Arf6 negatively controls the rapid recycling of the β2 adrenergic receptor

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Summary

β2-adrenergic receptor (β2AR), a member of the GPCR (G-protein coupled receptor) family, is internalized in a ligand- and β-arrestin-dependent manner into early endosomes, and subsequently recycled back to the plasma membrane. Here, we report that β-arrestin promotes the activation of the small G protein Arf6, which regulates the recycling and degradation of β2AR. We demonstrate in vitro that the C-terminal region of β-arrestin1 interacts directly and simultaneously with Arf6GDP and its specific exchange factor EFA6, to promote Arf6 activation. Similarly, the ligand-mediated activation of β2AR leads to the formation of Arf6GTP in vivo in a β-arrestin-dependent manner. Expression of either EFA6 or an activated Arf6 mutant caused accumulation of β2AR in the degradation pathway. This phenotype could be rescued by the expression of an activated mutant of Rab4, suggesting that Arf6 acts upstream of Rab4. We propose a model in which Arf6 plays an essential role in β2AR desensitization. The ligand-mediated stimulation of β2AR relocates β-arrestin to the plasma membrane, and triggers the activation of Arf6 by EFA6. The activation of Arf6 leads to accumulation of β2AR in the degradation pathway, and negatively controls Rab4-dependent fast recycling to prevent the re-sensitization of β2AR.

Key words: NFAT, TrpC6, c-Met, Drug screen, Ion flux, Metastasis

Introduction

The G-protein coupled receptors (GPCRs) constitute the largest family of protein with almost a thousand members (Fredriksson et al., 2003; Takeda et al., 2002). They play a central role in regulating various physiological functions such as neurotransmission, vision, olfaction, cardiac output, etc. Once stimulated by agonist binding, the receptors undergo conformational changes leading to trimeric G protein activation and then a cascade of signaling events inside the cell. Once internalized, the receptor is directed to intracellular endosomal compartments to be recycled rapidly back to the cell surface from early endosomes, or slowly from deeper late endosomes. Prolonged agonist treatment induces the sorting of the receptor to lysosomes and the degradative pathway leading to its cell surface downregulation.

The signaling of GPCRs is firmly regulated by arrestins. There are three isoforms of arrestins in humans, one is restricted to the visual system (v-arrestin) and the two others (β-arrestin1 and β-arrestin2) are ubiquitously distributed. Agonist stimulation promotes binding of β-arrestins to the G-protein receptor kinase (GRK)-dependent phosphorylated receptor that first induces the desensitization of the receptor by preventing the activation of G proteins. Second, β-arrestin controls receptor internalization through direct binding with AP-2 complexes and clathrin molecules. Indeed, it has been well established that the direct interaction of β-arrestins to ligand-activated and phosphorylated GPCRs leads to the exposure of β-arrestin C-terminal domain that contains the AP-2 and clathrin binding sites promoting receptor internalization (reviewed in Moore et al., 2007). In addition, β-arrestins could also participate in receptor signaling (reviewed in DeWire et al., 2007).

Arf6 belongs to the Arf family of small G proteins that are known to regulate intracellular vesicular transport. Arf6 has been involved in many cellular processes such as phagocytosis, exocytosis, cytokenesis, adhesion, migration and endocytic trafficking during which coordinating lipid membrane transport with F-actin structure reorganization is crucial (reviewed in D'Souza-Schorey and Chavrier, 2006). Arf6 functions in both clathrin-dependent and -independent pathways of endocytosis of different membrane cargos such as transferrin receptor, polymeric immunoglobulin receptor, E-cadherin and MHC class I (Altschuler et al., 1999; D'Souza-Schorey et al., 1995; Klein et al., 2006; Palacios et al., 2002; Radhakrishna and Donaldson, 1997). By stimulating the PIP_2 synthesis (Krauss et al., 2003) and by directly interacting with AP-2 and clathrin (Paleotti et al., 2005; Poupart et al., 2007), Arf6 could induce the membrane recruitment of the coated-pit components. The effect of Arf6 on clathrin-mediated endocytosis was also proposed to be regulated by the Arf6–GAP SMAP1, which interacts directly with the clathrin light chain (Tanabe et al., 2005). Arf6 depletion by siRNA seemed to affect the internalization of some GPCRs including the β2-adrenergic receptor (β2AR) (Houndolo et al., 2005) and to prevent clathrin and AP-2 recruitment onto the activated Angiotensin II receptor (Poupart et al., 2007). Arf6 was proposed to regulate the internalization of the β2AR by directly interacting in an agonist-dependent manner with the β-arrestins (Claing et al., 2001). Defects in β2AR internalization have also been observed following the expression of an Arf6–GAP GIT1 (Claing et al., 2000; Premont et al., 1998). In addition, ARNO, an Arf1 exchange factor that is recruited at the plasma membrane in an Arf6GTP-dependent manner (Cohen et al., 2007; Stalder et al.,

2011) has been shown to directly bind β-arrestins and to stimulate β2AR internalization when overexpressed (Claing et al., 2001). Taken together, these observations implied a role for Arf6 in GPCRs endocytosis.

Several studies have also implicated Arf6 in the post-endocytic transport of membrane receptors. However, the Arf6 recycling pathway seems to be dependent on the cell type, the endocytic pathway and the nature of the cargo (for a review, see D'Souza-Schorey and Chavrier, 2006), however no molecular mechanism has yet been described. Arf6 has also been shown to be involved in the regulated exocytosis in adipocytes and neuroendocrine cells (Aikawa and Martin, 2003; Galas et al., 1997; Yang and Mueckler, 1999), at least through its ability to activate the phospholipase D1 producing fusogenic lipid (Béglé et al., 2009; Caumont et al., 1998), and to interact with the vesicle tethering exocyst complex (Prigent et al., 2003). Until now, no molecular mechanism accounting for Arf6 activation in all these Arf6-dependent pathways has been identified.

The activation of Arfs is achieved by the Sec7-domain-containing protein family (Jackson and Casanova, 2000). This family is made up of at least fifteen members and each member seems to activate its cognate Arf isoform. EFA6, which is an Arf6 specific exchange factor, is involved in actin reorganization, neuronal morphogenesis, cellular polarization and migration processes (Choi et al., 2006; Franco et al., 1999; Li et al., 2006; Luton et al., 2004; Sakagami et al., 2007). By interacting directly with PIP_2 and actin filaments, EFA6 localizes to the plasma membrane where it coordinates cortical actin dynamics with plasma membrane remodeling (Macia et al., 2008). EFA6 seems to control the trafficking of some membrane receptors. Indeed, we have shown that the expression of EFA6 led to the redistribution of transferrin receptor as well as TWICK-1, a K^+ channel, to the cell surface suggesting that EFA6 together with Arf6 could regulate membrane receptor internalization and/or recycling (Decressac et al., 2004; Franco et al., 1999).

Here we identified a new Arf6-dependent pathway that couples the activation of the β2-adrenergic receptor with the inhibition of its recycling, contributing to the receptor desensitization and down-modulation. Agonist stimulation of the β2AR promoted the activation of Arf6 by EFA6 in a β-arrestin-dependent manner. The activated Arf6 led to the desensitization of β2AR by inhibiting the Rab4-dependent fast recycling pathway and relocalizing the receptor to the late endosomes/lysosomes.

Results

Ligand-induced stimulation of the β2AR leads to colocalization with β-arrestin and EFA6 at the plasma membrane

Since Arf6GDP was shown to interact with β-arrestin we asked whether its specific exchange factor EFA6 could also bind to this GPCR adaptor. We used the β2AR as a GPCR model because its endocytosis has been reported to be dependent on Arf6 (Houndolo et al., 2005). We established HEK293 cells stably expressing the β2AR fused to GFP. This cell line has been extensively used to study the β2AR signaling and trafficking (Gagnon et al., 1998; Kallal et al., 1998). We first examined the localization of myc–EFA6, β-arrestin1–HA and the β2AR–GFP in serum-starved cells and in the absence of isoproterenol, a β2AR agonist (–Iso.). As previously shown for the endogenous proteins, β-arrestin1–HA was soluble whereas β2AR–GFP and myc–EFA6 were mostly found at the plasma membrane (Fig. 1A, –Iso.) confirming that the tags did not affect localization of these proteins. It should be noted that numerous domains of the plasma membrane are enriched in myc–EFA6 and β2AR–GFP. These domains were found to be enriched in PIP_2, F-actin and ezrin (data not shown). When, isoproterenol (+Iso.) was added to the cells for 3 min, we observed a plasma membrane translocation of β-arrestin1–HA and a strong colocalization of the three proteins at the cell surface (Fig. 1A, +Iso., see arrowheads). We noticed that the stimulation of β2AR increased the number of membrane folds at the cell surface in which the three proteins are found. Thus, the ligand stimulation of the receptor leads to the concentration of the receptor, β-arrestin1 and EFA6 within discrete area of the plasma membrane.

EFA6 directly interacts with β-arrestin1

Next we examined whether EFA6 could physically interact with β-arrestin1 and more specifically with the C-terminal domain known to be exposed in an agonist-dependent manner and reported to interact with Arf6GDP. In glutathione *S*-transferase (GST) pull-down experiments we observed that GST fused to the C-terminal domain of β-arrestin1, but not GST alone, was able to pull-down EFA6 from a BHK cells lysate overexpressing VSV-G–EFA6 (Fig. 1B). Next, when purified proteins were used, we observed that His–EFA6 interacts directly and specifically with the C-terminus domain of β-arrestin1 (Fig. 1C). Furthermore, the C-terminal portion of EFA6 was sufficient for the interaction with β-arrestin1, while a longer C-terminal portion of EFA6 containing the PH domain could no longer interact with β-arrestin1 suggesting that the PH domain could regulate the accessibility to the β-arrestin binding site (supplementary material Fig. S1). In conclusion, these experiments demonstrate that EFA6 interacts directly with β-arrestin.

The V319–K357 fragment of β-arrestin1 interacts simultaneously with Arf6GDP and EFA6

To further map the β-arrestin binding sites for Arf6 and EFA6, we performed GST pull-down experiments with three fragments of the C-terminal of β-arrestin1. The portion of β-arrestin1 from V319 to K357 was necessary and sufficient for the binding of EFA6 (Fig. 2A). The same portion was also the main binding site for Arf6GDP, while a second region on the C-terminal of β-arrestin1 (from K357 to D383) bound to Arf6 with a lower affinity (Fig. 2B).

This sub-domain could contribute to bind the same Arf6 molecule or to bind a second one. New constructs and more quantitative experiments will be needed to discriminate between these two possibilities.

As both Arf6 and EFA6 bound to the same region of β-arrestin1, it was important to determine whether they bind simultaneously or exclusively. To this end, GST pull-down experiments were performed in the presence of a constant concentration of Arf6GDP and increasing concentrations of EFA6. As observed in Fig. 2, panel A and B, both Arf6GDP and His–EFA6 bound to the β-arrestin1 fragment V319–K357 directly (Fig. 2C, lanes 1 and 2). When both proteins were present, there was no decrease of the binding between Arf6GDP and β-arrestin1 even at a high concentration of His–EFA6. In contrary, the association seemed to be synergistic as higher amounts of Arf6GDP and His–EFA6 were bound to β-arrestin1 when all three proteins were present, compared to when Arf6GDP or His–EFA6 was absent (Fig. 2C, pull down,

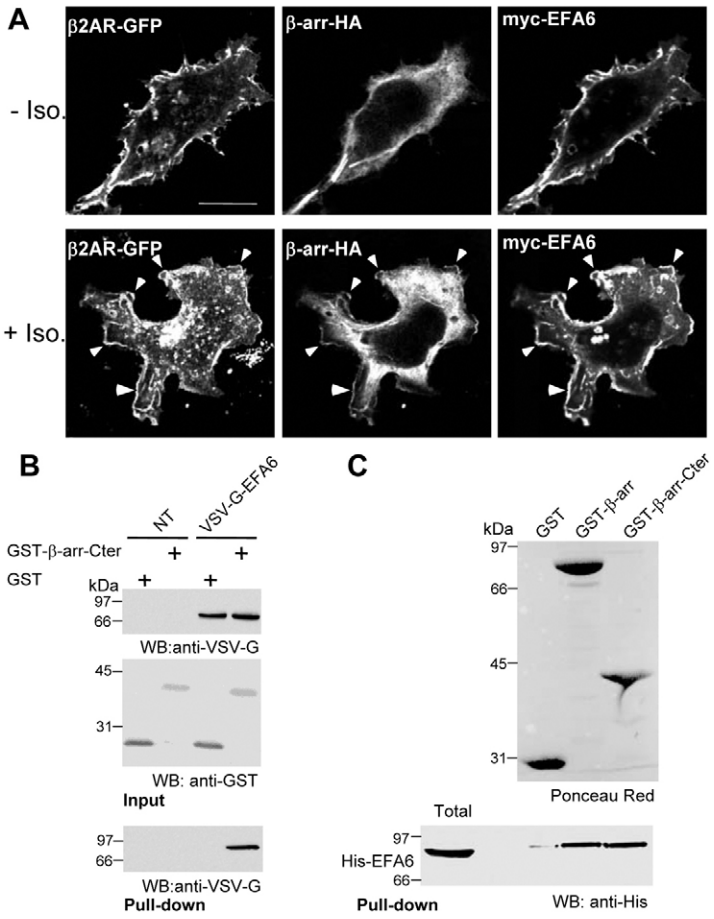

Fig. 1. β-arrestin1 directly interacts with EFA6. (**A**) HEK293 cells stably expressing β2AR–GFP and transiently expressing an HA-tagged β-arrestin (β-arr–HA) and myc–EFA6 were serum starved for 5 h and then stimulated (+Iso.) or not (–Iso.) with 100 nM isoproterenol for 2 min. Immunolocalization was performed as described in the Materials and Methods. (**B**) GST pull-down of VSV-G-tagged EFA6 expressed in BHK cells by the C-terminal domain of β-arrestin fused to GST protein. (**C**) GST pull-down of purified His–EFA6 by β-arrestin full length and its C-terminal domain fused to GST protein.

compare lane 3 to lanes 1 and 2). Therefore, β-arrestin1 can bind simultaneously to Arf6GDP and EFA6 to form a ternary complex.

The C-terminal part of β-arrestin1 stimulates EFA6-catalyzed activation of Arf6 in vitro

As both Arf6GDP and its exchange factor bind to β-arrestin1 simultaneously it was tempting to speculate that β-arrestin could act as a platform to facilitate the activation of Arf6. To this end, we examined in vitro the kinetics of GDP/GTP exchange in Arf6 in the presence or absence of His-tagged EFA6 and GST fusion of β-arrestin1 fragment V319–K357 containing the Arf6 and EFA6 binding sites. The presence of the β-arrestin1 fragment alone facilitated the spontaneous activation of Arf6GDP, possibly by decreasing the affinity of Arf6 for GDP. As expected, the addition of EFA6 strongly promoted Arf6 activation, and addition of the β-arrestin1 fragment further stimulated the EFA6-mediated Arf6 activation (Fig. 2D). We concluded that the C-terminal domain of the β-arrestin1 acts as a platform that allows for the activation of Arf6 by facilitating the gathering of the exchange factor EFA6 and its substrate Arf6GDP.

Agonist activation of the β2 adrenergic receptor leads to the activation of Arf6 in a β-arrestin-dependent manner

We hypothesized that the β-arrestin-dependent Arf6 activation could be triggered by β2AR activation, as β-arrestin is an adaptor of GPCR stimulation and internalization. To test this hypothesis, isoproterenol was added to serum-starved HEK293 cells stably expressing β2AR–GFP and transiently expressing RFP–EFA6, and Arf6GTP was specifically pulled down. In the absence of isoproterenol, a basal level of Arf6GTP was detected. Upon the addition of agonist, higher quantity of Arf6GTP was observed, and the activation peaked at 10 min (approximately twofold compared to basal level) and returned to the basal level after 30 min (Fig. 3A). To determine whether this agonist-mediated Arf6 activation was dependent on β-arrestin, the experiments were repeated on cells depleted of β-arrestin1 and 2 by siRNA. The addition of agonist increased the amount of Arf6GTP in control but not in β-arrestin-depleted cells (Fig. 3B), suggesting that Arf6 activation in response to β2AR activation is dependent on β-arrestin.

Thus, by co-recruiting Arf6GDP and EFA6, the activation of β-arrestin in response to agonist stimulation allows for the activation of Arf6 in vivo.

EFA6 inhibits the Rab4-dependent recycling of β2AR and relocalizes β2AR to late endosomes/lysosomes

Next we asked whether the β-arrestin-dependent activation of Arf6 by EFA6 upon agonist stimulation plays a role in the cellular trafficking of β2AR. We first assessed the effect of EFA6 exogenous expression on isoproterenol-stimulated β2AR internalization. HEK293 cells stably expressing β2AR–GFP were transiently transfected with a plasmid encoding for RFP–EFA6 and we monitored the internalization of the receptor after radio-ligand binding. The internalization rate of β2AR was similar in cells expressing RFP alone or RFP–EFA6, suggesting that Arf6

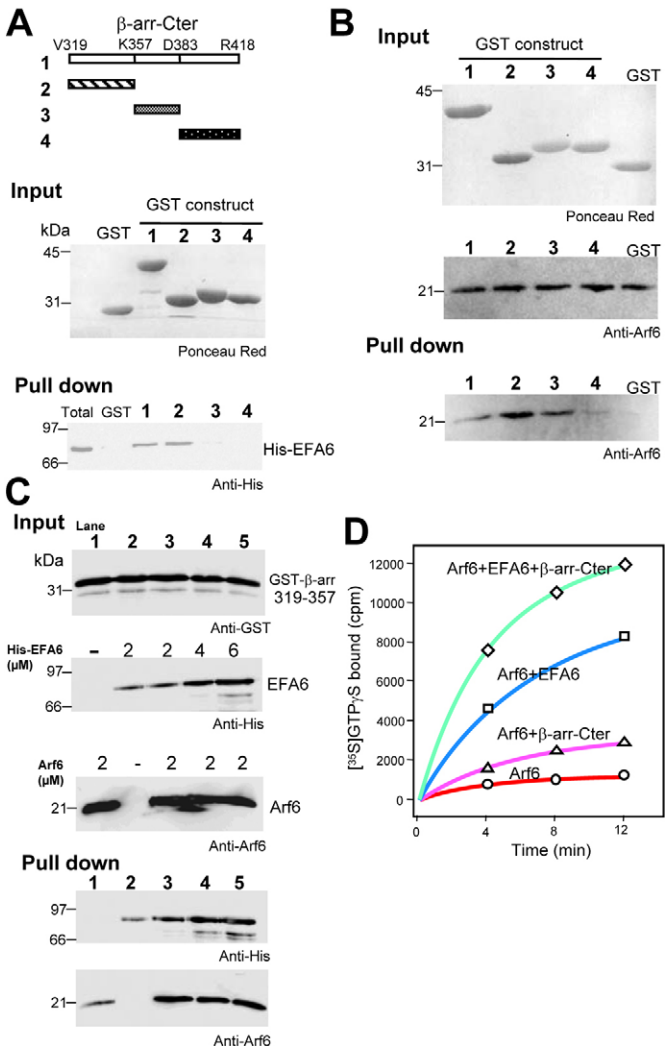

Fig. 2. The V319–K357 peptide of the β-arrestin C-terminal recruits both Arf6GDP and EFA6 and stimulates Arf6 activation in vitro. (**A**,**B**) GST pull-down of purified His–EFA6 (**A**) or purified Arf6GDP (**B**) with different constructs of the β-arrestin C-terminal fused to GST protein. (**C**) GST pull-down of ARF6GDP and His–EFA6 by the V319–K357 domain of β-arrestin C-terminal in the presence of increasing concentration of His–EFA6. (**D**) Kinetics of [^{35}S]GTPγS loading on Arf6GDP. Binding of [^{35}S]GTPγS to purified ARF6GDP (4 μM) was determined (as described in the Materials and Methods) in the presence or absence of purified His–EFA6 (0.5 μM) and the C-terminal domain of β-arrestin1 fused to GST (6 μM).

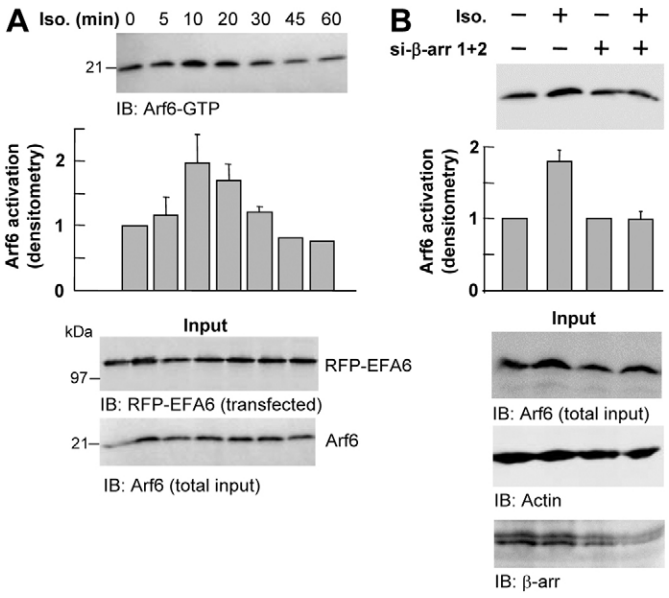

Fig. 3. Stimulation of β2AR by isoproterenol triggers a β-arrestin-dependent activation of endogenous ARF6. (**A**) HEK293 cells stably expressing β2AR–GFP and transiently expressing RFP–EFA6 were serum starved for 5 h and then stimulated with 100 nM isoproterenol (Iso.) for the indicated time. The amount of Arf6GTP was determined as described in the Materials and Methods. The means ± standard deviation (s.d.) of three separate experiments are shown. (**B**) HEK293 cells stably expressing β2AR–GFP and transiently expressing RFP–EFA6 were treated or not with siRNA against β-arrestin1 and 2 and stimulated (+Iso.) or not (–Iso.) with 100 nM isoproterenol for 15 min. Then, the amount of Arf6GTP was determined as described in the Materials and Methods. The means ± s.d. of four separate experiments are plotted.

activation is either not required or not limiting for β2AR internalization (Fig. 4A). We then analyzed the effect of EFA6 on β2AR recycling. We treated the cells with isoproterenol for 20 min at 37℃ to induce internalization, washed and further incubated the cells to allow for the β2AR to recycle. In control cells, 40% of internalized receptors recycled back to the plasma membrane after 30 min, but only 10% in EFA6 overexpressing cells indicating that the recycling is affected by EFA6 expression (Fig. 4B). We observed that during the first 10 min of incubation, the rate of fast recycling, presumably from peripheral early endosomes, was strongly decreased in the presence of RFP–EFA6, while during the following 20 min, overexpression of EFA6 did not affect the slow recycling, presumably from more internal compartments (reviewed in Seachrist and Ferguson, 2003).

As the recycling rate of β2AR was strongly decreased whereas its internalization rate remained unaffected upon Arf6 activation, we assessed the intracellular localization of the internalized receptors. To this end, EFA6 or the activated mutants of Arf6 were overexpressed in HEK293 cells stably expressing the β2AR–GFP and receptor localization was determined by immunofluorescence. When cells were serum starved (–FCS), as previously observed in Fig. 1, RFP–EFA6 and β2AR–GFP localized essentially at the plasma membrane and EFA6 expression did not affect the localization of the receptor (Fig. 5A). In contrast, when the serum was present (+FCS), and in spite of the absence of β2AR agonist, EFA6 expression led to a strong redistribution of the receptor from the cell surface to internal structures (Fig. 5A). This phenotype is reminiscent of the one observed after several minutes of agonist exposure (Kallal et al., 1998). Moreover, this serum-dependent relocalization of β2AR was strictly dependent on the expression of EFA6 and on the GEF activity of EFA6 on Arf6 (Fig. 5A, see quantification): in the presence of serum, β2AR remained at the plasma membrane when the cells did not express RFP–EFA6 (Fig. 5A), expressed a catalytically dead mutant of EFA6 (E242K) or expressed simultaneously the wild-type EFA6 (EFA6wt) and the dominant-negative mutant of Arf6 (Arf6T27N; not shown). These results demonstrate that the EFA6-mediated redistribution of the receptor is strictly dependent on the ability of EFA6 to

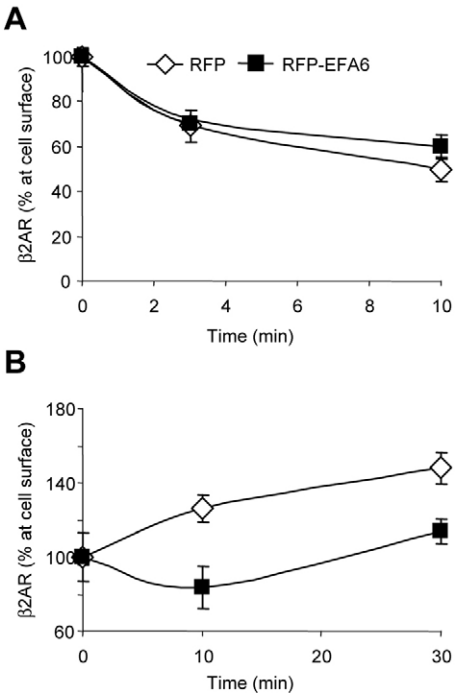

Fig. 4. EFA6 inhibits the fast recycling of the β2AR. (**A**) Kinetic analysis of β2AR internalization. HEK293 cells were transiently co-transfected with plasmids encoding β2AR–GFP and RFP alone or fused with EFA6wt. 24 h after transfection, β2AR internalization was triggered by the addition of isoproterenol (1 μM) and the amount of β2AR present at the cell surface at each time was determined as described in Methods and Methods. (**B**) β2AR recycling. Cells were treated with isoproterenol (1 μM) for 20 min to trigger receptor internalization, then washed twice with 37°C HEK medium and incubated for the indicated time. Surface receptor numbers were determined as described in the Materials and Methods.

activate Arf6. It should be noted that this EFA6- and serum-dependent redistribution of β2AR was β-arrestin dependent. Indeed the co-expression of the dominant negative form of β-arrestin with EFA6 strongly abolished the serum-induced redistribution of the β2AR (not shown). Moreover, our results suggest that the serum enhances the EFA6 GEF activity for Arf6. To test this hypothesis, a pull-down experiment was carried out to monitor the formation of Arf6GTP in HEK293 cells transiently expressing RFP–EFA6 (Fig. 5B). The addition of serum led to a slow but very robust activation of Arf6 (Fig. 5B). At the steady state, the presence of serum resulted in a significantly higher amount of activated Arf6 than when the cells were serum starved (about fourfold; Fig. 5B).

The expression of the fast cycling Arf6T157N-mCherry and the constitutively activated Arf6Q67L–mCherry induced a strong redistribution of the β2AR comparable to that of EFA6wt (Fig. 5A, +FCS). About 80% of the cells expressing either one of these three proteins exhibited a strongly modified pattern of β2AR distribution (Fig. 5C, see quantification). However, consistent with our model and in contrast to EFA6, this Arf6GTP-induced relocalization of the β2AR was independent on the presence of serum (Fig. 5C). Expression of the wt form or the dominant negative mutant of Arf6 did not modify the β2AR localization demonstrating that the accumulation of Arf6GTP was responsible for the internal redistribution of β2AR (Fig. 5C). Altogether these experiments demonstrate that the serum triggers the GEF activity of EFA6 on Arf6, and that the activation of Arf6 in turn inhibits the fast recycling of β2AR to the plasma membrane, resulting in its relocalization towards internal structures.

Then we sought to determine the identity of these internal structures. Co-staining of the receptor with EEA1 (Early Endosome Antigen 1), an early endosome associated protein, showed only a weak overlap between the two proteins, indicating that most of the receptor was not in early endosomes (Fig. 6). In contrast, CD63 a tetraspanin protein mainly located in late endosomes and lysosomes was found strongly colocalized with the β2AR positive internal structures (Fig. 6). This result suggested that the activation of Arf6 by EFA6 directs the β2AR toward the degradative pathway. This was confirmed by co-staining with Lamp-2 a lysosomal resident membrane protein (Fig. 6). The expression of the fast cycling Arf6 mutant Arf6T157N induced the β2AR redistribution into the same CD63 and Lamp-2 positive internal compartments (supplementary material Fig. S2). It should be noted that neither EFA6 nor Arf6 were found on these structures. However we could observe partial co-staining between Arf6T157N and EEA1 suggesting that a fraction of Arf6 was trafficking through early endosomal structures (supplementary material Fig. S2, see arrowheads). We concluded that the activation of Arf6 induces the accumulation of the β2AR in the degradative pathway. In addition it should be noted that prolonged agonist treatment (3 hours) also led to the relocalization of the β2AR to the degradative compartments (supplementary material Fig. S3). All together, these observations suggest that accumulation of Arf6GTP in response to β2AR stimulation leads to the sorting of the receptor to lysosomes.

Small G proteins of the Rab family are key regulators of membrane traffic and among them Rab4 and Rab35 have been described to regulate the fast recycling of membrane receptors. Rab4 controls the fast recycling of β2AR (Seachrist et al., 2000; Yudowski et al., 2009) whereas Rab35 is involved in the fast recycling of the transferrin receptor (Kouranti et al., 2006). If the accumulation of the β2AR in the late endosomes/lysosomes was caused by the inhibition of fast recycling of the receptor upon EFA6 overexpression/Arf6 activation, this phenotype should be rescued by overexpressing the constitutively active mutant of the small G protein Rab4. Indeed, in the presence of both Rab4 Q67L and Arf6T157N, β2AR was found on the plasma membrane, while Rab4Q67L alone did not affect the normal localization of β2AR in the absence of agonist (Fig. 7). On the contrary, the constitutively active mutant of the small G protein Rab35 was not able to rescue the intracellular accumulation of β2AR induced by the overexpression of Arf6T157N (Fig. 7), suggesting that the Rab4-dependent fast recycling of the β2AR was specifically regulated by the activation of Arf6.

Discussion

In this study, we demonstrated that the stimulation of the β2AR, a classical model for GPCR, led to the activation of Arf6 by EFA6 in a β-arrestin-dependent manner. This Arf6 activation did not affect the internalization rate of the receptor, but inhibited the Rab4-dependent fast recycling of the β2AR (Fig. 8). Prolonged activation of Arf6 relocalized the β2AR from the plasma membrane to the late endosomes/lysosomes, thus led to receptor desensitization and regulation of downstream signaling events.

Both EFA6 and Arf6 could bind directly and simultaneously to the C-terminal domain of β-arrestin, and this association facilitated Arf6 activation by EFA6. Biochemical and structural

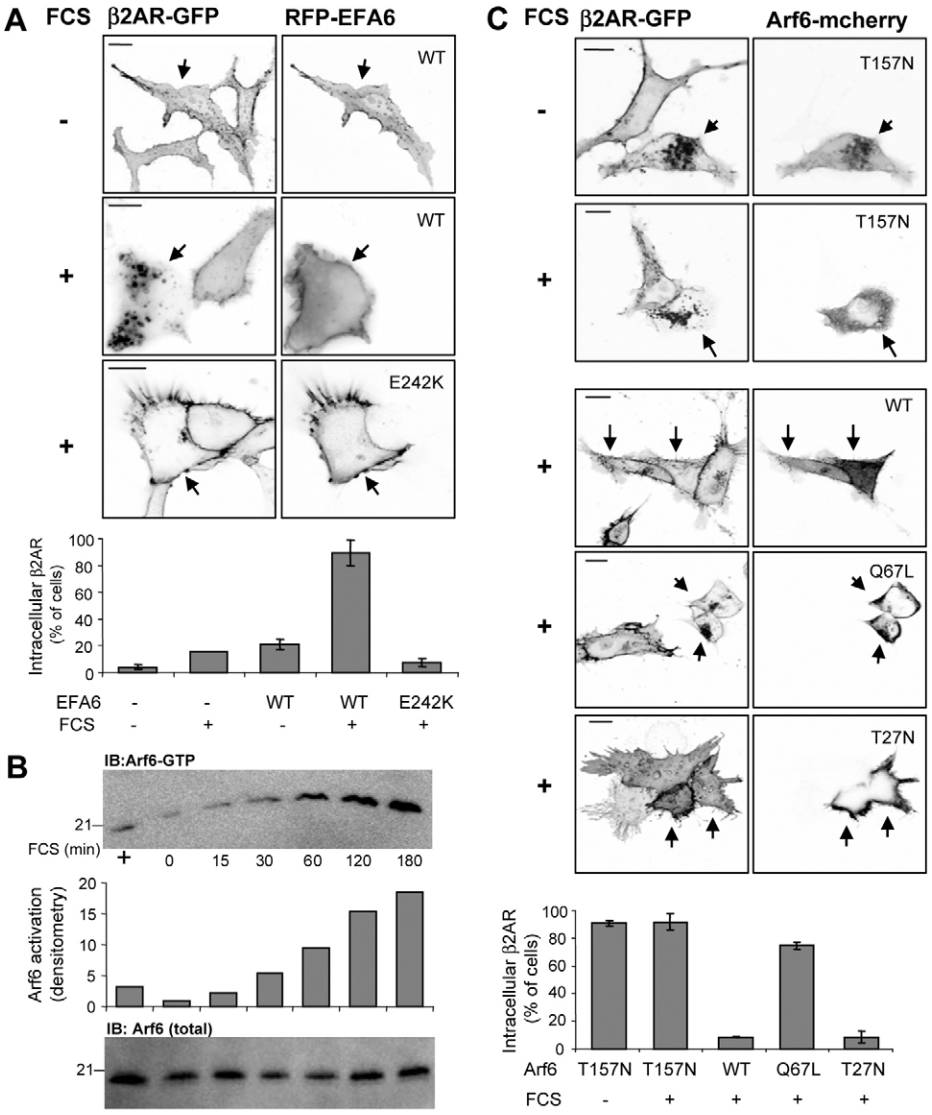

Fig. 5. Activation of Arf6 induces the redistribution of β2AR from the cell surface towards internal compartments. (**A**,**C**) HEK293 cells stably expressing β2AR–GFP were transiently transfected with plasmids encoding RFP-tagged EFA6 or EFA6(E242K (A) or mCherry-tagged Arf6 wt or mutants (C) and incubated with serum (+FCS) or serum-staved (–FCS) for 5 h. Scale bars: 10 μm. The percentage of HEK293 cells showing internal β2AR–GFP-labeled structures was determined for each plasmid combination in triplicate samples from three independent experiments. The error bars represent the standard deviations. (**B**) HEK293 cells stably expressing β2AR–GFP were transiently transfected with plasmids encoding RFP-tagged EFA6 and serum-starved or not (+) for 5 h. Then the serum (FCS) was added for the indicated time and the amount of activated Arf6 was determined as described in the Materials and Methods. The results shown are representative of three separate experiments.

studies have previously established that upon binding to the activated and phosphorylated receptor, β-arrestin undergoes a large conformational change and exposes its C-terminal tail which contains the binding sites for AP-2 and clathrin (reviewed in Moore et al., 2007). This activation of β-arrestin could also be necessary for the formation of the ternary complex (β-arrestin, Arf6 and EFA6), and the subsequent activation of Arf6 in response to receptor stimulation.

In contrast to Arf6, EFA6 interacted with both the full-length β-arrestin (mimicking the non-activated form) and the C-terminal domain of the protein (mimicking the activated form; Fig. 1C). As this interaction must be tightly regulated, conformational changes on EFA6 instead of β-arrestin may be required. Consistently, while the C-terminal domain of EFA6 bound to β-arrestin strongly, addition of the PH domain prevented this interaction. A strong interaction between the PH and the C-terminal domains of EFA6 was also observed (unpublished observation). Therefore, EFA6 needs to be activated and undergoes conformational changes, in order to reveal its β-arrestin binding site at the C-terminal domain, which is usually masked. What would be the activator of EFA6? In this study, addition of serum in cells expressing EFA6 led to Arf6 activation and β2AR redistribution into intracellular compartments. It should be noticed that the addition of serum did not trigger the internalization of the β2AR (not shown). This is the first description of a stimulus that reveals EFA6–GEF activity on Arf6. As the localization of EFA6 does not seem to be affected by agonist or serum exposure, the exchange activity could result from a structural conformational change of the protein. Specific serum-activated receptors would trigger post-traductional modifications of EFA6 such as phosphorylation or ubiquitylation (Théard et al., 2010). A PKC-induced phosphorylation of the Arf1–GEF Arno/cytohesin family has been previously described (Santy et al., 1999) and seems to negatively affect the exchange activity. GEP100/Brag2 has been shown to directly interact with the phosphorylated EGF receptor, but no EGF-dependent phosphorylation of the exchange factor has been reported (Morishige et al., 2008). We are now looking for the molecular mechanism that couples serum addition to the cells and EFA6–GEF activation.

Arno, another ArfGEF, has been shown to interact with the β-arrestin C-terminal region and to stimulate β2AR internalization

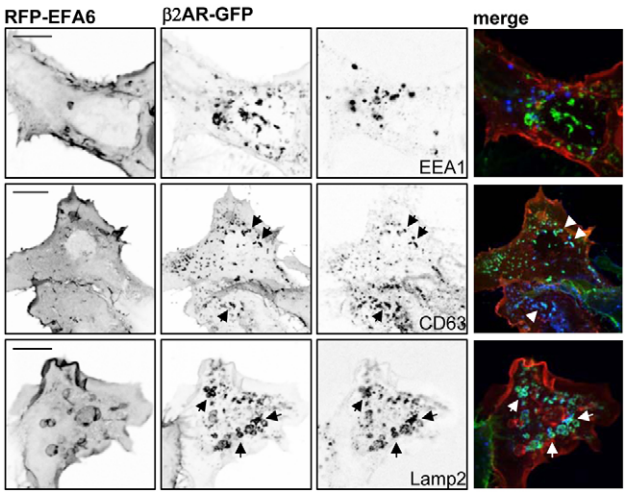

Fig. 6. EFA6 induces the accumulation of β2AR in the degradative pathway. HEK293 cells stably expressing β2AR–GFP were transiently transfected with plasmid encoding RFP-tagged EFA6. After 24 h in FCS-containing medium, cells were fixed and stained with anti-EEA1 (as a marker for early endosomes), anti-CD63 (as a marker for both late endosomes and lysosomes) and anti-Lamp2 (as a marker for lysosomes) antibodies. Scale bars: 10 μm.

after overexpression (Claing et al., 2001). It appears clear now from several studies that Arf6 could control the nucleotide exchange activity of Arno for Arf1 (Cohen et al., 2007; DiNitto et al., 2007; Stalder et al., 2011). Thus, this suggests that β-arrestin could recruit two ArfGEF, a first one, EFA6, as an Arf6 activator and a second one, Arno, as an Arf6 effector. The activation of the β2AR would be at the head of a molecular cascade involving Arf6 and Arf1. However a role for Arf1 in β2AR signaling and/or trafficking remains to be uncovered.

What is the function of the agonist-induced activation of Arf6? Arf6GTP may participate in clathrin coat assembly, by directly interacting with AP-2 and clathrin (Paleotti et al., 2005; Poupart et al., 2007) and by increasing the $PI(4,5)P_2$ level at the plasma membrane (Krauss et al., 2003). Consistently, depletion of Arf6 by siRNA affected the endocytosis of the β2AR. However, the internalization of β2AR was not affected by overexpression of EFA6 in this study, suggesting that the amount of Arf6GTP required for internalization is not limited. As the receptor internalization occurs usually within a few minutes, and the Arf6 activation upon agonist addition peaked around 10 minutes, the activated Arf6 could be required for later steps of receptor trafficking. Indeed, the recycling of β2AR was inhibited in the presence of overexpressed EFA6 or active mutants of Arf6, leading to the intracellular accumulation of β2AR in the endosomal/lysosomal compartments. The constitutively active mutant of Rab4 could rescue this intracellular accumulation of β2AR, suggesting that the Rab4-dependent fast recycling pathway was regulated by the activation of Arf6. This is in agreement with a recent an elegant study demonstrating that agonist removal produced a rapid increase in β2AR recycling (Yudowski et al., 2009). Thus, we propose that agonist removal stops the activation of Arf6 leading to the release from inhibition of Rab4-dependent fast recycling.

We have previously shown that EFA6 is involved in actin cytoskeleton reorganization and in epithelial cell polarity

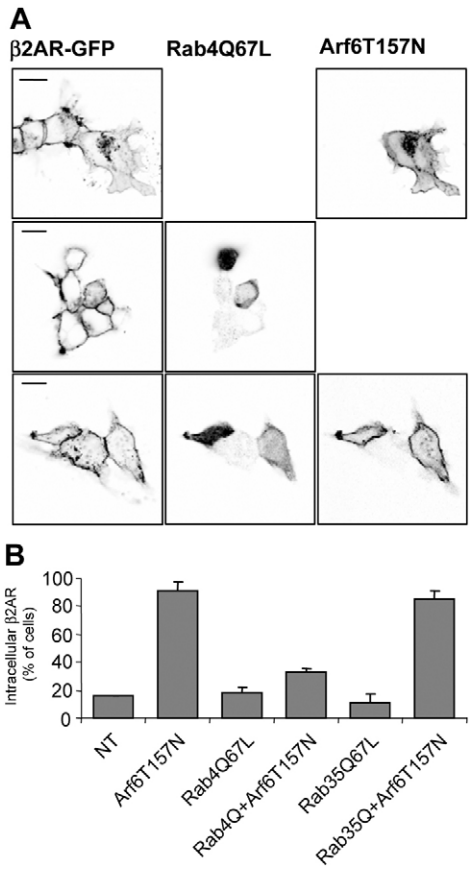

Fig. 7. Arf6GTP acts upstream Rab4 in the β2AR recycling pathway. (**A**) HEK293 cells stably expressing β2AR–GFP were transiently transfected with plasmid encoding mCherry-tagged Arf6(T157N) or myc-tagged Rab4(Q67L) or both plasmids in combination. Scale bars: 10 μm. (**B**) Quantification of the effect of Rab4 and Rab35 on the Arf6-induced redistribution of β2AR. The percentage of HEK293 cells showing internal β2AR–GFP-labeled structures was determined for each plasmid combination on duplicate samples in three independent experiments. The means ± s.d. of these three experiments are plotted.

(Derrien et al., 2002; Franco et al., 1999; Luton et al., 2004; Théard et al., 2010). In these two processes, the EFA6 C-terminal domain is strictly required presumably through its ability to directly interact and remodel F-actin organization (Macia et al., 2008), while Arf6GTP alone cannot reproduce the full function of EFA6 (Klein et al., 2008; Luton et al., 2004). In contrast, we show that the Arf6GTP mutants are fully competent to induce the β2AR intracellular redistribution suggesting that under agonist stimulation the EFA6 C-terminal domain is only required for β-arrestin binding and not for actin regulation.

Scarselli and Donaldson have recently demonstrated that in the absence of agonist, the β2AR constitutively internalizes in a clathrin-independent pathway (Scarselli and Donaldson, 2009). This internalization is slow (about four times slower than the agonist-induced internalization) but seems to be peculiar as the M2 receptor does not undergo any detectable internalization in the absence of ligand. Our study does not provide any information on the role of EFA6/Arf6 in this clathrin-independent constitutive internalization. However, as the expression of EFA6/Arf6 induces an intracellular redistribution

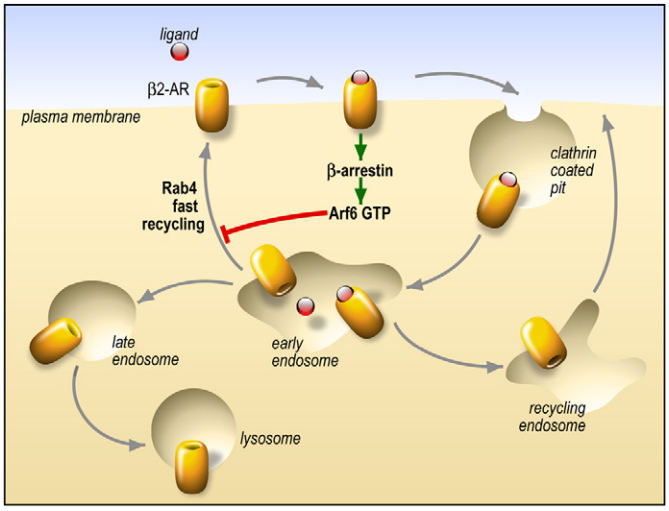

Fig. 8. Schematic model of the role of Arf6 in the control of β2AR rapid recycling.

of the β2AR in the absence of agonist and that their expression inhibits the fast recycling after agonist stimulation, we hypothesize that Arf6 could control both constitutive and agonist-dependent recycling.

Questions remain on how Arf6 activation is linked to Rab4-dependent recycling pathway. It is tempting to speculate that the Arf6GTP associated to AP-2/clathrin vesicles is transported to endosomal structures where it would regulate recycling by stimulating a Rab4–GAP. Consistently, whereas EFA6 is mainly located at the plasma membrane where Arf6 activation occurs, intracellular EEA1 positive staining of Arf6GTP was observed. Recently Chavrier and colleagues have demonstrated that Arf6GTP could be associated with clathrin-coated pits in an AP-2-dependent fashion (Montagnac et al., 2011). They also showed that the Arf6 effectors JIP3/4 became associated to the endocytic vesicles after the burst of auxilin (i.e. once the uncoating occurred), and that this Arf6-dependent recruitment of JIP3/4 was required for the fast recycling of the transferrin receptor (Montagnac et al., 2011). It will be interesting to determine if the Arf6-dependent recruitment of JIP3/4 also plays a role in β2AR fast recycling.

In conclusion, our results indicate that Arf6 is activated by EFA6 in a β-arrestin-dependent manner upon agonist-mediated activation of β2AR. The physical connection between the cargoes (here β2AR) and the activated Arf6 could ensure a specific accumulation of both molecules within the same micro-domains of the plasma membrane and subsequently in the same endocytic vesicles. This spatially and timely regulated accumulation would allow for the recruitment of Arf6 effectors required at later stages of trafficking to mediate receptor desensitization upon prolonged stimulation of agonists.

Materials and Methods

Cell culture, reagents and antibodies

Baby hamster kidney cells (BHK-21) were grown in BHK-21 medium (Gibco-BRL), containing 5% FCS, 10% tryptose phosphate broth, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine.

Human embryonic kidney cells (HEK293) were grown in HEK media corresponding to Dulbecco's modified Eagle's medium containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin. HEK293 stably expressing the β2-AR fused to GFP (HEK293-β2AR) were maintained in HEK medium complemented with 200 µg/ml G418.

Phospholipids were from Avanti Polar Lipids (Birmingham, AL), [^{35}S]GTPγS and [^{3}H]CGP12177 were from Perkin Elmer. Azolectin, isoproterenol, unlabeled nucleotides, were from Sigma Chemical (St Louis, MO).

The following antibodies were used: mouse monoclonal antibodies (mAb) against VSV-G epitope (clone P5D4; Roche Diagnostics GmbH, Mannheim, Germany), rabbit antiserum against GFP (Clontech), mouse monoclonal anti-His (Sigma), mouse mAb against Arf6 (clone 8A6-2 provided by S. Bourgoin, Sainte-Foy, Canada). Mouse monoclonal anti-Lamp2 antibody (clone AC17), goat anti-β-arrestin1 (K16 and C19) and anti-β-arrestin2 (C18) were from Santa Cruz Biotechnology (Santa Cruz, Heidelberg). Mouse monoclonal antibodies anti-EEA1 and anti-CD63 were from BD Biosciences (France). FITC, Texas-Red- and Cy5-conjugated antibodies were from Jackson ImmunoResearch (Interchim, France).

DNA constructs

pcDNA3-β2AR-GFP, pcDNA3-β-arrestin1 and pcDNA3-β-arrestin1 (319–418; named C-ter) plasmids were provided by C. Moore and J. Benovic (Thomas Jefferson University, Philadelphia). Sequences encoding residues 319–418; 319–357, 357–383, 383–418 of bovine β-arrestin1 were obtained by PCR and cloned into pGEX2T (GE Healthcare) for in-frame fusion with GST at the N-terminus. Plasmids encoding His–EFA6A, VSV-G-tagged EFA6A, GFP–EFA6A, His–Arf6 have been described elsewhere (Derrien et al., 2002; Franco et al., 1999; Macia et al., 2001). Sequences encoding EFA6A wt and E242K mutant were obtained by PCR and cloned into pRFP-C1 using HindIII/SacII cloning sites to for in-frame fusion with RFP at the N-terminus. Sequences encoding residues 1–645 (full length); 125–347 (Sec7); 490–645 (Cter); 351–645 (PH-Cter) of human EFA6A were obtained by PCR and cloned into pGEX-3X (GE Healthcare) for in-frame fusion with GST at the N-terminus. mCherry-tagged Arf6wt and T157N, Q67L, T27N Arf6 mutants were generated by PCR and inserted into p(mCherry-C1; Clontech). All constructs were verified by double-stranded DNA sequencing. pcDNA3 myc-tagged Rab4Q67L was provided by M. Cormont (I3M, Nice, France) and Rab35Q67L was obtained from A. Echard (Institut Pasteur, Paris, France).

RNA interference

A siRNA duplex (5′-ACCTGCGCCTTCCGCTATG-3′) that targets both human β-arrestin1 and β-arrestin2 (Gesty-Palmer et al., 2006). A siRNA (5′-GGUCUGCUCCUCAGAAGCU-3′) was used as a control. Both siβ-arrestin1/2 and siSiah were purchased from Eurogentec (Belgium).

HEK293 cells were plated at 40% confluency and transfected with the indicated siRNA (20 nM) using Interferin reagent according to the manufacturer's instruction (Polyplus transfection, Illkirch, France). To measure β-arrestin depletion after 72 h siRNA treatment, aliquots of total cell lysates were removed before GST-pull down experiment and analyzed by western blotting with anti-β-arrestin antibodies. Bound antibodies were detected by chemiluminescence with the ECL procedure and revealed using the Fujifilm LAS3000 fluorescence imaging system. The intensity of β-arrestin signals was then quantified using AIDA software.

Expression and purification of recombinant proteins

For the in vitro binding assays, recombinant Arf6GDP and recombinant myristoylated Arf6 wild-type with a C-terminal hexa-histidine tag were prepared as described elsewhere (Chavrier and Franco, 2001; Franco et al., 1995; Prigent et al., 2003). Recombinant his-tagged EFA6A was prepared as previously described (Macia et al., 2008).

Confocal immunofluorescence microscopy

BHK-21 or HEK293 cells plated on 11 mm round glass coverslips were transiently transfected with pcDNA3 or pEGFP-N1/C1 constructs using the Jet-PEI (Polyplus transfection, Illkirch, France) or Fugene 6 (Roche) transfection reagents as described by the manufacturers. Unless otherwise stated, the cells were washed twice in PBS 24 h after transfection, then fixed in 0.3% paraformaldehyde, and processed for immunofluorescence analysis as described previously (Franco et al., 1998). Confocal microscopy analysis was carried out with a Leica TCS-SP5 microscope (Leica Microsystems).

Co-immunoprecipitation

BHK-21 cells (2×10^6 cells in 100 mm tissue culture dishes) were transfected with various EGFP-EFA6A constructs or pEGFP-C1 alone. cells were lysed 48 h after transfection, in 0.5 ml of lysis buffer [20 mM Hepes, pH 7.4, 1% NP-40, 100 mM NaCl, 1 mM $MgCl_2$, 0.25 mM PMSF and a tablet of protease inhibitors (Roche)]. Lysates were clarified by centrifugation at 13,000 ***g*** for 10 min, and aliquots of the supernatant were kept for further analysis. The remaining supernatant was mixed with 5 µg of anti-GFP antibody and 20 µl protein-A–Sepharose CL4B for 4 h at 4℃. The beads were then washed in lysis buffer and immunoprecipitated proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes.

GST pull-down experiments

BHK-21 cells were mock-transfected or transfected with plasmids encoding VSV-G-tagged EFA6A using Fugene 6 reagent (Roche). After 24 h, cells were lysed in 50 mM Tris pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 0.05% sodium cholate, 0.005% SDS, 2 mM dithiothreitol (DTT) and a cocktail of protease inhibitors (Roche) and centrifuged at 15,000 ***g*** for 10 min at 4℃. Supernatants were incubated with 2 mM GST constructs for 15 min at 4℃ in the presence of 0.5% BSA. Then glutathione–Sepharose beads were added for 1 h at 25℃. Beads were washed and bound proteins were eluted using SDS sample buffer and separated on SDS-PAGE. The presence of EFA6 in the eluate was detected by western blot analysis using anti-VSV-G monoclonal antibody.

For direct binding assay with purified recombinant proteins, 2 μM GST or GST fusion proteins containing various fragments of β-arrestin1 were first immobilized onto glutathione–Sepharose beads in binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100 and 1 mM DTT), and then mixed with GDP-bound Arf6 and/or His-tagged EFA6 (2 μM of each unless otherwise stated) and incubated for 1 h at 25℃.

[^{35}S]GTPγS binding assay

Arf6GDP (4 μM) was incubated at 30℃ with [^{35}S]GTPγS (20 μM, ~2000 c.p.m./pmol) in 50 mM Tris/HCl (pH 8.0), 1 mM $MgCl_2$, 1 mM DTT, 120 mM NaCl, with or without (as indicated in the figure legend) His-tagged EFA6 (0.5 μM) and GST-β-arr1-Cter (6 μM). At the indicated times, samples of 25 μl were removed and measured for radioactivity as described previously (Franco et al., 1995).

Pull down assay for Arf6GTP

Activation levels of endogenous Arf6 proteins were assayed essentially as described previously (Niedergang et al., 2003). Briefly, 24 h after transfection (unless indicated otherwise), HEK293-β2AR cells (4×10^6 per condition) were lysed at 4℃ in 1 ml of lysis buffer (50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.05% sodium cholate, 0.005% SDS, 10% glycerol, 2 mM DTT with protease inhibitors). Lysates were clarified by centrifugation at 13,000 ***g*** for 10 min and incubated with 0.5% BSA and 40 μg of GST–Arf-binding-domain bound to glutathione–Sepharose beads (Amersham Pharmacia Biotech.) for 40 min at 4℃. The beads were washed three times in lysis buffer and bound proteins eluted in 30 μl of SDS-PAGE sample buffer. Normalized amounts of total Arf6 from the starting lysates were loaded on the gel and assessed for the presence of Arf6GTP by western blot using an anti-Arf6 antibody. After detection of bound antibodies by chemiluminescence (ECL Amersham procedure) and revelation using the Fujifilm LAS3000 fluorescence imaging system, the amount of Arf6GTP signals was quantified using the AIDA software.

β2AR internalization and recycling experiments

HEK293 cells (10^6) in six-well plates were transiently transfected and tested 24 h post-transfection. For the internalization assay, cell were washed with 37℃ HEK medium and incubated with 1 μM isoproterenol for the indicated times. For the recycling assay, cells were incubated with 1 μM isoproterenol for 20 min washed twice with 37℃ HEK medium and then incubated again. At various time points, cells were transferred to 4℃ and washed with ice-cold PBS to stop all membrane trafficking events. To measure the concentration of β2AR on the plasma membrane, cells were incubated with 10 nM [^{3}H]CGP12177 (Staehelin et al., 1983) for 5 h at 4℃, washed four times with ice-cold PBS, lysed in PBS, 0.1% Triton X-100, 2.5 mM EDTA, and measured for radioactivity using a scintillation liquid analyzer.

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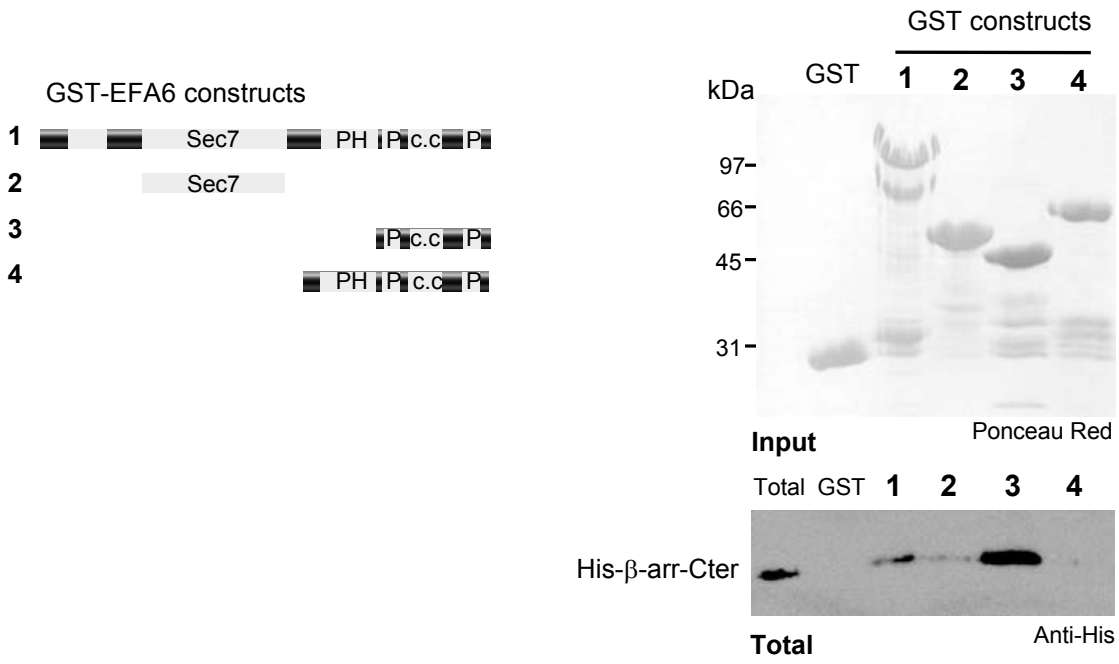

Fig. S1. The C-terminal domain of EFA6 is responsible for β-arrestin interaction. GST pull-down of purified His-tagged C-terminal domain of β-arrestin1 (1 μM) with different constructs of EFA6 fused to GST protein (2 μM).

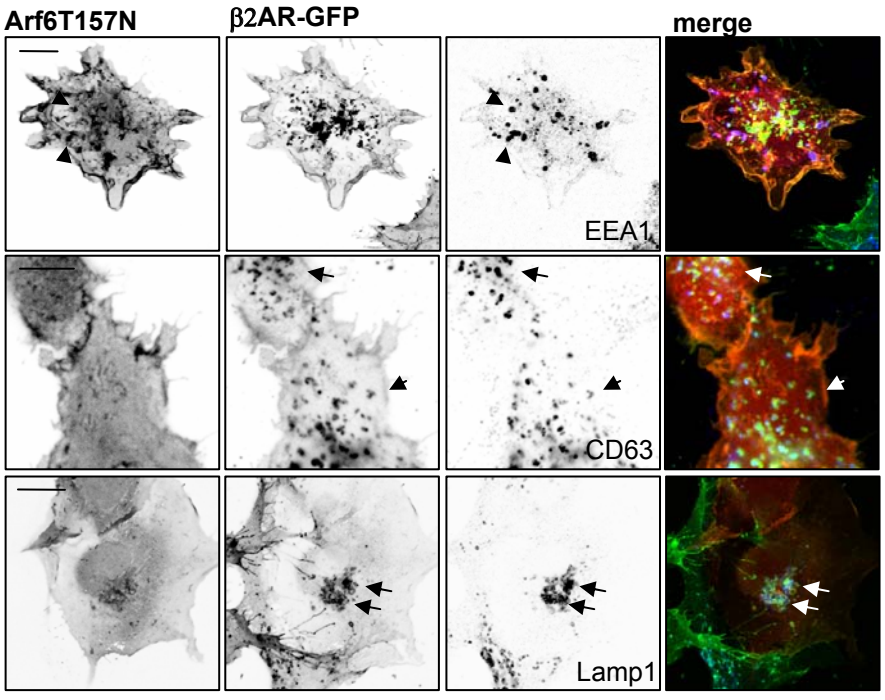

Fig. S2. Activated Arf6 induces the accumulation of the β2AR into the degradative pathway. HEK293 cells stably expressing β2AR-GFP were transiently transfected with plasmid encoding mcherry-tagged Arf6T157N. After 24 h, cells were fixed and stained with anti-EEA1 (as a marker for early endosomes), anti-CD63 (as a marker for both late endosomes and lysosomes) and anti-Lamp2 (as a marker for lysosomes) antibodies. Scale bars, 10 μm.

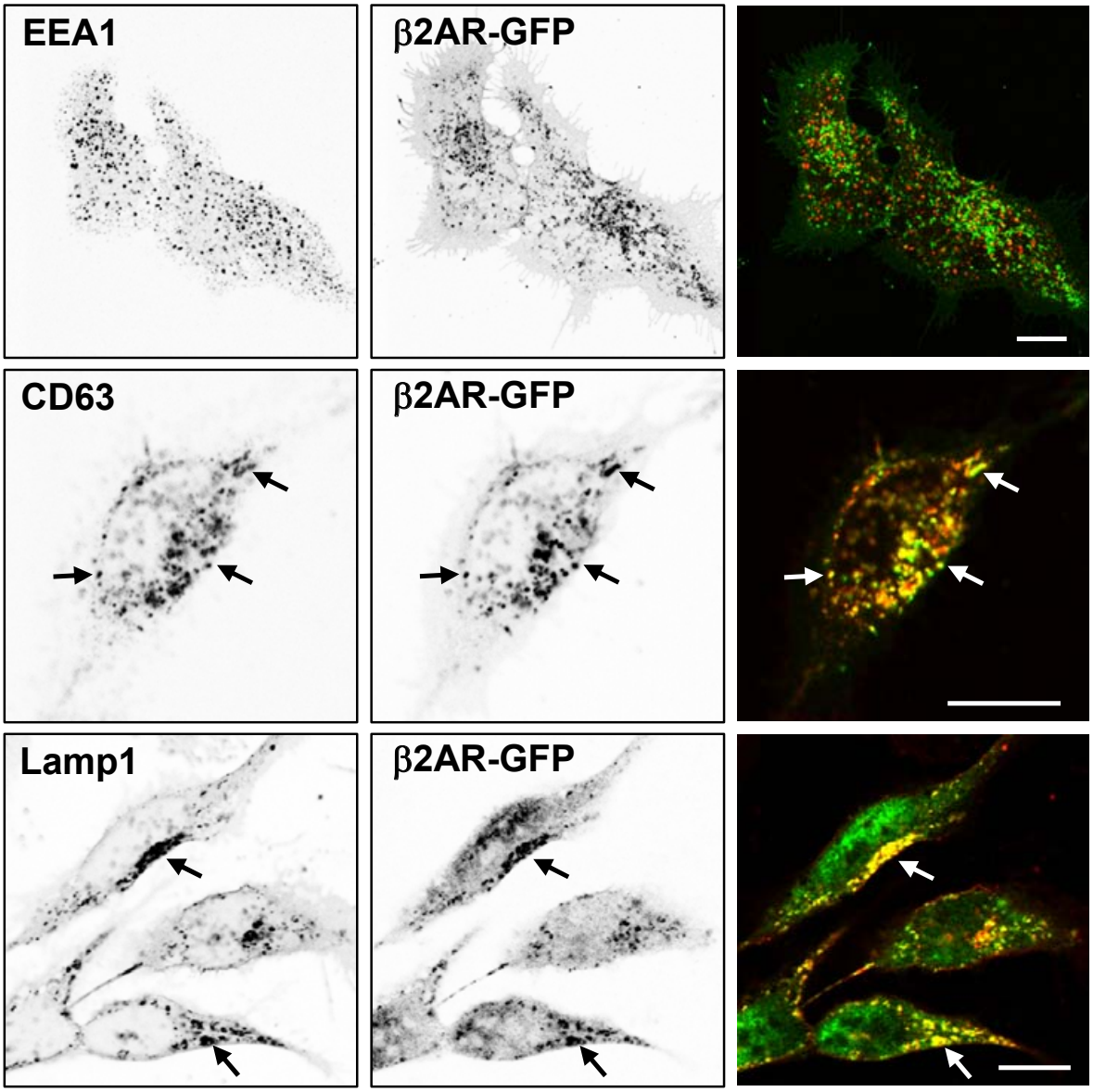

Fig. S3. Prolonged isoproterenol treatment induces the accumulation of β2AR into the degradative pathway. HEK293 cells stably expressing β2AR-GFP were treated with isoproterenol for 3h. Cells were fixed and stained with anti-EEA1 (as a marker for early endosomes), anti-CD63 (as a marker for both late endosomes and lysosomes) and anti-Lamp2 (as a marker for lysosomes) antibodies. Scale bars, 10 μm.