Src-dependent phosphorylation of β2-adaptin dissociates the β-arrestin–AP-2 complex

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Accepted 14 March 2007
Journal of Cell Science 120, 1723-1732 Published by The Company of Biologists 2007
doi:10.1242/jcs.03444

Summary

β-arrestins are known to act as endocytic adaptors by recruiting the clathrin adaptor protein 2 (AP-2) complex to G-protein-coupled receptors (GPCRs), linking them to clathrin-coated pits (CCPs) for internalization. They also act as signaling molecules connecting GPCRs to different downstream effectors. We have previously shown that stimulation of the angiotensin II (Ang II) type 1 receptor (AGTR1, hereafter referred to as AT1R), a member of the GPCR family, promotes the formation of a complex between β-arrestin, the kinase Src and AP-2. Here, we report that formation of such a complex is involved in the AT1R-mediated tyrosine phosphorylation of β2-adaptin, the subunit of AP-2 involved in binding β-arrestin. We identify a crucial tyrosine residue in the ear domain of β2-adaptin and show in vitro that the phosphorylation of this site regulates the interaction between β-arrestin and β2-adaptin. Using fluorescently tagged proteins combined with resonance energy transfer and image cross-correlation spectroscopy approaches, we show in live cells that β2-adaptin phosphorylation is an important regulatory process for the dissociation of β-arrestin–AP-2 complexes in CCPs. Finally, we show that β2-adaptin phosphorylation is involved in the early steps of receptor internalization. Our findings not only unveil β2-adaptin as a new Src target during AT1R internalization, but also support the role of receptor-mediated signaling in the control of clathrin-dependent endocytosis of receptors.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/10/1723/DC1

Key words: Src, β-arrestin, AP-2, GPCR, BRET, Image cross-correlation spectroscopy

Introduction

β-arrestins are adaptor molecules that have been first described in playing a role in G-protein-coupled receptor (GPCR) desensitization. They bind agonist-mediated GRK-phosphorylated sites on GPCRs in order to uncouple them from their G proteins. They also link the receptor to components of the clathrin-coated vesicles, such as clathrin and its adaptor protein 2 (AP-2), to mediate GPCR internalization (Claing et al., 2002; Lefkowitz, 1998). β-arrestin has been shown to interact directly with β2-adaptin (Kim and Benovic, 2002; Laporte et al., 1999), the β-subunit of the heterotetramic AP-2 complex that is known to play a role in the assembly of clathrin and in linking cargo to clathrin lattices. This interaction is important because it has been previously shown that β-arrestin mutants that lack the AP-2 binding sites are defective in their ability to target the GPCR–β-arrestin complexes to clathrin coated-pits (Laporte et al., 2000; Santini et al., 2002). Moreover, β2-adaptin mutants impaired in β-arrestin binding inhibit the agonist-induced internalization of certain GPCRs (Laporte et al., 2002).

Beyond their classical function, β-arrestins act as signaling adaptors linking receptors to multiple downstream effectors. The non-receptor tyrosine kinases of the Src family, such as Src, Hck and Yes, are amongst the numerous signaling proteins that are recruited to β-arrestin in an agonist-dependent manner (Lefkowitz and Shenoy, 2005; Luttrell and Luttrell, 2004). Src recruitment to the β2-adrenergic receptor (β2AR)–β-arrestin complex has been shown to activate the extracellular signal-regulated kinases 1/2 (ERK 1/2) (Ahn et al., 1999; Luttrell et al., 1999). Similarly, β-arrestin recruits Src to the neurokinin (NK-1) receptor (DeFea et al., 2000), Hck to the CXCR-1 chemokine receptor (Barlic et al., 2000) and Yes to the endothelin type A (ETA) receptor (Imamura et al., 2001) to regulate proliferative and antiapoptotic effects of substance P, granule release from human neutrophils and GLUT4 translocation in response to receptor activation, respectively. β-arrestin-mediated recruitment of Src following GPCR stimulation has been also shown to promote the phosphorylation of dynamin, a GTPase involved in the fission of clathrin-coated vesicles (CCVs) from the plasma membrane (Ahn et al., 1999; Miller et al., 2000) and to regulate the internalization of the β2AR and M1 muscarinic receptor (Ahn et al., 1999; Werbonat et al., 2000). Although, β-arrestin can assume both roles of signaling and endocytic adaptor for many

GPCRs, it is still largely unknown whether the recruitment of signaling molecules to β-arrestin impacts its endocytic function.

Our recent observation that, following receptor stimulation, Src and AP-2 are found in a complex with β-arrestin (Fessart et al., 2005), prompted us to investigate the role of Src in regulating the formation of the endocytic complex during internalization of angiotensin II (Ang II) type 1 receptor (AGTR1, hereafter referred to as AT1R). The data presented here not only support the idea that the recruitment of Src to the β-arrestin–AP-2 complex is necessary for the agonist-dependent phosphorylation of the β2-subunit, but that this phosphorylation event contributes in regulating the disassembly of the endocytic complex and AT1R internalization.

Results

AT1R activation induces Src-dependent phosphorylation of β2-adaptin

Since we have previously shown that AT1R activation in vascular smooth muscle cells (VSMCs) leads to the formation of a complex containing β-arrestin, Src and AP-2 (Fessart et al., 2005), we first assessed whether stimulation of the receptor also promoted tyrosine phosphorylation of AP-2. VSMCs were stimulated for different periods of time with Ang II, and the endogenous β-subunits of the AP complex were immunoprecipitated. The level of tyrosine phosphorylation in the immunopurified β-adaptin complexes was then analyzed by western blotting using an anti-phosphotyrosine antibody (Fig. 1A). Under conditions preventing dephosphorylation (using inhibitors of tyrosine phosphatases), Ang II induced the phosphorylation of a 105 kDa protein in a time-dependent manner. Densitometry analysis (Fig. 1A, graph) revealed that the phosphorylation of the 105 kDa protein increased after 5 minutes of Ang II stimulation, and was maintained after 15 minutes of agonist treatment. This 105 kDa protein could represent the β1 or β2 subunit of AP complexes, because the antibody used to immunoprecipitate the endogenous β-subunits does not differentiate between the two forms. To substantiate that the β2-subunit of adaptin – which was previously shown to directly bind β-arrestin (Kim and Benovic, 2002; Laporte et al., 1999) – was indeed a target for tyrosine phosphorylation, we used two other cell lines, HEK293 and COS-7 cells. Cells of both lines were transfected with AT1R and Flag-tagged β2-adaptin (Flag-β2-adaptin), and the phosphorylation status of the immunopurified β2-subunit was assessed using similar conditions as for VSMCs. Results show that in COS-7 cells an increase in tyrosine phosphorylation of the β2-subunit was detectable after 5 minutes of Ang II treatment (Fig. 1B) and reached a maximum level after 15 minutes. However, in HEK293 cells the level of β2-adaptin phosphorylation increased to maximum levels after 5 minutes and decreased after 15 minutes of agonist treatment (Fig. 1C). These results demonstrate that AT1R activation induces tyrosine phosphorylation of β2-adaptin in different cell types.

To verify whether Src is the kinase responsible for the phosphorylation of β2-adaptin, we used two different approaches. First, the phosphorylation status of β2-adaptin was assessed following Ang II stimulation of cells expressing AT1R and Flag-β2-adaptin pre-treated with either DMSO

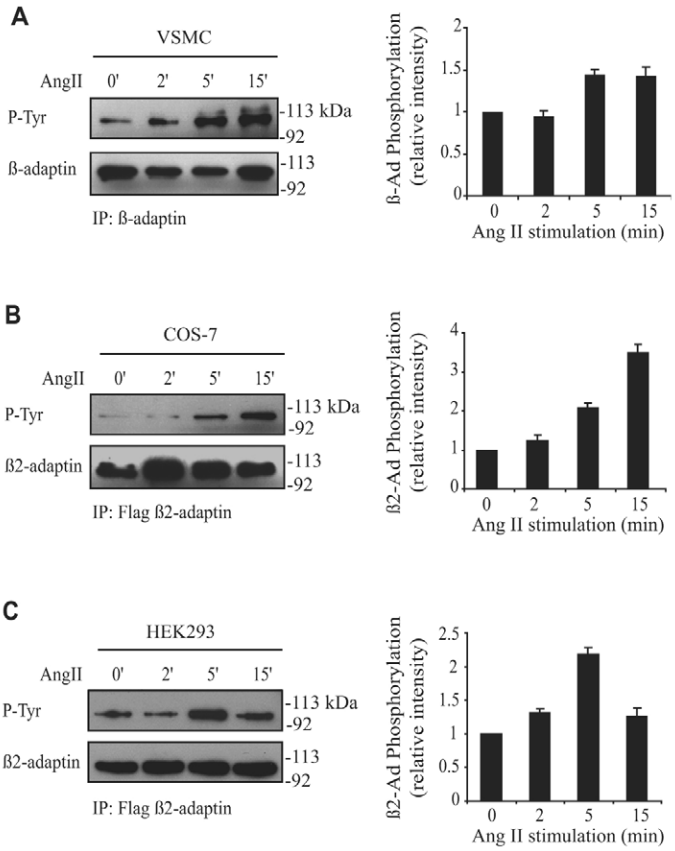

Fig. 1. Ang II induces the tyrosine phosphorylation of β2-adaptin. (A) VSMCs were stimulated with Ang II (1 μM) for the indicated time. Endogenous β-adaptins were immunoprecipitated using the anti-β-adaptin antibody (AP-1/2), and the immunoprecipitates were analyzed by western blot as described in Materials and Methods. (B,C) COS-7 (B) or HEK293 cells (C) transfected with HA-AT1R and Flag-β2-adaptin were stimulated with Ang II (1 μM) for the indicated time and immunoprecipitated Flag-β2-adaptin was analyzed as described above. Densitometry analyses are presented as the mean ± s.e.m. of at least three independent experiments. They represent the relative amount of β2-adaptin phosphorylated as compared with non-stimulated cells after normalizing for equal amounts of β2-adaptin.

(vehicle), the Src family kinase inhibitor (PP2), its inactive analogue (PP3) or the epidermal growth factor receptor (EGFR) inhibitor PD158780 (Fig. 2A). Results show that PP2 robustly inhibited (by more than 90%) the tyrosine phosphorylation of β2-adaptin compared with cells treated with vehicle, whereas PP3 had no significant inhibitory effect. When using the EGFR kinase inhibitor PD158780, we observed a reproducible but not significant reduction in β2-adaptin phosphorylation compared with PP2. Second, the effect of overexpressing a kinase-inactive mutant of Src, HA-tagged Src K298R (HA-Src-K298R) was assessed on β2-adaptin phosphorylation. Cells were transfected with AT1R, Flag-β2-adaptin and either Src or Src K298R. They were then left untreated or treated with Ang II for 5 minutes, and the level of β2-adaptin phosphorylation in the immunoprecipitates was evaluated by western blotting (Fig. 2B). Results show that, upon agonist addition β2-adaptin was phosphorylated in cells

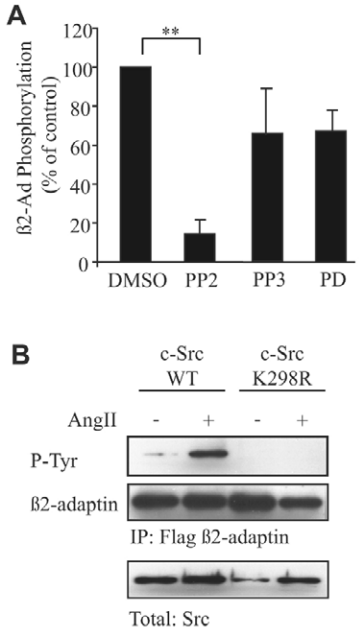

Fig. 2. Ang II-mediated phosphorylation of β2-adaptin involves Src. (A) COS-7 cells transfected with HA-AT1R and Flag-β2-adaptin were incubated for 30 minutes with either the vehicle (DMSO), the Src inhibitor (PP2, 4 μM), the EGFR inhibitor (PD158780, 50 nM) or the less active form of PP2 (PP3, 4 μM), before adding Ang II (1 μM). Flag-β2-adaptin was immunoprecipitated using an anti-Flag antibody, and the immunoprecipitates were analyzed by western blot as described in Materials and Methods. Data are presented as the mean ± s.e.m. of three independent experiments. They represent the percent of β2-adaptin phosphorylation as compared to vehicle, after normalizing for equal amounts of β-adaptin. ** indicates $P<0.01$ vs DMSO determined by one-way ANOVA, and was considered significant. (B) COS-7 cells transfected with AT1R, Flag-β2-adaptin and either Src wild type or Src K298R (HA-Src and HA-Src K298R) were left untreated (–) or stimulated (+) with Ang II (1 μM). Detection of the phosphorylated β2-adaptin in the immunoprecipitates was performed as described in (A).

expressing Src, whereas expressing the inactive kinase mutant prevented the Ang-II-mediated-phosphorylation of β2-adaptin.

Formation of a β-arrestin–AP-2–Src complex promotes the phosphorylation of β2-adaptin

We next sought to determine whether the agonist-mediated formation of a β-arrestin–AP-2–Src complex was necessary for β2-adaptin phosphorylation. We thus used a Src construct containing the β-arrestin-interacting Src homology domain 1 (SH1) that corresponds to the catalytic subunit of Src (amino acids 250-536), and its kinase-inactive mutant SH1-KD. Both constructs still bind β-arrestin and compete with the recruitment of endogenous Src to the β-arrestin–AP-2 complex (Miller et al., 2000). We hypothesized that, because its catalytic domain is inactive, SH1-KD presumably acts as a dominant-negative construct to inhibit β2-adaptin phosphorylation. Results show that in cells transfected with AT1R and Flag-β2-adaptin, Ang II stimulation induced the tyrosine phosphorylation of β2-adaptin as expected (Fig. 3A). The overexpression of SH1 induced the agonist-independent phosphorylation of β2-adaptin, whereas SH1-KD prevented the Ang-II-mediated phosphorylation of the β-subunit. To demonstrate the importance of AP-2 binding to β-arrestin in phosphorylation of the β-subunit, we next assessed whether β2-adaptin carrying the point mutation Glu902Ala (β2-adaptin E902A), rendering it deficient in binding β-arrestin (Kim and Benovic, 2002; Laporte et al., 2002), can still be phosphorylated upon AT1R stimulation. Fig. 3B shows that only wild-type β2-adaptin was phosphorylated upon AT1R activation. Taken together, these results suggest that the association of the β-arrestin–AP-2–Src complex is necessary for phosphorylation of β2-adaptin.

Tyrosine residues 737, 874 and 926 in the C-terminal domain of β2-adaptin are putative Src phosphorylation sites

We next sought to identify the phosphotyrosine residues within β2-adaptin, focusing on the C-terminal region of the ear domain where β-arrestin has been shown to bind (Laporte et al., 2002). As shown in supplementary material Fig. S1B, overexpression of Src resulted in a strong increase in phosphorylation of the HA-tagged β2-adaptin ear domain [HA-β2-ad (E); aa residues 664-937]. To determine which of the nine tyrosine residues is the preferred site for Src phosphorylation, we generated a series of GST

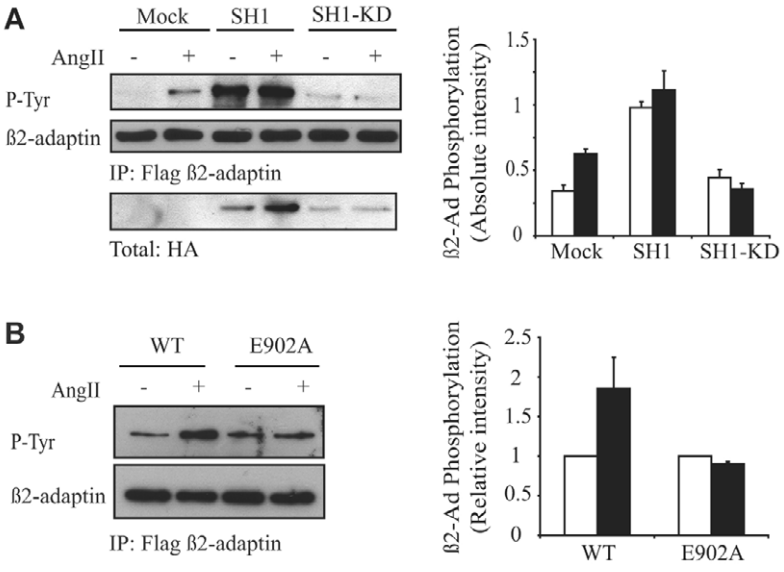

Fig. 3. β-arrestin–AP-2–Src complex is involved in β2-adaptin phosphorylation. (A,B) HEK293 cells were transfected with AT1R, Flag-β2-adaptin and either pcDNA3.1, HA-SH1 or HA-SH1-KD (A) or with AT1R and either Flag-β2-adaptin wild type or Flag-β2-adaptin E902A (B), before either being left untreated (–) or stimulated (+) with Ang II (1 μM). Flag-β2-adaptin were immunoprecipitated from cell lysates using an anti-Flag antibody, and the immunoprecipitates were analyzed by western blot as previously described. Whole cell extracts (Total: HA) were probed for the detection of HA-SH1 and HA-SH1-KD using an anti-HA antibody (A). Data are presented as the mean ± s.e.m. for three independent experiments.

fusion peptides containing different tyrosine residues in β2-adaptin. GST-fusion peptides were phosphorylated in vitro using purified Src, and their phosphorylation status was then analyzed by western blotting. Results reveal that only tyrosine residues in the aa sequences 733-741, 869-877 and 921-929 were substrates for Src (supplementary material Fig. S2B). To determine which of those tyrosine residues are phosphorylated, we used site-directed mutagenesis to replace tyrosine with phenylalanine residues in the GST-733-741-Y737F, GST-869-877-Y874F and GST-921-929-Y926F fusion peptides, and assessed the phosphorylation of the resulting constructs in vitro (supplementary material Fig. S2C). Results reveal that tyrosine to phenylalanine substitutions at these sites prevented their phosphorylation, thereby identifying them as Src phosphorylation sites.

Src phosphorylation of Y737 in β2-adaptin impairs its binding to β-arrestin

Since β-arrestin binds β2-adaptin through its C-terminal domain (ear domain) (Laporte et al., 1999), which includes tyrosine residues targeted by Src, we reasoned that phosphorylation of β2-adaptin might affect its association with β-arrestin. To test this hypothesis, GST-fusion protein of the β2-adaptin ear domain was either left unphosphorylated or phosphorylated in vitro with Src (Fig. 4A), and then incubated with increasing amounts of cell lysates expressing either Flag-β-arrestin1 or Flag-β-arrestin2. The amount of β-arrestin1 and β-arrestin2 associated with GST-fusion protein was then analyzed by western blotting (Fig. 4B). Results show that the level of β-arrestin1 and β-arrestin2 pulled down with unphosphorylated β2-adaptin increased proportionally with increasing amounts of β-arrestin. However, when β2-adaptin was first phosphorylated by Src and then incubated with β-arrestins, we observed a reduction in both proteins associated with β-arrestin1 and β-arrestin2, as compared with conditions where GST-fusion proteins were unphosphorylated. These results imply that Src-dependent phosphorylation of β2-adaptin limits its ability to bind β-arrestins.

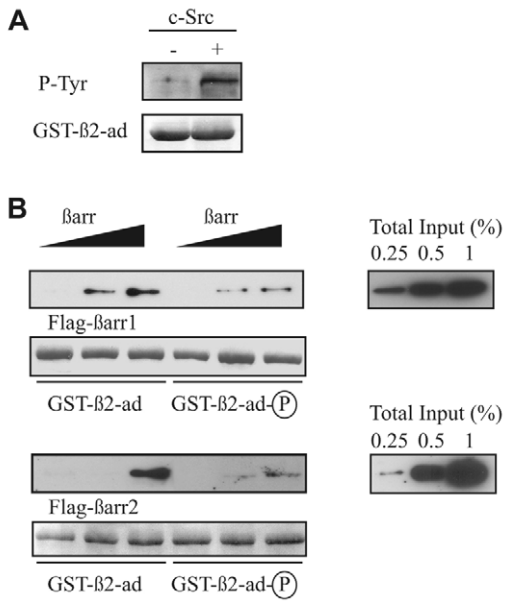

Fig. 4. Phosphorylation of β2-adaptin by Src reduces its ability to bind β-arrestins. (A) GST-fusion proteins of the ear domain of β2-adaptin were left unphosphorylated (–) or phosphorylated (+) in vitro with purified Src. The amounts of GST- β2-adaptin were assessed by Ponceau Red, and Src-phosphorylated proteins were detected by western blot using anti-phosphotyrosine antibody 4G10 (P-Tyr). (B) After removing Src from the reaction, the unphosphorylated or phosphorylated GST-proteins were incubated with increasing amounts of Flag-β-arrestin1 or 2 from cell lysates. The amounts of β-arrestin associated with the GST-proteins were determined by western blot using an anti-Flag antibody. Whole cell extracts (Total, right panels) were also blotted for detecting the level of Flag-β-arrestin1 or 2 expression using the anti-Flag antibody. Data are representative of three to five independent experiments.

To assess to which extent the three putative Src tyrosine residues identified above are involved in regulating binding of β2-adaptin to β-arrestin, we individually substituted them for phenylalanine (GST-β2-ad-Y737F, GST-β2-ad-Y874F and GST-β2-ad-Y926F), and assessed the ability of the mutant proteins to bind β-arrestin1 (Fig. 5). Since we found (as shown in Fig. 4B) that phosphorylation of β2-adaptin reduces its binding to β-arrestin, we reasoned that removal of an important regulatory tyrosine site would not change the ability of phosphorylated β2-adaptin to interact with β-arrestin as compared with its unphosphorylated form. Our results showed that when Src-phosphorylated GST-β2-adaptin-Y874F or GST-β2-adaptin-Y926F mutants were incubated with β-arrestin1, an important reduction in the interaction between the two proteins was observed, as compared with conditions where these mutants were left unphosphorylated. This reduction in binding of β-arrestin to the phosphorylated mutants was similar to that observed with Src phosphorylated GST-tagged wild type β2-adaptin (Fig. 4B), suggesting that aa Y874 and Y926 are not involved in regulating the binding between the two proteins. By marked contrast, phosphorylated GST-β2-adaptin-Y737F showed no changes in its ability to bind β-arrestin as compared with its unphosphorylated form, arguing for the loss of an important Src regulatory site in β2-adaptin. Similar results were also observed with β-arrestin2 (data not shown). Overall, these results demonstrate that Y737 is a crucial residue for the Src-dependent regulation of β-arrestin binding to β2-adaptin.

Tyrosine phosphorylation of β2-adaptin regulates the dissociation of the β-arrestin–AP-2 complex in clathrin-coated pits and the internalization of AT1R

Next, we sought to determine to what extent Y737 in β2-adaptin acts as a regulatory site for the dissociation of endocytic complexes in cells. We first verified that the mutation of Y737 in β2-adaptin did not alter the functionality of AP-2 complexes by assessing its cellular distribution and localization with β-arrestin following AT1R stimulation (Fig. 6B). Cells were transfected with AT1R, cyan fluorescent protein (CFP)-coupled β-arrestin2 (β-arrestin2-CFP) and either yellow fluorescent protein (YFP)-coupled β2-adaptin (β2-adaptin-YFP) or β2-adaptin-Y737F-YFP. In the absence of agonist, the fluorescence of both wild-type and mutant β2-adaptin was localized in punctuated areas at the plasma membrane, suggesting its adequate incorporation in clathrin coated-pits (CCPs), whereas β-arrestin2-CFP was uniformly distributed in the cytoplasm (Fig. 6B, upper panels). Stimulation of the cells with Ang II led to the translocation of β-arrestin from the cytosol to the plasma

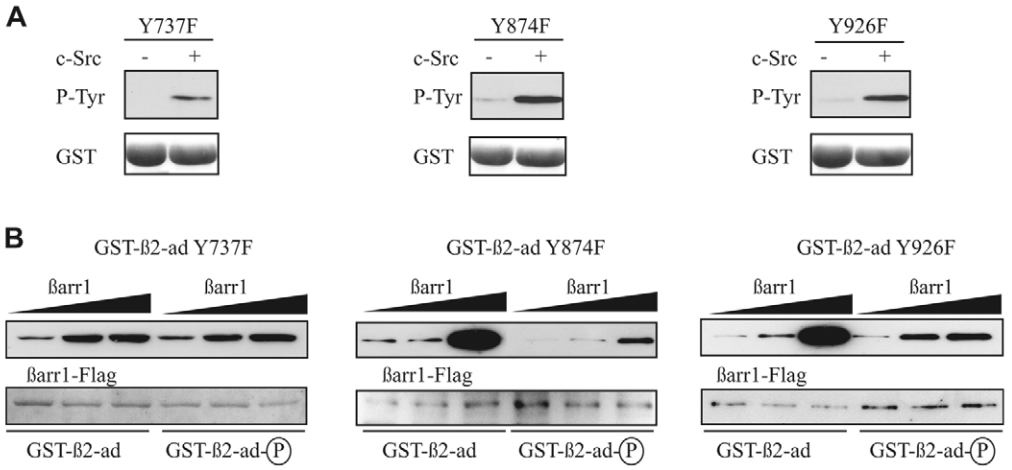

Fig. 5. Y737 in β2-adaptin is a regulatory site for Src-mediated binding of β-arrestin in vitro. (A) GST-fusion proteins of β2-adaptin with single substitutions (Y737F, Y874F and Y926F) were either left unphosphorylated (–) or phosphorylated (+) with purified Src. The amounts of GST-β2-adaptin were assessed by Ponceau Red and Src-phosphorylated proteins were detected by western blot. (B) After removing Src from the reaction, the unphosphorylated or phosphorylated GST- proteins were incubated with increasing amounts of Flag-β-arrestin1. The amounts of GST-proteins were detected using an anti-GST antibody and β-arrestin associated with the GST proteins was determined by western blot using anti-Flag antibody. Data are representative of three to five independent experiments.

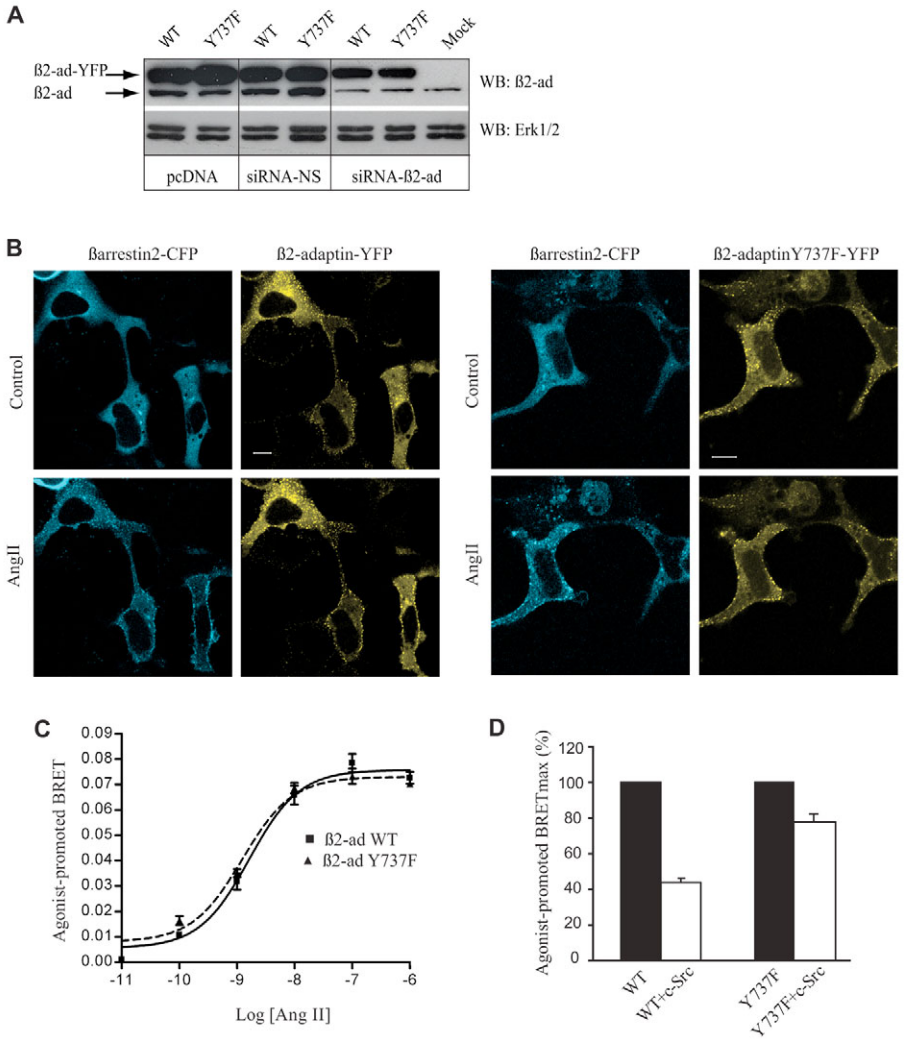

Fig. 6. Y737 in β2-adaptin is a Src regulatory site for β-arrestin–AP-2 dissociation in cells. (A) Expression of β2-adaptin-YFP in HEK293 cells. Cells were transfected with pcDNA3 (Mock), non-silencing siRNA (siRNA-NS) or siRNA targeting β2-adaptin (siRNA-β-ad) and either β2-adaptin-YFP (WT) or β2-adaptin-Y737F-YFP (Y737F) as described in Materials and Methods. Shown is the expression of endogenous and transfected β2-adaptin, and ERK1/2. (B) Colocalization of β-arrestin2 with wild type and mutant β2-adaptin in HEK293 cells. Cells expressing HA-AT1R, β-arrestin2-CFP, and either β2-adaptin-YFP wild type or Y737F mutant were treated with Ang II (1 μM) for 2 minutes and imaged live by confocal microscopy as described in Materials and Methods. Shown are representative images of β-arrestin2-CFP (cyan) and β2-adaptin-YFP proteins (yellow). (C) Ang II-promoted BRET between β-arrestin2 and β2-adaptin. HEK293 cells expressing Flag-AT1R, β-arrestin2-Rluc and either β2-adaptin-YFP wild-type or Y737F mutant were stimulated with increasing concentrations of Ang II before BRET measurements. (D) Effects of Src on agonist-promoted maximal values of BRET ($BRET_{max}$). HEK293 cells expressing Flag-AT1R, β-arrestin2-Rluc and either β2-adaptin-YFP wild type or mutant, in absence (black bars) or presence (white bars) of Src were stimulated with Ang II (1 μM). BRET measurements were performed as described in Materials and Methods. Data represent the average of three to five independent experiments.

membrane where it colocalized with both β2-adaptin-YFP constructs (Fig. 6B, lower panels). Interestingly, we observed that the size of the puncta resulting from the β-arrestin–AP-2 mutant complexes were bigger than the ones observed with the wild type. We also tested the functionality of the AP-2 complex by assessing its ability to support the internalization of another class of receptor, the transferrin receptor, which is known to internalize through the same clathrin pathway. In cells expressing β2-adaptin-YFP or the Y737F mutant no obvious difference in the uptake of transferrin was observed (supplementary material Fig. S3).

To confirm that β2-adaptin-Y737F functionally interacted with β-arrestin and that Src could regulate the interaction between β-arrestin and AP-2 in cells, we measured the agonist-induced formation of a complex using bioluminescence resonance energy transfer (BRET) assay. This approach, which consists of measuring the transfer of energy between *Renilla reniformus* luciferase (Rluc)-tagged β-arrestin2 (β-arrestin2-Rluc; the energy donor) and the full-length β2-adaptin-YFP (the energy acceptor), was recently used to characterize the clathrin-dependent internalization of different receptors (F.F.H., Moulay Driss Rochdi, Billy Breton, D.F., Douce Michaud, Pascale G. Charest, S.A.L. and M.B., unpublished). The BRET signal was measured after challenging HEK293 cells, expressing AT1R transfected with β-arrestin2-Rluc and either the wild-type or mutant β2-adaptin-YFP construct, with different concentrations of Ang II. As shown in Fig. 6C, a dose-dependent increase in BRET with similar EC_{50} and $BRET_{max}$ values was observed for β2-adaptin-YFP wild type and mutant, suggesting that the mutation of Y737 in β2-adaptin did not impair the ability of β-arrestin to associate with AP-2 complexes. To confirm that Y737 in β2-adaptin is a Src regulatory site for β-arrestin–AP-2 interaction, cells expressing AT1R with β-arrestin2-Rluc and either wild-type β2-adaptin fused to YFP or the β2-adaptin Y737F mutant (with or without Src) were stimulated with Ang II, and BRET signals were measured. Fig. 6D shows that overexpression of Src decreased the $BRET_{max}$ value by more than 60% for wild-type β2-adaptin, whereas for the Y737F mutant the $BRET_{max}$ value decreased by less than 22%. These results support our in vitro studies, showing that Y737 is a crucial target for Src in cells and that it regulates the interaction between β2-adaptin and β-arrestin.

To assess the consequence of Y737 phosphorylation in β2-adaptin on the stability of the AP-2–β-arrestin complexes in clathrin-coated pits, we used image cross-correlation spectroscopy (ICCS), an imaging variant of fluorescence cross-correlation spectroscopy (FCCS). Dual-color ICCS and FCCS have been previously applied to live cells to monitor protein complexes mobility, protein-protein interactions and to extract affinities between different protein interactions (association and dissociation rates) (Bacia et al., 2006; Comeau et al., 2006; Wiseman et al., 2004). Here we used ICCS analysis between β-arrestin2 and β2-adaptin, to assess the dynamic binding between the two proteins. HEK293 cells were transfected with AT1R, β-arrestin2-CFP, and either β2-adaptin-YFP or its Y737F mutant. The distribution of the CFP and YFP fluorescence was visualized before and after treatment of the cells with Ang II for several time points. Fig. 7A represents a typical confocal microscopy image of a HEK293 cell expressing β2-adaptin-YFP and β-arrestin2-CFP, 60 seconds after Ang II stimulation. The pseudo-color image corresponds

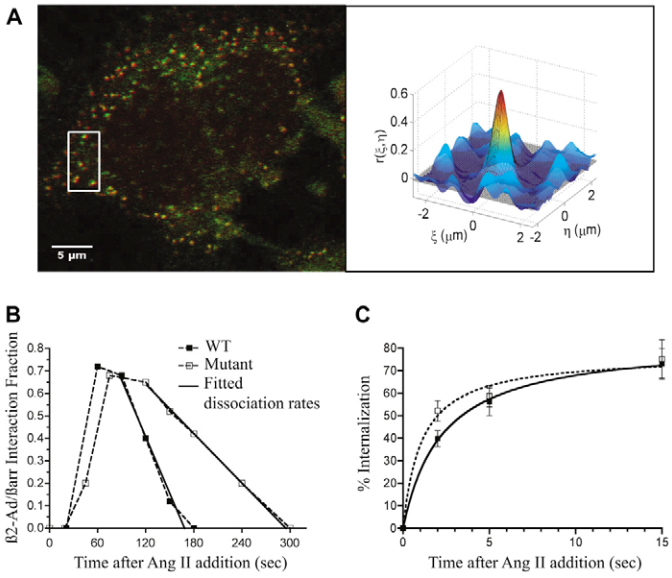

Fig. 7. Y737 in β2-adaptin regulates the dissociation of β-arrestin–AP-2 complexes in clathrin-coated pits and the internalization of AT1R. (A) Representative image of a cell expressing β-arrestin2 and β2-adaptin that was analyzed by ICCS. HEK293 cells transfected with HA-AT1R, β-arrestin2-CFP, and β2-adaptin-YFP wild-type were treated with Ang II (1 μM) and imaged live by confocal microscopy as described in Materials and Methods. Shown is a representative overlay confocal image taken after 1 minute of Ang II treatment and illustrated as pseudo color of β-arrestin2-CFP (green), β2-adaptin-YFP (red), and the colocalization of both proteins (yellow) (left panel). The region that was analyzed by ICCS is outlined in the white rectangle. The right panel represents the spatial autocorrelation (solid) and best-fit function (mesh) calculated for the image region outlined in the left panel. (B) Fraction of colocalization between β-arrestin2 and β2-adaptin wild type or its mutant was calculated from several image regions for different time points after Ang II treatment (1 μM) using ICCS as described in Materials and Methods. The dissociation rates were calculated from linear fits to the decay of the interaction fraction after reaching its maximum for five different cells from at least three different sets of experiments. (C) AT1R internalization was performed in HEK293 cells transfected with HA-AT1R and either β2-adaptin wild-type or Y737F mutant using [^{125}I]-Ang II. Percent of receptor internalization was calculated as described in Materials and Methods. Data represent the mean ± s.e.m. of at least eight independent experiments.

to the overlay of β-arrestin2 (green) and β2-adaptin (red) and shows that both proteins colocalize together in punctated structures at the plasma membrane after agonist stimulation. The fraction of colocalization between the two proteins was calculated from the image region outlined in Fig. 7A (white rectangle). Analysis of data from several images at different time points revealed that the fraction of AP-2 complexes containing wild-type or mutant β2-adaptin and β-arrestin2 reached a maximum after ~1 minute of agonist treatment (Fig. 7B), suggesting similar rates of complex formation. Dissociation rates were also extrapolated after maximal colocalization of β-arrestin with either β2-adaptin-YFP or Y737F mutant. Whereas complexes containing both wild-type β2-adaptin and β-arrestin were completely lost after only 1.5

minutes, total dissociation of β-arrestin and β2-adaptin mutant took 5.75 minutes, with rates almost four times lower than with β2-adaptin wild type (–0.011±0.005 second^{-1} versus –0.0029±0.0006 second^{-1} for β2-adaptin versus mutant, respectively; n=5). These results reveal that the phosphorylation of β2-adaptin by Src increases the rate of disassembly of the β-arrestin–AP-2 complex.

Finally, we assessed the effect of Y737 in β2-adaptin on receptor endocytosis. HEK293 cells were transfected with AT1R and either wild type β2-adaptin or the Y737F mutant, and receptor internalization was measured by radio-ligand binding assay using [^{125}I]-Ang II. Results show that after 2 minutes of agonist treatment 52% of receptors were internalized in cells expressing the β2-adaptin-Y737F mutant compared with 40% in cells expressing the wild type (Fig. 7C). This difference was, however, lost for longer periods of agonist stimulation, and reached similar levels of receptor internalization after 5 and 15 minutes. Taken together, these results point towards a role of β2-adaptin phosphorylation in the regulation of the early steps of AT1R internalization.

Discussion

Our study provides evidence for a new role of Src in the phosphorylation of the clathrin adaptor complex AP-2 (via the phosphorylation of the β-subunit, i.e. β2-adaptin). We identify Y737 in the ear domain of β2-adaptin as an important Src target. We show that this residue represents a regulatory site for controlling the dissociation of β-arrestin from AP-2 in clathrin-coated pits (CCPs). Our results not only extend the pleiotropic function of Src in GPCR internalization, but also highlight the importance of receptor-dependent signaling in the clathrin-mediated endocytosis of AT1R.

β-arrestins are multifunctional adaptors involved in the endocytosis and signaling of many GPCRs (Claing et al., 2002; Lefkowitz, 1998). They have been shown to target receptors to CCPs through binding to clathrin and the β-subunit of AP-2 (Goodman, Jr et al., 1996; Laporte et al., 1999), and to act as signal transducers by recruiting different kinases to GPCRs (Luttrell and Luttrell, 2004). We have previously shown that activation of AT1R induces the formation of a β-arrestin–Src–AP-2 complex, and reported that Src is involved in regulating the stability of the β-arrestin–AP-2 interaction (Fessart et al., 2005). Here, we provide new evidence that Src controls the binding of β-arrestin to AP-2 through the tyrosine phosphorylation of its β-subunit. We show that preventing Src activation by using pharmacological inhibitors or a dominant-negative mutant of Src inhibits the Ang-II-mediated phosphorylation of β2-adaptin. Although the major inhibitory effect on β2-adaptin phosphorylation was observed by using a Src inhibitor (PP2), we also found that blocking EGFR activity reduced the level of β2-adaptin phosphorylation. Our findings, however, suggest that the recruitment of Src kinases to an agonist-mediated β-arrestin–AP-2 complex plays a predominant role in β2-adaptin phosphorylation. Three lines of evidence support this premise. First, the time frame of the Ang-II-mediated formation of β-arrestin–Src–AP-2 complexes coincided with β2-adaptin phosphorylation (Fessart et al., 2005). Second, the expression of a dominant-negative construct of Src (SH1-KD), which prevents the interaction between β-arrestin and the endogenous kinase (Imamura et al., 2001; Miller et al., 2000), blocked the agonist-mediated phosphorylation of β2-adaptin (Fig. 3A). Third, we did not observe an increase in phosphorylation of the β2-adaptin E902A mutant, which does not interact with β-arrestin (Fig. 3B). Although we show that β2-adaptin phosphorylation can involve other potential receptor-mediated signaling events, such as the one suggested by the inhibition of EGFR activity, our findings strongly suggest that this phosphorylation is linked to the adaptor and signaling functions of β-arrestin (Luttrell and Luttrell, 2004). Of interest were the differences observed in the levels of β2-adaptin phosphorylation in various cell types (Fig. 1). The nature of these differences and their impact in regulating receptor endocytosis are, however, still unclear. Although we have used inhibitors to prevent the dephosphorylation of β2-adaptin in cells, we nonetheless observed a decrease in β2-adaptin phosphorylation in HEK293 cells following prolonged stimulation of the receptor. This suggests that β2-adaptin phosphorylation is regulated differently and may also influence the level of receptor internalization in different cell types.

We have identified Y737 in β2-adaptin as a Src target and as an important site for regulating the disassembly of the β-arrestin–AP-2 complex. Indeed, we provide evidence in vitro using GST-purified proteins that Src phosporylation of Y737 in the ear domain of β2-adaptin reduces its binding to β-arrestin. Using a BRET assay to assess the formation of the β-arrestin–AP-2 complex in live cells, we show that Src-dependent phosphorylation of Y737 reduces the binding of the two proteins. More importantly, we show that mutation of this crucial residue in β2-adaptin leads to a delay in the dissociation of β-arrestin–AP-2 complexes in clathrin-coated pits, as shown by ICCS.

How the phosphorylation of β2-adaptin and, in particular, Y737 regulates the dissociation of β2-adaptin from β-arrestin is still unclear. Y737 has not been reported to be part of the β-arrestin-binding domain of β2-adaptin per se (supplementary material Fig. S2A). Although phosphorylation is known to affect the folding of many proteins, structural analysis of the ear domain of β2-adaptin (Edeling et al., 2006; Schmid et al., 2006) does not support a conformational change of two subdomains of the ear and, thus, is unlikely to account for the mechanism that regulates binding of β-arrestin to β2-adaptin. Alternatively, phosphorylation of Y737 may serve to create a binding site for the recruitment of other accessory proteins that could participate in regulating the dissociation of the β-arrestin–AP-2 complex. Interestingly, Y737, which is highly conserved amongst the β2-adaptin forms from different species, is also part of a YMxM consensus motif for binding SH2-domain containing proteins (Garcia et al., 1993). The phosphorylation of Y737 in β2-adaptin could promote the recruitment of SH2 containing accessory proteins, and modulate the interaction between β-arrestin and AP-2. The recruitment of such regulatory proteins would, however, not only be solely dependent on the phosphorylation of β2-adaptin, but would also require the agonist-dependent stimulation of receptors. Indeed, in conditions where β2-adaptin phosphorylation is increased in an agonist-independent manner – either by overexpressing Src or its kinase domain – we did not observe the dissociation of the β-arrestin–AP-2 complex (Fessart et al., 2005). This implies that the formation of the β-arrestin–AP-2–Src complex and the resulting phosphorylation of AP-2 are not sufficient to trigger the disassembly of the

complex, and that a specific spatial and/or temporal context favored by the receptor activation is necessary. This also questions the involvement of Src in increasing the interaction between β-arrestin and AP-2 in vitro (Fessart et al., 2005). Although we cannot exclude that Src may stabilize the interaction between β-arrestin and AP-2 at some point during receptor internalization, our findings that depleting Src in cells increased the agonist-mediated formation of a β-arrestin–AP-2 complex (Fessart et al., 2005), combined with our most recent observations, favor a predominant role for Src in regulating the dissociation of AP-2 from β-arrestin.

Protein phosphorylation is a well-recognized mechanism for coordinating clathrin-coated vesicle formation and receptor internalization (Slepnev et al., 1998), but the actual involvement of tyrosine phosphorylation as a regulatory mechanism is only now starting to be appreciated (Ahn et al., 1999; Tosoni et al., 2005; Wilde et al., 1999). Studies on the β2AR revealed that receptor activation induces the Src-dependent tyrosine phosphorylation of dynamin, which controls receptor internalization (Ahn et al., 1999). Prevention of dynamin phosphorylation using pharmacological inhibitors or a phosphorylation-deficient dynamin mutant blocked the agonist-mediated internalization of the β2AR. Work on EGFR also reveals that in cells lacking Src, the internalization of this receptor is delayed by controlling the tyrosine phosphorylation of the clathrin heavy chain (Wilde et al., 1999). We also observed that depleting Src in cells reduced the rate of AT1R internalization (Fessart et al., 2005). However, our observations suggest that this effect does not result from the Src-dependent phosphorylation of AP-2. Indeed, when we express the β2-adaptin mutant (Y737F), we observe an increase in the initial rate of AT1R internalization. Most likely, the effect on AT1R internalization previously observed in Src-depleted cells resulted from the lack of other Src-dependent events.

How does Y737 phosphorylation in β2-adaptin increase the rate of AT1R internalization? One possible explanation may come from a recent report proposing that the formation of nascent CCPs at the plasma membrane can follow an abortive process, unless stabilized by either the internalizing receptor and/or the recruitment of accessory proteins into the forming vesicle (Ehrlich et al., 2004). For GPCRs, the agonist-mediated formation of receptor–β-arrestin–AP-2 complexes has been shown to be required for the proper clustering of receptors into CCPs (Laporte et al., 2000; Santini et al., 2002). Fluorescence and ultra-structural studies on the distribution of β-arrestin and AP-2 in cells reveal that the number and size of CCP clusters increase following GPCR activation (Laporte et al., 1999; Santini et al., 2002). Increasing the life time of β-arrestin–AP-2 complexes, such as in the case of the AP-2 mutant, would stabilize the receptors and other accessory proteins into the nascent CCPs, committing the vesicles to proceed for endocytosis, resulting in more efficient receptor internalization. This scenario would be consistent with our observation that the size of puncta resulting from the clustering of β-arrestin into CCPs in cells expressing the β2-adaptin-Y737F appeared generally bigger than those observed in cells expressing wild-type AP-2 (Fig. 6B).

Another pressing question concerns the role for regulating the dissociation of AP-2 from β-arrestin during receptor internalization. This may represent a fine mechanism to ensure the efficient and continuing internalization of newly activated receptors, by freeing AP-2 complexes for de novo formation of CCPs at the plasma membrane. Presumably, newly formed receptor–β-arrestin complexes would interact with available AP-2, ensuring the clustering of activated receptors into the forming CCP. However, this latter scenario would only be relevant in a cellular context where AP-2 becomes limiting. Alternatively, since AP-2 has been shown to bind many other accessory proteins through interactions that sometimes involve the same subunit (Bonifacino and Lippincott-Schwartz, 2003; Traub, 2003), the release of certain interactions would allow the coordinated recruitment and assembly of other accessory proteins (Edeling et al., 2006; Schmid et al., 2006).

Clathrin-dependent internalization can be described by a series of early and late events. Early events include the clustering of receptors in CCPs, the recruitment of accessory proteins and the initiation of the coat assembly. These are followed by maturation of CCPs, which involves the recruitment of additional accessory proteins, like dynamin that terminates the endocytic process by promoting the scission of the vesicle (Conner and Schmid, 2003). The herein work and previous findings on the role of Src in the phosphorylation of different proteins of the coat (Ahn et al., 1999; Miller et al., 2000; Wilde et al., 1999), suggest that this kinase is involved at different stages in the internalization process. They also underscore the difficulty of predicting the overall effect of blocking this kinase – by depleting Src expression, using kinase inhibitors or dominant-negative constructs of Src – on the biogenesis of CCPs and the internalization of receptors. This is also confounded by other protein-protein interactions within the coat that have been described to stabilize GPCRs into CCPs and to regulate receptor internalization (Diviani et al., 2003; Paing et al., 2006). The identification of an important tyrosine residue in β2-adaptin that is a Src target has allowed us to define a specific function for this kinase in the early steps of AT1R internalization. The extent to which the Src-dependent phosphorylation of β2-adaptin participates in the regulation of receptor internalization for other GPCRs, and the possible role for this kinase in other clathrin-dependent events, will require careful attention.

In summary, our findings unveil a new and unappreciated mechanism to explain how the β-arrestin–AP-2 complex is regulated during AT1R internalization. We show that receptor activation promotes the Src-dependent tyrosine phosphorylation of the β-subunit of AP-2. The functional consequence of β2-adaptin phosphorylation is to control the dissociation of β-arrestin from AP-2. This mechanism may represent an important step for the coordinated regulation of endocytic complexes, and to ensure efficient internalization of receptors through the clathrin pathway.

Materials and Methods

Materials

Angiotensin II was from Sigma Chemical Co. The inhibitors PP2, PP3, and PD158780 were from Calbiochem-Novabiochem. The antibody against β-adaptin was from BD Transduction Laboratories, the mouse anti-HA clone 12CA5 was from Roche, the FLAG, the AP-1/2 and the GST antibodies were from Sigma-Aldrich, the Src antibody GD11, the anti-phosphotyrosine 4G10 and purified Src were from Upstate Biotechnology. Coelenterazine-h was purchased from LUX Biotechnology (Edinburgh, UK).

Plasmids and constructs

Human wild-type AT1R, HA-AT1R, Flag-tagged β-arrestin1 and β-arrestin2 (Flag-

β-arrestin1 and Flag-β-arrestin2, respectively), CFP-tagged β-arrestin (β-arrestin2-CFP), *Renilla reniformus* luciferase (Rluc)-tagged β-arrestin2 (β-arrestin2-Rluc), Flag-tagged β2-adaptin (Flag-β2-adaptin), HA-tagged Src (HA-Src), inactive HA-tagged mutated Src K298R (HA-Src-K298R) and wild-type Src were described elsewhere (Fessart et al., 2005; Hamdan et al., 2005; Laporte et al., 1999; Laporte et al., 1996; Simaan et al., 2005). Constructs containing the Src homology domain 1 (SH1) tagged to HA, HA-SH1 and HA-SH1-KD, were provided by William Miller (University of Cincinnati, OH). The fusion protein of glutathione S-transferase (GST) and the ear domain [GST-β2-ad (E)] is also described elsewhere (Laporte et al., 2002). The GST-tagged β2-adaptin fusion constructs mutated at tyrosine residues Y737, Y874 and Y926 (β2-ad-Y737F, β2-ad-Y874F and β2-ad-Y926F, respectively) were generated using conventional polymerase chain reaction (PCR) and cloned into pGEX-5X2. For screening purposes, silent mutations creating a *Kpn*I or a *Pst*I sites were introduced in β2-adaptin-Y737F or Y874F, respectively. YFP-tagged β2-adaptin (β2-adaptin-YFP) constructs were generated using PCR and cloned into pEYFP-N1 using *Nhe*I and *Sal*I restriction sites. siRNA insensitive β2-adaptin-YFP and β2-adaptin-Y737F-YFP mutants were made by introducing silent mutations into nucleotide sequence 381-401 (aa 128-132) by PCR. All constructs were analyzed by DNA sequencing (Sequencing Service, Genome Quebec Innovation Centre, McGill University, QC, Canada).

Cell culture and transfection

HEK293 cells were grown in MEM (Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco) and gentamicin (100 μg/ml, Gibco). Cells seeded in a 100-mm dish, at a density of 2.5×10^6 cells per dish, were transfected using a calcium phosphate co-precipitation method as previously described (Fessart et al., 2005). COS-7 cells were grown in DMEM (Gibco) supplemented with 10% (v/v) heat-inactivated FBS and gentamicin. Transfection of COS-7 cells seeded in 60-mm dishes (at a density of 10^6 cells per dish) was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations using a 1:3 ratio of DNA:Lipofectamine in Opti-MEM (Gibco). All experiments were performed 36 hours post transfection. Vascular smooth muscle cells (VSMCs) were grown in DMEM with 10% (v/v) heat-inactivated calf serum (Gibco) and gentamicin. All cells were serum-starved overnight in DMEM before performing the experiments. HEK293 cells stably expressing Flag-AT1R were a generous gift from Richard Leduc (Université de Sherbrooke, QC, Canada).

Immunoprecipitation and western blot experiments

For phosphoprotein detection experiments, COS-7 cells, HEK293 or VSMCs were serum-starved overnight, and the next day incubated for 30 minutes at 37°C in DMEM buffered with 20 mM HEPES pH 7.4. Cells were then pretreated with 20 mM pervanadate for 10 minutes before adding Ang II (1 μM) for the indicated period of time. Stimulation was stopped on ice with cold PBS, and cells were solubilized in TGH buffer (50 mM HEPES pH 7.4, 1% Triton X-100 (v/v), 10% glycerol (v/v), 50 mM NaCl, 5 mM EDTA) containing protease and phosphatase inhibitors (0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptine, 25 μg/ml aprotinin, 1 mM pepstatin A). Supernatants were incubated for 1 hour at 4°C with anti-Flag-conjugated beads or with β-adaptin antibody AP-1/2 with 20 μl of a 50% slurry mixture of protein A/G Sepharose beads for 2 hours at 4°C. Beads were then washed with TGH and denatured in Laemmli buffer (2×) [250 mM Tris HCl pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), 0.01% Bromophenol Blue (w/v), 5% β-mercaptoethanol (v/v)]. Proteins were separated on a 10% SDS-polyacrylamide gel and protein detection was assessed using phosphotyrosine antibody (clone 4G10) and β-adaptin antibody using conventional western blot and chemiluminescence techniques according to the manufacturer's instructions (SuperSignal, Pierce).

GST fusion protein expression

GST fusion proteins were generated as previously described (Laporte et al., 2002). Briefly, BL21 cells transformed with pGEX-5X2 vector bearing the different constructs were grown overnight in LB medium. Cell cultures were then diluted to an OD_{600} of 0.2 and grown until an OD_{600} between 0.5 and 0.6, before being induced using 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3 hours at room temperature. GST proteins were then isolated by incubating cells previously sonicated in cold PBS containing 2 mM EDTA, 4 mM PMSF, 1% Triton X-100 (v/v) and 1 mg/ml lysozyme, with glutathione-Sepharose 4B beads. GST fusion proteins were analyzed by SDS-PAGE and protein concentration was determined using a DC protein assay kit (Bio-Rad).

In vitro Src kinase assays

GST-fusion proteins were resuspended in 50 μl of kinase reaction buffer (100 mM Tris HCl pH 7.2, 25 mM $MnCl_2$, 125 mM $MgCl_2$, 2 mM EDTA) supplemented with 10 mM ATP, 0.25 mM sodium orthovanadate, 2 mM DTT and 1 unit of purified Src. Proteins were incubated for 20 minutes at 30°C, and the reaction was stopped either by washing the beads with cold TGH buffer, or by adding an equal amount of Laemmli buffer (2×). Protein phosphorylation was analyzed by western blot using a phosphotyrosine antibody (4G10). To assess the effect of β2-adaptin tyrosine phosphorylation on β-arrestin binding, Src from the kinase reaction was first removed by washing the beads with cold TGH buffer. Phosphorylated and unphosphorylated proteins (0.5 μg) were incubated in 0.5 ml of TGH buffer with increasing amounts of cell lysates from HEK293 cells expressing either Flag-β-arrestin1 or Flag-β-arrestin2 for 1 hour at 4°C. Beads were resuspended in Laemmli buffer before being resolved by electrophoresis on a 10% SDS-PAGE gel and analyzed by western blot for β-arrestin detection or stained with Ponceau Red for GST-protein analysis.

Monitoring interaction between β-arrestin2 and β2-adaptin by BRET

HEK293 cells stably expressing Flag-AT1R were co-transfected in 6-well plates with β2-adaptin-YFP and β-arrestin2-Rluc, in presence or absence of Src. Eighteen hours later, cells were detached with trypsin and seeded (50,000 cells per well) into 96-well plates treated with poly-D-lysine. After 18 hours, the medium was replaced with 90 μl of PBS containing 0.5 mM $MgCl_2$ and incubated with or without various concentrations of Ang II for the indicated time at room temperature. BRET signals were measured after addition of coelenterazine-h to all the wells (5 μM final concentration) using the Mithras LB940 plate reader (Berthold) that allows the sequential integration of signals detected in the 480±20 nm and 530±20 nm windows for luciferase and YFP emissions, respectively. The BRET signal was calculated as a ratio of the light emitted by YFP to the light emitted by Rluc. Agonist promoted BRET was calculated by subtracting the BRET ratio obtained in absence of agonist from BRET signal in presence of agonist.

RNA interference

Double-stranded small interfering RNAs (siRNAs) were synthesized using the silencer siRNA Construction Kit (Ambion, Austin, TX) according to the manufacturer's instructions. The siRNA sequence targeting β2-adaptin is 5′-AAGUGCUUGAAGGAUGAGGAU-3′ and corresponds to nucleotides (381-401) relative to the start codon. The sequence was screened for unique sequence in the National Center for Biotechnology Information database using BLAST.

Confocal microscopy

HEK293 cells, seeded in 35-mm glass-bottomed culture dishes were transfected with HA-AT1R, β-arrestin2-CFP, siRNA β2-adaptin and wild-type siRNA resistant β2-adaptin-YFP or Y737F mutant. Cells were treated with Ang II (1 μM) for different time points. Images were collected on a Zeiss LSM-510 Meta laser scanning microscope with a 40× oil immersion lens in a multitrack mode using a dual excitation (458 nm for CFP and 514 nm for YFP) and emission (BP 470-500 nm for CFP and BP 530-600 nm for YFP) filter sets.

Image cross-correlation spectroscopy (ICCS)

For confocal laser scanning microscopy ICCS, two separate laser lines are used to excite two spectrally distinct fluorophores and the fluorescence emission is separated and collected in two detection channels. The number of interacting particles is then evaluated by determining the amplitude of the spatial cross-correlation function calculated between the two acquired images (equation 1):

$$r_{kl}(\epsilon,\eta) = \frac{\langle [I_k(x,y) - \langle I_k\rangle][I_l(x+\epsilon,y+\eta) - \langle I_l\rangle]\rangle}{\langle I_k\rangle\langle I_l\rangle}. \quad (1)$$

In equation 1, k and l represent the two separate detection channels, and ϵ and η are the corresponding spatial lag variables (pixel shifts). Equation 1 is a spatial cross-correlation function when $k\neq l$ (i.e. two distinct detection channels) and an autocorrelation function when $k=l$ (i.e. only one detection channel). The amplitude of the correlation functions must be determined by a non-linear least squares fit to a 2d-Gaussian function due to the noise contributions to the intensities of each pixel.

The amount of colocalization present in each set of dual color images is therefore evaluated by calculation of the autocorrelation functions for each noise-corrected detection channel image (total density in each channel), along with the cross-correlation function (interacting particle density). Each correlation function is fit to a 2d-Gaussian to obtain best fit $r_{11}(0,0)$, $r_{22}(0,0)$ and $r_{12}(0,0)$ values. Colocalization coefficients, defined as the ratio of the number of interacting particles (N_{kl}) to the total number of particles per beam area for a particular detection channel (N_{kk} or N_{ll}), are determined by using equations 2 and 3:

$$M1_{ICCS} = \frac{r_{kl}(0,0)}{r_{ll}(0,0)} = \frac{N_{kl}}{N_{kk}}, \quad (2)$$

$$M2_{ICCS} = \frac{r_{kl}(0,0)}{r_{kk}(0,0)} = \frac{N_{kl}}{N_{ll}}. \quad (3)$$

A more detailed description of the theory of ICCS can be found in the original articles (Comeau et al., 2006; Petersen et al., 1993; Wiseman et al., 2000).

Receptor internalization

Internalization was performed as described previously (Fessart et al., 2005; Laporte et al., 1996). HEK293 cells seeded in 24-well plates were transfected with HA-AT1R, siRNA β2-adaptin and either wild-type siRNA-resistant β2-adaptin-YFP or the Y737F mutant. Seventy-two hours post-transfection cells were incubated at 37°C in DMEM containing 20 mM HEPES (pH 7.4), 0.1 mg/ml Bacitracin (Sigma) and 0.2% BSA (w/v), in presence of 0.11 nM of [^{125}I]-Ang II for the indicated period of time. Incubation was stopped on ice by rapidly washing the cells with either ice-cold PBS or ice-cold acid buffer [0.2 N acetic acid (pH 3.5), 150 mM NaCl]. Cells were then solubilized in 0.5 N NaOH, 0.05% SDS (w/v), and the radioligand content was evaluated by γ-counting. Percent of receptor internalization was calculated from the ratio of acid-resistant binding over total binding (PBS wash). Data were analyzed by nonlinear regression using Prism4 (GraphPad Software, San Diego, CA).

Data analysis

Intensity of the signals from western blots was determined by densitometric analysis using the MetaMorph Software (Universal Imaging Corporation), and is presented as the mean ± s.e.m. of at least three independent experiments. Densitometry of western blot data was analyzed statistically by one-way ANOVA followed by a Bonferroni test for multiple comparisons. Means were considered significantly different when $P<0.05$.

We thank C. Proulx and R. Leduc and G. Guillemette (Université de Sherbrooke) for providing [^{125}I]-Ang II. This work was supported by a Canadian Institutes of Health Research Grant (MOP-49447) to S.A.L. and a NSERC Discovery grant to PWW. D.F. and M.S. hold a Fonds de Recherche en Santé du Québec (FRSQ) awards. S.A.L. and M.B. are Canada Research Chairs in Molecular Endocrinology, and in Signal Transduction and Molecular Pharmacology, respectively.

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