Human calcitonin receptor is directly targeted to and retained in the basolateral surface of MDCK cells

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Nussenzveig, Daniel R., Maria De Fatima C. Matos, and Colette N. Thaw. Human calcitonin receptor is directly targeted to and retained in the basolateral surface of MDCK cells. Am. J. Physiol. 275 (Cell Physiol. 44): C1264–C1276, 1998.—The human calcitonin receptor (hCTR) is expressed in polarized cells of the kidney, bone, and nervous system. In the kidney, hCTRs are found in cells of the distal nephron to which blood-borne calcitonin has access only at the basolateral surface. We expressed hCTR subtypes 1 and 2 in Madin-Darby canine kidney (MDCK) cells to establish a cell model useful for delineating the molecular mechanisms underlying hCTR polarity. Selective cell surface incubation demonstrated functional polarity of hCTRs by equilibrium binding or cross-linking of radioiodinated salmon calcitonin (125I-sCT) and cAMP accumulation stimulated by sCT. We estimated that at the steady state there are 40-fold more hCTRs on the basolateral than on the apical side. Domain-selective cell surface biotinylation followed by immunoblotting of streptavidin-agarose-fractionated biotinylated glycoproteins independently confirmed the polarized distribution of FLAG epitope-tagged hCTR-2 in the basolateral domain. Confocal microscopy of immunostained receptors revealed that hCTRs are concentrated on a lateral subdomain of the basolateral membrane. Cell surface arrival assay of newly formed receptors demonstrated that direct delivery to the basolateral domain is the mechanism by which hCTRs become polarized. Measurement of receptor turnover on the basolateral surface showed that retention contributes to hCTR distribution at the steady state.

Madin-Darby canine kidney cells; renal epithelial cells; G protein-coupled receptor; basolateral sorting; polarized cells

CALCITONIN RECEPTORS (CTRs) belong to a recently defined subfamily of the large superfamily of seven transmembrane-spanning G protein-coupled receptors (GPCRs) (31). Other members of the CTR subfamily include important cell function regulators such as receptors for parathyroid hormone/parathyroid hormone related peptide (PTH/PTHrP), secretin, glucagon, glucagon-like peptide, vasoactive intestinal peptide (VIP), pituitary adenyl cyclase-activating peptide, growth hormone-releasing hormone, and corticotropin-releasing hormone. Although receptors of the CTR subfamily appear to share the same general structure as members of the better-studied rhodopsin/oβ2-adrenergic receptor (rho/oβ2-ADR) subfamily of GPCRs, they do not exhibit any homology to the members of the rho/oβ2-ADR subfamily at the amino acid sequence level (31). Consequently, many of the studies aiming to understand the biology of the molecular level of members of the rho/oβ2-ADR subfamily of GPCRs are not necessarily directly applicable to the members of the CTR subfamily.

There are three isoforms of human CTRs (hCTRs) that are formed by alternative splicing of the product of a single gene (Ref. 1 and references within). Two of them are identical except for the presence (hCTR-1) or absence (hCTR-2) of a 16-amino acid insertion in the putative intracellular loop 1 (24). These two hCTR isoforms have different signaling properties: hCTR-2 stimulates both the adenylyl cyclase and the phosphoinositide-specific phospholipase C (PPI-PLC) signaling pathways, whereas hCTR-1 can signal via adenylyl cyclase but not through PPI-PLC (24). A third isoform, which lacks the first 47 amino acids that include the putative endoplasmic reticulum signal translocation peptide sequence, displays a biology that is indistinguishable from the hCTR-2 (1).

Stimulation of CTRs by calcitonin in vivo leads to important effects in Ca2+ metabolism. The major biological activity recognized for calcitonin is to inhibit osteoclastic bone resorption (2) through a potent direct action on osteoclasts. It acts on renal tubular cells of the thick ascending limb of the loop of Henle and in the bright portions of the distal tubule (10) to regulate urinary Ca2+ excretion (5). Calcitonin also acts as a neurotransmitter or neuromodulator in the nervous system (2). In all these tissues, CTRs are expressed in highly polarized cells. For blood-borne or presynaptic calcitonin to have access to its receptors, it is necessary for CTRs to be expressed on the blood-facing side, i.e., basolateral plasma membrane of renal tubular cells or the somatodendritic region of neurons.

Although the most extreme manifestation of cellular polarity is found in the structural organization of neurons, the best-studied type of polarity is the one present in epithelial cells (reviewed in Refs. 4, 22, 28). This is due to the availability of suitable cell models, like the Madin-Darby canine kidney (MDCK) cell line, which becomes polarized in culture. In these cells, genesis of two distinct plasma membrane domains involves, most frequently, the sorting of apical and basolateral proteins from each other, presumably in the trans-Golgi network (TGN), followed by their direct delivery to a distinct plasma membrane domain. Other mechanisms include targeting first to one domain and then transport to the other domain or random delivery to both plasma membrane domains but a selective retention in one of the domains. Maintenance of the polarity depends not only on the separation of the two plasma membrane domains, which is achieved by tight junctions that act as a fence to hold separate the
elements of each domain, but also on the faithful recycling to the correct plasma membrane domain of endocytosed elements ("static and dynamic" selective retention; reviewed in Refs. 4, 22, 28).

Most of the studies involving the mechanism of polarity and identification of the molecular sorting signals of membrane proteins utilized molecules that are either anchored to the membrane by linkage to a glycosylphosphoinositide lipid or are single-pass integral membrane proteins. Although less is known about the molecular signals determining polarized sorting of proteins that span the membrane multiple times, it appears, at least for some molecules, that single cytoplasmic domains are sufficient to direct their plasma membrane insertion. It was demonstrated that the dog betaine transporter (26) and the junctional protein occludin (21) contain a basolateral targeting signal in their respective carboxy-terminal domains. For the GPCRs, a numerous and important family of polytopic membrane proteins, no definitive evidence for a domain responsible for cell targeting has been found so far. The plasma membrane distribution of only a limited number of GPCRs has been determined. In the proximal tubule of the nephron, receptors for PTH/PTHrP (13), ANG II (30), VIP (16), and dopamine (9) have been found on both apical and basolateral membrane domains, whereas α2A-adrenergic receptors (α2A-ADR) have been localized mainly to the basolateral membrane domain (15). Luteinizing hormone (LH)/human chorionic gonadotropin receptors are expressed on the luminal side of the endothelium of rat testicular microvasculature, where they were shown to be involved in the transcytotic transport of hormone through the endothelial cells (11). The expression in MDCK cells of a variety of endogenous and transfected GPCRs showed differences in the steady-state distribution for each individual protein. Whereas bradykinin and A1 adenosine receptors appear to be expressed only at the apical membrane (19, 29), serotoninergic receptors (5-HT1C) (20), the three α2-adrenergic receptors (α2A, α2B, α2C) (33), thyrotropin-releasing hormone (TRH) receptors (34), follicle-stimulating hormone (FSH) receptors, LH receptors, and thyrotropin receptors are present predominantly on the basolateral side (3). The basolateral-to-apical distribution of α2A-ADR is 9:1, and that of TRH receptors is 4:1, showing variability among different GPCRs. The mechanism of polarization of a few GPCRs has been investigated in MDCK cells. For example, although all three subtypes of α2-ADR achieve a basolateral localization on the cell surface at steady state, their targeting modes are distinct. Whereas α2A-ADR is targeted directly to the basolateral membrane, α2B-ADR is delivered randomly into both plasma membrane domains but is retained longer on the basolateral side. The α2C-ADR is inserted directly on the basolateral membrane, but a pool of receptors remains trapped in intracellular compartments (33). Targeting elements within GPCRs have only begun to be evaluated, and so far none have been identified (14).

In the present work, we have stably expressed hCTR-1 and -2 in polarized MDCK cells to study their cell trafficking and resulting polarized distribution. Both isoforms are highly polarized to the basolateral plasma membrane domain, with a basolateral-to-apical ratio of ~40:1. Targeting experiments showed that this polarized expression is in part achieved by the direct delivery of newly formed hCTRs to the basolateral surface. Comparison of the overall metabolic half-life of hCTRs with their turnover on the basolateral surface showed that hCTRs are retained in the basolateral plasma membrane domain.

EXPERIMENTAL PROCEDURES

Materials

Salmon calcitonin (sCT) was a generous gift from Dr. R. Gamse (Sandoz Pharma, Basel, Switzerland). The following materials and substances were purchased commercially: 24-mm and 12-mm Transwell inserts with polycarbonate membrane filters (0.4-μm pore size), from Corning Costar (Cambridge, MA); FCS, DMEM, antibiotic/antimycotic, and G418 (Geneticin) from Gibco Life Sciences; acetonitrile, trifluoroacetic acid, biotin-LC-hydrazide, streptavidin-agarose beads, N-ethylmaleimide (NEM), tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl), and disuccinimidyl suberate (DSS) from Pierce Chemical (Rockford, IL); carrier-free Na125I, EasyTag EXPRESS protein labeling mix, [3H](3-methyl-hystidinyl)TRH (MeTRH), and myo-[2-3H(N)]inositol from DuPont NEN (Boston, MA); restriction endonucleases and DNA modifying enzymes from New England Biolabs (Beverly, MA); M2 anti-FLAG monoclonal antibody from Kodak/Bi (New Haven, CT); Texas red-conjugated donkey anti-mouse IgG from Jackson Immunoresearch (West Grove, PA); and nitro blue tetrazolium (NBT) and 5-bromo-1-chloro-3-indolyl phosphate (BCIP) from Promega (Madison, WI). All other reagents were obtained from Sigma (St. Louis, MO).

Plasmid Constructions

Construction and expression of the synthetic cDNAs encoding the wild-type hCTRs were described previously (24). Subclonings were performed using standard molecular biological techniques, as described (24). To improve the ability of the M2 anti-FLAG monoclonal antibody to immunoprecipitate hCTRs specifically and quantitatively, we produced an hCTR-2 construct containing two FLAG epitope tags (pFLAG2-hCTR-2; DYKDDDDKGDYKDDDDK) attached in tandem to its amino-terminal segment (see Fig. 3). To accomplish this, we used a previously described construct that contains a DNA sequence encoding the leader sequence of the pre-pro hormone prolactin, followed by a FLAG epitope tag immediately upstream of the putative mature sequence for hCTR-2 (pFLAG-hCTR-2) (6). In brief, pFLAG-hCTR-2 was cut with the restriction endonuclease Hind III and ligated with a double-stranded DNA cassette produced by annealing the pair of oligonucleotides FLAG-Hind III sense (5′-AG CTT GGG GAC TAC AAG GAC GAC GAC A-3′) and FLAG-Hind III antisense (5′-AG CTT GTC GTC GTC GTC GTC TTC GTA GTC CCA A-3′). Correct orientation of the cassette was verified by automated DNA sequencing. pFLAG-hCTR-2 cDNA was subcloned into the EcoRI I and Not I sites of the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA), which contains a neomycin (G418) resistance gene, to allow for the selection of stable transfectants.
Culture and Transfection of MDCK Type II Cells

Parental MDCK type II cells (clone no. 9) were kindly provided by Dr. David Sabatini (Department of Pathology, New York University Medical Center). MDCK II cells were grown at 37°C in a humidified atmosphere containing 5% CO2 in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 mg/ml amphotericin B. MDCK II cells were stably transfected with the wild-type and FLAG epitope-tagged hCTRs either by electroporation (clones A and B only; see Polarity of Wild-Type hCTR-2 in Stably Transfected MDCK Cells) or by the calcium phosphate precipitation method. Stable transfectants were selected for resistance to G418. Individual cell lines were cloned using cloning cylinders. Cell lines or pooled clones stably expressing a wide range of hCTRs were used in these experiments (from 1.4 fmol to 5.3 pmol receptors/mg of protein). MDCK cell lines were seeded on polycarbonate filter supports (1 × 10^6-1.5 × 10^9 cells/24-mm filter or 250,000 to 300,000 cells/12-mm filter) and cultured for 1 wk with medium changed daily before they were used in an experiment.

Leak Test

The integrity of the impermeable barrier formed by the MDCK monolayer grown on polycarbonate filters was tested by measuring the Transwell leakage of 0.1 nM [3H]MeTRH from the upper (apical) chamber to the lower (basolateral) side over time. MeTRH is a peptide analog of TRH that normally is not transported by the epithelial cells and to which no receptors are found to be endogenously expressed in MDCK cells (34). The test was performed routinely before each experiment at 37°C with [3H]MeTRH dissolved in growth medium. Passage of radioactivity from the apical to the basolateral side typically amounted to between 0.3 and 1% of counts added to the apical chamber. This passage of radioactivity is due in part to degradation of the trace added to the growth medium and occurrence of passage of the breakdown products to the basolateral chamber. This is attested to by an increase in TCA solubility of the radioactivity present in medium from ~5% apical to ~50% basolateral. Filters that allowed passage of >2% of dpm added/h were not used in experiments.

cAMP was generated as follows. Cells were incubated in Hanks' balanced salt solution (HBSS) containing 1 mM IBMX and varying concentrations of sCT for 1 h at 37°C. cAMP was measured as described (24).

Methods to Determine Steady-State Distribution of hCTRs in Polarized MDCK Cells

Receptor binding studies. Receptor binding was performed in cells grown in replicate 12- or 24-mm Transwell filters essentially as described (24), except that the binding solution containing the trace was incubated vectorially (either in upper or lower chambers). Equilibrium was reached in 3 h at 4°C. Binding solution consisted of HBSS supplemented with 1 mg/ml BSA, 1 mg/ml bacitracin, and 1 mM phenylmethylsulfonyl fluoride. Binding was performed with ~0.7 nM [125I]-sCT (specific activity ~2,200 Ci/mmol) and the indicated concentrations of unlabeled sCT. Nonspecific binding was determined in the presence of ≥1,000-fold molar excess of unlabeled sCT (1 µM). Nonspecific binding amounted to <5% of the total binding on the basolateral plasma membrane domain. Due to the very low level of expression of hCTRs on the apical side, nonspecific binding was ~60% of the total binding on the apical plasma membrane domain. To rule out the possibility that low specific binding of [125I]-sCT to the apical plasma membrane (see Fig. 1) was due to hydrolysis of the radioligand by the activity of proteases present on the apical side, we performed 10% (wt/vol) TCA-1% (wt/vol) phosphotungstic acid precipitation of free [125I]-sCT collected from both apical and basolateral sides. After incubation with polarized cells at 4°C for up to 180 min, there was no difference in the TCA-precipitable radioactivity present in the medium collected from the two chambers. They were not different from TCA precipitation of [125I]-sCT before it made contact with cells, suggesting that there was not a significant degradation of the ligand under the conditions of the binding experiment.

Receptor cross-linking. Cells grown on 24-mm Transwell filters were submitted to selective binding of [125I]-sCT, as described above. At equilibrium, free [125I]-sCT was removed by washing, and cells were selectively incubated for 2 h at 4°C with 1 mM DSS dissolved in PBS (pH 7.0) containing 0.1 mM CaCl2, 1 mM MgCl2, and 10% DMSO. Then, excess DSS was quenched by washing cells with PBS supplemented with 50 mM Tris·HCl (pH 7.5). Cells were lysed at 70°C with SDS-PAGE sample buffer containing 20 mM TCEP·HCl and 5 mM EDTA. Then, lysates received NEM to a final concentration of 50 mM, followed by incubation at 50°C for 45 min to alkylate free sulfhydryls before resolution of the samples in a 7.5% SDS-PAGE gel.

Vectorial biotinylation followed by cell surface domain-specific precipitation of proteins in polarized MDCK cells. MDCK cells grown on 24-mm Transwell filters had their cell surface glycoproteins of either the apical or the basolateral plasma membrane domain covalently labeled by biotin-LC-hydrazide as described by Lisanti et al. (18). In short, the carbohydrate moieties of the cell surface glycoproteins were oxidized by vectorially incubating cells with 10 mM sodium metaperiodate in PBS containing 0.1 mM CaCl2 and 1 mM MgCl2. Then, oxidized sugars reacted with 2 mM biotin-LC-hydrazide in 100 mM sodium acetate buffer (pH 5.5) containing 0.1 mM CaCl2 and 1 mM MgCl2. After this procedure, cells were solubilized with RIPA buffer [150 mM NaCl, 50 mM Tris·HCl (pH 8.0), 0.5 mM EDTA, 0.5% deoxycholate, 1% Nonidet P-40, and 0.1% SDS] and plasma membrane-domain-specific proteins were fractionated using streptavidin-agarose affinity chromatography. After elution with SDS-PAGE sample buffer, fractions were resolved by SDS-PAGE, followed by electrophoretic transfer to nitrocellulose filters. Filters were probed with M2 anti-FLAG monoclonal antibody and rabbit anti-mouse IgG antibodies conjugated to alkaline phosphatase to develop color, using NBT and BCIP as substrates.

Immunocytochemical analyses of polarized MDCK cells grown on polycarbonate filters. Cells grown on 12-mm Transwell filters were washed with PBS containing 0.1 mM CaCl2 and 1 mM MgCl2 and fixed with 2% paraformaldehyde in PBS buffer for 15 min at room temperature. Paraformaldehyde was quenched with 0.05 M NH4Cl in PBS, and cells were permeabilized with 0.075% saponin in PBS. Permeabilized cells were blocked with PBS containing 2% BSA and then incubated with 10 µg/ml M2 anti-FLAG monoclonal antibody and rabbit anti-mouse IgG antibodies conjugated to alkaline phosphatase to develop color, using NBT and BCIP as substrates. Immunocytochemical analyses of polarized MDCK cells grown on polycarbonate filters. Cells grown on 12-mm Transwell filters were washed with PBS containing 0.1 mM CaCl2 and 1 mM MgCl2 and fixed with 2% paraformaldehyde in PBS buffer for 15 min at room temperature. Paraformaldehyde was quenched with 0.05 M NH4Cl in PBS, and cells were permeabilized with 0.075% saponin in PBS. Permeabilized cells were blocked with PBS containing 2% BSA and then incubated with 10 µg/ml M2 anti-FLAG monoclonal antibody and rabbit anti-mouse IgG antibodies conjugated to alkaline phosphatase to develop color, using NBT and BCIP as substrates. Immunocytochemical analyses of polarized MDCK cells grown on polycarbonate filters. Cells grown on 12-mm Transwell filters were washed with PBS containing 0.1 mM CaCl2 and 1 mM MgCl2 and fixed with 2% paraformaldehyde in PBS buffer for 15 min at room temperature. Paraformaldehyde was quenched with 0.05 M NH4Cl in PBS, and cells were permeabilized with 0.075% saponin in PBS. Permeabilized cells were blocked with PBS containing 2% BSA and then incubated with 10 µg/ml M2 anti-FLAG monoclonal antibody and rabbit anti-mouse IgG antibodies conjugated to alkaline phosphatase to develop color, using NBT and BCIP as substrates.
After the starvation period, cells were pulse labeled in the cysteine-free DMEM supplemented with 10% dialyzed FCS. Newly synthesized hCTRs. MDCK cells grown on 24-mm Transwell filters (3 filters-time point-1-plasma membrane domain-1) were incubated for 120 min in methionine- and cysteine-free DMEM supplemented with 10% dialyzed FCS. After the starvation period, cells were pulse labeled in the same medium to which 1 mCi/ml of [35S]methionine-cysteine mixture (EasyTag EXPRESS protein labeling mix) was applied basolaterally for increasing periods of time. Cells were chilled by washing with PBS (pH 7.4) supplemented with 0.1 mM CaCl2 and 1 mM MgCl2 at 4°C to halt cell trafficking immediately following the pulse period, and cell surface glycoproteins were biotinylated on each domain using biotin-LC-hydrazide. Then, cells were detergent solubilized with RIPA buffer, and cell lysate was preclarified with Sepharose 4-B (100 µl packed beads-lysat). The total receptor pool was immunoprecipitated by adding 10 µg/ml lysate of M2 anti-FLAG monoclonal antibody and goat anti-mouse polyclonal IgG conjugated to agarose beads sequentially. After the agarose beads were washed six times with lysis buffer, the immunoprecipitate was eluted two times from the beads with RIPA buffer containing 2% SDS at 95°C. The vectorial appearance on the plasma membrane (apical or basolateral) was determined by fractionation of the immunoprecipitate, diluted with SDS-free RIPA buffer to 0.1% SDS, by streptavidinagarose affinity chromatography to detect plasma membrane domain-specific receptors. Beads were eluted twice with Laemmli sample buffer supplemented with 20 mM TCEP-HCl and 5 mM EDTA (pH 6.8) at 100°C. Then, lysates received NEM to a final concentration of 50 mM followed by incubation at 50°C for 45 min to alkylate free sulfhydryls before resolution of the samples in a 7.5% SDS-PAGE gel. The gel was exposed to a phosphor-screen, and specific bands were quantified using the phosphorimag software.

Determination of overall metabolic and cell surface-specific half-lives of hCTRs. MDCK cells grown on 24-mm Transwell filters were labeled for 36 h with 0.2 mCi/ml of [35S]methionine-cysteine mixture in methionine- and cysteine-free DMEM supplemented with 10% dialyzed FCS and 10% normal growth medium. After labeling, cells were incubated with DMEM supplemented with 2 mM methionine and 4 mM cysteine (chase medium) for the indicated periods of time. Then, cells were vectorially biotinylated, as described above. The total receptor pool was immunoprecipitated, and a fraction of immunoprecipitated receptors was used to determine the overall metabolic half-life of hCTRs. The plasma membrane domain-specific receptor half-lives were determined by fractionation of the immunoprecipitate using streptavidin-agarose affinity chromatography, as described above.

RESULTS

Polarity of Wild-Type hCTR-2 in Stably Transfected MDCK Cells

We first showed that untransfected MDCK type II cells do not express CTRs. Radioligand binding assays using ~0.3 nM [125I]-sCT (specific activity ~2,200 Ci/mmol) showed no specific binding. Furthermore, incubation with high concentrations (1–10 µM) of sCT of cells prelabeled with myo-[3H]inositol in the presence of 10 mM LiCl and 1 mM IBMX did not show any increase of intracellular cAMP or inositol phosphates (IPs) over the basal levels (24). These results were identical using MDCK cells growing on normal tissue culture plastic substrates and on polycarbonate filters (polarized cells). Therefore, polarized MDCK cells constitute a good model system to study the heterologous expression of CTRs by transfecting cDNAs of wild-type and mutant CTRs, without the complications of endogenously expressed receptors.

We established permanent cell lines expressing two different levels of the wild-type hCTR-2, as determined by the specific binding of [125I]-sCT. Clone A expresses ~1.4 fmol receptors/mg protein, and clone B expresses ~17.6 fmol receptors/mg protein. These cell lines, when grown on polycarbonate filters, maintain the polarized characteristics of the parental cell line, with a transepithelial electrical resistance between 200 and 300 Ω·cm² (34). We determined the distribution of CTRs on polarized MDCK cells expressing these two different levels of hCTR-2. Binding of ~0.7 nM [125I]-sCT applied either to the apical or basolateral domains of cells grown on 24-mm Transwell filters showed a very asymmetrical distribution of specifically bound radioactivity that was independent of the level of expression of hCTR-2. Whereas there was only very little specific binding of [125I]-sCT to the apical plasma membrane, there was clearly distinct specific binding to the basolateral domain, suggesting that >98% of the CTRs are expressed on the basolateral side at the steady state. Comparison of the dose-dependent inhibition of binding of [125I]-sCT by sCT between apical and basolateral sides of clone B (Fig. 1) revealed that these cells express on the apical plasma membrane domain ~27% of the number of receptors expressed on the basolateral side. This represents ~0.42 ± 0.2 fmol receptors/mg protein on the apical side and 15.1 ± 1.7 fmol receptors/mg protein on the basolateral side (means ± SE, n = 14 filters). Calculated Michaelis-Menten inhibition constant (Ki) values were identical for receptors present on both plasma membrane domains: Ki = 3.3 nM on basolateral side (n = 2; range 3.2–3.4 nM) and Ki = 3.4 nM on the apical side (n = 2; range 2.8–4.0 nM). These results indicate that the difference in binding between the two domains is due to a difference in the number of receptors expressed at each side rather than a difference in the affinity for binding [125I]-sCT by hCTR-2 expressed at each plasma membrane domain. Therefore, the expression of hCTR-2 in MDCK cells is distributed with a ratio of receptors on the basolateral plasma membrane domain to receptors on the apical plasma membrane...
domain of ~40:1. Similar experiments performed with an MDCK cell line expressing hCTR-1, with ~350 fmol receptors/mg protein, showed that this other isoform is also highly polarized, with equivalent basolateral-to-apical ratios of ~40:1 (Fig. 2).

FLAG2-hCTR-2 is Functionally Indistinguishable From Wild-Type hCTR-2 When Expressed in Polarized MDCK Cells

One of the essential requirements for analyzing targeting mechanisms of expressed proteins in MDCK cells is the ability to immunoprecipitate these proteins quantitatively. With this objective, we have produced an hCTR-2 construct made to express a double FLAG epitope tag at its amino-terminal segment (Fig. 3). The double FLAG epitope tag improves the ability of the M2 anti-FLAG monodonal antibody to immunoprecipitate hCTRs specifically and quantitatively. We selected this location because it would be unlikely to interfere with receptor function. Epitope tags in the amino terminus of GPCRs have worked well in many instances (12, 15). We have previously shown that a similar construct containing a single FLAG epitope tag hCTR-2 is functionally indistinguishable from the wild-type receptor (6, 8).

We established permanent cell lines expressing different levels of the FLAG2-hCTR-2, as determined by saturation binding of 125I-sCT. We elected to work with a cell line expressing between 3.5 and 5.3 pmol receptors/mg protein, a much higher receptor density than we used previously, to ensure detectability by the methods we used to study trafficking of membrane proteins in polarized epithelial cells. We characterized the FLAG2-hCTR-2 construct in this cell line. It behaves in a manner identical to its parental wild-type hCTR-2 counterpart with respect to ligand binding for 125I-sCT and second messenger formation. In fact, unlike the MDCK cell lines expressing much lower levels of the wild-type hCTR-2, this cell line expressing FLAG2-hCTR-2 showed a distinct dose-response for stimulation by sCT of both cAMP (see Fig. 6) and IP formation [EC50 = 11.82 ± 1.33 nM (mean ± SE); 2.82-fold stimulation; not shown]. These results indicate, similarly to what was observed with other GPCRs with dual signaling capacity, that stimulation of the PPI-PLC signaling pathway is highly sensitive to receptor number. Activation of this signaling pathway at low levels of receptor expression, as manifested by an increase in IP levels, is usually not detectable (25). We demonstrated, by determining the differential distribution of the endogenous apical (gp114) (17) and basolateral (E-cadherin) (32) markers, that monolayers of this particular cell line become polarized in a fashion that is indistinguishable from their parental MDCK cell line. Immunostaining of gp114 and E-cadherin in this cell line expressing FLAG2-hCTR-2 showed predominant apical and basolateral distribution, respectively (not shown). Immunoblot of plasma membrane domain-specific cell surface membrane proteins obtained by vectorial biotinylation of each plasma membrane domain, followed by streptavidin-agarose chromatography, shows identical basolateral distribution of E-cadherin in both the parental cell line and in cells expressing FLAG2-hCTR-2 (Fig. 4). This cell line, when grown on polycarbonate filter support, expresses FLAG2-hCTR-2 with a distribution of receptors that is ~40-fold more basolateral than apical, as determined by binding of 125I-sCT. This level of polarized expression is similar to what was determined for the wild-type hCTR-2 expressed at much lower levels of expressed receptors (Fig. 1). These results demonstrate that the polarized distribution of hCTRs is independent of the expression levels. Taken together, these results indicate that the tagged receptors are processed by MDCK cells identically to the wild-type hCTRs and that the tag does not interfere with receptor function. Figure 5 demonstrates by cross-linking of radioiodinated ligand to its receptor that the steady-state distribution of FLAG2-hCTR-2 protein on polarized MDCK cells is highly polarized to the basolateral plasma membrane.
domain. $^{125}$I-sCT was applied either to apical or to basolateral plasma membrane domains, and bound radioligand was cross-linked to the receptor with the homobifunctional cross-linker DSS. The basolateral $^{125}$I-sCT-bound FLAG 2-hCTR-2 migrated in an SDS-PAGE gel with an apparent molecular mass of 86 kDa. The apparent molecular mass was higher than the calculated molecular mass of the receptor based on its amino acid sequence. This is because hCTR-2 is glycosylated, a characteristic frequently found in the amino terminus of other GPCRs (8). Also, glycosylation of the receptor is suggested by the broad appearance of the specific band. There was a very slight labeling of FLAG2-hCTR-2 present on the apical side, consistent with the low expression level on the apical domain as determined by binding alone.

cAMP Signaling by hCTR-2 in Polarized MDCK Cells

We investigated the functional polarity of expressed FLAG2-hCTR-2 by comparing stimulation of cAMP formation caused by the separate application of sCT to the apical and basolateral domains of MDCK cells grown on permeable filter supports. Similar results were obtained with MDCK cells expressing wild-type hCTR-2 (clone B with ~17.5 fmol receptors/mg protein; not shown). We found a dose-related increase in generation of intracellular cAMP by stimulation with sCT on both apical and basolateral domains (Fig. 6). Figure 6A shows the dose-response curves of cAMP generation produced by sCT stimulation of the basolateral receptors. The EC$_{50}$ for stimulation of intracellular cAMP generation was 0.32 ± 0.12 nM (mean ± SE). Figure 6B shows that, as expected, the efficacies of cAMP stimulation by the population of receptors present at each plasma membrane side are different. This comparison was performed using 1 nM sCT, a concentration of agonist that caused near maximal stimulation of cAMP generation by the basolateral receptors (see Fig. 6A). Whereas basolateral application of 1 nM sCT produced an increase in cAMP levels of ~20-fold over basal, apical application of sCT increased cAMP levels only ~3-fold over basal.
Anti-FLAG M2 Monoclonal Antibody Confirms That Steady-State Distribution of Epitope-Tagged hCTR-2 Is Polarized to Basolateral Plasma Membrane Domain of MDCK Cells

Because there was a possibility that, for an undetermined reason, a proportion of apically expressed hCTR-2 was not detectable by binding of radiiodinated sCT, we used a radioligand-independent method to confirm that the steady-state distribution of FLAG$_2$-hCTR-2 is polarized to the basolateral surface of MDCK cells grown on filters. Plasma membrane domain-specific cell surface membrane glycoproteins were separately labeled by vectorial biotinylation of each plasma membrane domain with biotin-LC-hydrazide. Then, cells were solubilized with RIPA buffer, and cell surface membrane domain-specific glycoproteins were separated by precipitation of biotinylated glycoproteins with streptavidin-agarose beads. By performing Western blot using the anti-FLAG M2 monoclonal antibody, we demonstrate that the FLAG$_2$-hCTR-2 protein is detectable only on the basolateral plasma membrane domain of cells expressing the epitope-tagged hCTR-2 (Fig. 7).

Immunolocalization of expressed FLAG$_2$-hCTR-2, visualized either on an epifluorescence microscope or on a confocal microscope, corroborated our findings that the FLAG$_2$-hCTR-2 protein is highly polarized to the basolateral plasma membrane domain. Immunostaining with M2 anti-FLAG monoclonal antibody and a Texas red-conjugated anti-mouse second antibody of a clonal

Fig. 5. Steady-state distribution of double FLAG epitope-tagged hCTR-2 expressed in MDCK cells is polarized to basolateral domain, as determined by cross-linking of prebound $^{125}$I-sCT to apical and basolateral surfaces. MDCK cells either expressing (lanes 1–4; ~3.5 pmol receptors/mg protein) or not expressing (lanes 5 and 6; control, untransfected) FLAG$_2$-hCTR-2 grown on 12-mm Transwell filters were incubated with ~1 nM $^{125}$I-sCT as described for Fig. 1 and in EXPERIMENTAL PROCEDURES. At equilibrium, cells were washed and cross-linked (X-linked) with disuccinimidyl suberate as described in EXPERIMENTAL PROCEDURES. Some Transwell filters were further processed by domain-selective biotinylation procedure using biotin-LC-hydrazide (Biot; lanes 3, 4, and 6), as described in EXPERIMENTAL PROCEDURES. Cells were extracted in Laemmli sample buffer containing 20 mM TCEP·HCl, and free sulfhydryls were alkylated with N-ethylmaleimide before resolution of samples in a 7.5% SDS-PAGE gel. Dried gels were exposed to a storage phosphor autoradiography screen and analyzed by Storm system (Molecular Dynamics). Right: molecular mass markers (kDa). Arrow, position of cross-linked $^{125}$I-sCT:FLAG$_2$-hCTR-2 complex showing that >95% of FLAG$_2$-hCTR-2 are labeled from basolateral surface. There are bands that migrate at higher positions on gel (similar to experiments in Figs. 7 and 9) that probably correspond to receptors that either aggregate to each other or to other proteins to which they may be tightly associated. Biotinylation procedure did not in any respect change pattern of migration of cross-linked $^{125}$I-sCT:FLAG$_2$-hCTR-2 complexes (lanes 2 and 4 for comparison).

Fig. 6. Functional polarity of agonist-induced production of cAMP second messenger evidenced by separate application of sCT to apical or basolateral membranes of MDCK cells expressing FLAG$_2$-hCTR-2. A: polarized MDCK cells stably expressing FLAG$_2$-hCTR-2 grown on 12-mm Transwell filters for 7 days were incubated in Hank’s balanced salt solution containing 1 mM IBMX and indicated concentrations of sCT applied to basolateral cell surface for 1 h at 37°C. Intracellular accumulation of cAMP was measured by radioimmunoassay as described in EXPERIMENTAL PROCEDURES. B: comparison of intracellular cAMP accumulation in cells cultured as in A, incubated for 1 h at 37°C with 1 nM sCT from either apical or basolateral chamber. Data are means and range for duplicate determinations in a representative experiment. Total of 3 experiments produced similar results.
cell lines. We examined several different clones expressing FLAG2-hCTR-2. We also used the immunomagnetic cell separation technique with anti-FLAG M2 monoclonal as the first antibody and Dynabeads M-450 (Dynal, Great Neck, NY) coated with sheep anti-mouse IgG1 or recloned cells expressing FLAG2-hCTR-2 without a significant increase in uniformity of expression. We think that this heterogeneity did not distort our results on the polarized expression of hCTR. Positive cell surface staining was also obtained by supravital incubation with the first antibody on the basolateral side, indicating that the amino-terminal domain of FLAG2-hCTR-2 is normally exposed to the medium (data not shown). These results also show that cellular processing and insertion in the plasma membrane of these receptors did not remove their amino-terminal tags. Cells that were not transfected or that were transfected with the wild-type (not tagged) hCTR-2 did not show any specific or similar staining (not shown), proving the specificity of the procedure.

Figure 8A shows a computer-generated projection image of horizontal sections obtained by confocal microscopy. It shows a characteristic honeycomb pattern, which indicates that FLAG2-hCTR-2 is present predominantly on the basolateral plasma membrane domain of polarized MDCK cells grown on filters. Figure 8D shows a confocal image of an optical section (x-y-section) 0.31 mm thick at mid height of the monolayer of polarized MDCK cells. There was marked staining of the lateral aspect of the basolateral plasma membrane domain. Examination of a stack of sections going from the filter (basal) to the top (apical) of the monolayer along the z-axis (not shown) indicates that the specific staining is limited almost exclusively to the lateral plasma membrane domain. This specific and restricted

Fig. 7. Immunoblot of cell surface fractionated glycoproteins corroborates that hCTR-2 is polarized to basolateral side of MDCK cells at steady state. Parental cells (control; lanes 2 and 3) or cells expressing FLAG2-hCTR-2 (lanes 4 and 5) grown on Transwell filters were domain-selective biotinylated by applying biotin-LC-hydrazide to either apical (lanes 2 and 4) or basolateral (lanes 3 and 5) cell surface. Then, they were processed exactly as described for Fig. 4, except that blot was probed with anti-FLAG M2 monoclonal antibody. Arrow, —86-kDa-specific FLAG2-hCTR-2 band detected only on basolateral blot was probed with anti-FLAG M2 monoclonal antibody. Arrow, Then, they were processed exactly as described for Fig. 4, except that their expression of FLAG2-hCTR-2. This allowed for some cells that did not stain, suggesting that they lost intensity of the staining varied among cells. There were membrane. The majority of the cells were stained, albeit the surface, suggesting localization in the plasma membrane domain-selective biotinylated by applying biotin-LC-hydrazide to either apical (lanes 2 and 4) or basolateral (lanes 3 and 5) cell surface. Then, they were processed exactly as described for Fig. 4, except that blot was probed with anti-FLAG M2 monoclonal antibody. Arrow, —86-kDa-specific FLAG2-hCTR-2 band detected only on basolateral side of cells expressing FLAG2-hCTR-2. Results are representative of 3 independent experiments.
lateral plasma membrane staining can best be visualized in computer-generated cross sections (x-z sections, Fig. 8C). There was no significant staining either of the apical plasma membrane domain or of any intracellular structure. Figure 8B shows an optical section (x-y section) at the basal region of the cells. The basal region of the membrane did not stain, except for some spots of intense specific staining. These spots, visualized in the projection images (Fig. 8A) or under the epifluorescence microscope (not shown), are clearly present on the cell surface. They are caused either by irregularities in the basalmost height at which the lateral plasma membrane staining starts to appear or by patches of receptors restricted to the basal membrane (x-y section, Fig. 8B; x-z sections, Fig. 8C). Some of these possibly correspond to cell processes that emanate from the lateral membrane and extend to the intercellular space, as can usually be seen by electron microscopy of this columnar epithelium. The lack of intracellular localization of FLAG2-hCTR-2 is in direct contrast to what we observed in COS-1 cells transiently expressing epitope-tagged hCTR-2 (8). These results show that transfected MDCK cells expressing very high levels of receptor protein are better able than COS-1 cells to synthesize, process, and deliver hCTR-2 to the cell surface without being overwhelmed in the folding, processing, and transport machinery.

Polarized Cell Surface Delivery of Newly Synthesized FLAG2-hCTR-2 in Polarized MDCK Cells

We performed separate measurements of the direct cell surface delivery of newly synthesized FLAG2-hCTR-2 from the biosynthetic pathway to each plasma membrane domain. Polarized MDCK cells grown on Transwell filters expressing or not expressing (control) FLAG2-hCTR-2 were pulse labeled with [35S]methionine-cysteine mixture for increasing periods of time. Then, immediately following the pulse period, plasma membrane glycoproteins were biotinylated using the domain-specific (apical or basolateral) biotinylation procedure described in EXPERIMENTAL PROCEDURES. The biotinylation was performed at 4°C to halt cell trafficking. Cells were detergent solubilized, and the total receptor pool was immunoprecipitated as described in EXPERIMENTAL PROCEDURES. After elution from goat antimouse beads, the immunoprecipitate was further fractionated by streptavidin-agarose affinity chromatography to detect plasma membrane domain-specific receptors. Samples were resolved by SDS-PAGE, and specific bands were visualized by autoradiography using a phosphorimager screen. Figure 9 demonstrates that delivery of newly formed FLAG2-hCTR-2 is directed to the basolateral plasma membrane domain with essentially no detectable appearance, at this level of sensitivity, of de novo-synthesized FLAG2-hCTR-2 protein to the apical side. At 30 min of pulse, there was already a faint specific band running at ~86 kDa, with a molecular mass similar to that we detected in the steady-state studies (Figs. 5 and 7). The intensity of the specific band increases progressively up to the last pulse time point. In addition, there are specific bands of higher molecular mass than the ~86 kDa band that appears with identical kinetics. In all experiments that we performed to detect the FLAG2-hCTR-2, including metabolic labeling with [35S]methionine-cysteine (Fig. 9), cross-linking with radiolabeled agonist (Fig. 5), and immunoblotting with the anti-FLAG antibody (Fig. 7), we have always detected a significant proportion of the specific signal present as higher molecular mass species. In the cross-linking experiment shown in Fig. 5, we eliminated the biotinylation procedure as a cause of the observed aggregation, as has been suggested to occur with the FSH, LH, and thyrotropin receptors (3), because there was no difference in the profile of FLAG2-hCTR-2 cross-linked with 125I-sCT followed or not by biotinylation. This suggested to us that, following cell lysis, the receptor molecules aggregate either to each other or to other proteins with which they may be tightly associated. In general, our attempts to reduce the amount of high-molecular-mass material by denaturing our samples in the presence of a strong reducing agent (TCEP·HCl) followed by alkylation of the free sulfhydryls with NEM were only partially successful.
Basolaterally Delivered FLAG2-hCTR-2 Are Retained in the Basolateral Plasma Membrane Domain With a Half-Life Similar to Overall Metabolic Half-Life of Receptor

To verify to what extent basolateral plasma membrane domain retention contributes to the steady-state distribution of hCTRs in polarized MDCK cells, we determined the half-life of FLAG2-hCTR-2 in each plasma membrane domain. Polarized MDCK cells grown on Transwell filters were labeled with [35S]methionine-cysteine mixture for 36 h and then chased in the presence of excess unlabeled cysteine and methionine for increasing periods of time. Then, plasma membrane proteins were biotinylated using the selective (apical or basolateral) biotinylation procedure. Cells were detergent-solubilized, and the total receptor pool was immunoprecipitated. Figure 10A shows total [35S]-labeled immunoprecipitated proteins obtained from cells that were biotinylated at the apical or basolateral side at each chase period. This demonstrates that recovery of [35S]FLAG2-hCTR-2 was similar for each biotinylation condition at each time point. More importantly, it permits calculation of the metabolic half-life of total cell FLAG2-hCTR-2. The FLAG2-hCTR-2 shows an overall metabolic half-life of 9–12 h. Immunoprecipitated proteins were further fractionated by streptavidin-agarose affinity chromatography to detect plasma membrane domain-specific receptors. Figure 10B shows retained [35S]FLAG2-hCTR-2 on the apical or on the basolateral surfaces over time. It is apparent that [35S]FLAG2-hCTR-2 are retained in each plasma membrane domain for an extended period of time. Calculated half-lives of FLAG2-hCTR-2 in each plasma membrane domain are not very different from the overall metabolic half-life of the total cell population of receptors.

**DISCUSSION**

In this report, we show that two isoforms of hCTR, hCTR-1 and hCTR-2, are expressed at the steady state in a highly polarized manner on the basolateral plasma membrane domain of MDCK cells. The polarized distribution of wild-type hCTRs was demonstrated by equilibrium binding of 125I-sCT in intact cells grown on permeable polycarbonate filter supports. The polarity of sCT-induced cAMP accumulation attested to the asymmetrical distribution of functional hCTRs. Because the function and polarized expression of hCTRs were not disturbed by attaching a double FLAG epitope tag into the amino terminus of hCTRs, we were able to use immunologic methods to study the distribution and trafficking of hCTRs in MDCK cells. Selective cell surface biotinylation followed by immunoblotting of streptavidin-agarose-fractionated cell surface membrane glycoproteins confirmed the polarized distribution of hCTR-2 on the basolateral plasma membrane. Immunolocalization of double FLAG epitope-tagged receptors showed that hCTRs are concentrated on the lateral aspect of the basolateral membrane. These results independently confirmed that the expression of hCTRs in MDCK cells is polarized. These findings corroborated the notion that the observed differences obtained by radioligand binding methods are indeed due to differences in the actual number of receptors expressed in each plasma membrane domain rather than to a population of apically located receptors that do not bind radioligand with detectable affinity. We were thus able to use simple equilibrium binding assays with radiiodinated ligand in intact cells to detect and precisely quantify the distribution of hCTRs between the basolateral and apical sides. This method we could estimate that the expression of hCTRs in MDCK cells is polarized, with a basolateral-to-apical receptor ratio of ~4:1.

The exclusive direct delivery of de novo-synthesized receptors to the basolateral side demonstrates that the predominant mechanism underlying polarity of hCTRs in MDCK cells is by sorting newly formed hCTRs into vesicles, most likely in the TGN, that are destined to be delivered to the basolateral plasma membrane. The almost exclusive basolateral distribution of hCTRs at the steady state suggests that basolateral delivery of hCTRs from the biosynthetic pathway is associated...
with retention of these proteins in the basolateral plasma membrane domain. This was evaluated by comparing the total receptor population overall metabolically half-life with the rate of turnover of basolateral hCTRs. Indeed, we found that the majority of basolaterally delivered receptors are retained in this domain. Consequently, retention makes an important contribution to the maintenance of the polarized distribution of hCTRs.

What is the origin of the apical hCTRs that we detected by binding or cAMP stimulation? There are three distinct mechanisms that alone or in combination could account for the apical expression of hCTRs. First, polarized expression of molecules in MDCK cells is usually not absolute. There is always a proportion of molecules with primary targeting to one domain but which is found in the opposite domain. The proportion of molecules found in each domain would depend, among other factors, on the dominance of the targeting signal to its respective domain (reviewed in Refs. 4, 22, 28). The cell surface arrival assay of newly formed receptors did not show the origin of the apical hCTRs. It is very likely that the number of counts of [35S]FLAG2-hCTR-2 incorporated into the apical plasma membrane domain during the pulse experiments was too low to be measurable in the targeting experiments, impeding an accurate assessment of delivery of hCTRs to the apical membrane. Therefore, it remains possible that the receptors detected in the apical plasma membrane domain result, at least in part, from direct apical delivery of hCTRs. Alternatively, the sorting of hCTRs in the TGN may not be as striking as the polarity observed at the steady state, but the small fraction of receptors delivered to the apical plasma membrane domain may be rapidly removed by endocytosis and may be either transported to the opposite plasma membrane domain by transcytosis or targeted to lysosomes for degradation. This possibility was virtually eliminated by the observation that the half-life of hCTRs on the apical surface is as long as the half-life of hCTRs on the basolateral surface. Last, apical receptors may be derived in part from basolateral receptors that have been internalized and transported to the apical side by a limited transcytosis, as has been observed to occur with gonadotropin receptors (LH and FSH receptors) (3). At present, we cannot differentiate between the first and the last possibilities, but whatever is the mechanism by which hCTR are delivered to the apical plasma membrane domain, hCTRs are also retained in this domain.

Confocal microscopy of immunostained MDCK cells expressing FLAG2-hCTR-2 protein showed that basolateral receptor distribution is restricted to a lateral subdomain of the plasma membrane. Although basal immunofluorescence images may be quenched by the filter, quenching alone cannot account for the observed pattern. Indeed, spots of intense fluorescence localized on the basal membrane may be formed by patches of membrane containing receptors with restricted mobility. Restricted distribution to a lateral subdomain was observed in other GPCRs expressed in MDCK cells, like the three subtypes of α2-ADR (33). The lateral subdomain localization is shared by other polarized basolateral membrane proteins, such as Na+/K+-ATPase, transforming growth factor-α, or the transmembrane forms of the Ca2+-independent neural cell adhesion molecule (7, 23, 27). Na+/K+-ATPase was shown to form a specific complex involving ankryin, the epithelial spectrin protein fodrin, and the cell adhesion molecule uvomorulin. Several other glycoproteins appear to interact with these membrane-cytoskeleton complex proteins, which may spatially organize proteins on the plasma membrane (23). We do not know the reason for the restricted localization of FLAG2-hCTR-2 to the lateral aspect of the basolateral plasma membrane domain. The possibilities are either that hCTR-2 interacts with structural proteins, probably from the cytoskeleton, to maintain them at the lateral region of the basolateral plasma membrane or that the basal region of this domain is normally saturated by other proteins that themselves interact with the cytoskeleton and exclude other proteins from that site.

Sorting and direct delivery of a protein to a specific domain requires information built into the protein sequence or structure itself (reviewed in Refs. 4, 22, 28). Bona fide sorting signals have been identified for both apically and basolaterally directly targeted proteins. For single-span membrane proteins, two types of basolateral sorting signals were identified. One type overlaps, but is not identical to, either tyrosine-containing cytoplasmic sequences or a dileucine motif located upstream of acidic amino acids that mediate endocytosis via clathrin-coated pits. The important feature of these signals is a tight β turn formed by a conserved tetrapeptide, usually located at a distance (7 or more amino acids) from the transmembrane domain. This structure is recognized by cytosolic adaptor proteins that are constituents of clathrin coats. The other type of basolateral sorting signal does not overlap with endocytosis signals, but these signals tend to form a tight β turn as well, and it is followed by a nascent helix structure. This type of signal was identified in polymeric immunoglobulin receptor, transferrin receptor, vesicular stomatitis virus G protein, and low-density lipoprotein receptor (reviewed in Refs. 4, 22, 28). In the case of GPCRs, there are multiple domains that could potentially be involved in targeting the protein to a specific domain. These domains may combine to form a three-dimensional structure that serves as a sorting signal. The identification of the sequences or structural elements in targeting to and retention of hCTRs at the basolateral domain will involve the use of mutant receptors. So far, there have been only a few attempts to identify sorting signals in GPCRs through mutagenesis, and they were all negative. For example, extensive mutagenesis analysis of the basolaterally targeted α2A-ADR involving different functional domains, either alone or in combination, was essentially negative. It was concluded that, in this receptor, basolateral sorting information must be present within its membrane-spanning domains (14). In the TRH receptor, the steady-state basolateral distribution was not dependent on...
either coupling with G proteins or of a major endocytic motif that is present in its carboxy-terminal domain (34). In hCTRs, identical steady-state distribution of hCTR-1 and hCTR-2 indicates that the 16-amino acid insertion in the putative first intracellular loop of hCTR-1, although capable of disrupting receptor activation of PLC (24), does not interfere with polarized expression of hCTR. CTR was shown to couple to both Gαs and Gαq/11 families of α-subunit (25). We do not know the mechanism by which the 16-amino acid insertion interferes with coupling to Gαq/11, but our findings indicate that the steady-state distribution of hCTR is not reliant on coupling to this signaling pathway. It is conceivable that these negative results are caused in part by the presence of built-in redundancies in the system, with multiple and perhaps even competing signals that are utilized with a certain hierarchy, whose summation results in the final destination of the transported protein.

In summary, our results demonstrate that hCTR-2 is targeted preferentially to the basolateral plasma membrane domain but that retention on the basolateral side must contribute importantly to produce the striking polarity observed with hCTRs at the steady state. We can predict that there are specific amino acid residues and/or structures within the sequence of hCTRs that, alone or in combination, are presented to the intracellular sorting machinery as signal(s) that direct the final fate of hCTRs during vesicular traffic. These pathways are intimately related to the function of hCTRs, because hCTRs are normally expressed in polarized cells (renal tubule epithelia, neurons, osteoclasts) (2), where CTRs need to be expressed at the basolateral plasma membrane domain to have access to the incoming agonist from bloodstream or paracrine origin and to exert their regulatory function in the correct subcellular domain. The unveiling of signals involved in receptor targeting may help to better understand not only the physiology of hCTR, but also that of other members of the CTR subfamily, as well as of the entire GPCR superfamily.

NOTE ADDED IN PROOF

A basolateral targeting signal was recently localized in the carboxy-terminal domain of the follicle-stimulating hormone receptor (I. Beau, M. T. Groyer-Picard, A. Le Bivic, B. Vannier, H. Loosfelt, E. Milgrom, and M. Misrahi. The basolateral localization signal of the follicle-stimulating hormone receptor. J. Biol. Chem. 273: 18610–18616, 1998). We are grateful to Dr. M. C. Gershengorn for helpful discussion in the course of the work and for critical review of the manuscript. We thank Dr. B. M. A. Fontoura for comments on the manuscript.

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