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Endoplasmic reticulum export of adrenergic and angiotensin II receptors is differentially regulated by Sar1 GTPase

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Abstract

The molecular mechanism underlying the export of G protein-coupled receptors (GPCRs) from the endoplasmic reticulum (ER) remains largely unknown. In this manuscript, we investigated the role of Sar1 GTPase, which coordinates the assembly and budding of COPII-coated vesicles, in the cell-surface targeting, signaling and ER export of α_{2B}-adrenergic (α_{2B}-AR), β_2-AR and angiotensin II type 1 receptors (AT1R). The cell-surface expression of α_{2B}-AR, β_2-AR and AT1R, and receptor-mediated ERK1/2 activation were significantly attenuated by the GTP-bound mutant Sar1H79G, suggesting that export from the ER of these receptors is mediated through the Sar1-dependent COPII-coated vesicles. Interestingly, subcellular distribution analyses showed that α_{2B}-AR and AT1R were highly concentrated at discrete locations near the nucleus in cells expressing Sar1H79G, whereas β_2-AR exhibited an ER distribution. These data indicate that Sar1-catalyzed efficient GTP-hydrolysis differentially regulates ER export of adrenergic and angiotensin II receptors. These data provide the first evidence indicating distinct mechanisms for the recruitment of different GPCRs into the COPII vesicles on the ER membrane.

1. Introduction

G protein-coupled receptors (GPCRs) are a superfamily of cell surface receptors which couple to heterotrimeric G-proteins and regulate multiple downstream effectors such as adenylyl cyclases, phospholipases, protein kinases and ion channels [1-4]. All GPCRs share common structural features with a hydrophobic core of seven transmembrane-spanning α-helices, three intracellular loops, three extracellular loops, an N-terminus outside the cell and a C-terminus inside the cell. It has been well documented that the magnitude of receptor-elicited cellular response to a given signal is modulated by elaborately regulated intracellular trafficking which dictates the level of the receptor expression at the plasma membrane and the targeting of the receptor to specific subcellular compartments [5-8]. The life of GPCRs begins at the endoplasmic reticulum (ER) where they are synthesized, folded and assembled. Properly folded receptors are transported from the ER to the ER-Golgi intermediate complex (ERGIC), the Golgi apparatus and the trans-Golgi network (TGN). During their migration, receptors undergo post-translational modifications to attain mature status [5]. Mature receptors then move from the TGN to the plasma membrane, their functional destination. At the plasma membrane

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GPCRs may undergo internalization upon stimulation by their ligands. The internalization involves phosphorylation of GPCRs by G protein receptor kinases and subsequent binding of the phosphorylated receptors to arrestins, which serve as adaptor proteins recruiting components of the transport machinery to the clathrin-coated pits, initiating formation of the early endosome [9]. The internalized receptors in the early endosome may be sorted to the lysosome for degradation or to the recycling endosome for return to the plasma membrane [10].

Protein export from the ER is mediated through COPII-coated vesicles containing of a small GTPase Sar1 and the heterodimeric Sec23/24 and Sec13/31 complexes [11-14]. It has been well demonstrated that GDP/GTP exchange and GTP hydrolysis by Sar1 GTPase play a crucial role in the regulation of formation and budding of COPII-coated vesicles on the ER membrane. Assembly of the COPII coat takes place on the ER membrane at discrete locations called ER exit sites and is initiated by the exchange of GDP for GTP on Sar1 GTPase, which is catalyzed by the ER-localized transmembrane guanine nucleotide exchange factor Sec12. GTP activation of Sar1 GTPase then recruits the Sec23/24 complex to the ER membrane forming a functional prebudding complex. The prebudding complex containing GTP-bound Sar1 and Sec23/24 are then clustered by Sec13/31 forming the COPII vesicles. Hydrolysis of GTP to GDP by Sar1 GTPase results in the dissociation of Sar1 GTPase from the prebudding complex and facilitates the release of the COPII vesicles from the ER membrane [14-18].

ER export of GPCRs represents the first step in intracellular trafficking of the receptors and influences the kinetics of receptor maturation and cell-surface targeting [19,20]. Interestingly, recent studies have demonstrated that β_2-adrenergic receptor (β_2-AR) may form signaling complexes with G protein $\beta\gamma$ subunits and adenylyl cyclase II in the ER [21,22]. However, compared with extensive studies on the events involved in the endocytic and recycling pathways, molecular mechanism underlying the ER export and targeting to the cell surface of GPCRs and regulation of receptor signaling by export trafficking are relatively less understood. The progress achieved over the past few years indicates that GPCR export from the ER is a highly regulated process. First, GPCR export from the ER is directed by highly conserved motifs identified exclusively in the membrane-proximal C-termini [23-27]. Second, GPCR dimerization (homo- and hetero-dimerization) plays an important role in proper receptor folding and assembly to pass through the ER quality control mechanism, in addition to regulating ligand binding, signal transduction and internalization [28,29]. Third, GPCR export from the ER is modulated by direct interactions with multiple regulatory proteins such as ER chaperones, accessory proteins and receptor activity modifying proteins, which may stabilize receptor conformation, facilitate receptor maturation and promote receptor delivery to the plasma membrane [8,19].

To define the mechanism underlying the export from the ER and transport from the ER to the cell surface of GPCRs, we focused our effort on angiotensin II type 1 receptor (AT1R), β_2-AR, α_1-AR and α_2-AR, representative members of the GPCR superfamily, which couple to different G proteins and initiate distinct signaling pathways. We previously determined the role of Rab1, a Ras-like small GTPase that specifically coordinates protein transport from the ER to the Golgi [30,31], in the transport of these receptors. We demonstrated that the transport from the ER to the cell surface of AT1R, β_2-AR and α_1-AR is dependent on Rab1, whereas the transport of α_{2B}-AR is independent of Rab1 [32-34]. We have recently reported that the transport of α_{2B}-AR from the ER to the cell surface is also independent of Rab6 [35], a small GTPase involved in regulation of retrograde protein transport from the Golgi to the ER. These data indicate that the cell surface targeting of different GPCRs may be mediated through distinct pathways and suggest a novel pathway mediating α_{2B}-AR targeting to the cell surface. We have identified the highly conserved $F(X)_6LL$ motif which is required for the export of AT1R and α_{2B}-AR from the ER [26,27] and the YS motif in the N-termini close to the first

transmembrane domain which is essential for α_2-AR export from the Golgi [36]. In this manuscript we sought to further define the mechanism underlying the export of GPCRs from the ER by investigating the role of the ER-derived COPII transport vesicles in mediating GPCR export from the ER through manipulating the function of Sar1 GTPase. We demonstrated that expression of the constitutively active GTP-bound mutant Sar1H79G similarly inhibits the cell-surface expression and signaling of α_{2B}-AR, β_2-AR and AT1R, but differentially modifies their subcellular localization. These data indicate that efficient GTP hydrolysis by Sar1 GTPase selectively regulates GPCR export from the ER and provide the first evidence indicating different regulatory roles for Sar1-coordinated COPII vesicle assembly on the ER membrane in the export of distinct GPCRs.

2. Experimental procedures

2.1. Materials

Rat α_{2B}-AR in vector pcDNA3, human β_2-AR in vector pBC and rat AT1R in vector pCDM8 were kindly provided by Dr. Stephen M. Lanier, Dr. John D. Hildebrandt (Department of Pharmacology and Experimental Therapeutics, Medical University of South Carolina, Charleston, SC) and Kenneth E. Bernstein (Department of Pathology, Emory University, Atlanta, GA), respectively. AT1R, α_{2B}-AR and β_2-AR tagged with green fluorescent protein (GFP) at their C-termini and α_{2B}-AR tagged with the HA epitope at its N-terminus were generated as described previously [32]. These epitope tagged receptors have characteristics similar to their wild-type receptors [32]. Sar1, the GTP-bound mutant Sar1H79G and antibodies against Sar1 were gifts from Dr. William E. Balch (Institute for Childhood and Neglected Diseases, Scripps Research Institute, La Jolla, CA). Antibodies against phospho-ERK1/2 and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and antibodies against ERK1/2 from Cell Signaling Technology. High affinity fluorescein-conjugated anti-HA antibody 3F10 was from Roche Applied Science (Mannheim, Germany). Anti-GM130 antibodies were from Transduction Laboratories (San Diego, CA). Alexa Fluor 594-labeled anti-mouse antibodies and 4,6-diamidino-2-phenylindole were obtained from Molecular Probes, Inc. (Eugene, OR). pDsRed2-ER, an ER marker, was from BD Biosciences (Palo Alto, CA). Isoproterenol (ISO) and UK14304 were obtained from Sigma (Saint Louis, MO). Human angiotensin II (Ang II) was purchased from Calbiochem. Penicillin/streptomycin, L-glutamine, and trypsin/EDTA were from Invitrogen. [^{3}H]-CGP12177 (specific activity = 51 Ci/mmol), [^{3}H]-RX821002 (41 Ci/mmol) and (3-([^{125}I)-iodotyrosyl 4) angiotensin II ([^{125}I]-Ang II) (2000 Ci/mmol) were purchased from Amersham Biosciences/GE Healthcare. All other materials were obtained as described elsewhere [26,32].

2.2. Cell Culture and Transient Transfection

HEK293T cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transient transfection of the HEK293T cells was carried out using LipofectAMINE 2000 reagent (Invitrogen) as previously described [32]. The transfection efficiency was estimated to be greater than 70% based on the GFP fluorescence.

2.3. Radioligand Binding

Cell-surface expression of α_{2B}-AR, β_2-AR and AT1R in HEK293T cells was measured by ligand binding of intact live cells as previously described using [^{3}H]-RX821002, [^{3}H]-CGP12177 and [^{125}I]-Ang II [33-37], respectively. Briefly, HEK293T cells were cultured 6-well dishes and transfected with 200 ng of the α_{2B}-AR or β_2-AR plasmid together with 800 ng of pcDNA3, Sar1 or Sar1H79G. After 6 h the cells were split into 12-well plates at a density of 4×10^5 cells/well and cultured for an additional 24 h. For measurement of β_2-AR expression at the cell surface, the cells were incubated with DMEM containing the ligand [^{3}H]-CGP12177

at a concentration of 20 nM for 90 min at room temperature. Non-specific binding was determined by pre-incubation with alprenolol at a concentration of 20 μM for 30 min followed by incubation with [^{3}H]-CGP12177 (20 nM) in the presence of alprenolol (20 μM). For measurement of α_{2B}-AR expression at the cell surface, the cells were incubated with DMEM plus [^{3}H]-RX821002 at a concentration of 20 nM for 90 min at room temperature. The non-specific binding was determined in the presence of rauwalscine (10 μM). The cells were washed twice with 1 ml of ice-cold PBS, and the cell surface-bound ligands were extracted by 1M NaOH treatment for 2 h. The radioactivity was counted by liquid scintillation spectrometry in 3.5 ml of Ecoscint A scintillation solution (National Diagnostics, Inc., Atlanta, GA).

For measurement of AT1R expression at the cell surface, HEK293T cells were cultured on 6-well plates and transfected with 0.5 μg of AT1R together with 1.5 μg of pcDNA3 or Sar1H79G. The cells then were split into 12-well plates and starved for 24 h followed by an incubation with 400 μl DMEM containing ^{125}I-Ang II at a concentration of 20 pM for 5 h at 4°C to avoid AT1R internalization. The non-specific binding was determined in the presence of nonradioactive Ang II (3 μM). After washing the cells twice with 1 ml DMEM, the bound ligand was extracted by mild acid treatment (2 × 5 min with 0.5 ml buffer containing 50 mM glycine, and 125 mM NaCl, pH 3,). The radioactivity was counted in a γ-counter. All radioligand binding assays were performed in triplicate.

2.4. Flow Cytometric Analysis of Receptor Expression

For measurement of total receptor expression, HEK293T cells transfected with individual receptors were collected and resuspended in PBS containing 1% fetal calf serum at a density of 8×10^6 cells/ml. Total receptor expression was determined by measuring total GFP fluorescence on a flow cytometer (BD Biosciences FACSCalibur) as described [32].

2.5. Immunofluorescence Microscopy

For fluorescence microscopic analysis of receptor subcellular localization, HEK293T cells were grown on coverslips pre-coated with poly-L-lysine in 6-well plates and transfected with 100 ng of GFP-tagged receptors with or without co-transfection with 400 ng of the pcDNA3 vector, Sar1 or Sar1H79G. For co-localization of GFP-tagged receptors with the ER marker pDsRed2, HEK293T cells grown on coverslips were transfected with 100 ng of GFP-tagged receptors and 100 ng of the ER marker pDsRed2-ER with or without 400 ng of the pcDNA3 vector or Sar1. The cells were fixed with 4% paraformaldehyde-4% sucrose mixture in PBS for 15 min and stained with 4, 6-diamidino-2-phenylindole for 5 min. The coverslips were mounted, and fluorescence was detected with a Leica DMRA2 epifluorescent microscope as described previously. Images were deconvolved using SlideBook software and the nearest neighbor deconvolution algorithm (Intelligent Imaging Innovations, Denver, CO) as described previously [27,32]. For co-localization of GFP-tagged receptors with HA-tagged receptors or GM130, HEK293T cells were permeabilized with PBS containing 0.2% Triton X-100 for 5 min, and blocked with 5% normal donkey serum for 1 h. The cells were then incubated with antibodies against the epitope HA or GM130 (1:50) for 1 h. After washing with PBS (3 × 5 min), the cells were incubated with Alexa Fluor 594-labeled secondary antibody (1:2000 dilution) for 1 h at room temperature, and the fluorescence was analyzed as described above.

For co-localization of GFP-tagged receptors with the ER marker pDsRed2-ER in live cells, HEK293T cells were plated on 35-mm glass dishes pre-coated with poly-L-lysine and transiently transfected with GFP-tagged receptor and the ER marker pDsRed2-ER mixed in equal amounts (100 ng) with LipofectAMINE 2000 reagent as described above. One hour before imaging, culture medium was replaced with CO_2-independent medium (Invitrogen) [36]. Images were obtained in a Zeiss Axiovert microscope (200M)

2.6. Measurement of ERK1/2 Activation

HEK293T cells were cultured on 10-cm dishes and transfected with 2 μg of receptor plasmids with or without con-transfection with 8 μg of the pcDNA3 vector, Sar1 or Sar1H79G. At 6-8 h after transfection, the cells were split into 6-well dishes and cultured for 24-30 h. The cells were starved for at least 2 h and then stimulated with agonists as indicated in the figure legends. Stimulation was terminated by addition of 1 × SDS gel loading buffer as described previously [32]. After solubilizing the cells, 20 μl of total cell lysates was separated by 12% SDS-PAGE. ERK1/2 activation was determined by Western blotting measuring the levels of phosphorylation of ERK1/2 with phospho-specific ERK1/2 antibodies.

2.7. Immunoblotting

Western blot analysis of protein expression was carried out as previously described [32]. HEK293T cell lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The signal was detected using ECL Plus (PerkinElmer Life Sciences) and a Fuji Film luminescent image analyzer (LAS-1000 Plus) and quantitated using the Image Gauge program (Version 3.4).

2.8. Statistical Analysis

Differences were evaluated using Student's *t* test, and $P < 0.05$ was considered as statistically significant. Data are expressed as the mean ± S.E.

3. Results

3.1. Effect of Transient Expression of Sar1H79G on the Cell-surface Expression and Signaling of AT1R, α_{2B}-AR and β_2-AR

Sar1 GTPase, through its abilities to undergo GDP/GTP exchange and hydrolyze GTP to GDP, plays a crucial role in regulating the assembly of the COPII-coated vesicles, which mediate the transport of newly synthesized proteins exclusively from the ER to the ERGIC. The function of Sar1 GTPase was manipulated by transiently expressing the constitutively active GTP-bound mutant Sar1H79G, which has been well characterized to inhibit protein transport from the ER [14-18]. We first determined the effect of transient expression of Sar1 and Sar1H79G on the overall and cell surface expression of α_{2B}-AR, β_2-AR and AT1R. Sar1 and Sar1H79G expression was determined by Western blotting using Sar1-specific antibodies (Fig. 1A). The total receptor expression was determined by measuring GFP signal using flow cytometry and the cell-surface expression of α_{2B}-AR, β_2-AR and AT1R in HEK239T cells quantitated by ligand binding in intact live cells. The cell-surface expression of α_{2B}-AR, β_2-AR and AT1R was markedly attenuated in cells expressing Sar1H79G and the individual receptor compared with cells expressing receptor alone. Sar1H79G similarly inhibited the cell-surface expression of α_{2B}-AR, β_2-AR or AT1R by 48%, 59% and 55%, respectively (Fig. 1B). In contrast, expression of wild-type Sar1 did not significantly influence the cell-surface expression of all three receptors (Fig. 1B). The overall expression of α_{2B}-AR, β_2-AR or AT1R was also not significantly altered by either Sar1 or Sar1H79G expression (Fig. 1B). These data indicate that the export from the ER and transport to the cell surface of α_{2B}-AR, β_2-AR and AT1R is mediated through Sar1-coordinated COPII-coated vesicles.

We then determined if transient expression of Sar1 and Sar1H79G could influence receptor signaling by measuring activation of the mitogen-activated protein kinase pathway in response to stimulation with receptor agonists. The agonists UK14304 (Fig. 2A), ISO (Fig. 2B) and Ang II (Fig. 2C) stimulated ERK1/2 activation in a dose-dependent fashion in cells expressing α_{2B}-AR, β_2-AR and AT1R, respectively. ERK1/2 activation by the agonists was markedly compromised in cells expressing Sar1H79G and individual receptor as compared with cells

expressing receptor alone (Fig. 2A, 2B and 2C). In contrast, agonist-mediated ERK1/2 activation was similar in cells expressing Sar1 and individual receptor as compared with cells expressing receptor alone (Fig. 2D) These data are consistent with the effect of Sar1 and Sar1H79G on the cell-surface expression of these receptors, and further indicate that cell-surface targeting and signaling of α_{2B}-AR, β_2-AR and AT1R are dependent on the function of Sar1 GTPase.

3.2. Differential Regulation of the Subcellular Distribution of AT1R, α_{2B}-AR and β_2-AR by Sar1H79G

We then determined the effect of Sar1H79G on the subcellular distribution of α_{2B}-AR, β_2-AR and AT1R. The receptors were conjugated with GFP at their C-termini and their subcellular localization at steady state was revealed by fluorescence microscopy analysis. As anticipated, AT1R, α_{2B}-AR and β_2-AR were expressed at the cell surface in cells transfected with the pcDNA3 vector and Sar1 (Fig. 3), which was confirmed by co-localization with tetramethylrhodamine-conjugated concanavalin A, a plasma membrane marker (data not shown). Consistent with the marked reduction in the cell-surface expression as measured by intact cell radioligand binding, α_{2B}-AR, β_2-AR and AT1R lost their cell-surface expression pattern in cells transfected with Sar1H79G. Interestingly, the subcellular localization of α_{2B}-AR, β_2-AR and AT1R was apparently different. Whereas α_{2B}-AR and AT1R were highly concentrated at discrete locations near the nucleus, β_2-AR exhibited an even distribution in the perinuclear region.

To further confirm that the Sar1H79G mutant was capable of arresting α_{2B}-AR, β_2-AR and AT1R at distinct subcellular compartments, we determined the effect of Sar1H79G on the subcellular localization of two receptors tagged with different epitopes being expressed in the same cell. HA-α_{2B}-AR and Sar1H79G were expressed together with AT1R-GFP or β_2-AR-GFP in HEK293T cells. The effect of Sar1H79G on the subcellular distribution of AT1R-GFP and HA-α_{2B}-AR or β_2-AR-GFP and HA-α_{2B}-AR was determined in the same transfected cells by fluorescence microscopy analysis following staining with anti-HA antibodies. β_2-AR-GFP and AT1R-GFP were nicely co-localized with HA-α_{2B}-AR at the cell surface in cells transfected with the pcDNA3 vector (Fig. 4). HA-α_{2B}-AR was also extensively co-localized with AT1R-GFP in cells transfected with Sar1H79G (Fig. 4B). In contrast, HA-α_{2B}-AR was not co-localized with β_2-AR-GFP in Sar1H79G-transfected cells (Fig. 4A). Expression of Sar1 did not disrupt the co-localization of HA-α_{2B}-AR with β_2-AR-GFP (Fig. 4A) or AT1R-GFP (Fig. 4B). These data demonstrate that expression of GTP-bound Sar1H79G mutant induced an arrest of AT1R and α_{2B}-AR in the same intracellular compartment, which is different from that where β_2-AR accumulated. These data indicate that manipulation of the GTP hydrolysis of Sar1 GTPase differentially modifies the subcellular distribution of α_{2B}-AR, β_2-AR and AT1R,.

3.3. Colocalization of α_{2B}-AR, β_2-AR and AT1R with organelle markers in the presence of Sar1H79G

To precisely define the sites at which α_{2B}-AR, β_2-AR and AT1R, are arrested by expression of Sar1H79G, we first co-localized the receptors with GM130. GM130 is a cis-Golgi protein which redistributes into ER exit sites in the presence of Sar1H79G [38]. GFP-tagged α_{2B}-AR, β_2-AR and AT1R were expressed in HEK293T cells and their co-localization with GM130 was visualized by microscopy analysis following staining with anti-GM130 antibodies. α_{2B}-AR (Fig. 5A) and AT1R (Fig. 5B) were markedly co-localized with GM130 in cells expressing Sar1H79G. In contrast, β_2-AR did not co-localize with GM130 in cells transfected with Sar1H79G (data not shown). Both α_{2B}-AR and AT1R transported to the cell surface in cells expressing Sar1 (Fig. 5).

We then sought to define the site where β_2-AR accumulated during co-expression with the GTP-bound mutant Sar1H79G. In the first series of experiments, GFP-tagged β_2-AR, α_{2B}-AR and AT1R were transiently transfected together with the ER marker pDsRed2-ER. Co-localization of the receptors with the ER marker was revealed by microscopy analysis in fixed cells. β_2-AR extensively co-localized with the ER marker DsRed2-ER in cells transfected with Sar1H79G, but not in cells transfected with the pcDNA3 vector or Sar1 (Fig. 6A). In contrast, α_{2B}-AR and AT1R were not co-localized with the ER marker in cells expressing Sar1H79G (data not shown).

To eliminate the possible non-specific influence of cell fixation on the subcellular localization of the receptors, in the second series of experiments we determined the effect of transient expression of Sar1H79G on the co-localization of α_{2B}-AR, β_2-AR and AT1R with the ER marker DsRed2-ER in live HEK293T cells. Similar to the results obtained from the fixed cells, β_2-AR strongly co-localized with the ER marker DsRed2-ER in live cells (Fig. 6B). In contrast, α_{2B}-AR (Fig. 6C) and AT1R (data not shown) were not co-localized with the ER marker. These data demonstrate that expression of the GTP-bound Sar1H79G mutant resulted in the selective arrest of β_2-AR in the ER, indicating that GTP hydrolysis by Sar1 GTPase differentially modulates the export of α_{2B}-AR, β_2-AR and AT1R from the ER.

4. Discussion

The molecular mechanism underlying the recruitment and package of GPCRs into the ER-derived COPII-coated vesicles, which transport cargo proteins exclusively from the ER to the ERGIC, remains undefined. To address this issue we investigated the function of Sar1 GTPase, which plays a crucial role in the assembly and budding of the COPII-coated vesicles on the ER membrane, in regulating the export from the ER and transport to the cell surface of α_{2B}-AR, β_2-AR and AT1R. Loss-of-function of Sar1 GTPase was achieved by transiently expressing the GTP-restricted mutant Sar1H79G. Inhibition of normal Sar1 function by Sar1H79G has been demonstrated to attenuate protein transport to the cell surface [14-18]. Our data demonstrated that transient expression of Sar1H79G significantly inhibited the cell-surface expression of all GPCRs examined as determined by intact cell ligand binding. Consistent with attenuation of receptor expression at the cell surface, ERK1/2 activation in response to stimulation with the receptor agonists was also blocked**.** These data indicate that ER export of α_{2B}-AR, β_2-AR and AT1R is mediated through the Sar1-dependent COPII vesicles. In contrast, expression of wild-type Sar1 GTPase did not significantly influence the cell-surface expression of all three receptors and receptor-mediated ERK1/2 activation, suggesting that endogenous Sar1 expression is not a rate-limiting factor for the transport of the receptors to the cell surface.

Microscopic analysis on the subcellular distribution of the receptors revealed that Sar1H79G clearly arrested α_{2B}-AR, β_2-AR and AT1R in distinct subcellular compartments. Co-localization of the receptors with different intracellular compartment markers revealed that α_{2B}-AR and AT1R did not co-localize with the ER marker DsRed2-ER, but co-localized strongly with GM130 in Sar1H79G-transfected cells. GM130 has been demonstrated to translocate from the cis-Golgi to ER exit site in the presence of Sar1H79G. These data suggest that α_{2B}-AR and AT1R were still able to export from the ER and reached ER exit sites. These data are consistent with the function of GTP hydrolysis by Sar1 GTPase in the assembly and disassembly of the COPII-coated transport vesicles. It has been shown that Sar1H79G supports the budding of the COPII vesicles on the ER membrane, but blocks protein transport further, and therefore, arrests transported cargo proteins at ER exit sites [38]. In contrast to α_{2B}-AR and AT1R, β_2-AR exhibited marked co-localization with the ER in cells expressing Sar1H79G. These data indicate that efficient GTP hydrolysis by Sar1 GTPase differentially modulates the export of α_{2B}-AR, β_2-AR and AT1R from the ER. Such a differential requirement for Sar1

GTP hydrolysis has been reported for ER export of glycosylphosphatidylinositol-anchored protein, vesicular stomatitis virus glycoprotein and soluble lumenal GFP [17].

There are several possibilities regarding the differential regulation by Sar1H79G of targeting of different GPCRs to ER exit sites. It is possible that export from the ER of α_{2B}-AR, β_2-AR and AT1R is mediated through distinct populations of COPII vesicles or at distinct sites on the ER membrane, which have different sensitivities to the GTP hydrolysis by Sar1 GTPase. Consistent with this possibility, exit of glycosylphosphatidylinositol-anchored proteins and other membrane proteins from the ER is mediated through distinct COPII vesicles [39]. Furthermore, it has been recently demonstrated that GTP hydrolysis by Sar1 GTPase can be linked to the specificity of cargo incorporation into budding structures [16]. AT1R, α_{2B}-AR and β_2-AR may have different affinities for components of the COPII vesicles (such as active GTP-bound Sar1). α_{2B}-AR and AT1R may bind to Sar1 or other components of COPII vesicles with higher affinity than β_2-AR, resulting in the recruitment of the receptors to ER exit sites. This possibility is supported by the fact that many cargo proteins directly interact with the COPII components to facilitate their export from the ER [40-43]. It is also possible that export from the ER of different GPCRs is differentially regulated by their interaction with the ER-resident proteins. The ER retention of β_2-AR may be caused by strong interaction between the receptor and ER-localized proteins, which traps the newly synthesized β_2-AR in the ER and limits packaging of the receptor into ER exit sites. This possibility is supported by interaction of GPCRs with a number of ER-localized proteins to modulate their proper folding and assembly and to facilitate their maturation during biosynthesis of the receptors [8,19].

Export trafficking of GPCRs from the ER to the cell surface is a highly regulated, dynamic process and the specificity/selectivity of export trafficking can be modulated by multiple regulators at each stage of the transport process along the secretory pathway. We have demonstrated that AT1R and α_{2B}-AR use the same $F(X)_6LL$ motif to export from the ER, implying that these receptors may be sorted from other GPCRs with different ER exit motifs [26,27]. The YS motif in the N-termini dictates the export from the Golgi of α_2-AR, providing a means for the segregation of GPCRs at the level of the Golgi [36]. We have also demonstrated that the transport of AT1R, β_2-AR and α_{2B}-AR from the ER to the cell surface is selectively regulated by Rab1 and Rab6, indicating multiple routes by which GPCRs move to their functional destination [32-35]. In this manuscript, our data have showed that export from the ER of α_{2B}-AR, β_2-AR and AT1R is differentially modulated by GTP hydrolysis of Sar1 GTPase, suggesting that GPCRs could be sorted from one another at the level of ER exit sites. Therefore, selective export trafficking of GPCRs with similar structural features may be achieved at discrete intracellular levels and are modulated by multiple regulatory molecules.

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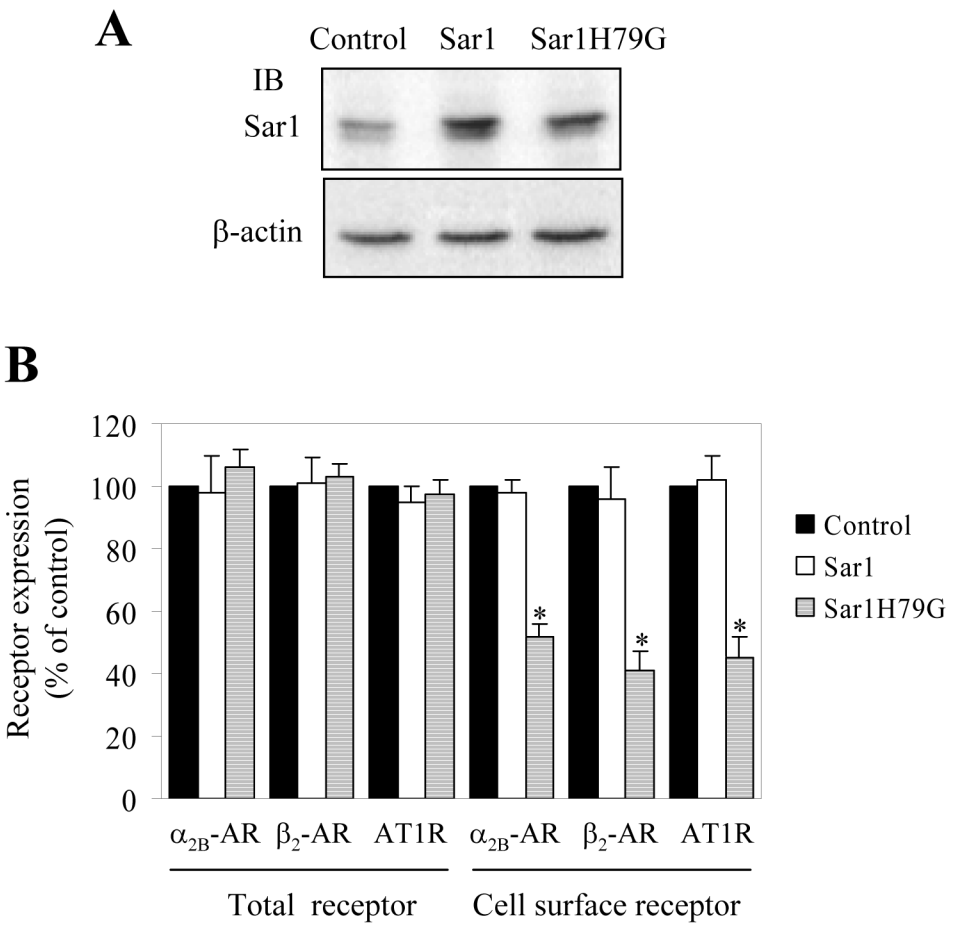


Fig. 1.

Effect of transient expression of Sar1 and Sar1H79G on the cell-surface expression of α_{2B}-AR, β_2-AR and AT1R. A, Western blot analysis of Sar1 and Sar1H79G expression. HEK293T cells cultured on 6-well plates were transfected with the pcDNA3 vector (control), Sar1 or Sar1H79G. Cell homogenates (30 μg) were separated by 12% SDS-PAGE and expression of endogenous Sar1 and transfected Sar1 and Sar1H79G was detected by Western blotting using anti-Sar1 antibodies. Two bands with similar apparent molecular weight revealed by Sar1 antibodies are probably Sar1a and Sar1b isoforms (upper panel). Expression of β-actin is used as a control for equal loading (lower panel). *B*, inhibition of the cell-surface expression of α_{2B}-AR, β_2-AR and AT1R by Sar1 and Sar1H79G. HEK293T cells were transfected with GFP-conjugated α_{2B}-AR, β_2-AR or AT1R together with the pcDNA3 vector (control), Sar1 or Sar1H79G mutant. The expression of α_{2B}-AR, β_2-AR or AT1R at the cell surface was determined by intact cell ligand binding using [^{3}H]-RX821002, [^{3}H]-CGP12177 and [^{125}I]-Ang II, respectively, and total receptor expression by measuring GFP fluorescence using a flow cytometer as described under "Experimental procedures." The mean values of specific ligand binding were 14863 ± 901, 15372 ± 1248 and 10583 ± 230 cpm (n = 3, each in triplicate) from cells transfected with α_{2B}-AR, β_2-AR or AT1R together with the pcDNA3 vector, respectively. The data shown are percentages of the mean value obtained from cells transfected with individual receptor and the pcDNA3 vector and are presented as the means ± S.E. of three experiments. *, $P < 0.05$ versus the cells transfected with respective receptor and the pcDNA3 vector.

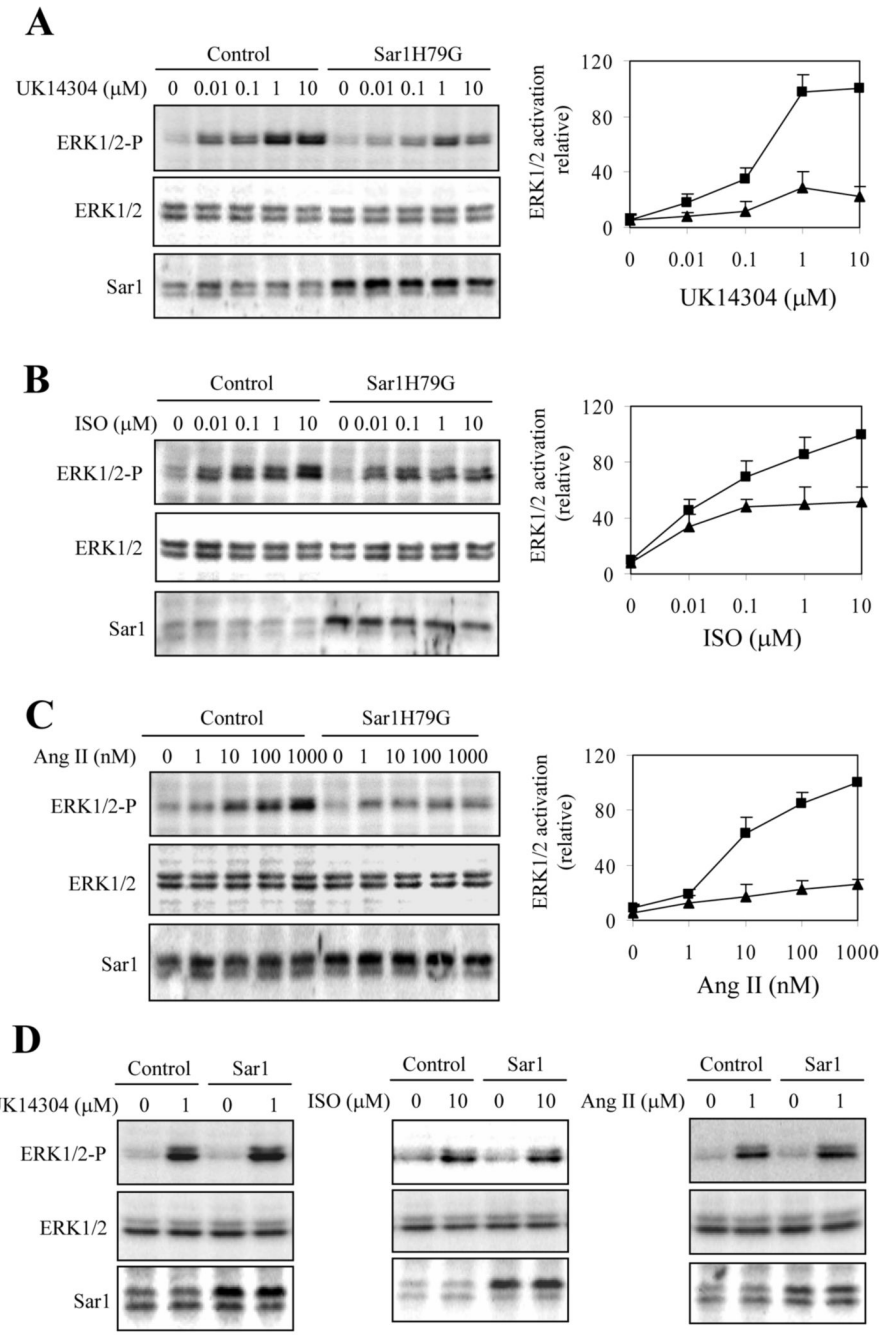


Fig. 2.

Effect of Sar1 and Sar1H79G on ERK1/2 activation by α_{2B}-AR, β_2-AR and AT1R. A, B and C. Effect of Sar1H79G on ERK1/2 activation. HEK293T cells transfected with GFP-tagged α_{2B}-AR (A), β_2-AR (B) or AT1R (C) together with pcDNA3 (control; squares) or Sar1H79G (triangles) were stimulated with increasing concentrations of UK14304 (0.01-10 μM) for 5 min (A), or isoproterenol (ISO; 0.01-10 μM) for 5 min (B) Ang II (1-1000 nM) for 2 min (C) at 37 °C. ERK1/2 activation was determined by Western blot analysis using phospho-specific ERK1/2 antibodies (ERK1/2-P). Left panels, representative blots of ERK1/2 activation (upper panels), total ERK1/2 expression (middle panels), and Sar1H79G expression (bottom panels); right panels, quantitative data expressed as percentages of ERK1/2 activation obtained from

cells transfected with individual receptors and stimulated with 10 μM UK14304 (A), 10 μM ISO (B) or 1 μM Ang II (C) and are presented as the mean ± S.E. of three experiments. D. Effect of Sar1 on ERK1/2 activation. HEK293T cells transfected with GFP-tagged α_{2B}-AR (left panel), β_2-AR (middle panel) or AT1R (right panel) together with pcDNA3 (control) or Sar1 were stimulated with UK14304, isoproterenol and Ang II, respectively. Each experiment was repeated three times with similar results.

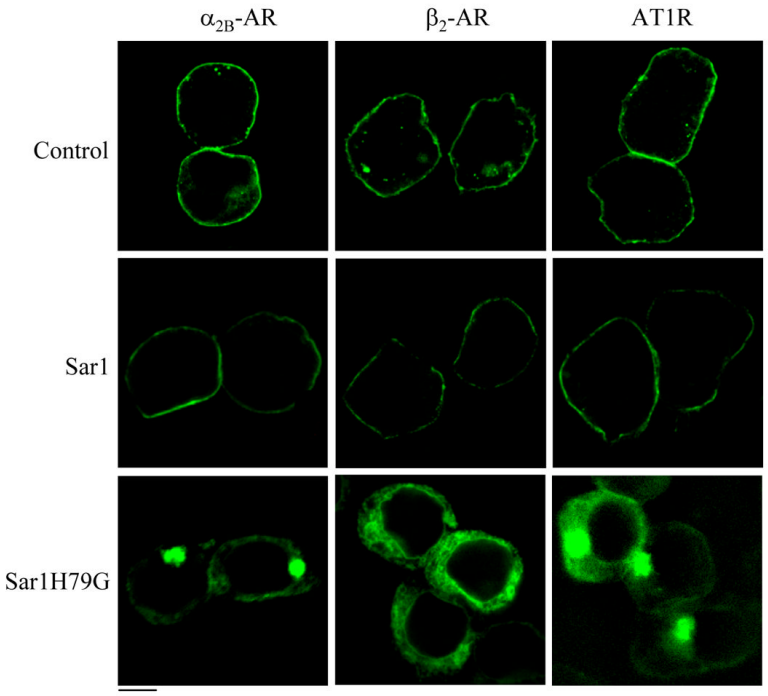


Fig. 3.

Effect of Sar1 and Sar1H79G on the subcellular distribution of α_{2B}-AR, β_2-AR and AT1R. HEK293T cells cultured on coverslips were transfected with GFP-conjugated α_{2B}-AR, β_2-AR or AT1R (40 ng) together with the pcDNA3 vector (control), Sar1 or Sar1H79G (400 ng). The subcellular distribution of the receptors was revealed by detecting GFP fluorescence as described under "Experimental procedures." The data are representative images of at least five independent experiments. Scale bar, 10 μm.

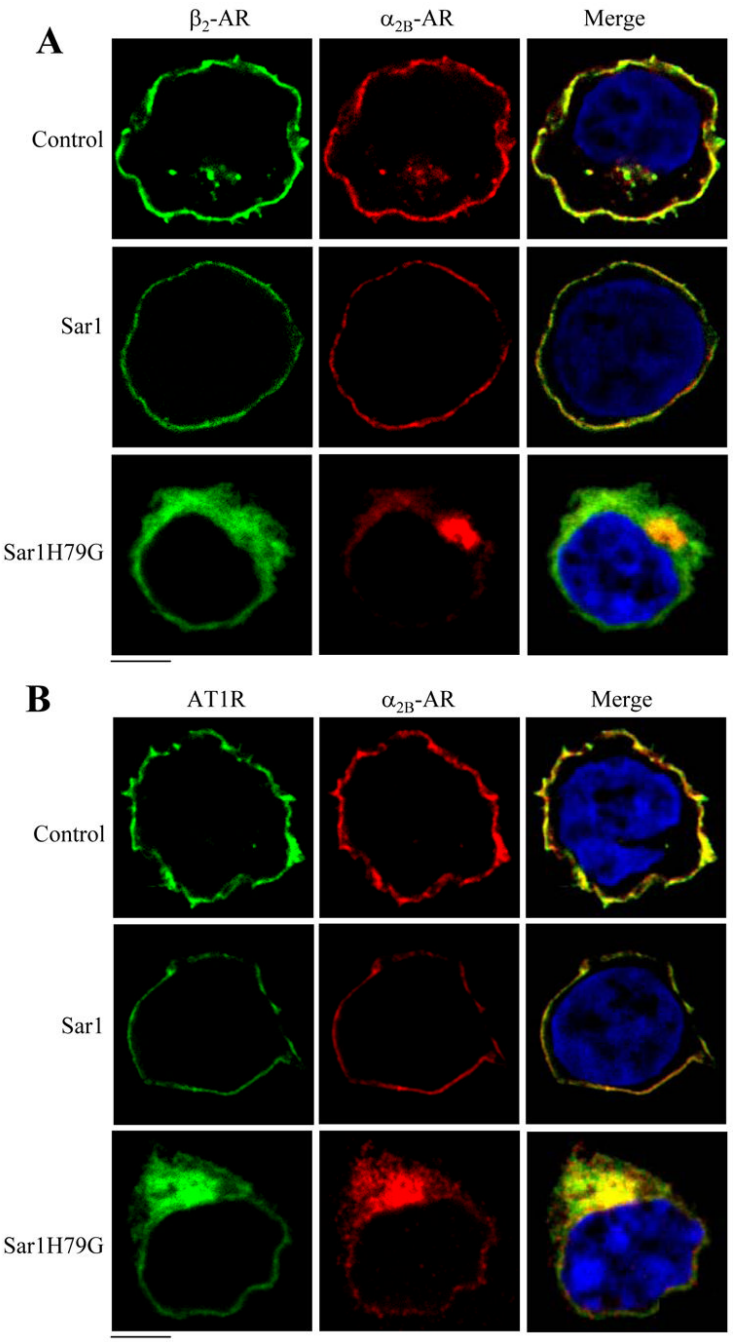


Fig 4.

Effect of Sar1 and Sar1H79G on the subcellular distribution of α_{2B}-AR, β_2-AR and AT1R in the same transfected cells. HEK293T cells were transfected with β_2-AR-GFP and HA-α_{2B}-AR (A) or AT1R-GFP and HA-α_{2B}-AR (B) together with pcDNA3 (control, upper panels), Sar1 (middle panels) or Sar1H79G (lower panels). The subcellular distribution of the receptors was detected by fluorescence microscopy following immunostaining with rhodamine-conjugated anti-HA antibodies as described under "Experimental procedure." Green, β_2-AR-GFP (A) and AT1R-GFP (B); red, HA-α_{2B}-AR; blue, DNA staining by 4,6-diamidino-2-phenylindole (nucleus); yellow, co-localization of HA-α_{2B}-AR with β_2-AR-GFP (A) and with AT1R-GFP

(B). The data are representative images of at least three independent experiments. Scale bars, 10 μm.

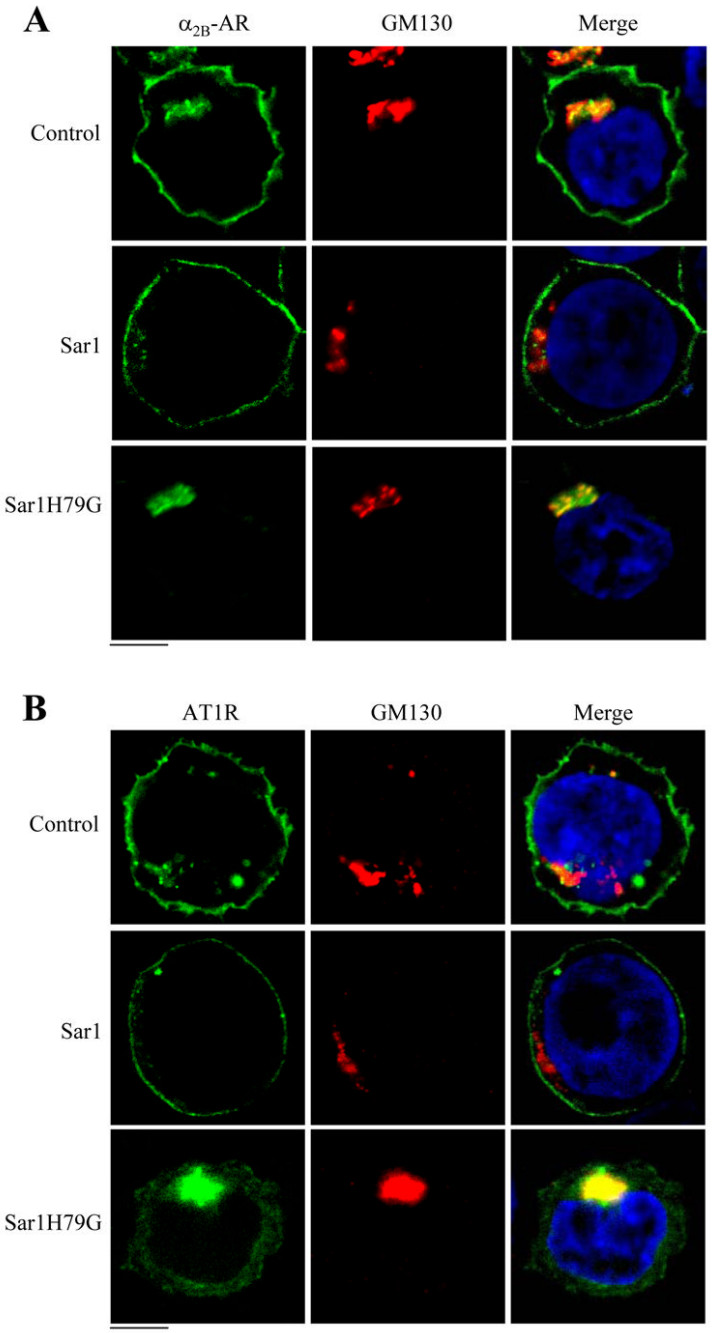
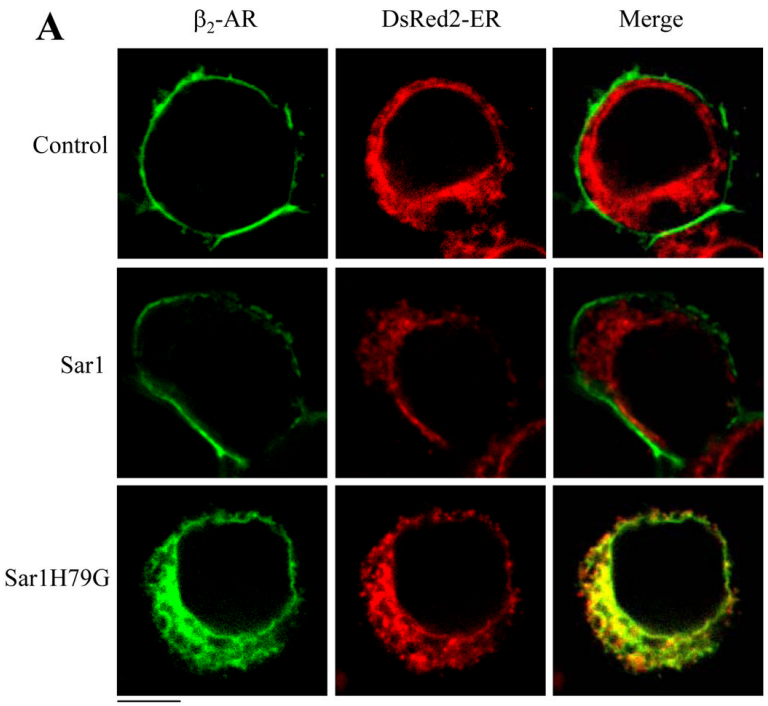


Fig. 5.

Co-localization of α_{2B}-AR and AT1R with GM130. HEK293T cells were transfected with α_{2B}-AR-GFP (A) or AT1R-GFP (B) (100 ng) together with the pcDNA3 vector (control), Sar1 or Sar1H79G (400 ng) and then stained with antibodies against GM130 (1:50 dilution) as described under "Experimental procedure." Subcellular distribution and co-localization with GM130 of α_{2B}-AR and AT1R were revealed by fluorescence microscopy. The data are representative images of four independent experiments. Green, GFP-tagged receptors; red, GM130; blue, DNA staining by 4,6-diamidino-2-phenylindole (nucleus); yellow, co-localization of the receptors and GM130. Scale bars, 10 μm.



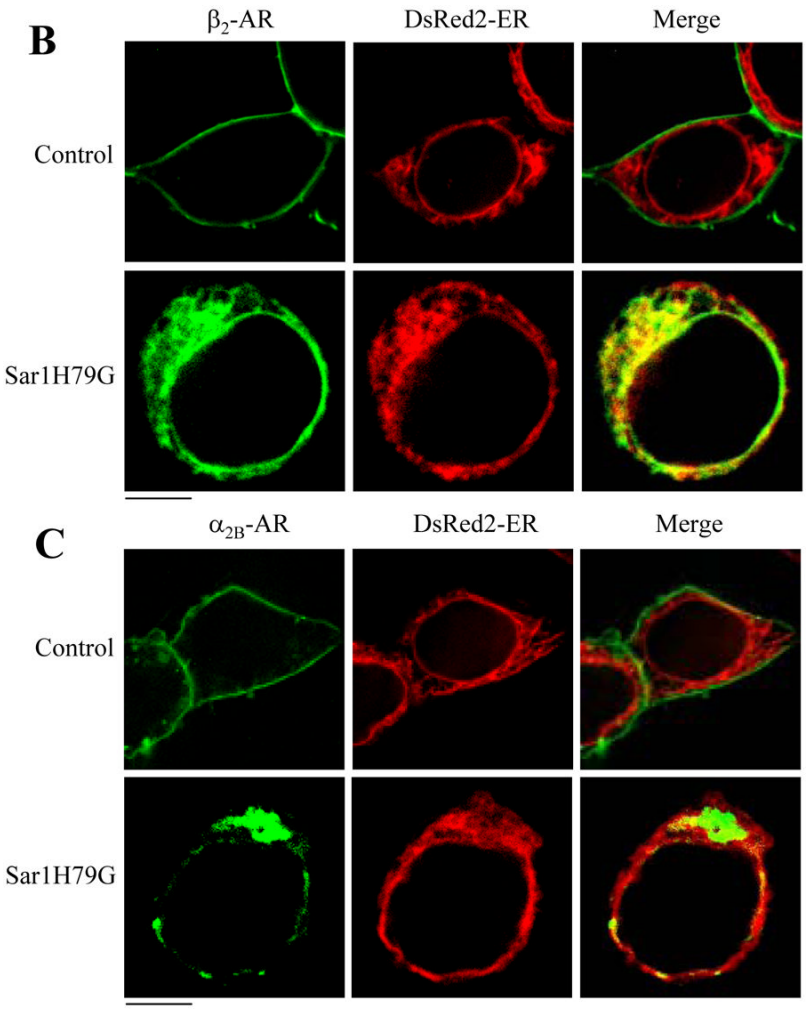


Fig. 6.

Colocalization of β_2-AR with the ER marker DsRed2-ER. HEK293T cells cultured on coverslips were transiently transfected with pDsRed2-ER (100 ng) and GFP-tagged β_2-AR (100 ng) or GFP-tagged α_{2B}-AR together with the pcDNA3 vector (control), Sar1 or Sar1H79G (400 ng). Subcellular distribution and co-localization with DsRed2-ER of the receptors in fixed cells (A) and in live cells (B and C) were revealed by fluorescence microscopy as described under "Experimental procedures." The data are representative images of five independent experiments. Green, β_2-AR-GFP (A and B) and α_{2B}-AR (C); red, the ER marker DsRed2-ER; yellow, colocalization of β_2-AR (A and B) or α_{2B}-AR (C) with DsRed2-ER. Scale bars, 10 μm.