER exit and transport. A simplistic explanation is that these mutations affect the correct folding of the luminal hCaR ECD and thus the ER quality control checkpoints prevent ER exit of these secreted cargo. The results also raise interesting questions regarding a role of hCaR amino-terminal ECD in a receptor-mediated mechanism for ER export. hCaR may have to display a specific assembly by disulfide-linked dimer formation and N-linked glycosylation of the ECD to serve as a signal for incorporation of fully assembled hCaR cargo into the COPII vesicles. The roles of the amino-termini motifs in regulating GPCR export trafficking have been much less investigated and remain controversial. A novel ER export signal consisting of a proline residue at the +2 position from the signal peptide cleavage site in nucleobindin-1 (NUCB1) a Golgi-localized soluble protein has been reported recently [13]. A Tyr-Ser motif within the amino-terminus of

α_2 -adrenergic receptor has been demonstrated to modulate trafficking of the receptor [14]. Because amino-terminal domains of secreted proteins cannot rely on a direct interaction with the COPII coat for topological reasons, selective export of some soluble luminal cargo require specific transmembrane cargo receptors, such as ERGIC53, p24 family of proteins, to mediate the interaction of cargo in the ER lumen with COPII components in the cytosol [6,11]. Thus, it remains to be determined whether the hCaR ECD and family-3 GPCRs require transmembrane cargo receptor interactions, specific signal motifs, combination of glycosylation and homodimeric association to be packaged into COPII vesicles.

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Fig. 1.

Sar1-dependent cell surface expression of hCaR. (A) Schematic representation of the full length hCaR and truncated receptor variants. The location of the rhodopsin epitope is depicted as a small circle for r-hCaR, r-T903 and T903-Rhoc. The ECD contains a venus-flytrap motif (VFTM) shown as a chevron and a nine cysteine-rich domain (CRD). The seven transmembrane helices are shown as barrels labeled as 7TMD. (B) Western blot analysis in HEK293 cells transfected with hCaR (1 μ g) plus pcDNA (control), Sar1 (0.7 μ g) or Sar1H79G mutant (0.7 μ g) detected by the anti-hCaR monoclonal antibody, ADD, after 48 h transfection. The samples were either untreated (–) or digested with EndoH (+) prior to SDS-PAGE. *Inset*, expression of Sar1 and Sar1H79G detected with anti-VSVG antibody. (C) Data from

blot in (A) and three additional experiments quantified by densitometric scans and band-C was normalized to that of the control. Values presented are the means $\pm$ SEM (n=4). *, $P < 0.05$. (D) Intact cell ELISA of r-hCaR (1 μ g) plus pcDNA, Sar1 or Sar1H79G (0.7 μ g) in transiently expressed HEK293 cells. Absorbance values were normalized to that for the control cells. Values are the means $\pm$ SEM for two independent experiments (n= 6). *, $P < 0.05$. (E) Untransfected HEK293 cells (labeled as none) or cells transiently expressing hCaR (control), or in combination with Sar1 and Sar1H79G was assessed by PI assay after addition of 10 mM extracellular Ca^{2+} . Values were obtained from quadruplicate determinations and presented as mean $\pm$ SEM normalized to the hCaR control. *, $P < 0.05$. *Inset*, expression of hCaR proteins from the same transfection. (F) HEK293 cells were transfected with no siRNA, control low G/C content siRNA, or Sar1-specific siRNA together with VSVG epitope tagged Sar1 or hCaR. Cell lysates separated by 4–12% gradient SDS-PAGE and Sar1, hCaR and α -actin were detected by anti-VSVG, anti-hCaR and anti- α -actin specific monoclonal antibodies, respectively. α -actin was used as loading control. (G, H) HeLa cells transiently expressing r-hCaR plus Sar1 or Sar1H79G and the subcellular distribution of these proteins were visualized using mouse anti-rhodopsin antibody B6-30N (1:2000) and rabbit anti-VSVG antibodies. hCaR labeled at the cell surface in non-permeabilized cells is shown in green and intracellular receptor in permeabilized cells in red. Sar1 and Sar1H79G mutant were visualized after permeabilization of cells and their distributions are shown in blue. The co-localized intracellular hCaR and Sar1 or Sar1H79G are shown in pink.

Fig. 2.

Effect of mutation of putative DXE-like diacidic motifs or carboxyl-tail truncation in hCaR expression. (A) Sequence comparison of amino acids 1019–1052 of human calcium receptor with those of other mammalian species. Conserved DXE-like acidic residues (Asp and Glu) are shaded in bold type. In r-AXA-2 mutant receptor, the indicated Asp and Glu acidic residues has been changed to Ala. (B) r-hCaR or r-AXA-2 mutant receptors were transfected in HEK293 cells and receptor expression was analyzed by Western blot. The cell extracts were either untreated (–) or digested with EndoH (+). (C) Cell surface expression of r-hCaR and r-AXA-2 mutant receptors were quantified by intact cell ELISA with anti-rhodopsin antibody B6-30N. Absorbance values normalized to that for r-hCaR control and presented as mean $\pm$ SEM from a representative experiment (n = 6). (D) Western blot analysis of HEK293 cells expressing T903 receptor in the presence of Sar1 or Sar1H79G under similar transfection conditions described in Fig 1 A. (E) Cell surface expression of r-T903 in the presence of Sar1 or Sar1H79G in HEK293 cells were quantified by intact cell ELISA. Absorbance values are normalized to that for r-T903 control and presented as mean $\pm$ SEM (n = 6). *, $P < 0.05$.

Fig. 3.

Trafficking of membrane-anchored and secreted hCaR ECD variants. (A) Surface expression of r-T903 and T903-Rhoc measured by intact cell ELISA with anti-rhodopsin B6-30N antibody. The absorbance were expressed as absorbance value $\times$ 1000 of the mean value (shown as Arbitrary Unit, AU) and measurements are shown as mean $\pm$ SEM. (B) Western blot analysis of TM1 receptor under similar experimental conditions described in Fig 1. B. (C) Analysis of T613 ECD variants transiently expressed in HEK293 cells. To detect secreted T613, 1 ml of conditioned cell culture medium from cells transiently expressing T613 was collected and concentrated to 100 μ l using a Millipore ultrafiltration membrane (CM 30) in an Amicon concentrator. 10 μ l of the concentrate was loaded per lane for Western blot and

detection with anti-hCaR-specific ADD antibody. Cell lysates were analyzed to detect intracellular forms. The samples were either untreated (–) or digested with EndoH and PNGaseF as indicated in Panel I. Panel II shows secreted and intracellular T613 transiently transfected in HEK293 cells alone or in combination with Sar1 or Sar1H79G. Panel III shows secreted and intracellular T613 detected in cells transfected with T613 as control, T613 mutant C129,131S and T613 N-glycosylation mutant N4 expression.