Rab5 Association with the Angiotensin II Type 1A Receptor Promotes Rab5 GTP Binding and Vesicular Fusion*

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The angiotensin II type 1A receptor (AT1AR) is a member of the large superfamily of G protein-coupled receptors (GPCRs). AT1ARs are coupled via Gq to the stimulation of phospholipase CB leading to increases in intracellular inositol 1,4,5-triphosphate formation, the release of calcium from intracellular stores, and the activation of protein kinase C (1). Agonist activation also initiates the feedback phosphorylation and desensitization of the AT1AR in response to phosphorylation by both second messenger-dependent protein kinases and G protein-coupled receptor kinases (2, 3). G protein-coupled receptor kinase phosphorylation promotes the membrane translocation and binding of β-arrestins to the AT1AR, which serves both to uncouple the receptor from heterotrimeric G proteins and to target the receptor for endocytosis (3–7). β-Arrestins act as intermediary GPCR endocytosis motif proteins through their association with clathrin and the β2-adaptin subunit of the heterotrimeric AP2 adaptor complex (8, 9). However, β-arrestin-directed internalization of GPCRs in clathrin-coated vesicles may represent only one of many mechanisms contributing to GPCR endocytosis (4, 10, 11). For example, AT1AR internalization may also involve a pathway that is relatively insensitive to dominant-negative β-arrestin mutants (4).

The AT1AR is a member of a class of GPCRs that remain associated with β-arrestins during clathrin-mediated endocytosis. Moreover, the AT1AR is targeted to enlarged hollow core vesicular structures (3, 12) but is neither dephosphorylated nor recycled efficiently back to the cell surface (3). In contrast, endocytosis is required for the dephosphorylation and desensitization of GPCRs like the β2-adrenergic receptor (β2AR) but does not appear to contribute to the desensitization of AT1AR responses (3, 13–15). Although β2AR internalization is exquisitely β-arrestin-dependent, β-arrestin is excluded from β2AR-bearing vesicle structures allowing dephosphorylation in endosomes (3, 5, 16). The apparent difference in the ability of the β2AR and AT1AR to internalize in a complex with β-arrestin appears to be regulated by their carboxyl-terminal tails (3). These observations have led to the suggestion that β-arrestins may also regulate the reestablishment of GPCR responsiveness (16). However, although the determinants regulating the stable high affinity association of β-arrestin with GPCRs like the AT1AR have been characterized (12), little is known about the mechanism(s) regulating the intracellular trafficking and retention of these receptors in endosomes. To identify additional molecular components contributing to the regulation of both AT1AR internalization and intracellular trafficking, we used the AT1AR carboxyl-terminal tail as bait in the yeast two-hybrid system to screen for novel AT1AR-interacting proteins. Here we describe that the interaction of Rab5α with the carboxyl-terminal tail of the AT1AR not only promotes the vesicular sorting of the AT1AR into enlarged vesicular structures but also activates the GDP/GTP cycle of Rab5α.
**EXPERIMENTAL PROCEDURES**

**DNA Construction**—The truncated AT1R mutants were constructed by polymerase chain reaction. A 5’–oligonucleotide primer hybridized upstream from a unique EcoRI site within the coding sequence of the AT1R-encoding gene, and 3’-oligonucleotides introduced a stop codon after residues 319, 329, 339, and 349 in the AT1R carboxyl-terminal tail. GST-Rab5a fusion proteins were constructed by subcloning Rab5a, Rab5a-S34N, and Rab5a-Q79L into the eukaryotic expression vector pEBG4. The V2 vasopressin carboxyl-terminal tail (residues 320–371) and the AT1R carboxyl-terminal tail constructs (297–359, 310–359, and 297–249) were subcloned by PCR in to the yeast vector pAS2-1.

**Yeast Two-hybrid Screen**—The truncated AT1R mutants were constructed by a modified lithium acetate method according to the manufacturer's instructions (CLONTECH). Double transformants were plated on synthetic yeast drop-out medium lacking leucine and tryptophan, uracil, and histidine in the absence and presence of 25 mM 3-aminotriazole to test for 3-aminotriazole were grown in selective liquid medium to constitute GAL4 activation domain library clones were analyzed by DNA sequencing. Isolated library clones were cloned by DNA sequencing. All other cDNA constructs used have been reported previously (3, 5, 17).

**Yeast Two-hybrid Screen**—The rat AT1R carboxyl-terminal tail (residues 297–359) GAL4 binding domain fusion protein construct was co-transformed into the yeast strain PJ69-4A with a GAL4 activation domain fusion library of rat whole brain cDNA in pGAD10 (CLONTECH). Yeast two-hybrid screening was carried out according to the CLONTECH MATCHMAKER protocol. Clones expressing both the bait domain fusion library of rat whole brain cDNA in pGAD10 (CLONTECH). Yeast two-hybrid screening was carried out according to the CLONTECH MATCHMAKER protocol. Clones expressing both the bait.
13,000 X g for 20 min. Cleared supernatants were incubated with 40 μl of glutathione 4B-Sepharose beads (Amersham Biosciences, Inc.) for 3 h at 4 °C. The samples were counted for 35S activity (cpm) in a β-scintillation counter. Aliquots (20 μl) of each cleared supernatant were analyzed for relative expression of GST-Rab5a by immunoblotting using an anti-GST antibody.

Receptor Internalization Assays—Radioligand binding measurements of AT1R internalization were assessed as described previously (20). Briefly, HEK 293 cells transiently transfected to overexpress FLAG-AT1R in the absence and presence of Rab5a constructs were washed with serum-free MEM with 10 mM Hepes, pH 7.4, and then were incubated at 37 °C in the same medium containing 100 nM Ang II for 1 h. The cells were then washed in cold MEM containing 10 mM Hepes and were incubated in acid wash solution (90 mM NaCl, 50 mM sodium citrate, pH 5.0) for 20 min to dissociate bound ligand. The cells were then washed and incubated with 250 μl [125I-Sar1-Ile8] Ang II for 3–4 h at 14 °C. Nonspecific binding values were obtained in the presence of 10 μM Losartan. The cells were washed on ice and solubilized in 0.1% NaOH, and radioactivity was counted using β-scintillation. Receptor expression ranged from 250 to 500 fmol/mg protein.

Confocal Immunofluorescence Microscopy—Confocal microscopy was performed on a Zeiss LSM-510 laser scanning microscope using a Zeiss 63X 1.3 NA oil immersion lens. Live cell imaging of HEK 293 cells expressing FLAG-AT1R and Rab5a, Rab5a-S34N, or Rab5a-Q79L and β-arrestin2-GFP was performed using cells plated on 35-mm glass-bottomed culture dishes. The cells were kept warm at 37 °C in serum-free MEM on a heated microscope stage as described previously (3, 17). FLAG-AT1R staining of HEK 293 cells grown on glass coverslips and fixed with 3% paraformaldehyde in Hanks’ balanced salt solution with 0.1% Triton X-100 for 20 min was performed using monoclonal anti-FLAG (M2) antibody (1:250 dilution) in conjunction with a rhodamine red-conjugated goat anti-mouse secondary antibody (1:500 dilution) (Molecular Probes). Co-localization studies of GFP-Rab5a and rhodamine-labeled FLAG-AT1R fluorescence were performed using dual excitation (488, 568 nm) and emission (515–540 nm, GFP; 590–610 nm, rhodamine) filter sets. The specificity of labeling and the absence of signal cross-over were established by examination of single-labeled samples.

Data Analysis—The means ± S.E. are shown for the values obtained for the number of independent experiments indicated in the figure legends. The data were analyzed for statistical significance using GraphPad Prism software. Statistical significance was determined by an unpaired two-tailed t test.

RESULTS AND DISCUSSION

Identification of Rab5 as an AT1R-interacting Protein—To identify novel proteins that interact with the AT1R, we screened ~20 × 106 independent clones of a rat brain cDNA library using the AT1R carboxyl-terminal tail (Ct), amino acid residues 297–359, as bait in the yeast two-hybrid system. Six of the clones isolated for growth on Leu−/Trp−/Ade plates were positive for both partial and full-length Rab5a sequences. Rab5a is a member of a large family of Rab GTP-binding proteins (~60 members) that regulate the trafficking of vesic-
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Fig. 5. Agonist-stimulated formation of AT1AR-Rab5a complexes and Rab5a GTP binding. COS7 cells were transiently transfected with 2 μg each of plasmid cDNAs encoding FLAG-AT1AR with either GST-Rab5a, GST-Rab5a-S34N, or GST-Rab5a-Q79L. A and B, representative autoradiograph (A) and densitometric analysis (B) of the means ± S.D. of four independent experiments demonstrating the relative amounts of GST-Rab5a, GST-Rab5a-S34N, and GST-Rab5a-Q79L co-immunoprecipitated with FLAG-AT1AR in the absence of agonist (−) and following agonist treatment (+) with 100 nM Ang II for 20 min at 37 °C. IP, immunoprecipitation. *, p < 0.05 compared with Rab5a receptor binding in the absence of agonist stimulation; **, p < 0.001 compared with Rab5a-S34N receptor binding in the absence of agonist stimulation. C, COS7 cells expressing AT1AR either alone (Control) or with either FLAG-AT1AR or FLAG-β2AR were stimulated with and without either 100 nM Ang II or 10 μM isoproterenol for 15 min. The binding of [35S]GTPγS to GST-Rab5a-purified on glutathione-Sepharose beads from COS7 cells was assessed by β-scintillation counting. The data represent the means ± S.D. of three independent experiments and are expressed as fold over basal control binding (324 ± 55 cpm). *, p < 0.05 compared with GTPγS binding of GST-Rab5a expressed in COS7 cells in the absence of FLAG-AT1AR; †, p < 0.05 versus AT1AR GTPγS binding in the absence of agonist.

ular cargo between intracellular compartments (21). In particular, Rab5a regulates the formation, trafficking, and fusion of clathrin-coated vesicles with early endosomes (22–24).

Specificity of Rab5a/AT1AR Interactions—Growth of a canine Rab5a clone on −Leu/−Trp/−Ade plates was dependent upon the AT1AR Ct GAL4 BD fusion protein and was not observed in the presence of either empty vector or a GAL4 BD fusion protein of the tail of the vasopressin type 2 receptor (V2R) (Table I). To determine whether Rab5a physically interacts with the AT1AR, GST-Rab5a and FLAG-AT1AR were both expressed in COS7 cells and the co-immunoprecipitation of these proteins was assessed. We found that GST-Rab5a and not GST alone was co-immunoprecipitated from COS7 cells transfected with FLAG-AT1AR (Fig. 1A). Furthermore, co-immunoprecipitation of Rab5a was specific to the AT1AR, because Rab5a was not co-immunoprecipitated with FLAG-β2AR (Fig. 1B). Taken together, these observations indicate that Rab5a represents a novel AT1AR-interacting protein.

Effect of Rab5a Mutants on AT1AR Internalization and Vesicular Distribution—Rab5a has previously been shown to regulate the endocytosis and intracellular trafficking of the β2AR, D2 dopamine receptor, and neurokinin 1 receptor (17, 25, 26). Therefore, we sought to examine whether Rab5a was involved in the endocytosis and vesicular trafficking of the AT1AR. We find that even though a Rab5a-S34N mutant defective in GTP binding blocks the internalization of several other GPCRs (17, 25, 26), co-expression of either wild-type Rab5a, Rab5a-S34N, or constitutively active Rab5a-Q79L has no apparent effect on the agonist-stimulated (100 nM angiotensin II) internalization of FLAG-AT1AR (Fig. 2). In the absence of agonist stimulation, GFP-Rab5a is neither co-localized with FLAG-AT1AR at the cell surface nor localized to large hollow vesicular structures (Fig. 3A). Rather, GFP-Rab5a is limited to small endocytic vesicles (Fig. 3A). However, upon agonist stimulation of the AT1AR we observed enlarged GFP-Rab5a-positive vesicular structures and that the AT1AR is completely co-localized with GFP-Rab5a within these enlarged hollow core vesicular structures (Fig. 3B). These observations suggest that the proposed association of Rab5a with the AT1AR may contribute to the regulation of AT1AR trafficking and/or vesicular fusion rather than AT1AR endocytosis.

We have previously demonstrated that β-arrestin2-GFP is internalized in a physical complex with the AT1AR making this a useful tool for following the temporal dynamics of AT1AR internalization and trafficking in live cells (3, 12). In the absence of agonist stimulation, β-arrestin2-GFP is diffusely distributed throughout the cytosol of FLAG-AT1AR-expressing HEK 293 cells (Fig. 4A). In response to agonist stimulation, we observe that AT1AR-β-arrestin2-GFP complexes first redistribute to clathrin-coated pits (Fig. 4A, 5 min) and are then localized in small endocytic vesicles (Fig. 4A, 10 min), which subsequently fuse into the enlarged hollow core vesicular structures (Fig. 4A, 15 min) in which the receptor was previously demonstrated to co-localize with GFP-Rab5a (Fig. 3B). To determine whether Rab5a may regulate the intracellular trafficking and fusion of AT1AR-containing endocytic vesicles, we utilized β-arrestin2-GFP to follow the subcellular localization of the AT1AR in the presence of wild-type Rab5a, dominant-negative Rab5a-S34N, or constitutively active Rab5a-Q79L. In either the absence (Fig. 4A, 15 min) or presence (Fig. 4B) of wild-type Rab5a, β-arrestin2-GFP-labeled FLAG-AT1ARs are localized to large hollow core endocytic vesicular structures. In contrast, the expression of Rab5a-S34N prevents the agonist-stimulated redistribution of β-arrestin2-GFP labeled FLAG-AT1ARs to enlarged hollow core vesicular structures (Fig. 4C). Instead, β-arrestin2-GFP fluorescence remains limited to smaller endocytic vesicles (Fig. 4C). The expression of Rab5a-Q79L did not alter the formation of enlarged vesicles contain-
ing β-arrestin2-GFP-labeled FLAG-AT_{1A}R (Fig. 4D). Taken together, these data suggest that the GTP-bound form of Rab5a is required for fusion of AT_{1A}R containing endocytic vesicles into enlarged hollow core endocytic structures.

**Functional Consequence of the Agonist-stimulated Formation of AT_{1A}R-Rab5 Complexes**—We find that Rab5a forms a physical complex with the AT_{1A}R in the absence of agonist and also regulates the agonist-promoted intracellular trafficking and fusion of AT_{1A}R-bearing endocytic vesicles. Therefore, we examined whether AT_{1A}R activation stimulates both GST-Rab5a association with the receptor and GTP\'S loading of GST-Rab5a in COS7 cells. We observe that agonist stimulation increases the relative amount of GST-Rab5a, GST-Rab5a-S34N, and GST-Rab5a-Q79L co-immunoprecipitated with the FLAG-AT_{1A}R (Fig. 5A). Consistently more Rab5a-S34N than Rab5a and Rab5a-Q79L is co-immunoprecipitated with the FLAG-AT_{1A}R under basal conditions (Fig. 5, A and B), which may reflect preferential interaction of the receptor with the GDP-bound form of Rab5a. Angiotensin II stimulation of the AT_{1A}R increases GTP\'S binding to GST-Rab5a by 6.6 ± 1.7-fold above agonist-stimulated GST-Rab5a expressing control cells lacking AT_{1A}R (Fig. 4C). Basal GTP\'S binding to GST-Rab5a in AT_{1A}R-expressing cells is also increased 3.2 ± 1.6-fold above GST-Rab5a-expressing control cells lacking AT_{1A}R (Fig. 4C). The increased basal GST-Rab5a GTP\'S binding likely reflects the observed agonist-independent association of the GTPase with the AT_{1A}R. Nonetheless, agonist activation of the AT_{1A}R increases GTP\'S binding to GST-Rab5a 2.4 ± 0.9-fold above basal. In contrast, β\textsubscript{AR} expression and activation has no effect on GTP\'S binding to GST-Rab5a (Fig. 4C). These data not only demonstrate that the association of Rab5a with the AT_{1A}R is regulated by agonist activation of the receptor but also suggest that the AT_{1A}R may function as a guanine nucleotide exchange factor for Rab5a, thereby serving a function that is analogous to its role as a heterotrimeric G protein-coupled receptor.

**Characterization of Rab5a Interactions with the AT_{1A}R Tail and AT_{1A}R Internalization**—To identify the regions of the AT_{1A}R carboxyl-terminal tail contributing to Rab5a binding, we used GAL4 BD fusion proteins of different regions of the AT_{1A}R Ct in the yeast two-hybrid system with Rab5a fused to the GAL4 4D. We find that the removal of either residues 297–310 or residues 349–359 prevents the transactivation of yeast reporter genes in a β-galactosidase activity assay (Fig. 6A). This observation suggests that the association of Rab5a with the AT_{1A}R requires Rab5a binding to multiple regions of the AT_{1A}R Ct. Furthermore, amino acid residues 349–359 do not contribute to AT_{1A}R endocytosis, because the truncation of the AT_{1A}R by 10 and 20 amino acid residues had no effect on the agonist-stimulated internalization of the AT_{1A}R (Fig. 6B). The internalization of the AT_{1A}R was not altered until 30 amino acids were deleted from the carboxyl-terminal tail, AT_{1A}R-(1–329) (Fig. 6B). Therefore, the regions critical for Rab5a binding to the AT_{1A}R Ct are distinct from those residues (residues 327–332) required for stable β-arrestin binding and AT_{1A}R endocytosis (16).

**Effect of AT_{1A}R Carboxy-terminal Truncations on AT_{1A}R Trafficking**—We observed that the removal of either residues 297–310 or residues 349–359 from the AT_{1A}R Ct prevented the interaction of Rab5a with the AT_{1A}R Ct. However, because the removal of residues 297–310 would result in a nonfunctional receptor, we tested whether the deletion of the last 10 amino acid residues from the AT_{1A}R carboxyl-terminal tail might alter the agonist-stimulated redistribution of the mutant AT_{1A}R into enlarged hollow core vesicular structures. Although the truncation of the last 10 amino acid residues from the AT_{1A}R carboxyl-terminal tail has no effect on the internalization of AT_{1A}R-β-arrestin2-GFP complexes (Fig. 7A), β-arrestin2-GFP labeled AT_{1A}R-(1–349) is no longer observed to redistribute into large hollow core vesicles (Fig. 7A). Rather, the β-arrestin bound AT_{1A}R remains localized in small endocytic vesicular structures (Fig. 7A). Moreover, similar to what was previously observed for the β\textsubscript{AR} (17), the AT_{1A}R (1–349) mutant remains localized to small endocytic vesicles and no longer completely co-localizes with GFP-Rab5a (Fig. 7B). Consequently, the redistribution of the AT_{1A}R into enlarged endocytic structures appears to be dependent both of Rab5a activity and the last 10 amino acids of the AT_{1A}R carboxyl-terminal tail that contributes to AT_{1A}R/Rab5a interactions.

Taken together, our data indicate that Rab5a is an AT_{1A}R binding protein and that the association of Rab5a with the AT_{1A}R appears to regulate the intracellular trafficking and fusion of AT_{1A}R-bearing endocytic vesicles into enlarged hollow core vesicular structures. The association between the AT_{1A}R and Rab5a appears to be interactive, in that activation of the AT_{1A}R increases Rab5a GTP binding and activated Rab5a is required for homotypic fusion of AT_{1A}R-bearing endocytic vesicles into enlarged hollow core vesicular structures. Rab5a binding to the AT_{1A}R requires both the distal and proximal ends of the AT_{1A}R Ct. Because the proximal end of the AT_{1A}R Ct is implicated in heterotrimeric G protein coupling (27), it stands to reason that its carboxyl-terminal tail may allow the AT_{1A}R to act as a guanine nucleotide exchange factor for Rab5a. As a consequence, acti-
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Fig. 7. Effect of C terminus truncations on AT1AR intracellular trafficking. A, representative laser scanning confocal micrographs demonstrating the agonist-stimulated trafficking of β-arrestin2-GFP with the AT1AR and AT1AR-(1–349) in live HEK 293 cells in response to a 30-min treatment with 100 nM Ang II. B, the distribution and co-localization (yellow) of monoclonal anti-FLAG antibody labeled FLAG-AT1AR-(1–349) (red) and GFP-Rab5a (green) following 30 min of stimulation with 100 nM Ang II. The data are the representative images of four independent experiments. The bars represent 10 μm.

vated AT1AR exhibits the capacity to promote the Rab5a-dependent homotypic fusion of endocytic vesicles.

The trafficking of proteins between intracellular organelles is a highly regulated process involving several membrane budding and fusion events between donor and acceptor membranes (22–24, 28). Rab GTPases are important regulators of intracellular vesicular transport and endosomal fusion (22–24, 28). Recent studies have indicated that Rab5 contributes to the formation, trafficking, and fusion of clathrin-coated vesicles with early endosomes (29). The expression of the dominant-negative Rab5a-S34N mutant blocks the internalization and trafficking of β2AR-bearing endocytic vesicles (17), but Rab5a does not associate with the β2AR. In contrast, although Rab5 binds the AT1AR, similar to what is observed for β-arrestin dominant-negative, the internalization of the AT1AR appears to also be insensitive to inhibition by a dominant-negative Rab5a-S34N mutant. The reason why the internalization of the AT1AR is insensitive to Rab5a-S34N remains unknown. It is possible that the β2AR and AT1AR are internalized by distinct endocytic mechanisms and/or vesicular populations or that the AT1AR recruits additional proteins involved in clathrin-mediated endocytosis.

Multiple effector proteins regulate the distribution of Rab5 between membrane compartments and the cytosol. For example, the Rab GDP dissociation inhibitor regulates Rab5 membrane localization where the exchange of GDP for GTP is regulated by specific guanine nucleotide exchange factors (30, 31). We propose that the AT1AR also functions as a novel Rab5a guanine nucleotide exchange factor (30) and that AT1AR signaling and intracellular trafficking. In particular, AT1AR activation promotes Rab5a GTP binding, which is correlated with enhanced vesicular fusion. Agonist activation of the epidermal growth factor receptor (EGFR) also results in the recruitment of intracellular proteins involved in endocytic trafficking. In particular, EGFR stimulation leads to the activation and plasma membrane translocation of Rab5a (32). However, unlike what we observe for the AT1AR, EGFR internalization is blocked by Rab5a-S34N. Furthermore, EGFR-dependent Rab5a activation requires the stimulation of a tyrosine kinase signal transduction cascade that leads to the activation of uncharacterized intermediary Rab5a regulatory proteins (32). In contrast, the activation of Rab5a by the AT1AR appears to involve the direct association of the receptor with the GTPase.

There is emerging evidence to suggest that Rab GTPases may be required for selecting cargo proteins for sequestration into clathrin-coated vesicles (33–35). Recently, Carroll et al. (36) demonstrated that Rab9 sequesters cargo proteins into vesicles shuttling from endosomes back to the trans-Golgi network. Specifically, this involves an intermediary Rab9 effector protein, TIP47, which acts as a molecular link between the mannos-6-phosphate receptor cargo protein and Rab9 (36, 37). These studies indicate that Rab GTPases not only function to recruit vesicular effector proteins but may also regulate their activities. In contrast, the observation that Rab5/AT1AR interactions may stimulate Rab5-dependent homotypic fusion of early endosomes suggests that vesicular cargo proteins, such as the AT1AR, may control their own targeting between intracellular compartments by controlling the activity of specific components of the intracellular trafficking machinery in the absence of an intermediary effector protein.

It is now appreciated that the termination of GPCR signaling via heterotrimeric G proteins does not abrogate the potential for “desensitized” GPCRs to signal via other cascades. Through their association with regulatory proteins such as β-arrestins, GPCRs serve as scaffolds for the assembly and compartmentalization of multi-molecular signaling protein complexes (38–41). Understanding why and how GPCRs regulate the redistribution of these complexes into different intracellular compartments will improve our comprehension of the molecular and physiological consequences of GPCR signaling. The identification of an interdependent relationship between AT1AR activation and Rab5a-mediated intracellular vesicular trafficking and fusion provides the first indication that GPCR subtypes may specify the compartment to which these signaling complexes are mobilized. Because there is evidence that multiple small GTP-binding proteins interact with GPCRs (42), future studies should reveal whether GPCRs both interact with and regulate additional components of the intracellular trafficking machinery.

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REFERENCES

1. Touyz, R. M., and Schiffrin, E. L. (2000) Pharmacol. Rev. 52, 639–672
2. Oppermann, M., Freedman, N. J., Alexander, R. W., and Lefkowitz, R. J. (1996) J. Biol. Chem. 271, 13266–13272
3. Anborgh P. H., Seachrist, J. L., Dale, L. B., and Ferguson, S. S. G. (2000) Mol. Endocrinol. 14, 2040–2053
4. Zhang, J., Ferguson, S. S. G., Barak, L. S., Menard, L., and Caron, M. G. (1996) J. Biol. Chem. 271, 18302–18305
