Regulation of the endocytic adaptor proteins ßarrestin and AP-2 during clathrin-mediated internalization of Angiotensin II type 1 receptor.

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Your file Votre référence
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To my family and friends

ABSTRACT

G protein-coupled receptors (GPCRs) are the largest family of cell-surface receptors. They transduce the signals mediated by a diverse range of signalling molecules, including ions, amines, and peptides, as well as photons, to mediate intracellular functions. These receptors play a fundamental role in many physiological responses such as cardiovascular functions. To remain responsive to their environment, cells must find a way to rapidly desensitize and resensitize their activated GPCRs. Desensitization of receptors, for instance, involves the phosphorylation of receptors by G protein-coupled receptor kinase (GRKs) followed by the recruitment of βarrestin. This interferes with the binding of the G protein (the signalling effector). βarrestin then targets the receptors to the clathrin endocytosis pathway, and serves as an adaptor linking receptors to other signalling pathways. Internalization of receptors serves not only to remove desensitized receptors from the plasma membrane, but also to engage receptors in the resensitization pathway.

The internalization of Angiotensin II (Ang II) type 1 receptor (AT1R) is controversial and poorly described. Therefore, our laboratory studies the mechanisms behind AT1R internalization. The agonist-induced internalization of AT1R begins with the formation of a complex including βarrestin, the clathrin adaptor AP-2, and the tyrosine protein kinase, c-Src. In turn, this c-Src recruitment regulates the clathrin-mediated internalization of AT1R by controlling the formation of endocytic complexes during endocytosis. Indeed, the recruitment of c-Src is involved in the dissociation of AP-2 during receptor internalization. Based on our evidence that AP-2 and c-Src can be found in the same complex, we suggested that AP-2 could be phosphorylated by c-Src. Indeed, we found that Ang II induced the c-Src-mediated tyrosine phosphorylation of the beta-subunit of AP-2 (β2-adaptin). We were able to map one of the tyrosines in β2-adaptin and assess its role in regulating the binding of its principal partner: βarrestin. The phosphorylation state of β2-adaptin dictates its association profile with βarrestin: when phosphorylated it reduces its binding to βarrestin. Finally, we proposed a model for AT1R internalization. Overall, these studies are significant because they allow a better understanding of the underlying mechanism that regulates the initial steps of clathrin-coated vesicle endocytosis of AT1R.

RÉSUMÉ

Les récepteurs couplés aux protéines G (RCPG) représentent la plus grande famille de récepteurs. Ces récepteurs exercent un rôle fondamental dans toutes les grandes fonctions de l'organisme comme la régulation du système cardiovasculaire. La désensibilisation se traduit par une perte progressive du signal cellulaire induit par un récepteur lors d'une stimulation répétée. Ce phénomène de régulation est essentiel pour contrôler la durée de l'activation cellulaire induite par un récepteur et empêcher les effects toxiques dû à une stimulation excessive. L'événement le plus précoce au cours de la désensibilisation est la phosphorylation des RCPG par les kinases GRK. Ces phosphorylations favorisent la translocation des βarrestins vers les récepteurs permettant ainsi de bloquer la liaison des protéines G. Les beta-arrestins servent alors de pont moléculaire entre les récepteurs et les molécules de la machinerie de l'endocytose. L'internalisation des récepteurs, non seulement sert pour oter de la membrane plasmique le récepteur, mais également pour engager le récepteur dans la voie de resensibilisation.

Les mécanismes d'internalisation du récepteur de l'Angiotensine II de type 1 (AT1R) étant peu décrite et très controversée, nous avons décidé d'étudier son mode d'internalisation. Nous avons tout d'abord démontré que βarrestin sert de molécule adaptatrice pour le récepteur AT1R; puis que la stimulation de ce récepteur induit le recrutement de la kinase c-Src et de la protéine adaptatrice de la clathrine AP-2, formant ainsi un complexe. C-Src régulerait la formation de ces complexes par l'intermédiaire de son activité kinase en induisant la dissociation de AP-2 pendant l'internalisation. Étant donné que Src et AP-2 sont retrouvés dans le même complexe, Src pourrait alors phosphoryler AP-2. En effet, nous avons observé que l'activation de AT1R induit aussi la phosphorylation sur tyrosine de la sous-unité β de AP-2 (β2-adaptine). Nos études nous ont permis d'identifier le site de phosphorylation qui a pour rôle de diminuer son association avec les βarrestines. Finalement, nous proposons un modèle pour l'internalisation du récepteur AT1. L'ensemble de ces études nous a permis d'accroître nos connaissances dans la compréhension des mécanismes régulant les étapes précoces de l'endocytose du récepteur AT1R par les vésicules tapisées de clathrine.

CONTRIBUTION OF AUTHORS

This thesis is assembled in accordance with the regulations of the Faculty of Graduate Studies and Research, McGill University. It is written in manuscript format, and comprises two original manuscripts in Sections III and IV.

The two manuscripts that are included in this work are co-authored. This section details to what extent the co-authors contributed to these studies. Section III consists of a first article, "c-Src regulates AP-2 interaction with beta-arrestin and the Angiotensin II type 1 receptor during clathrin-mediated internalization", published in 2005, in *Molecular Endocrinology* **19:** 491-503. M. Simaan helped me with the preparation of the manuscript. S.A. Laporte helped analyze and interpret the data, as well as the preparation of the manuscript.

In Section IV "c-Src-mediated phosphorylation of AP-2 regulates its dissociation from ßarrestin during Angiotensin Type 1 Receptor Internalization." F. Hamdan assisted with the Bioluminescence Resonance Energy Transfer experiments. M. Bouvier helped interpret the BRET data and with the discussion. S.A. Laporte supervised all the experiments.

I am also co-author of several other manuscripts/publications to which I contributed:

Simaan M, Bédard-Goulet S, Fessart D, Gratton JP, Laporte.SA, Dissociation of ß-arrestin from internalized Bradykinin B2 receptor is necessary for receptor recycling and resensitization. *Cellular Signalling* 2005 **17**, 1074-1083.

Hamdan FF*, Rochdi MD*, Breton B, Fessart D, Charest P, Laporte SA, and Bouvier M., Real-time monitoring of agonist-promoted interaction between ß-arrestins and AP-2 by BRET reveals clathrin-mediated endocytosis and G protein-coupled receptor activation. *Manuscript in Preparation.*

Poupart ME*, Fessart D*, Houndolo T, Cotton M, Laporte SA and Claing A, ARF6 regulates Angiotensin II type 1 receptor endocytosis by controlling the recruitment of AP-2 and clathrin. * Contributed equally to this work. *To be submitted.*

Finally, a list of abstracts (posters and orals) that were submitted to local, national and international scientific meetings throughout my graduate studies include:

Oral presentations

Regulation par tyrosine phosphorylation du complexe endocytique AP-2/ ßarrestin lors de l'internalisation du récepteur AT1. Club de Recherches Cliniques du Québec. Sept 2005. Bromont (Québec)

Beta-arrestin/c-Src scaffold: role of phosphorylation of proteins of the clathrin coat in the Internalization of the Angiotensin II Type I Receptor. Endocrine Retreat. May 2005. Montréal (Québec)

Recruitment of c-Src to the Angiotensin II type 1 receptor: Role in the formation of a ßarrestin/AP-2 complex. GPCR Meeting Oct. 2003 Toronto (Ontario)

Poster presentations

Fessart D, Laporte SA, Role de la phosphorylation en tyrosine du complexe AP-2 lors de l'internalisation du récepteur de l'Angiotensine II de type 1 dans les vesicules de clathrine. 74ème Congrès de l'Acfas. May 2006 Montréal (Québec).

Fessart D, and Laporte SA, Tyrosine phosphorylation of the beta-subunit of AP-2: role in the dissociation of endocytic complex During Angiotensin II Type 1 Receptor Internalization. GPCR Meeting Oct. 2005 Montebello (Québec).

Fessart D, Simaan M, and Laporte SA, c-Src regulates the binding of AP-2 to the receptor/ ßarrestin complex during angiotensin II type 1 receptor internalization. 5th Annual McGill Biomedical Graduate Conference. Feb. 2005 Montréal (Québec).

Fessart D, and Laporte SA, Tyrosine phosphorylation of the beta-subunit of AP-2 regulates AT1R internalization. GPCR Meeting Oct. 2004 Bromont (Québec).

Fessart D, Simaan M, and Laporte SA, c-Src Regulates the Binding of AP-2 to the AT1R/ ßarrestin Complex During Endocytosis. 12th International meeting for 2nd messenger and Phosphoproteins. Aug. 2004 Montréal (Québec).

Fessart D, and Laporte SA, Recruitment of c-Src to the Angiotensin II type 1 receptor: Role in the formation of a ßarrestin/AP-2 complex. 4th Annual McGill Biomedical Graduate Conference. Feb. 2004 Montréal (Québec).

ABBREVIATIONS

5-HT_{2A}	5-hydroxytryptamine 2A
7TM	Seven-transmembrane receptors
AAK1	α-adaptin-associated kinase-1
AC	Adenylyl cyclase
ACE	Angiotensin-converting Enzyme
ADH	Antidiuretic Hormone
Ang II	Angiotensin II
AP-(1-4)	Clathrin adaptor protein (1-4)
ARF6	ADP-ribosylation factor 6
ARH	Autosomal recessive hypercholesterolemia protein
ARNO	ARF nucleotide binding site opener
Arg	Arginine
ASK1	Apoptosis signal-regulating kinase 1
Asp	Aspartic acid
AT1R	Angiotensin II type 1 receptor
AT1R-i2m	Angiotensin II type 1 receptor intracellular second loop mutant
AT2R	Angiotensin II type 2 receptor
β_2AR	β_2-adrenergic receptor
βARK	β-adrenergic receptor kinase
B2R	Bradykinin type 2 receptor
BRET	Bioluminescence resonance energy transfer
BSA	Bovine serum albumin
Btk	Bruton's tyrosine kinase
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
CCPs	Clathrin-coated pits
CCVs	Clathrin-coated vesicles
CHC	Clathrin heavy chain
CKD	Chronic kidney disease
CLC	Clathrin light chain

CRF	Chronic renal failure
CTX	Cholera toxin
CXCR1	CXC-Chemokine receptor R1
DAG	1,2-diacylglycerol
DOR	Delta-opioid receptor
EGFR	Epidermal growth factor receptor
ERK	Extracellular-signal regulated kinase
ETA	Endothelin type A
ETB	Endothelin type B
FRET	Fluorescence resonance energy transfer
G protein	Guanine nucleotide binding protein
GABA	γ-aminobutyrate
GAP	GTPase-activating protein
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
GDP	Guanosine 5'-diphosphate
GDS	GDP dissociation stimulator
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GIRK	G protein-coupled inwardly rectifying potassium channels
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GST	Glutathione S-transferase
GTP	Guanosine 5'-triphosphate
Hg	Hedgehog
His	Histidine
IGF1R	Insulin-like growth factor 1 receptor
Ile	Isoleucine
IP_3	Inositol 1,4,5-trisphosphate
JAK	Janus kinase
JNK3	c-jun N-terminal kinase type 3
KO	Knockout

LPA	Lysophosphatidic acid
LH-Hcg	Luteinizing hormone-Human chorionic gonadotropin
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
MKP7	Mitogen-activated protein kinase (MAPK) phosphatase
mGluR	Metabotropic glutamate receptor
NHERF	Na+/H+ exchanger regulatory factor
NK1	Neurokinin-1
NSF	*N*-ethylmaleimide-sensitive fusion protein
PACAP	Pituitary adenylate cyclase-activating peptide
PAR	Protease-activated receptor
PDE	Phosphodiesterase
PDZ	PSD95/DLG/ZO-1
PH	Pleckstrin homology domain
Phe	Phenylalanine
PI3K	Phosphatidylinositol 3-kinase
PI 4,5-P_2	Phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PP2A	Protein phosphatase 2A
Pro	Proline
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone related peptide
PTX	Pertussis toxin
RFP	Red fluorescent protein
RGS	Regulator of G protein signalling
SH1	Src Homology domain 1
SH2	Src Homology domain 2
SH3	Src Homology domain 3
siRNA	Short interfering RNA

Src	Sarcoma Rous
STAT	Signal transducers and activators of transcription
TfR	Transferin receptor
TGN	Trans Golgi Network
TM	Transmembrane domain
Tyr	Tyrosine
Val	Valine
V2R	Vasopressin receptor type 2
VIP	Vasoactive intestinal peptide
VSMC	Vascular smooth muscle cell
YFP	Yellow fluorescent protein

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SECTION I

LITERATURE REVIEW

1. G Protein-coupled Receptors : an overview

The evolution of multicellular organisms has been highly dependent on the capacity of cells to perceive their environment. A major discovery of the last 15 years was the membrane-bound receptors which are dedicated to recognizing extracellular messenger molecules (such as hormones, neurotransmitters, growth and developmental factors), and several other sensory messages (such as light, odours and gustative molecules). These receptors belong to the most common family of surface receptor: the G protein-coupled receptor (GPCR) family. G protein-coupled receptors form a large superfamily of over 800 genes (1) encoding seven-transmembrane (7TM) receptors [also referred to as seven-membrane-spanning receptors]. They mediate a wide variety of biological processes ranging from neurotransmission and hormonal control of virtually all physiological responses, to perception of taste, smell, light and pain. 7TM receptors are commonly referred to as GPCRs, based on the notion that most of them interact with one or more members of the intracellular guanine nucleotide binding signal transducing proteins (*i.e.* G proteins). Upon stimulation, heptahelical receptors undergo conformational changes that allow binding of heterotrimeric G proteins, usually within the intracellular domains of the GPCRs, leading to the activation of different effectors and signalling pathways (2, 3). Effectors generate second messengers, which in turn regulate a wide variety of cellular processes including cell growth and differentiation.

1.1. Classes of G Protein-coupled Receptors

The general structure of a GPCR consists of a central core domain comprising seven transmembrane helices (TM-I through -VII) connected by three intracellular (i1, i2 and i3) and three extracellular (e1, e2 and e3) loops (4) (Figure 1). According to their genetic and structural characteristics, GPCRs can be grouped into three distinct families: A, B and C (Figure 2). Each family shares over 25% sequence identity in the transmembrane core region and also a set of highly conserved residues and motifs.

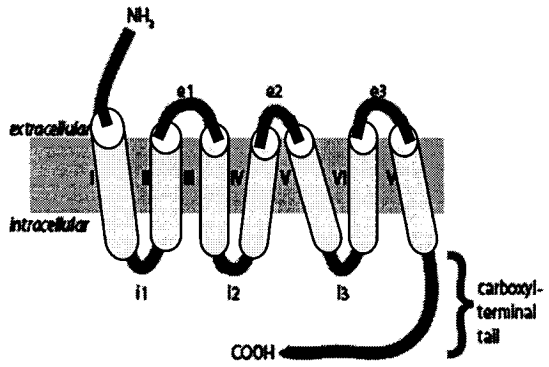


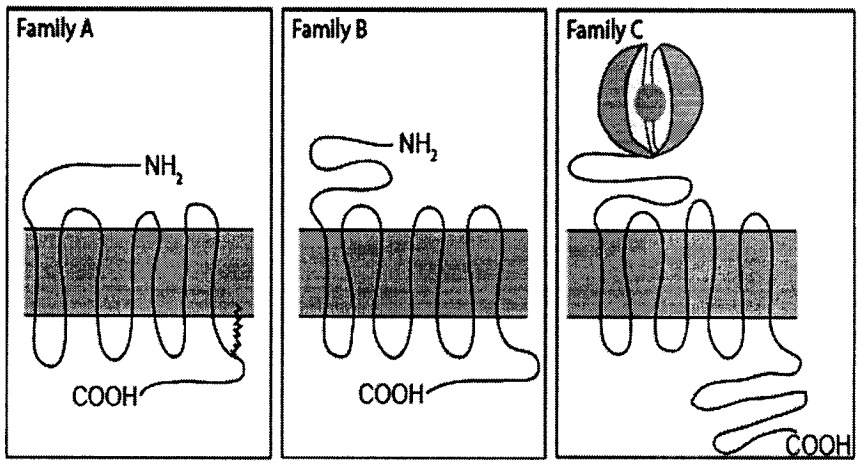
Figure 1. *Schematic representation of the seven transmembrane spanning domains (I-VII) of a characteristic G protein-coupled receptor, forming transmembrane helices and alternating extracellular (e1-e3) and intracellular (i1-i3) sequences.*

Family A is the largest group so far. It contains receptors that are activated by a broad variety of stimuli including photons for the light receptor (rhodopsin), odorants, as well as hormones and neurotransmitter agonists (such as dopamine and serotonin). These include the adrenergic receptor and most other 7TM receptor types which encompass the olfactory subgroup. The olfactory subgroup contains nearly 350 non-olfactory 7TM, recognizing more than 80 ligands (5). This class of receptor is characterized by several highly conserved amino acids and a disulphide bridge connecting the first and second intracellular loop (i1 and i2). Most of theses receptors also have a palmitoylated cysteine in the C-terminal tail, which serves as an anchor to the plasma membrane (represented with zigzag on Family A, Figure 2).

Family B contains only about 25 members, including the gastrointestinal peptide hormone receptor family (secretin, glucagon, vasoactive intestinal peptide (VIP) and growth-hormone-releasing hormone), corticotropin-releasing hormone, calcitonin and parathyroid hormone. For this receptor family, which is activated by large peptides, the relatively long N-terminal domain contains at least six highly conserved cysteine residues which play a role in binding of the ligand (6). The palmitoylation site is missing and the conserved residues and motifs are different from the conserved residues in family A.

Family C contains the metabotropic glutamate receptor family (mGluRs), the $GABA_B$ receptor, and a receptor activated by extracellular Ca^{2+} (calcium-sensing

receptor), as well as some taste receptors. This family is very particular in that the different members share a large extracellular N-terminus and C-terminal tail that seems to be crucial for ligand binding and activation. The ligand binding domain (represented as a circle on Family C, Figure 2) is located in the N-terminus, which was demonstrated by the resolution of the mGluR crystal structure (7). A unique characteristic of these receptors is that the third intracellular loop (i3) is short and highly conserved.



Family A	Family B	Family C
Rhodopsin-like	Glucagon-like	Metabotropic-like
Rhodopsin	α-latrotoxin	mGluR4
LH-hCG	PTH and PTHRP	mGluR2
Angiotensin	Secretin	mGluR1
Thrombin	VIP	Ca^{2+}sensing
Adenosine (A2)	PACAP	Pheromone VR1
Adrenergic (β2)	Glucagon	Pheromone GoVN2
Serotonin (5-HT)	CRF	Pheromone GoVN1
Muscarinic		Taste
Bradykinin		$GABA_B$
Dopamine		

Figure 2. *Three main GPCR families and their principal members*

1.2. GPCRs ligands

The idea that drugs bind to specific sites or receptors on cell surfaces stemmed originally from the work of Paul Ehrlich, who postulated that the cells of a mammalian organism carry a great number of side chains (receptors) on their surface (reviewed in (8)). The term 'receptive substance' was then used by John Langley and further developed by Sir Henry Dale as molecules in cells that bound a pharmacological substance (9). The further development of quantitative methods by which the properties of different ligands can be evaluated from the measurement of functional responses in isolated tissues led to the classification of GPCR ligands into three classes (see Figure 3) : 1) agonists, which increase the number of active receptor states to induce a biological response; 2) inverse agonists, which decrease the proportion of active receptor states and thereby, reduce basal receptor activity; and 3) antagonists, which inhibit the action of other ligands (reviewed in (10)). The majority of the drugs classified today as antagonists are actually inverse agonists (11). Only a ligand inhibiting drug action without an effect on the receptor coupling to G protein should be referred to as a neutral antagonist, instead of an antagonist.

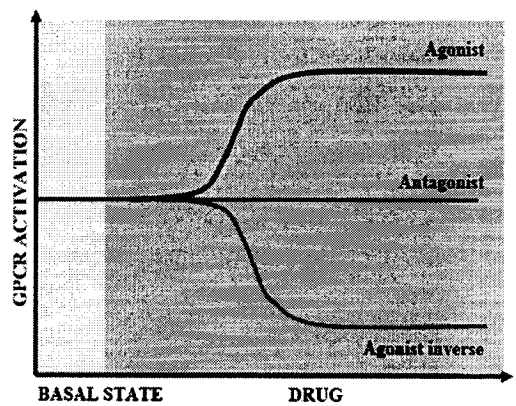


Figure 3. *Classification of GPCR ligands*

Although the chemical diversity of GPCRs ligands is exceptional, some receptors are still orphan receptors. These are GPCRs identified within the human genome that encodes proteins that possess the caracteristics of classic seven transmembrane spanning domains, but for which no natural nor synthetic ligands have

been identified. Although current estimates vary, there are probably still more than 150 human orphan receptors.

1.3. GPCR signalling

In the late1950s, studies by Sutherland and others demonstrated that a range of hormones was able to stimulate the production of cyclic AMP (cAMP) through the adenyl (now adenylyl) cyclase enzyme (12). It was not evident at that time, however, that this was a GTP-dependent process that required the intermediate G protein. This led to a minimalist model from a multi-subunit enzyme formed by a regulatory subunit for hormone binding, and a catalytic subunit for the production of cAMP. This model was inferred by Rodbell *et al* (13-15). Their studies with isolated fat cells showed that five different hormones acted at independent binding sites to stimulate a common adenylyl cyclase molecule. The role of GTP in hormonal stimulation of adenylyl cyclase was then discovered by Rodbell's group. In 1971, Rodbell *et al.* first proposed the existence of a guanine nucleotide regulatory protein that served as a transducer between hormone receptors and effectors (16). The basic findings were that without GTP in the medium, glucagon exerted little effect on adenylyl cyclase activity, and that GTP dramatically decreased the binding kinetics of the hormone to its receptor at the same concentrations required for hormonal stimulation of the enzyme. In 1974, Rodbell's group proposed that the actions of hormone and GTP in stimulating adenylyl cyclase were interdependent (17), and thus suggested that hormones act by regulating the effect of GTP on adenylyl cyclase. Later, independent studies from the laboratories of Lefkowitz (18) and Gilman (19) revealed that the effects of GTP on the β-adrenergic receptor were restricted to the binding of agonists. The G protein was later purified, named G_s, and shown to be a heterotrimeric complex comprising α, β and γ subunits (reviewed in (20)). Today, many studies on different types or classes of receptors have shown that receptor binding affinities for agonists are regulated by guanine nucleotides (21).

In the classical model of GPCR action in their activated (agonist-bound receptor) conformation, they catalyze exchange of guanosine 5'-diphosphate (GDP) tightly bound to the Gα for guanosine 5'-triphosphate (GTP). This in turn leads to the

activation of the α subunit and its dissociation from the G protein βγ complex (Figure 4) (20). However, much controversy has been raised concerning the issue of whether physical dissociation of α from βγ actually occurs during G protein activation. The issue remains unclear. The nature of the second messenger pathways activated in response to agonist binding to a G-protein-linked receptor is essentially determined by the type of G protein or other proteins coupled to each particular receptor. To date, 20 α, 6 β, and 11 γ subunits have been cloned. On the basis of sequence similarity, the Gα-subunit has been divided into four families (G_s, G_i, $G_{11/q}$ and $G_{12\text{-}13}$) and this classification has served to define both receptor and effector coupling.

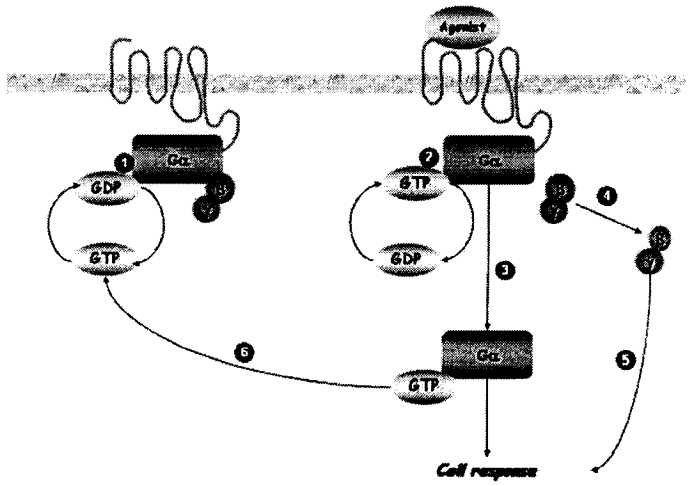


Figure 4. *The regulatory cycle of a G protein involving both GTP-induced activation and subunit dissociation and GTPase-dependent inactivation and subunit reassociation. The unoccupied receptor (1) interacts with a specific agonist leading to the activation of the receptor. The receptor can then interact with the trimeric Gαβγ complex (2) promoting Mg^{2+}-dependent GDP->GTP exchange and subunit dissociation (4), allowing interaction with effectors (3,5).*

The G_s proteins are coupled to the stimulation of adenylyl cyclase (AC); G_i proteins are coupled to the inhibition of AC as well as to activation of G protein–coupled inwardly rectifying potassium (GIRK) channels; $G_{11/q}$ proteins are coupled to the activation of phospholipase Cβ; and $G_{12\text{-}13}$ proteins are coupled to the activation of Rho guanine nucleotide exchange factors (GEFs).

1.3.1. Gs pathway

Gs activates adenylyl cyclase (AC) (22) to enhance the synthesis of the second messenger cyclic AMP (cAMP) (Figure 5). This increase in cAMP in turn activates protein kinase A (PKA), which is a serine/threonine kinase that phosphorylates many different substrates, including GPCRs, other protein kinases like mitogen-activated protein kinase (MAPK), and transcription factors. This $G\alpha_s$ protein subclass is ADP-ribosylated by bacterial toxins from *Vibrio cholerae* (cholera toxin or CTX) (23). Cholera toxin catalyses adenosine diphosphate (ADP)-ribosylation of an arginine residue present within the GTP-binding domain of $G\alpha_s$ in a ligand-independent fashion. The CTX-induced modification of the Gα-subunit considerably decreases its intrinsic GTPase activity, causing constitutive activation of the protein. Mutation of the same arginine residue also inhibits GTPase activity, leading to a comparable constitutive activation (24).

1.3.2. Gi pathway

This pathway was originally identified by the ability of $G\alpha_i$ to inhibit AC and thus reduce cAMP (Figure 5). Signalling through this pathway is inhibited by pertussis toxin, which (ADP)-ribosylates the Gα-subunit at its C-terminal region and thus prevents it from interacting with the receptor. This $G\alpha_i$ protein subclass is ADP-ribosylated by bacterial toxins from *Bordetella pertussis* (pertussis toxin or PTX) (25) on the fourth cysteine residue from the C-terminus. This modification leads to uncoupling of the G protein from the receptor without modifying other functions such as the guanine nucleotide exchange or GTPase activity.

1.3.3. Gq pathway

The G_q pathway is the classical pathway that is activated by calcium-mobilizing hormones and stimulates phospholipase Cβ (PLC-β) (reviewed in (26)) which catalyzes the hydrolysis of the lipid phosphatidylinositol 4,5-biphosphate (PI 4,5-P_2) to form two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Figure 5). The effect of IP_3 is to increase intracellular Ca^{2+} through release from internal stores, and DAG recruits protein kinase C (PKC) to the membrane and activates it.

1.3.4. G_{12} and G_{13} pathway

The G_{12} and G_{13} proteins were discovered through sequence similarity to known Gα proteins. In many cases, direct interactions with effectors are not yet fully established. So far, $G\alpha_{12}$ has been reported to directly interact with a GTPase-activating protein for Ras (Figure 5), RasGAP, and Bruton's tyrosine kinase (Btk) (27). $G\alpha_{13}$ directly interacts with and activates a guanine nucleotide exchange factor for the GTPase Rho (Figure 5), p115RhoGEF, and thus activates Rho leading to a variety of effects such as Na^+H^+ exchange (reviewed in (28)).

1.3.5. Gβ- and γ pathways

Gβγ-subunits are tightly bound to each other through noncovalent hydrophobic interactions; the complex is resistant to tryptic cleavage and can be dissociated only by treatment with denaturating agents (29). Although the diversity in Gβ and γ-subunits may theoretically yield up to 30 or more different βγ complexes, not all the possible pairs can combine, and some β-subunits are more able to interact and form stable combinations with particular γ-subunits than others (29). In some systems, the activation of particular effectors by the Gβγ complex is conditioned by Gα, whereas in others it seems to be independent. The Gβγ heterodimer also has the capacity to directly couple to at least four effectors including PLC-β, K^+ channels, AC, and phosphatidylinositol 3-kinase (PI3K) (30) and also proteins containing a PH domain. Numerous proteins involved in cellular

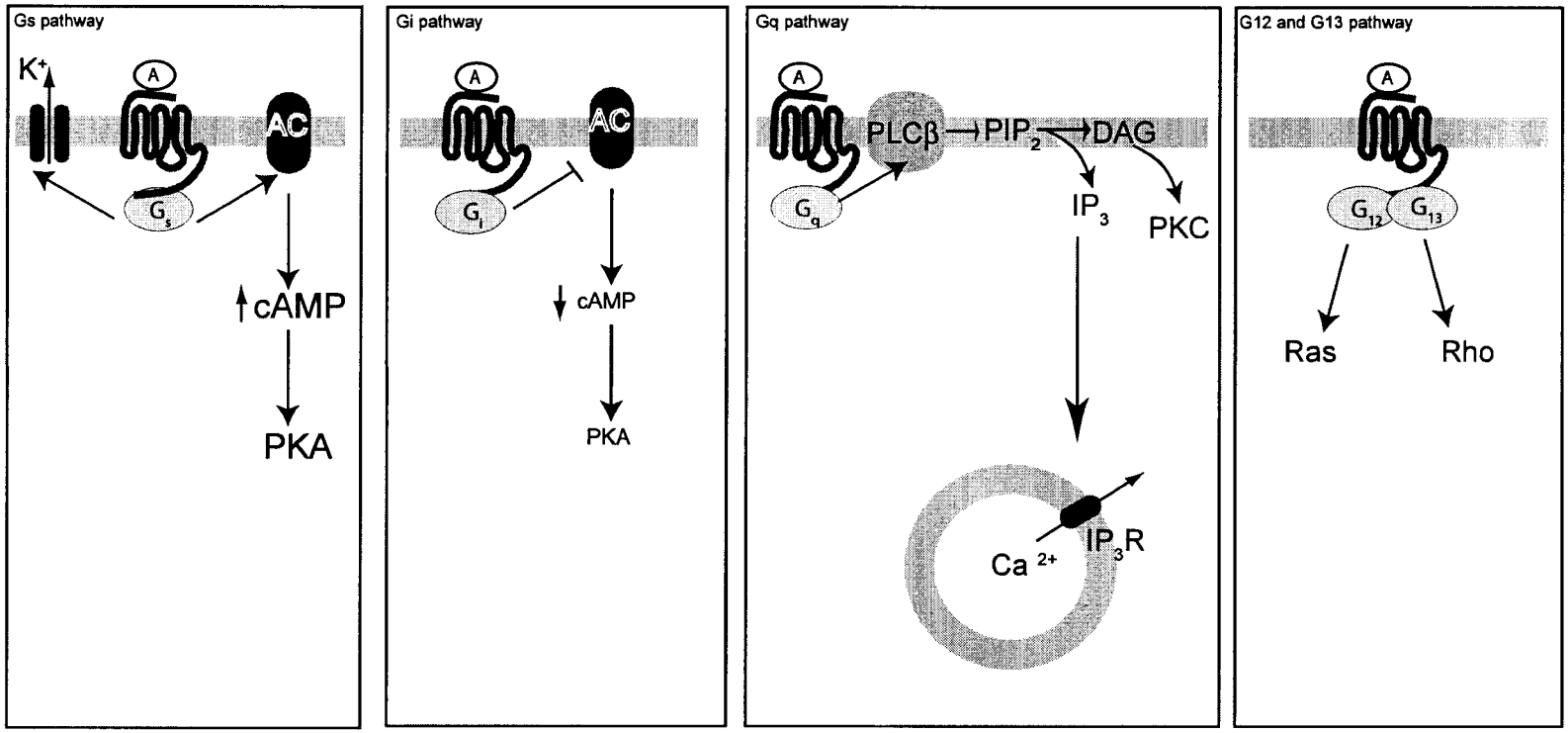


Figure 5. *The different G-protein coupling and their signaling pathways*

signal transduction have been shown to contain sequences homologous with a "domain" originally identified in the protein "pleckstrin" (pleckstrin homology domain; PH domain) and subsequently found in the G beta gamma interaction region of the beta ARK sequence (31). Recently, it has also been shown that Gβ can migrate between subcellular compartments (32), such as between the cytoplasm and the nucleus, or plasma membrane, depending on the type of stimuli (33). The Gβγ complex as a negative scaffold can bind, for example, the glucocorticoid receptor, and suppress its transcriptional activity (33).

2. Inactivation mechanisms of GPCRs

GPCRs are subject to three principal modes of regulation : 1) **desensitization**, in which receptors become refractory to continued stimuli; 2) **endocytosis or internalization,** whereby receptors are removed from the cell surface, and 3) **down regulation**, in which total receptor levels are decreased (reviewed in (34)).

2.1. GPCR desensitization

Following receptor activation by an agonist, the receptor undertakes the process of desensitization, defined as the "off switch" of all receptor functions triggered by the agonist at the plasma membrane. Therefore, GPCRs control their own responsiveness to dampen the physiological response to the continued presence of the stimulus (35). This process is the consequence of a combination of different mechanisms. One of the first steps involves the functional "uncoupling" of the G proteins from the receptors. The cloning of the β2-adrenergic receptor (β2-AR) in 1986 and its shared homology with the rhodopsin receptor (36), allowed the discovery that both receptors become phosphorylated in a stimulus-dependent way, and that this phosphorylation seemed to be related to the process of receptor desensitization. Desensitization is fairly rapid (seconds to minutes) and thought to be mediated through phosphorylation of serine and threonine residues by G protein-coupled receptor kinases (GRKs), second messenger-

dependent protein kinases such as cAMP-dependent kinase (PKA), and/or protein kinase C (PKC) (37-41).

2.1.1.Receptor phosphorylation by second messengers : heterologous desensitization

PKA and PKC are phosphotransferases that catalyze the transfer of the γ-phosphate group of ATP to serine and threonine residues contained within specific amino acid consensus sequences of proteins. They can phosphorylate a receptor even in the absence of agonist occupancy of the receptor, resulting in "heterologous" desensitization (reviewed in (42)). These protein kinases are activated in response to GPCR-stimulated increases in intracellular second messengers such as cAMP, Ca^{2+}, and diacylglycerol (DAG), and participate in GPCR signalling by mediating the phosphorylation of the receptor itself or other downstream target proteins. In the latter case, receptors that have not been activated by a ligand may become desensitized by activation of these protein kinases. PKA-mediated receptor phosphorylation has also been shown, in the case of the β2-AR, to switch the coupling of the receptor away from G_s in favour of enhanced coupling to G_i (43). Several other receptors, including the prostacyclin receptor, also seem to undergo such PKA-mediated switching in their G protein-coupling specificity (44).

2.1.2.Receptor phosphorylation by G protein-coupled receptor kinases: Homologous desensitization

By the mid-1980s, it had become clear that both rhodopsin (45) and the β2-AR (46) were phosphorylated in a stimulus-dependent manner, and that this phosphorylation seemed to be related to receptor inactivation or desensitization. In the case of rhodopsin, the enzyme responsible for the phosphorylation was referred to as rhodopsin kinase (47). In the case of the β2-AR, a novel cAMP-independent kinase [called the β-adrenergic receptor kinase (βARK)] appeared to contribute to agonist-dependent phosphorylation of the receptor (48). Now 7TM receptors are traditionally

thought to signal by means of activation of heterotrimeric G proteins and then, to be desensitized by G protein-coupled receptor kinases (GRKs) which are recruited to and specifically phosphorylate only agonist-occupied receptors leading to "homologous desensitization" (48, 49).

2.1.2.1.GRK Family

GRKs phosphorylate GPCRs at both serine and threonine residues localized within the third intracellular loop (i3) or C-terminal tail domain (50). Although no putative GRK phosphorylation consensus motifs have been identified, localization of amino acidic residues flanking repeated serines/threonines to the site of phosphorylation seems to favour GRK2-mediated phosphorylation (51). So far, only GRKs selectively phosphorylate agonist-activated receptors, whereas second messenger-dependent kinases can phosphorylate receptors in the presence or absence of an agonist. Seven mammalian GRK genes have been identified, some of which undergo alternative splicing to generate different isoforms (50, 52). The GRKs consist of three distinct domains: the kinase domain, the N-terminal RGS (regulator of G protein signalling) domain and the C-terminal domain which contributes to the plasma membrane targeting of the kinase (Figure 6). The function of RGS proteins is to inhibit the activity of the Gα-subunit by acting as GTPase-activating proteins (GAPs) (53). The specificity of GRKs *in vitro* has not yet been defined, but tissue distribution as well as levels of expression probably contribute to their specificity *in vivo* (54-56). However, recent studies show that different GRKs induce distinct signalling upon Angiotensin II type 1 receptor (AT1R) or V2 vasopressin receptor (V2R) activation, and suggest that GRK2 and -3 will antagonize the effects of GRK5 and -6 (57, 58). Several factors control the activity of the kinases towards the receptors. Principally the activated conformations of the receptors themselves, and allosterically activate the enzymes (55).

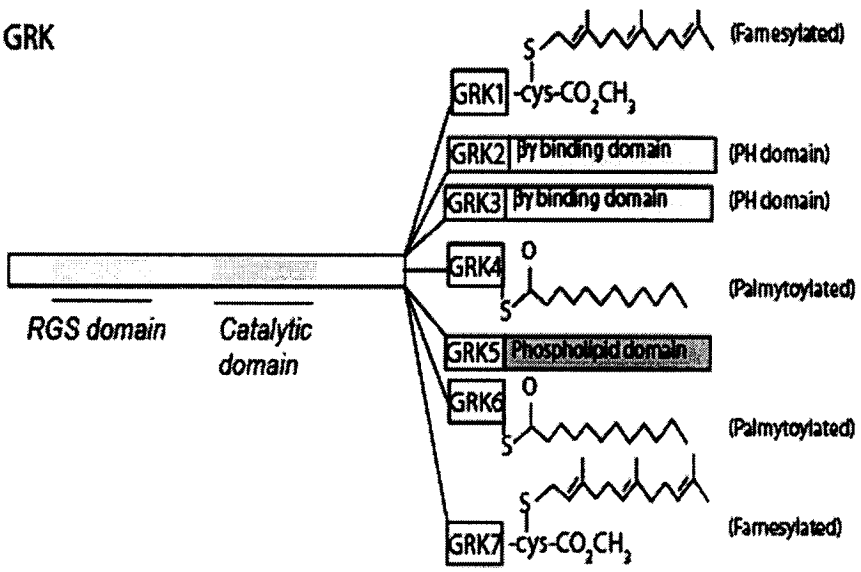


Figure 6. *Schematic representation of the domains for GRK1-GRK7. The Regulatory G protein signalling domain (RGS) and the catalytic domain are common to all GRKs. The C-terminal domain contains either a PH domain and a farnesylated or palmytoylated sequence, depending of the GRK subtype (modified from Fergusson SS (34)).*

GRKs also associate with the plasma membrane by various mechanisms, in order to be in close proximity with the activated-receptor. For example, GRK2 and GRK3 interact with the prenylated Gβγ subunits, whereas GRK1 is farnesylated, and GRK4 and GRK6 are palmyotylated for their insertion into the plasma membrane. A regulatory complexity is added by the fact that both PKA and PKC can phosphorylate and activate GRK2 by promoting its Gβγ-mediated membrane association (59, 60).

Based on sequence homology, the seven GRKs have been divided into three subfamilies : i) GRK1 (Rhodopsin kinase) and -7 are localized to retina, ii) GRK2 (β-adrenergic receptor kinase, βARK1) and -3 (βARK2), which interact with βγ ; iii) and the membrane-associated GRK4, -5 and -6 (55).

(i) GRK1 and GRK7 subfamily

In unstimulated cells, GRK1 is localized to the cytosol and translocates to bind its substrate in response to agonist activation of the rhodopsin receptor. This

translocation at the plasma membrane is facilitated by the post-translational farnesylation of its carboxyl-terminal CAAX motif (61), whereas its activity can be regulated by the calcium sensor protein recoverin (62). Protein farnesylation is catalyzed by protein farnesyltransferase, and plays important roles in the membrane association and protein-protein interaction of a number of eukaryotic proteins (reviewed in (63)).

(ii) GRK2 and GRK3 subfamily

For these protein kinases, the translocation to the plasma membrane is regulated in part by their association with the βγ-subunit of G proteins (64) which is mediated by a 125 amino acid region stretch in the C-terminus of the kinases. Plasma membrane targeting is also influenced by phosphatidylinositol 4,5-bisphosphate binding to the pleckstrin homology (PH) domain of the kinases (65). The PH domain acts as a membrane adaptor, linking proteins to the membrane surface via its binding to different phospholipids (66). GRK2 activity can also be influenced by phosphorylation. MAPK decreases its efficacy towards GPCR substrates (67). In contrast, PKC phosphorylation potentiates GRK2 activity (59, 68). Moreover, c-Src, via its recruitment to the agonist-dependent binding of βarrestin to GPCRs, phosphorylates GRK2 on tyrosine residues and targets GRK2 for degradation (69, 70).

(iii) GRK4, GRK5 and GRK6 subfamily

In the absence of GPCR activation by an agonist, these GRKs are localized to the plasma membrane. Both GRK4 and GRK6 are palmitoylated on C-terminal cysteine residues (54) facilitating their functional activity to phosphorylate GPCRs (71). GRK5 association with the plasma membrane is thought to be mediated by electrostatic interaction between 46 highly basic amino acid residues contained in the C-terminus of the kinase and membrane phospholipids (54). GRK5 activity can be influenced by phosphorylation of different protein kinases such as PKC, which reduces its activity (72).

GRK-phosphorylation of receptors is not sufficient for desensitization, but rather serves to create high affinity sites to promote the recruitment of the cytoplasmic accessory proteins, arrestins, and target the receptors for internalization via clathrin-coated pits. The arrestin protein was first identified by Herman Kuhn in 1986 as, "a 48K protein", bound to the phosphorylated rhodopsin, thereby interfering with its coupling to transducin (73). The protein was later renamed arrestin (74). Therefore it was postulated that the GRK-mediated phosphorylation of clusters of serine and threonine residues in the C-terminal tails of some receptors may regulate the stability of receptor/arrestin complexes (75). For most receptors, the determining factor for βarrestin interaction is the phosphorylation status of the activated GPCR. Thus impairment of receptor phosphorylation by mutagenesis of key serine/threonine residues generally leads to diminished βarrestin binding after stimulation of the M2 muscarinic cholinergic receptor, rhodopsin, the AT1R or the V2R (75-78). The arrestin recruitment in turn induces desensitization by preventing further coupling to G proteins (79, 80).

2.1.2.2.Arrestin Family

βarrestins are divided into two major classes, visual and nonvisual, on the basis of their localization. Four distinct mammalian arrestin proteins are known, two of which (visual and cone arrestins) are restricted to the phototransduction pathway (42). Two somatic forms, βarrestin 1 (arrestin 2) and βarrestin 2 (arrestin 3) are ubiquitously expressed and are thought to regulate signalling as well as internalization of many different GPCRs (52). βarrestin 1 and 2 have highly homologous structures, sharing 78% amino acid identity (81, 82). Several alternative splice variants have been described including variants of visual arrestin (83), βarrestin 1 and βarrestin 2 (84). Numerous reports have addressed whether these two isoforms serve different roles in receptor internalization or whether they are functionally redundant (85-88).

A-Beta-arrestins and their interacting proteins

Beta-arrestins preferentially bind to agonist-activated and GRK-phosphorylated GPCRs, as opposed to second messenger protein kinases that recognize phosphorylated or non-phosphorylated receptors (79). βarrestins contain a specific region that links GPCRs to components of the clathrin-dependent endocytic machinery. The general model is that βarrestins function as adaptor proteins that support the assembly of a multi-protein complex on the receptor and initiate both the internalization of receptor and signalling via the recruitment of other signalling molecules independent of second messengers. The first indication that βarrestins specifically target GPCRs for endocytosis via the clathrin-coated vesicles was confirmed by studies which showed that βarrestin interacts directly with clathrin (89) and AP-2 (90, 91), both components of the endocytic machinery. Since the discovery of βarrestins as proteins that desensitize receptor dependent-second-messenger signalling, several new roles and βarrestin interacting proteins have been identified (Table 1). They also bind to other cellular components involved in the modulation of vesicular trafficking, including the ATPase protein NSF (N-ethylmaleimide-sensitive fusion protein) (92), small GTPases such as ARF6 (93) and the E3 ubiquitin ligase Mdm2 (94). Recently, βarrestins were also described to homo- and hetero-oligomerize in living cells; however the biological relevance of this interaction *in vivo* remains unclear (95).

βarrestin isoform	Binding Partner	Residues	References
βarrestin1,2	**Src-SH1, Src-SH3**	1-185, Pro^{91} Pro^{121}	(96, 97)
βarrestin 2	ASK1	1-185	(98)
βarrestin2	JNK3	Arg^{196} Ser^{197} Ser^{198}	(98, 99)
βarrestin2	Mdm2	1-185	(100)
βarrestin2	Mdm2 (E3 Ub lig)	1-260	(101)
βarrestin2	Smo		(102, 103)
βarrestin2,1	**βadaptin2**	Arg^{394} Arg^{396}	(90, 104)
βarrestin1,2	Clathrin	Leu^{374} Ile Glu Phe^{377}	(105)
βarrestin2	Phosphoinositides	Lys^{233} Arg^{237} Lys^{251}	(106)
βarrestin1	NSF		(92)
βarrestin1,2	ARF6	(1) 319-418	(93)
βarrestin2	PP2A/Akt		(107)
βarrestin1,2	PDE4D		(108)
βarrestin1,2	IkBα		(109, 110)
βarrestin1	Fgr		(111)
βarrestin1	Hck		(111)
βarrestin1	Yes		(112)
βarrestin1,2	ERK2		(113, 114)
βarrestin1,2	cRaf1		(113)
βarrestin1	RhoA		(115)
βarrestin1,2	Ral GDS		(116)
βarrestin2	ARNO		(93)
βarrestin2	MKP7	165-239	(99)
βarrestin1,2	βarrestin1,2		(95)
βarrestin2	Filamin A	1-80	(117)
visual arrestin	Microtubules		(118)

***Table 1**: The different βarrestin-interacting proteins*

As shown in figure 7, the βarrestin domain involved in clathrin binding is localized to amino acids 373-377 in βarrestin 2 (105). The βarrestin domain involved in binding the β2- subunit of AP-2 (β2-adaptin) is in the C-termini of βarrestin 1 and 2 (91). In particular, two arginine residues (R394 and R396) in βarrestin 2 mediate the binding of β2-adaptin *in vitro* (90, 104). A phosphate sensor region localizes to the linker between the N and C domains and forms part of the polar core of the protein. Interactions between the C-terminal tail and the phosphate sensor region that maintain arrestin in an inactive state are disrupted upon receptor binding, allowing arrestin to bind with high affinity to the phosphorylated receptor (reviewed in (119)).

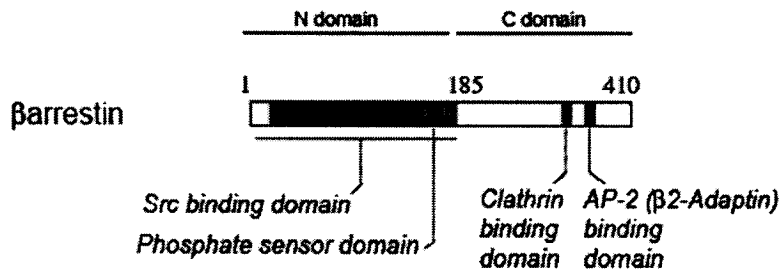


Figure 7. *Schematic representation of the principal binding sites on βarrestin.*

Based on their capacitiy to bind phosphorylated-GPCR, clathrin or AP-2, three dominant-negative arrestin mutants have been described in sequestration studies. The carboxyl-terminal clathrin-binding domain of either βarrestin 1 (residues 319-418) or βarrestin 2 (residues 284-409) binds to clathrin but interacts only weakly with phosphorylated receptors which are completely devoid of ligand binding. The βarrestin 1 V53D mutant, which contains a Val to Asp mutation in its N-terminus, binds well to clathrin, but is defective in interacting with phosphorylated agonist-activated GPCR.

However, how the specific βarrestin 1 V53D mutation functions as a dominant negative remains to be elucidated.

B-Differential affinities of the arrestins for GPCRs

Following agonist-dependent GRK-phosphorylation of β2-AR as well as other receptors, the βarrestin proteins are recruited from the cytosol to the plasma membrane (75, 120, 121). Using fluorescence microscopy to visualize the distribution of GFP-tagged βarrestin 1 or 2 (*i.e.* translocation assay), it was shown that these proteins possess distinct preferences for different receptors, allowing them to distinguish two classes of G protein-coupled receptors based on their differential binding avidities for βarrestin 1 and 2 (121). The first class receptors (*i.e.* Class A) including β2-AR, μ-opioid, endothelin type A (ETA), dopamine type 1, and α1b-adrenergic receptors (Table 2), were demonstrated to interact with βarrestin 2 at a higher affinity than with βarrestin 1. However, because the interaction is of relatively low affinity for the receptor, both βarrestins dissociate from the receptor as the coated pit pinches off as a coat vesicle, and the receptor internalizes without the βarrestins. A second class of receptors (*i.e.* Class B) including AT1R, V2R, neurotensin, thyrotropin-releasing hormone, and neurokinin type 1 receptors, did not discriminate between βarrestin 1 and 2, and showed high affinity for both proteins (121) (Table 2). βarrestin does not dissociate from receptors at the plasma membrane, and internalizes with them into endosomes where they remain stably associated for prolonged periods (Table 2). Apparently, the density of GPCR phosphorylation sites within the C-terminus may be involved in regulating the stability of the βarrestin interaction with receptors (122). The ability of beta-arrestin to remain associated with receptors was shown to be mediated by specific clusters of serine and threonine residues located in the receptor carboxyl-terminal tail. These clusters are remarkably conserved in their position within the carboxyl-terminal domain and serve as primary sites of agonist-dependent receptor phosphorylation (122). The C-terminal domain of βarrestin also determines the stability of the interaction. Indeed, a βarrestin mutant truncated at residue 383, thus

lacking the AP-2 binding site, binds the β2-AR (class A) with high affinity and trafficks into endosomes (122). Removal of the βarrestin carboxyl terminus may constitutively expose the high affinity receptor binding site and allow the mutant βarrestin to bind with higher avidity to GPCRs.

Class A	*Class B*
ß2-adrenergic (121)	angiotensin II type 1 (121)
μ-opioid (121)	vasopressin type 2 (121)
endothelin type A (121)	neurotensin 1 (121)
dopamine type 1 (121)	thyrotropin-releasing hormone (121)
α1b-adrenergic (121)	neurokinin type 1 (121, 123)
lysophosphatidic acid (124)	proteinase-activated receptor 2 (114)
leukotriene B_4 receptor 1 (125)	orexin-1 (126)
apelin (126)	

Table 2. *Classification of receptors according to their affinities for βarrestin 1 and 2.*

Another level of complexity maybe added by the fact that some receptors form an intermediate (*i.e.* Class C) between class A and class B. These receptors are internalized with βarrestin into endosomes, suggesting a higher avidity for βarrestin. However, βarrestin dissociates rapidly from the receptors in endosomes, and receptors are recycled back to the plasma membrane (127).

C-Arrestins: their relevance in the internalization

βarrestin plays a key role in coordinating processes during receptor internalization across different species such as *Mus musculus* (87, 128, 129), *Drosophila melanogaster* (130) and the zebrafish *Brachydanio rerio* (103). The functional specificity of the various arrestins in the overall desensitization is supported by *in vivo* experiments derived from knockout animals in which arrestin genes have been inactivated by homologous recombination. βarrestin 1 knockout (-/-) mice

display altered cardiac responsiveness to β2-AR stimulation (128). βarrestin 2 knockout (-/-) mice exhibit a potentiation and prolongation of the analgesic effect of morphine, suggesting a role in μ-opioid receptor desensitization in the central nervous system (129). However, a double knockout of both βarrestins is embryonic lethal (113). Similarly, knockout (-/-) of Kurtz, a βarrestin analogue in *Drosophila melanogaster*, is lethal during embryogenesis (130). Recently, Wilbanks *et al.* (103) were able to show that knocking-down βarrestin2 in zebrafish embryo affects the Hedgehog (Hg) signalling pathway during embryogenesis. The Hg signalling pathway uses the GPCR, Smoothened, and is fundamental in development in which any deregulation in its signalling is associated with malignancies.

In addition, the recent discovery of short interfering RNAs (siRNA) to induce sequence-specific gene silencing in cells without initiating the non-specific gene silencing pathways (131) has become a remarkable tool to study the real impact of depleting one protein in cells without generating knockout animals. Using a different approach with antisense to reduce βarrestin levels in cells it was found that reduction βarrestin impairs β2-AR desensitization and internalization, but has no effect on the internalization of either M2 or M3 muscarinic receptors (132). However, because βarrestin expression was not completely eliminated by this method, Kohout *et al.* (87) generated mouse embryonic fibroblast (MEF) cell lines from knockout (KO) animals, which lack one of the βarrestins (βarrestin 1-KO and βarrestin 2-KO) or both (βarrestin 1/2-KO). Internalization of the β2-AR was compared to the AT1R for the different cell lines described above. They demonstrate that the sequestration of various receptors is regulated differently by the two βarrestin proteins. The internalization of β2-AR is dramatically affected in βarrestin 2-KO while in βarrestin 1-KO the levels of receptor internalization are normal. For AT1R, in contrast internalization is slightly reduced in the βarrestin 1-KO cells and unaffected in the βarrestin 2-KO cells. However, in the βarrestin 1/2-KO cells both receptors show a dramatically reduced internalization, which can be restored by reintroducing βarrestin 1 and 2, suggesting that both isoforms are able to support receptor internalization. Similarly, siRNA

depletion of βarrestin 1 and 2 shows patterns in desensitization and internalization identical to those observed in MEF cells (133), confirming the important role for βarrestin in GPCR internalization. Today, it is well-appreciated that most GPCRs are regulated by GRKs and arrestins. Details of which kinases phosphorylate which receptors in any given tissue, however, remain largely unknown.

3. Receptor internalization

3.1. Molecular mechanisms involved in G protein-Coupled Receptor Endocytosis

Several mechanisms modulate GPCR responsiveness following agonist occupancy, including agonist-induced receptor desensitization, endocytosis (or sequestration), and resensitization (or degradation). The intrinsic properties of receptors to interact with other regulatory proteins such as arrestin control the rate of agonist-induced turnover, as well as the fate of receptors (recycling versus degradation) after endocytosis. Although arrestins play a pivotal role in agonist-stimulated signalling and regulation of most GPCRs, several alternative pathways have been described (134). These include internalization in arrestin- and clathrin-independent manners.

3.1.1. Arrestin- and dynamin-dependent endocytosis

Clathrin-mediated endocytosis is the well-described mechanism for the entry of molecules into cells. This pathway is characterized by the recruitment of soluble clathrin from the cytoplasm to the plasma membrane. Clathrin-binding adaptors, such as the adaptor-protein 2 (AP-2), are the key components of this pathway. They bind directly to clathrin (135), as well as other endocytic regulatory proteins and cargo to stimulate the formation of the clathrin coat (136). In this pathway, receptor phosphorylation by GRKs enhances binding of βarrestins, which serves to quench agonist-mediated G protein signalling, and acts as a scaffold intermediate with two components of the clathrin-coated-pit machinery: AP2 and

clathrin, to stabilize the association with clathrin-coated pits (CCPs). In this model, the clathrin adaptor complex AP-2 plays a central role in CCP formation and function, being responsible for the assembly of clathrin triskelia at the plasma membrane and selection of cargo receptors that will be internalized by forming clathrin-coated vesicles (CCVs). CCVs are so-called because the main component of the coat is clathrin, which forms a polymeric scaffold on the vesicle inner surface.

3.1.1.1.Adaptor clathrin protein family

Clathrin coats were identified by their distinctive appearance in electron microscopy. The presence of clathrin lattices that form characteristic open hexagonal and pentagonal facets of the coat are depicted in Figure 8A. A clathrin coat is a three-dimensional (3D) array of triskelia. Each triskelion is made of three 1,675-residue clathrin heavy chains (CHCs, approximately 190-kDa) and three 25–29-kDa clathrin light chains (CLCs) (Figure 8B).

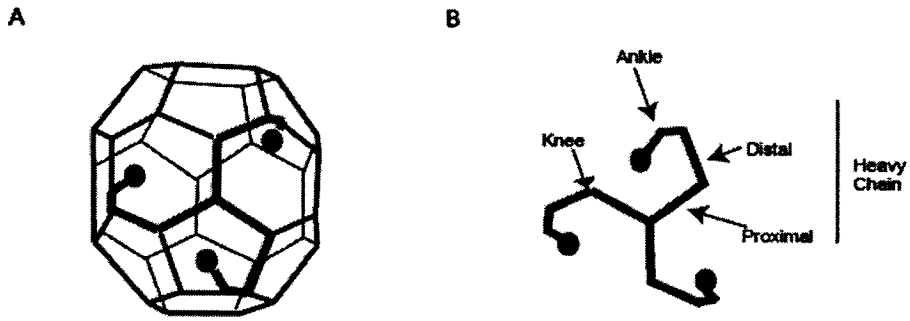


Figure 8: *The architecture of clathrin.* A, *Clathrin barrel with a single triskelion highlighted.* B, *Schematic representation of a clathrin triskelion. The triskelion is composed of 3 heavy chains and 3 light chains (not shown). Each heavy chain comprises ankle, distal, knee and proximal segments to form a clathrin leg (137).*

In vitro, purified clathrin can spontaneously assemble into cages or, in the presence of adaptor proteins, into coats. The clathrin coat is assembled on the cytoplasmic face of the plasma membrane by the recruitment of the adaptor complex AP. The characteristic of an adaptor is to bind endocytic cargo and serve as a link between the substrate for internalization and coat components (138). At least 20 endocytic accessory factors contribute to clathrin-mediated endocytosis, and many of these factors are clathrin adaptors themselves (reviewed in (139)). The AP-2 complex was the first protein characterized as an 'adaptor'(136), and it is a member of the highly conserved adaptor family. Within this family, AP-2 is specifically found at the plasma membrane whereas, AP-1, -3 and -4 have been shown to localize both to the endosomes and the Trans Golgi Network (140). APs are heterotetrameric proteins, and to date, four APs are identified (AP1–4). All APs have two large subunits of 100–130 kDa (γ and β1 in AP-1, α and β2 in AP-2, δ and β3 in AP-3, and ε and β4 in AP-4). They also contain a medium subunit of approximately 50 kDa (μ1 for AP-1, μ2 for AP-2, μ3 for AP-3 and μ4 for AP-4) and a small subunit of about 20 kDa (σ1 for AP-1, σ2 for AP-2, σ 3 for AP-3, and σ4 for AP-4). The large subunits can each be divided into a trunk domain separated from an appendage domain (referred to as an 'ear' domain) by a protease-sensitive linker (referred as a 'hinge' domain) (Figure 9).

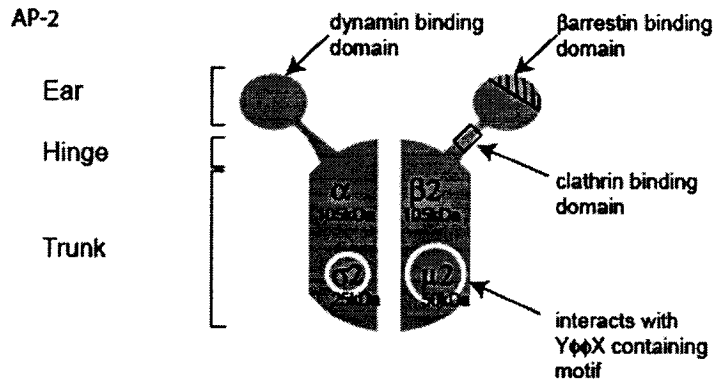


Figure 9. *Representation of the heterotetrameric AP-2 complex with its α, β2, μ2 et σ2 subunits. AP-2 links the clathrin to the membrane through interactions of its μ2 and α subunits with lipids.*

AP-2 adaptors play a central role in CCV formation by linking the endocytic cargo to the clathrin coat, and also by interacting with a number of proteins involved in the regulation of CCV formation (See Table 3) (138). The assembly of the CCP is facilitated by cytosolic proteins that form a dynamic network of protein-protein interactions by associating with multiple partner proteins during the different steps of endocytosis. For example, a βarrestin R396A mutant, which cannot interact with AP-2, does not support the sequestration of agonist-activated β2AR into plasma membrane puncta (90) and suggests that the interaction of βarrestin with AP-2 is important for endocytosis.

Each AP-2 subunit has a distinct function (See Table 3). Via its μ2 subunit, AP-2 binds to the tyrosine-base internalization motifs on cargo molecules, NPXY (where N, P and Y are asparagine, proline and tyrosine residues, respectively, and x represents any amino acid) (141, 142). These motifs determine which vesicular traffic pathway is used to transport a particular molecule, and hence determine its final destination.

AP-2 subunit	Bind to	References
α	Auxilin	(143)
α	AAK1	(143)
α	Stonin	(143)
α	Synaptojanin 1	(143)
α	NECAP1	(143)
α	Eps15	(144, 145).
α	AP180	(146)
α	PIP_2	(147)
α	Dynamin	(148)
α	Amphiphysin	(146)
α	Clathrin	(149)
α	Sorting nexin 9	(150)
α	Dab2	(151)
β2 (E^{849} and E^{902})	βarrestin1,2	(152)
β2	Epsin	(153)
β2	AP180	(153)
β2 (LΦxΦD/E)	Clathrin	(135)
β2 (D^{253} F^{259} L^{262} and R^{266})	ARH	(154)
β2	Sorting nexin 9	(150)
μ	PIP_2	(147)
μ (YxxΦ)	TfR	(155)
μ (YxxΦ)	EGFR	(156)
μ	AAK1	(157, 158)
μ (YxxΦ)	α1a AR + PAR	(159, 160)

***Table 3**: The different AP-2-interacting proteins and their identified interacting regions (Φ is an amino acid with a hydrophobic side group).*

The interaction of clathrin with AP-2 is via the clathrin box motif LΦxΦD/E (where L, D and E are leucine, aspartic acid and glutamic acid respectively; x represents any amino acid and Φ any hydrophobic amino acid), in the hinge region of β2-adaptin (135). The α-subunit binds to clathrin, dynamin (148) and Eps15 (144, 145). Some studies also indicate that the phosphorylation of AP-2, induced by α-adaptin-associated kinase-1 (AAK1), could regulate its binding to endocytic cargo (157, 158, 161). The β2-adaptin subunit is essential for clathrin coat formation and interacts with clathrin through dileucine motifs (reviewed in (162)). Furthermore, the use of siRNA against the α and μ2 subunits has shown that the AP-2 complex is required for the formation of most plasma membrane-associated CCPs (163, 164). The depletion of the different subunits of the AP-2 complex has been reported to be embryonically lethal (See Table 4) and thus supports different role of AP-2 such as embryogenesis.

Complex	Organism	Methodology	Phenotype
α	*D. melanogaster*	P-element enhancer trap insertion	Several alleles - most severe are embryonic lethal, synapses devoid of vesicles
α	*C. elegans*	dsRNAi	Embryonic lethal, inhibition of yolk endocytosis
μ2	*C. elegans*	dsRNAi	50% embryonic lethal, dumpy phenotype
σ 2	*C. elegans*	dsRNAi	50% embryonic lethal, dumpy phenotype
α2	*S. cerevisiae*	Targeted disruption	None observed
α	*S. cerevisiae*	Targeted disruption	None observed
β2	*S. cerevisiae*	Targeted disruption	None observed
μ 2	*S. cerevisiae*	Targeted disruption	None observed
μ 2	*M. musculus*	Targeted disruption	Embryonic lethal

Table 4. *Genetic analysis of the function of AP-2 complex.*

3.1.1.2.Src

The list of GPCR-activated signalling pathways in which βarrestins have been implicated includes not only components of the clathrin-endocytic machinery, but also signalling molecules such as the non-receptor tyrosine protein kinase Src family (165-171). The Src family of non-receptor protein-kinases consists of 9 members: Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, Yes and Yrk. Src family protein kinases exhibit a highly conserved structural organization that includes a myristoylated N-terminal domain to facilitate its attachment at the plasma membrane, followed by the SH3 domain recognizing a proline rich region in target proteins, the SH2 domain recognizing tyrosine phosphorylated residues, the tyrosine kinase domain (SH1) and a C-terminal tail (Figure 10) (172). Thus, targeting of Src family protein kinase is accomplished through protein–protein interactions involving the phosphotyrosine-binding Src homology domain 2 (SH2) and proline-rich domain-binding SH3 domains common to all members of the family.

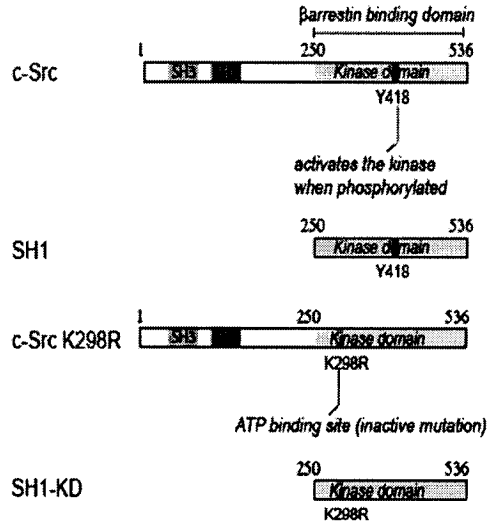


Figure 10. *Representation of the non receptor tyrosine kinase c-Src and its different domains. The SH3 domain is located between amino acids 91 and 151, the SH2 domain is located between 151 and 250 and the catalytic domain is located between 250 and 536. SH1 corresponds to the isolated catalytic subunit containing the kinase activity. An additional mutant, c-Src K298R, contains a point mutation in the ATP binding site and in its small catalytic region SH1-KD (KD: Kinase Dead) (96).*

Apart from targeting c-Src, the SH2/SH3 domain mediated-interaction also induce c-Src activation by destabilizing the domain that maintains it inactive. The Src tyrosine kinase activity is regulated by tyrosine phosphorylation at two sites, Tyr^{418} and Tyr^{530}, with opposing effects. First, under basal conditions Tyr^{530} is phosphorylated and induces a conformational change in the C-terminal tail of the molecule to interact with the SH2 domain of Src. This interaction results in the suppression of its protein kinase activity (173). The dephosphorylation of Tyr^{530} opens the conformation of c-Src, thereby allowing the protein kinase activity (174). Second, the phosphorylation of Tyr^{418} in the activation loop of the protein kinase domain up-regulates the enzyme activity. When this site becomes phosphorylated, it is displaced from the substrate binding pocket, allowing the kinase access to substrates. Thus, when Tyr^{418} is phosphorylated, it is a positive regulator of Src activity, and when dephosphorylated, it is a negative regulator of c-Src activity. Introduction of a kinase dead mutation (K298R, where K represents a lysine and R an arginine residue) into the ATP binding site of c-Src protein also completely eliminates kinase activity by blocking the hydrolysis of ATP (96, 171). Once activated, protein kinases catalyze the following reaction:

$$MgATP + protein\text{-}OH \rightarrow Protein\text{-}OPO_3^{2-} + MgADP + H^+$$

Recent evidence suggests that c-Src recruitment to the endocytic complexes may play a role in the activation of the endocytic machinery (170, 171, 175). Upon stimulation of the β2AR, c-Src rapidly associates with the receptor in a βarrestin-dependent manner (97). c-Src mutants devoid of catalytic activity that can interact with βarrestin (SH1-KD) function as dominant-negative inhibitors of clathrin-mediated internalization of receptors (96, 170). Also, several new substrates for the c-Src phosphorylation of proteins involved in the endocytic machinery have been identified (Table 5).

Src family	Phosphorylation Substrates	References
Src	Dynamin Y^{231} Y^{597}	(170)
Src	Caveolin Y^{14}	(176)
Src	GRK2	(69, 177)
Src	Angiotensin II type 1 receptor	(178)
Src	ASAP1	(179)

Table 5. *Src and its principal substrates involved in endocytosis of receptors*

Today, c-Src is described as a protein kinase playing pleiotropic roles such as the regulation of the early steps of GPCRs internalization, but also as a signalling protein regulating MAPK activation following GPCR activation. Indeed, c-Src activity is required not only for GPCR-mediated stimulation of the Ras pathway leading to the activation of MAPK (165-169), but also for β2AR endocytosis, where in MEF cells deficient of Src-family tyrosine kinases (SYF cells) the internalization was abolished (180).

3.1.1.3. Dynamin

During receptor internalization, it was initially thought that βarrestin participated in the *de novo* formation of CCPs (89, 181). However, recent evidence shows that βarrestin targets receptors to existing pits at the cell surface and engages them in the internalization process (182, 183). Although this issue remains controversial, the general model for receptor internalization from the plasma membrane is that the formed pits progressively invaginate and are finally released into the cytosol as a free CCV, a step which requires the activity of dynamin. Dynamin is a GTPase involved in the pinching off of clathrin-coated vesicles from the plasma membrane (184). Dynamin was discovered in a mutant of *Drosophila melanogaster*, known as *Shibire*, which became paralysed at high temperatures (185). By performing an electron microscopy study of *Shibire* flies, Kosaka *et al.*, discovered that high

temperatures blocked the endocytosis of clathrin-coated pits and thus paralyzed flies. This makes cells unable to recycle synaptic vesicles leading to the depletion of these vesicles, blockage of synaptic transmission, and then paralysis. Using microscopy, the clathrin-coated pits appeared as deep coated pits with long necks formed on presynaptic membranes (186). Apparently, *Shibire* cells had a defect in the fission of coated vesicles from synaptic membranes. When the mutated gene in *Shibire* was finally identified and cloned (187), it was found to encode dynamin, a large GTPase that was originally identified as a microtubule-binding protein (188).

Dynamin is now considered to activate effectors of the fission machinery that yield CCV. Three mammalian dynamin isoforms have been identified : neuronal dynamin-1, ubiquitously expressed dynamin-2, and dynamin-3, which is expressed in testes, neurons, and lungs (189). Comparison of the primary sequences shows that all dynamin isoforms contain three highly conserved GTP-binding motifs necessary for guanine-nucleotide binding and hydrolysis. The GTPase activity of dynamin is stimulated by self-oligomerization once 'a critical mass is reached' (reviewed in (190)). A lysine-to -alanine substitution (K44) in the first GTP-binding motif yields a dominant negative dynamin mutant, which is strongly impaired in GTPase activity and reduced in GTP affinity (187). A second important regulator of dynamin function is PIP_2 (191, 192). All three dynamins contain a PH domain that is able to bind PIP_2 (Figure 11).

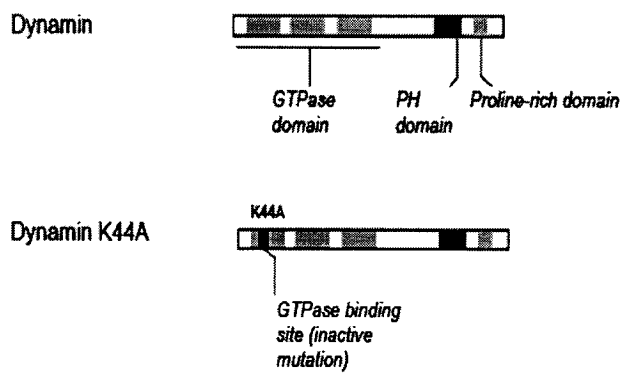


Figure 11. *Representation of Dynamin and its dominant negative (K44A) construct.*

The binding of PIP_2 to dynamin strongly increases GTPase activity and serves to target dynamin to the plasma membrane for its assembly at the neck of the clathrin-coated vesicles (191, 192). A single point mutation (K535A) in the PH domain of dynamin-1 has a dominant negative effect on endocytosis in cells (193). There is also a proline-rich domain that binds to a SRC-homology 3 (SH3) domain, which is found in proteins of the endocytic machinery such as amphiphysin, and endophilin (194). Dynamin is also regulated by tyrosine phosphorylation during β2-AR internalization. The proposed model is that βarrestin will act as an adaptor/scaffold to bind c-Src, allowing the phosphorylation of dynamin (170). Ahn and collaborators identified two tyrosines (Y231F/Y597F) in dynamin and, when mutated, cannot be tyrosine phosphorylated by c-Src and thus inhibited the agonist-induced internalization of the β2-AR. These data suggest that tyrosine phosphorylation of dynamin plays an important role in endocytosis.

3.1.2. Arrestin independent, dynamin-dependent internalization

Although the general model for GPCR internalization involves both βarrestin and dynamin, some receptors are internalized via the clathrin-coated vesicle pathway independently of βarrestin (195-199) using an alternative endocytic pathway. This pathway is characterized by blockage of GPCR internalization with dominant-negative dynamin, but not with dominant negative βarrestin. For example, overexpression of the dominant negative mutants βarrestin (βarr1 V53D, truncated βarr1 (319-418) and βarr2 (284-409)) do not affect the internalization of the 5-Hydroxytryptamine 2A ($5\text{-}HT_{2A}$) receptors, while overexpression of dominant negative dynamin (K44A) significantly impairs receptor endocytosis (200). Similar results were obtained for M1 and M3 muscarinic receptors (195) and suggest that these receptors might use a dynamin-dependent internalization pathway independent of βarrestin. Interestingly, recent evidence suggests that the $5\text{-}HT_{2A}$ (201), the muscarinic (202) and the Endothelin type B (ETB) receptors are internalized via specific

structures called caveolae (203). Caveolae are morphologically distinct from CCVs, but their formation is reported to be dynamin-dependent (204). Caveolae are specialized domains of the plasma membrane containing as a principal component the integral membrane protein caveolin-1. One biochemical piece of evidence that caveolae mediates the uptake of molecules in cells comes from the observation that some GPCRs were found in caveolae structure : M2 muscarinic receptor (205), β2-AR (206), bradykinin receptor (B2R) (207), ETA receptor (208) and ETB receptor (203). There is also evidence that caveolin-1 directly interacts with a number of signalling proteins, including Gα-subunits, non-receptor tyrosine kinases-like Src, PKA, and PKC (209, 210). For the cholecystokinin (CCK) receptor, which is internalized via both caveolae and clathrin-coated pit pathways, treatment with inhibitors of the clathrin-coated pit pathway induced GPCR internalization for almost all CCK receptors (211). These results suggest that the caveolae pathway is able to fully support internalization, and also that the CCK receptor can utilize both endocytic pathways.

It has long been known that GPCRs can internalize by different pathways in distinct cells (212), but the physiological significance of these differences has only recently begun to emerge. For example, it has been shown that, in the case of CCK receptor type A, the clathrin-coated pit-dependent pathway leads to lysosomal compartments, whereas caveolae-mediated endocytosis does not (211). However, switching the internalization pathway of the ETA receptor from caveolae to CCP does not alter its lysosomal destination (213). Finally, the molecular basis of this internalization switch has been elucidated for β1-AR, which enters via both the caveolae and the CCPs. In this case, PKA phosphorylation directs the receptor to a CCP, whereas GRK phosphorylation of the receptor directs it to caveolae-mediated endocytosis (214). These findings may explain how the levels of βarrestins or GRKs in distinct cells lines determine the different endocytosis pathways of specific GPCRs, as has been shown for the GnRH receptor (215). The receptor GnRH, when expressed in both COS-7 and HEK 293 cells, preferentially undergoes a rapid agonist-induced internalization in a caveolae-like, dynamin-dependent manner, but upon βarrestin overexpression GnRH-Rs appeared to mobilize to the βarrestin-mediated endocytic

pathway (215). However, the exact molecular mechanisms of the caveolae-mediated endocytosis remain to be defined.

3.1.3. Arrestin and dynamin independent endocytosis

Other receptors appear to be internalized via a third endocytic pathway, which does not require βarrestin or dynamin. Upon agonist activation, the secretin receptor is phosphorylated by GRKs and stimulates translocation of βarrestin to the cell surface. Expression of dominant negative βarrestin and dynamin, however, does not affect internalization (216). Similarly, the M2 muscarinic (77, 217, 218), the B2R (219), and the *N*-formyl peptide receptors (220) are other examples of G protein-coupled receptors, which are internalized in a βarrestin- and dynamin-independent fashion (219-222). The exact mechanism and nature of vesicles responsible for the internalization of these receptors remain to be defined.

3.2. The fate of internalized receptors

Once internalized, receptors are targeted to specialized intracellular compartments where they are dephosphorylated and recycled back to the plasma membrane, or processed by the degradation pathway. Clearly, the sequences of the exposed intracellular domains of a GPCR, and the pattern of phosphorylation sites, and thus the binding properties of βarrestins, coordinate the intracellular trafficking of the internalized receptor. Recently, βarrestin ubiquitination and de-ubiquitination were shown to correlate with different receptor trafficking patterns (101, 223). Conjugation of ubiquitin, a 76-amino acid polypeptide, to lysine residues, was found to function as a sorting signal for receptor degradation in lysosomes (reviewed in (224)). Transient βarrestin ubiquitination correlates with class A and more permanent βarrestin ubiquitination with class B receptor endocytosis (101). It has also been shown that Mdm2-mediated ubiquitination of βarrestin 2 is necessary for β2AR internalization but

ubiquitination of the receptor by a second ubiquitin ligase mediates receptor down-regulation (94). Similarly, other GPCRs were found to be ubiquitinated, such as CXCR4 (225), μ- and δ-opioid (226), and V2 receptors (227).

Two different types of GPCRs internalized via the same endocytic pathway may be targeted to different intracellular compartments and recycled to the cell surface with different kinetics. For example, in HEK 293 cells, β2-adrenergic and δ-opioid receptors are internalized via the clathrin-coated vesicle pathway (228). While almost all of the β2-ARs are recycled to the cell surface within minutes, δ-opioid receptors are targeted to lysosomes for degradation (228). Interestingly, studies of chimeric receptors have shown that interchanging the C-terminal tail can change the fate of receptors. Indeed, a mutant β2-AR containing the V2R C-terminal tail is recycled to the cell surface with kinetics similar to the vasopressin receptor (75). Moreover, the same phenomenon has been observed with other receptor chimeras such as the β2-AR and the AT1R (229), the ETA and ETB receptors (230), the gonadotropin-releasing hormone and the thyrotropin-releasing hormone receptors (231); as well as, the protease-activated receptor 1 and substance P receptor (160, 232). Therefore, molecular determinants present within the C-terminal tail of GPCRs appear to contain domains responsible for determining the fate of receptor.

Studies of the V2R as well as other GPCRs have implicated a cluster of serine residues in the receptor tail as an important motif involved in intracellular receptor trafficking (75, 233-235). Receptors possessing this domain interact with high affinity to βarrestin. Upon agonist binding, phosphorylation of the V2R within the serine cluster is believed to promote the formation of a stable complex between the receptor and βarrestin. Together, the receptor/βarrestin complex is internalized and targeted to endosomes where they are retained for long periods of time before being recycled to the cell surface. Removal or addition of such serine/threonine clusters in certain receptors such as the β2-AR, the neurotensin, the oxytocin, the AT1R, and the neurokinin type 1 receptors is sufficient to alter the kinetics of receptor recycling (122)

Other proteins have been shown to bind to the tail of a variety of GPCRs thereby regulating receptor trafficking (236-238). One such example is the binding of the Na+/H+ exchanger regulatory factor (NHERF), which binds to the β2-AR tail via

protein-protein interaction domains (*i.e* PDZ domain) (236-238). PDZ domains contain ~80-90 residues and bind to the C-terminal 4-5 residues of their target proteins, frequently transmembrane receptors or ion channels. The consensus binding sequence contains a hydrophobic residue, commonly Val or Ile, at the very C-terminus. This interaction is important for post-endocytic sorting of the receptor (237). More recently, determinants including the last three amino acids of the C-terminal tail of the β2-AR also bind to *N*-ethylmaleimide-sensitive fusion protein (NSF), another protein involved in vesicular trafficking (239). Mutations in the β2-AR tail that affect NSF binding, but not the interaction with NHERF, reduce receptor internalization and totally prevent receptor recycling from the endocytic compartments. Thus, the post-endocytic fate of the β2-AR appears to be dependent on the ability of the receptor to interact directly with different intracellular proteins. These interactions between receptors and sorting molecules remain to be characterized.

3.3. Internalization for desensitization, resensitization or signalling

It is well-recognized that the clathrin-mediated endocytosis serves a number of purposes, including regulation of cell surface protein expression levels in order to modulate cellular responsiveness to ligands (82), localization of intracellular signalling complexes to receptor-bearing endosomes (119), degradation (also known as down regulation) of GPCRs to effect long term desensitization (212), and processing of receptors for recycling to the cell surface to initiate continued signalling (34).

3.3.1. GPCR internalization for desensitization

For a long time, GPCR internalization has been considered a mechanism for receptor desensitization, due to the uncoupling of the receptor from its effector (240). In this way, GPCR removal from the cell surface could provide a mechanism to protect cells against receptor overstimulation. In response to agonist-stimulation, many GPCRs are internalized but are not recycled back to the plasma membrane (75, 160, 229, 232). In some cases, internalized GPCRs such as the

protease-activated receptor (PAR) (160) and ETB (241) are targeted to lysosomes for degradation. Other GPCRs, such as AT1R, are internalized and retained within the endosomes (229, 232) (Figure 12).

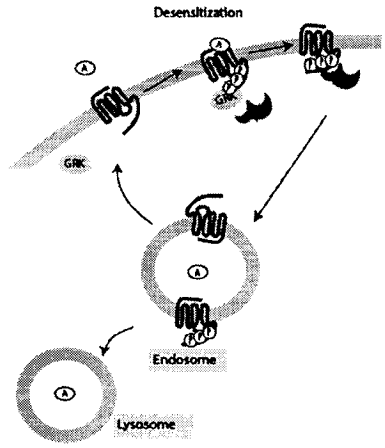


Figure 12. *GPCR internalization for desensitization*

GPCR internalization for desensitization is mainly controlled by βarrestins. Since the discovery of βarrestin as proteins acting in concert with GRK to desensitize receptors (242), βarrestin 2 was cloned (79), the crystal structure was solved (243), and βarrestin 2 was originally classified as a desensitization protein (42). The desensitization function of βarrestin has been demonstrated by Attramadal *et al.* (81), using purified βarrestin 1 and βarrestin 2 proteins. They were able to block the GTPase activity of G_s induced by the β2-AR with each βarrestin *in vitro*. In addition, numerous reports show that reduction of endogenous βarrestin expression affects receptor desensitization (87, 132, 133).

3.3.2. GPCR internalization for resensitization

Resensitization can blocked by pharmacological treatment, such as hypertonic sucrose which is used to block receptor internalization without affecting G protein coupling or desensitization (244-246). GPCR resensitization thus protects cells against prolonged receptor unresponsiveness. The mechanism by which the resensitization of many GPCRs is achieved is thought to be agonist-dependent internalization and targeting of receptors to endosomes. It has been shown that β2-AR

isolated from endosomes exhibited a lower phosphorylation stoichiometry than β2-AR isolated from the plasma membrane (36), and that endosomes are enriched in phosphatase activity (247). These results suggest that GPCRs are dephosphorylated in endosomes and recycled back to the cell surface to be available for a new round of agonist activation (232, 248) (Figure 13). Therefore, GPCR resensitization not only requires the βarrestin-dependent targeting of receptors to the CCPs and their endocytosis to endosomes, but receptors also require βarrestin release to be dephosphorylated and then recycled back to the cell surface. The exclusion of βarrestin from CCVs was proposed to facilitate the β2-AR dephosphorylation by allowing receptor exposure to specific protein phosphatase (PP2A) (229). Furthermore, the association of phosphatase with the β2-AR requires the acidification of the receptor in the endosomes (248).

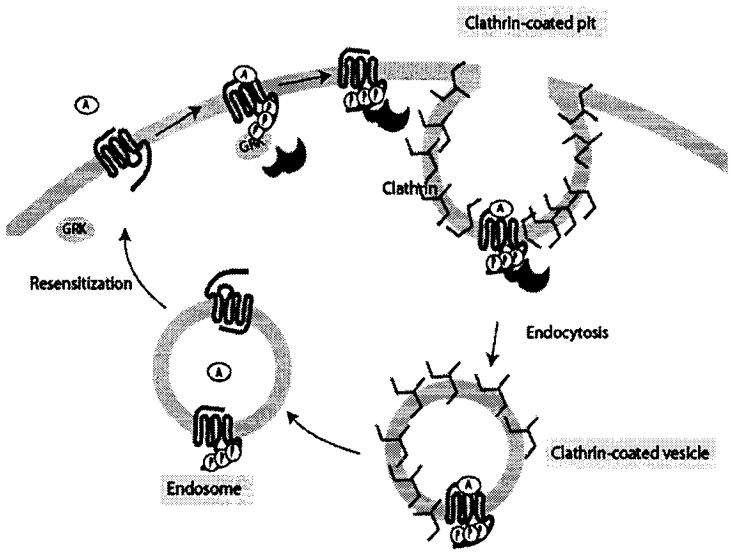


Figure 13. *GPCR internalization for resensitization*

GPCR internalization and dephosphorylation in endosomes is not the only mechanism by which GPCR resensitization is achieved. Indeed, because the protease-activated receptors (PARs) are degraded in lysosomes (249), these receptors use alternative mechanisms for resensitization. The first mechanism is the *de novo* synthesis of new

receptors, and the second is the maintenance of an intracellular pool of receptors that can be mobilized to replace degraded receptors (250, 251).

Interestingly, in COS-7 cells, β2-AR resensitization does not occur unless βarrestins are overexpressed (181) suggesting that βarrestin not only plays a role in GPCR desensitization but also in resensitization. Therefore, the capacity of a GPCR to become resensitized may depend on the protein expression levels of different regulators of intracellular trafficking. Moreover, heterodimerization of receptors, shown between the μ-opioid and substance P (NK1) receptors can alter the μ-opioid resensitization profile (252). While heterodimerization revealed no changes of the ligand binding and signalling properties, μ-opioid/NK1 heterodimerization change the trafficking with βarrestin of the μ-opioid from class A to class B and thus, delays recycling and resensitization. In addition to βarrestin, the internalization of the β2-AR and the D2 dopamine receptor depends on the small GTPases Rab5 and Rab4 (253, 254). Rab5 regulates the movement and fusion of vesicles to endosomes and also the formation of vesicles at the plasma membrane (254). Rab4 regulates the recycling of β2-AR back to the plasma membrane and is required for β2-AR resensitization (254). Therefore, receptors may use distinct mechanisms to be resensitize.

3.3.3. GPCR internalization for signalling

Activation of MAPKs via G protein-mediated pathways has been studied for many GPCRs (255). The activation of MAPK (p44/p42) by β2-AR involves the PKA-phosphorylation-dependent switch of β2-AR coupling from G_s to G_i activation (43). It has also been proposed that the MAPK activation induced by GPCR requires both endocytosis and βarrestin proteins (111, 218).

As mentioned previously, arrestin proteins were originally identified based on their role in GPCR desensitization. The roles of βarrestin have since been expanded to include several additional functions. One function is its ability to serve as a scaffold for two mitogen-activated protein kinase (MAPK) cascades by linking

GPCRs to the extracellular-signal regulated kinase (ERK) cascade (113, 114, 256) and the c-jun N-terminal kinase type 3 (JNK3) cascade (257) (Figure 14).

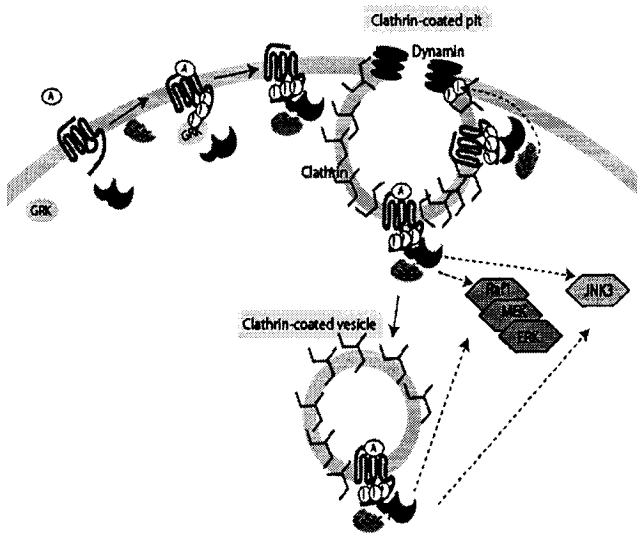


Figure 14. *GPCR internalization for signalling*

The MAP kinases are a family of conserved serine/threonine kinases that are involved in the transduction of signals regulating cell growth, division, differentiation and apoptosis. MAP protein kinase activity is regulated by a series of parallel kinase cascades comprising three protein kinases that successively phosphorylate and activate the downstream effector. Raf phosphorylates and activates MEK, and MEK phosphorylates and activates ERK/MAPK, causing dissociation from MEK and translocation to the nucleus. Recent data shows that βarrestin interacts directly with both Raf-1 protein kinase and MAPK in response to the activation of PAR2 (114). The activation of PAR2 prevents the translocation of MAPK to the nucleus; this in turn blocks cell proliferation. In contrast a PAR2 receptor mutant defective in βarrestin-binding, stimulates MAPK activation and cell proliferation. Similar results were also observed for other GPCRs such as AT1R, V2R and β2AR (113, 258, 259). However, in the case of AT1R, it has been suggested that βarrestin 1 may inhibit the βarrestin 2-mediated ERK activation (260), indicating that the two forms of βarrestin might have different functions in regulating ERK signalling. A

recent study showed that GRK5 and -6 induced βarrestin 2-mediated ERK signalling upon V2R activation, and suggested that the specificities of GRK phosphorylation can coordinate βarrestin-mediated signalling (58). Using G_s knockout MEF cells, Shenoy *et al* also demonstrated that the β2AR can signal to ERK via a GRK5/6 and βarrestin-dependent pathway, independently of G protein coupling (261). However, the roles of βarrestin as endocytic versus scaffolding protein in GPCR-mediated activation of MAPK remains understudied.

In addition to MAPK, βarrestin was shown to be essential for the agonist-dependent formation of a complex containing β2AR, βarrestin and c-Src (97). Similar results have been obtained with the NK1 (262) and the CXCR-1 (111). The βarrestin/c-Src interaction involves the association of the βarrestin N-terminus with the Src homology 1 (SH1) domain of c-Src (96). βarrestin-mediated Src recruitment has been implicated in several GPCR-mediated signalling events, including tyrosine phosphorylation of dynamin (96); and the activation of the ERK/MAPK cascade (97, 114). Furthermore, βarrestin mutants defective in their ability to interact with c-Src, blocked β2-AR-mediated MAPK activation (97). These results suggest that βarrestin initiates alternative signal transduction cascades in which desensitized receptor and βarrestin act as scaffolds. Recent reports have extended the role of signalling for βarrestin to other receptors such as IGF1R (263) and the single-TM Notch (264). In these particular cases, βarrestin allows the recruitment of the ubiquitin-ligase to promote receptor degradation.

Another function for βarrestin is to serve as a messenger in controlling transcription of target genes. This hypothesis comes from the observation that βarrestin was able to shuttle between the cytoplasm and the nucleus (85, 86). This was further demonstrated for the δ-opioid receptor (DOR) where the agonist-activation of DOR was shown to induce the nuclear translocation of βarrestin 1, and thus the βarrestin-dependent histone H4 acetylation resulting in the transcription of specific target genes (265). However, it remains to be determined by which mechanism βarrestin mediates this epigenetic signalling pathway.

4. One particular GPCR : The Angiotensin II type 1 receptor

4.1. The discovery of AT1R

In 1897, Tiegerstedt and Bergman found that crude saline extracts from the kidney contained a pressor substance that they named *renin.* In 1940, Braun-Menendez, as well as Page and Helmer reported that renin was an enzyme that acted on a plasma protein substrate to catalyze the formation of the actual pressor material, a peptide that was named *hypertensin* by the former group and *angiotonin* by the latter. These two terms persisted for nearly 20 years until it was agreed to rename the pressor substance *angiotensin* and to call the plasma substrate *angiotensinogen.* By the mid-1950s, two forms of angiotensin were recognized: a decapeptide (angiotensin I) and an octapeptide (angiotensin II) formed by proteolytic cleavage of angiotensin I by an enzyme termed *angiotensin-converting enzyme* (ACE). As with other peptide hormones, Ang II was postulated to act on a receptor located on the plasma membrane of its target cells. It was not until the end of the 1980s that tools became available to demonstrate the existence of at least two receptor types in many tissues (reviewed in (266)). These receptors are termed AT1R, for Angiotensin II type 1 receptor which encompasses two subtypes ($AT1_A$ and $AT1_B$, in rat and mice) and the AT2R, for the Angiotensin II type 2 receptor.

4.2. Angiotensin II

Clinical and experimental studies show that angiotensin II (Ang II) (Figure 15), the major renin-angiotensin system effector, has an important role in biological processes. These processes include blood pressure control through vascular muscle contractility, aldosterone secretion from adrenal glomerulosa cells, and ion transport in renal tubular cells (Figure 16) (reviewed in (267)). Pharmacological inhibition of the renin-angiotensin system attenuates the development of renal lesions in several experimental models of renal injury and progressive loss of renal function in individuals with chronic kidney disease (CKD) (268). It has been suggested that Ang II causes renal injury through renal hemodynamic effects and stimulation of kidney

growth, but the molecular pathways underlying these phenomena remain largely unknown.

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

Figure 15. *The sequence of the human Angiotensin II*

There is considerable information on the structure-activity relationships of angiotensin-related peptides with regard to activity at receptors for angiotensin II (269). In general, phenylalanine in position 8 is critical for most agonist activity, and the aromatic residues in positions 4 and 6, the guanido group in position 2, and the C-terminal carboxyl are thought to be involved in binding to the receptor site. Position 1 is not critical, but replacement of aspartic acid in position 1 with sarcosine enhances binding to angiotensin receptors and slows hydrolysis by rendering the peptide refractory to a subgroup of aminopeptidases (angiotensinase A). Such a substitution, combined with that of alanine or isoleucine in place of phenylalanine in position 8, yields potent angiotensin II receptor antagonists.

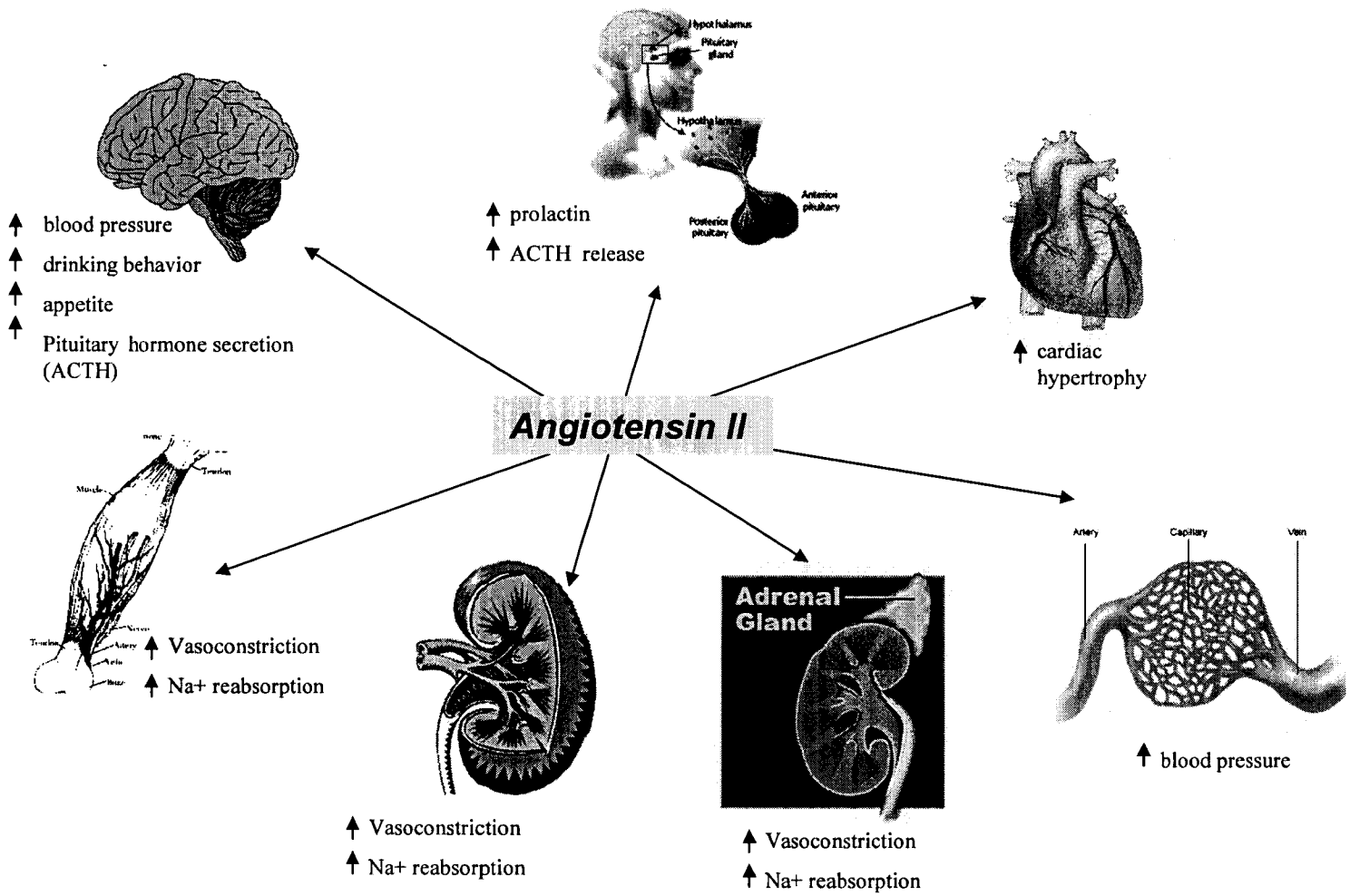


Figure 16. *The biological functions of Angiotensin II*

The various components of the cascade leading to the formation of Angiotensin II were characterized, including angiotensinogen, angiotensin converting enzyme (ACE), and angiotensin I, II (Figure 17) (266). Briefly, sympathetic stimulation (acting via β_1-adrenergic receptors), renal artery hypotension, and decreased sodium delivery to the distal tubules stimulate the release of renin by the kidney. Renin is an enzyme released from the kidney that acts upon the circulating substrate angiotensinogen, which undergoes proteolytic cleavage to form the decapeptide angiotensin I. Then angiotensin-converting enzyme (ACE) removes the carboxy-terminal dipeptide of angiotensin I to produce the octapeptide angiotensin II. The major determinant of the rate of angiotensin II production is the amount of renin released by the kidney.

Ang II has several very important functions, which are depicted in figure 16 (266). It constricts resistance vessels thereby increasing systemic vascular resistance and arterial pressure; acts upon the adrenal cortex to release aldosterone, which in turn acts upon the kidneys to increase sodium and fluid retention; stimulates the release of vasopressin (antidiuretic hormone, ADH) from the posterior pituitary which acts upon the kidneys to increase fluid retention; stimulates thirst centers within the brain; facilitates norepinephrine release from sympathetic nerve endings and inhibits norepinephrine re-uptake by nerve endings, thereby enhancing sympathetic adrenergic function; and stimulates cardiac and vascular hypertrophy. Angiotensin II is also involved in the development of cardiovascular diseases such as hypertension and atherosclerosis.

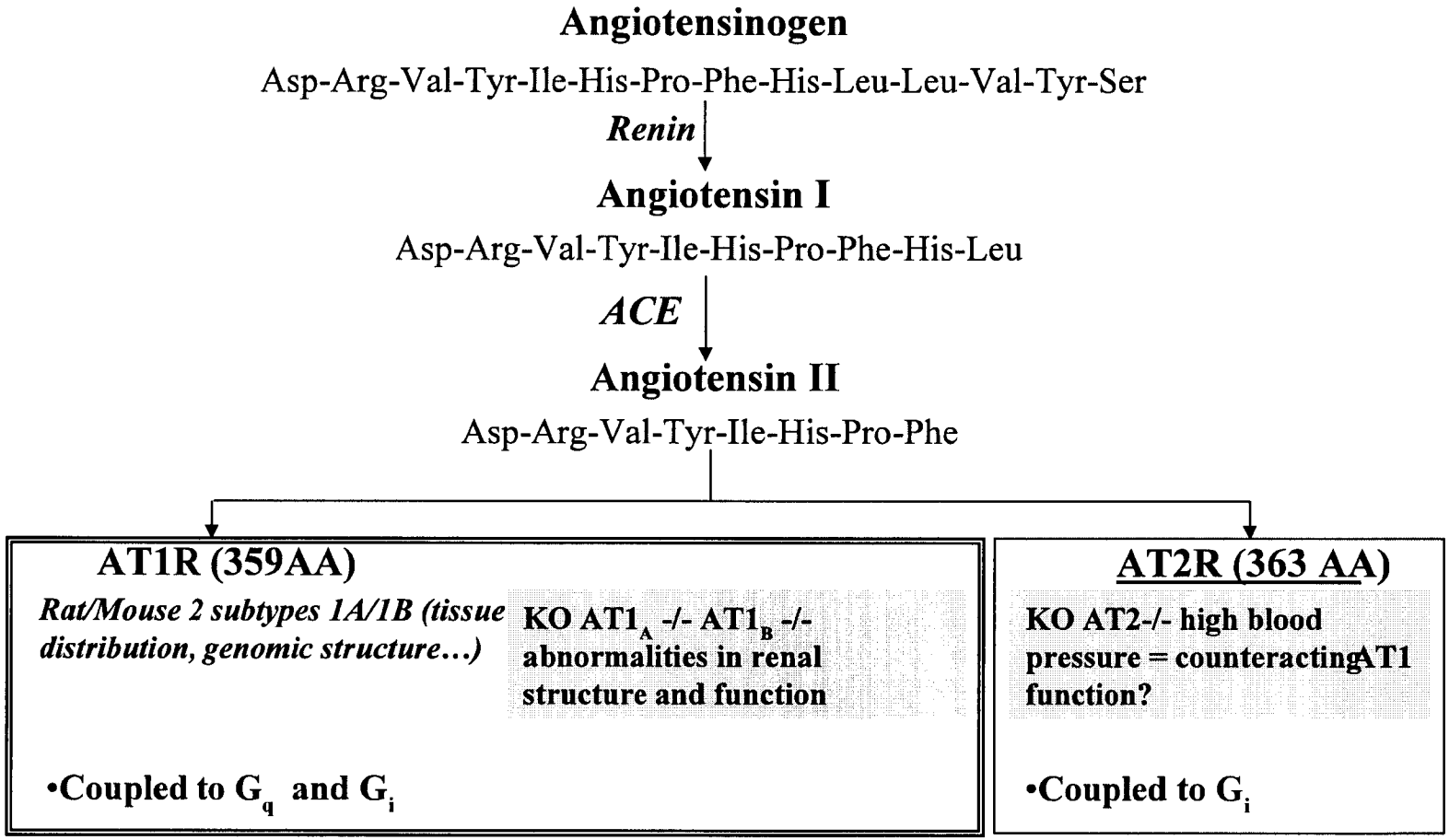


Figure 17. *The synthesis of Angiotensin II*

4.3. AT1R signalling

Two major subtypes of Ang II receptors expressed in mammalian tissues are the AT1R and AT2R, with about 30% sequence identity (267). The AT1 receptor is 359 amino acids long, while the AT2 receptor consists of 363 amino acids. AT1R is principally coupled to G_q, leading to the activation of phospholipase C. It may also be coupled to G_i. Agonist-induced G_q activation results in activation of PLCβ, which catalyses the PIP_2 into IP_3 and DAG. DAG directly activates PKC, whereas IP_3 induces the release of Ca^{2+} from intracellular stores (reviewed in (267)). For AT2R, the precise functions and intracellular signalling pathways have not been clearly defined.

AT1R also activates intracellular signalling pathways such as ERK1/2 (258) and JAK/STAT (270). Ang II was shown to activate ERK1/2 by two distinct mechanisms (258). Upon agonist stimulation, ERK1/2 are immediately and transiently activated via the G protein-dependent pathway, whereas βarrestin-mediated ERK1/2 activation is relatively slow but persistent (258). These results suggest that the cellular response mediated by two distinct pools of ERK1/2 activated via βarrestin and G protein must be distinct. However, the specific downstream substrates for the βarrestin-dependent ERK activity remain to be determined. Stimulation of AT1R induces the βarrestin-recruitment of the MAPK and JNK3 (257). However, βarrestin's role in recruiting this protein kinase remains to be determined. The AT1R-mediated activation of ERK1/2 can also occur via different pathways that include PKC, Ras/Raf (271), Pyk2 (272) and growth factor tyrosine protein kinases (273).

AT1R also induces the activation of the JAK/STAT pathway (274).The JAK protein kinases activate STAT (signal transducers and activators of transcription) and promote their migration to the nucleus to induce gene transcription (275). The binding of AT1R to the intracellular tyrosine protein kinase JAK 2 requires a motif in the AT1R C-terminus, $Y^{319}IPP^{322}$ (274). However, no correlation between βarrestin and JAK activation has been reported yet for the internalization of receptor.

Interestingly, the AT1 receptor can form homodimers (276) and heterodimers with various other receptors, such as the bradykinin B2 receptor (277), the AT2R (278) and the β2AR (279). These dimers may affect receptor signalling as well as receptor internalization or cell surface targeting (280). Some pathophysiological evidence

suggests that the AT1R homodimers contribute to an enhanced monocyte adhesiveness of hypertensive patients, thereby possibly sustaining the process of atherogenesis by chronic sensitization of circulating monocytes (276).

4.4. AT1R endocytosis

Ang II binding causes rapid internalization of the AT1R (reviewed in (281)). However, studies on endogenous AT2R have demonstrated that AT2R is internalization deficient (282). So, this present paragraph will focus on the agonist-induced internalization of AT1R. The internalization of AT1R was previously shown to be unaffected by dominant negative forms of βarrestin or dynamin (222, 283). However, contradictory data have recently suggested that βarrestin proteins regulate endocytosis of this receptor. In βarrestin double knockout cells (βarr 1/2-KO), internalization decreased by approximately 80%. These results suggest that in these cells, the AT1R is primarily internalized in a βarrestin-dependent manner (87). Furthermore, this pathway was also confirmed by Gaborik *et al.* (284), who found that in three different cell types, the internalization of the AT1R is dependent on βarrestin and dynamin. These contradictory data suggest that multiple mechanisms might exist for the internalization of this receptor. However, at physiological hormone concentrations, endocytosis of AT1R occurs predominantly via a βarrestin- and dynamin-dependent mechanism (76, 87, 229, 284).

4.5. Molecular basis of AT1R internalization

Ang II binding to the AT1R induces a conformational change that results in isomerization of the receptor to its active conformation and acceleration of its internalization. Antagonists such as Losartan impair the AngII-mediated receptor internalization (285). Several point mutations of the AT1R cause impairment of receptor internalization and signal generation (Figure 18):

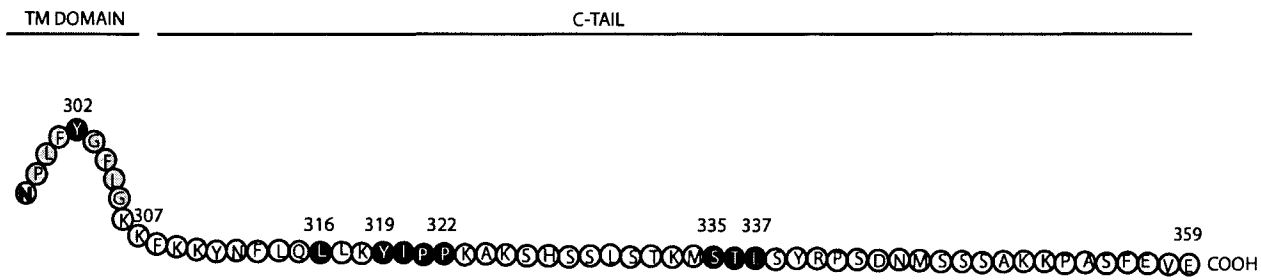


Figure 18. *Schematic representation of the amino sequence of AT1R C-terminal tail. Black circles represent residues that are important for receptor internalization.*

Substitution of aspartic acid residue (Asp^{74}) in the second transmembrane helix disrupts G protein coupling of the AT1R (286), but the internalization is slightly impaired (285). The AT1R mutation of the conserved tyrosine (Tyr^{302}) or other amino acids in the NPXY sequence in the seventh transmembrane helix, causes a relatively minor effect on receptor internalization but affects the G protein activation (287). A serine-threonine-rich region in the cytoplasmic tail, which includes the Ser^{335}-Thr^{336}-Leu^{337} sequence and the presence of proximal amino acids in the cytoplasmic tail , including leucine (Leu^{316}) and tyrosine (Tyr^{319}) have also been shown to be important for AT1R internalization (288). Despite the difficulties in detecting endogenous AT1R, it has been shown using epitope-tagged receptors that AT1R is indeed phosphorylated. These studies have shown that the agonist-induced phosphorylation of AT1R is initially mediated by PKC, GRK2 and GRK5 (289, 290). Furthermore, two residues Ser^{335}-Thr^{336} were found to be rapidly phosphorylated after receptor activation (291).

4.6. The fate of the receptor

AT1R is co-localized in endosomes with βarrestin, indicating that the receptor maintains its active conformation for some time after the formation of endocytic vesicles (232). AT1R are then trafficked through early endosomes (Rab5) and some of them associate with Rab7 in late endosomes (292). During this transition, vesicular acidification can promote the dissociation of the ligand and receptor recycling through Rab11 in recycling vesicles (292). The AT1R possibly recycles to the plasma membrane in a state uncoupled from G proteins; the affinity of the recycled-AT1R for the Ang II was shown to be reduced (293).

4.7. Role of AT1R internalization in transgenic mice

Mice lacking both isoforms, $AT1_AR$ and $AT1_BR$, have a severe phenotype with abnormalities in renal structure/function, and low blood pressure (294). In previous *in vitro* studies, AT1-i2m (a receptor mutant in its intracellular second loop) is incapable of activating $G\alpha_q$ and $G\alpha_i$, but retains the ability to activate effectors such as Src and ERK (295). However, the physiological roles of these activated-effectors following AT1R stimulation remain unclear. Recently, it was demonstrated that the i2m mice display a pronounced cardiac phenotype as compared to wild-type mice (296). These data suggest a possible role for AT1R internalization independently of G protein signalling in the regulation of cardiac ion channels. These results may reveal an unexpected physiologic role for AT1R internalization in cardiac function, which remains to be defined.

SECTION II

RATIONALE AND OBJECTIVES

The general aim of this thesis is to advance our understanding of the mechanisms regulating GPCR internalization. Growing interest in this field is due to their involvement in the regulation of a number of functions, including cardiovascular responsiveness. Following their activation, these receptors enter the cells, a process named receptor **internalization**. This process requires receptor **desensitization** which is achieved by receptor phosphorylation, sequestration and internalization. In this manner, receptors are removed from the surface and transferred into cells. Inside the cells, receptors are embanded into small membrane vesicles (**endosomes**) which may be recycled back to the plasma membrane in order to renew their fully functional coupling with proteins and effectors (*i.e.* **resensitization**). GPCR expression can also be regulated by a process of **down-regulation** which is a terminal stage of receptor life where receptors are degradated in lysosomes.

The current proposed model for GPCR internalization is essentially based on β2-AR studies. Briefly, following agonist stimulation, GPCRs undergo conformational changes that allow binding of G proteins, leading to the activation of different effectors and signalling pathways (2). The desensitization process is then activated (Figure 1). One of the first steps involves the functional "uncoupling" of the G proteins from the receptors. The receptor is subsequently phosphorylated by GRKs to enhance the binding of βarrestins (step 1). βarrestins act as scaffolding intermediates with components of the clathrin-coated-pit machinery, thus stabilizing association with clathrin-coated pits (CCPs) (steps 2 and 3). The formed pits progressively invaginate and are finally released into the cytosol as a free CCV (step 4), a step which requires the GTPase activity of dynamin to pinch off clathrin-coated vesicles from the plasma membrane (184). This signal comes from phosphorylation of dynamin by Src, which is itself recruited to and activated by GPCR-bound βarrestin (96). Clathrin-coated vesicles are then uncoated after endocytosis and fuse with early endosomes (step 5). The early endosome controls the activity and the destination of proteins in the compartment. Therefore endosomes are a key control point for sorting receptors, which can be directed to recycling endosomes and back to the cell surface (step 6), or directed to lysosomes for degradation (step 7).

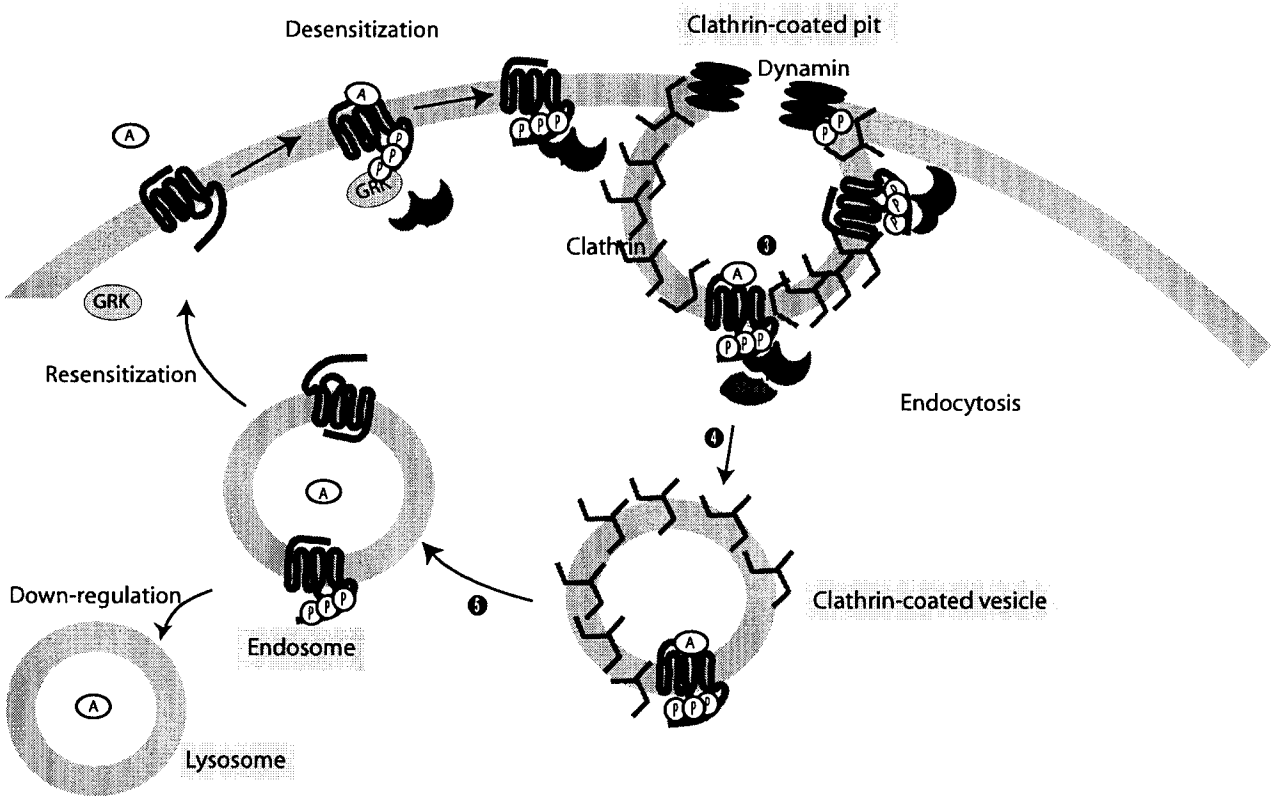


Figure 1. *Model for βarrestin-mediated internalization of GPCR via clathrin-coated pits*
Internalization and recycling of GPCRs. Upon agonist binding, receptors are phosphorylated by GRKs leading to the recruitment of βarrestins (step1). Beta-arrestins, through their interaction with clathrin and AP-2, target the receptor/arrestin complexes to clathrin coated pits (step2). Beta-arrestins also bind c-Src (2 and 3). Dynamin (step3), a GTPase, regulates the pinching off from the cell surface of clathrin coated-pits (step3 and 4). Once clathrin coated vesicles are formed (step4), the receptor is then internalized into endosomes (step5) where it is dephosphorylated before returning to the cell surface (step6), or the receptor is degradated to lysosomes (step7).

The mechanisms that activate and deactivate AT1R are complex, involving βarrestin binding to the phosphorylated receptors and thus, promoting G-protein uncoupling and internalization. Beta-arrestins are also now thought to function as scaffolds that recruit regulatory molecules to the receptor. The proteins that interact with AT1R via βarrestin are unknown and important to characterize. For instance, the βarrestin-mediated internalization of AT1R is controversial and poorly described. In addition, the involvement of signalling molecules such as c-Src in receptor internalization has only been reported for a few receptors. In an attempt to better characterize AT1R internalization, we searched, in our first study, for possible functional protein interactions regulating the endocytic complex formation during receptor internalization. Two main studies were critical in establishing our hypothesis. The first study describes the recruitment of c-Src protein kinase to βarrestin to further regulate β2-AR internalization (97). The second study shows that the activation of the β2-AR promotes the recruitment of the clathrin adaptor AP-2 to βarrestin for CCV formation (90). These findings constituted evidence that the formation of an endocytic complex tightly regulates GPCR internalization. Thus, we investigated whether βarrestin could serve as an adaptor for both AP-2 and c-Src during the internalization of a controversial receptor: the AT1R. We first decided to determine if the agonist-stimulation of AT1R could lead to the formation of βarrestin/AP-2 complex with c-Src. We worked on the assumption that AP-2, c-Src and βarrestin could be present in the same endocytic complex and indeed, we found, in cells, that Angiotensin II promotes this endogenous complex formation. Using active or inactive c-Src protein kinase mutants, we determined that c-Src functions as an adaptor to stabilize the AP-2/βarrestin association but also as a protein kinase to regulate this interaction. To further understand the importance of this interaction, we performed siRNA experiments to reduce c-Src expression in cells and determine its impact on the complex formation and also on receptor internalization. This work is presented, in section III, with the first manuscript entitled, 'c-Src regulates AP-2 interaction with beta-arrestin and the Angiotensin II type 1 receptor during clathrin-mediated internalization'. This paper shows that βarrestins interact with both c-Src and AP-2 in response to the activation of AT1R to regulate receptor internalization.

In our first study, we have demonstrated the agonist-mediated formation of an endocytic complex containing c-Src, βarrestin and AP-2 during AT1R internalization. Thus, our objective in section IV was to understand the regulation of that complex formation. A few examples of the phosphorylation mechanisms occurring during GPCR internalization were previously reported. Indeed, dynamin was shown to be tyrosine phosphorylated, and to regulate β2-AR internalization (170). We started our study by investigating whether the activation of AT1R could induce the phosphorylation of the endocytic βarrestin partner: AP-2, and, if so, to identify the tyrosine residue(s). Interestingly, we found that activation of AT1R with Ang II promotes the tyrosine phosphorylation of AP-2. We then further characterized the role of this phosphorylation during receptor internalization. One possibility was that the phosphorylation of AP-2 could modify the interaction between proteins in the endocytic complex. Because of our previous findings showing that the protein kinase activity of c-Src controls the βarrestin/AP-2 association, we decided to assess the effect of AP-2 phosphorylation on its binding with βarrestin first *in vitro* and secondly *in vivo* by BRET assay. This study is presented in section IV with the second manuscript entitled, 'c-Src-mediated phosphorylation of β2-adaptin reveals a role in the formation of the ßarrestin/AP-2 endocytic complex during Angiotensin Type 1 Receptor Internalization'. This paper describes how c-Src regulates the early steps of AT1R internalization via the clathrin-coated vesicles and shows that the beta-subunit of AP-2 (β2-adaptin) is tyrosine phosphorylated upon AT1R activation to regulate its interaction with ßarrestin.

In light of the literature review presented in a section I, and the two manuscripts in section III and IV, I will propose a model for AT1R internalization via clathrin-coated vesicles. The possible mechanisms for c-Src activation, the implication of these findings, and their physiological relevance are discussed in section V.

CONNECTING TEXT

The following section III entitled, "c-Src regulates AP-2 interaction with beta-arrestin and the angiotensin II type 1 receptor during clathrin-mediated internalization", demonstrates the now well-appreciated role for βarrestin as an adaptor during GPCR endocytosis. Our objective was to show that c-Src was also a key player in βarrestin-dependent internalization. Indeed, in the literature, it has only been shown for β2-AR that βarrestin serves as a scaffold to recruit c-Src (97) but it was unclear whether other GPCRs such as the AT1R could also induce the βarrestin-mediated c-Src recruitment. It was reported separately for the β2-AR that βarrestin, in an agonist-dependent fashion, recruits the clathrin-adaptor AP-2 (91). However, it was unknown if the AT1R also recruits AP-2 and if so, whether the βarrestin/c-Src complex was necessary for AP-2 association. Taken together, it was of great interest to study:

1) whether AP-2 could be recruited to the AT1R/βarrestin complex.

2) c-Src effects on the early events of AT1R internalization: the βarrestin/AP-2 complex formation

2) the role for c-Src in the clathrin-mediated AT1R internalization

SECTION III

C-SRC REGULATES AP-2 INTERACTION WITH BETA-ARRESTIN AND THE ANGIOTENSIN II TYPE 1 RECEPTOR DURING CLATHRIN-MEDIATED INTERNALIZATION.

ABSTRACT

Beta-arrestins are multifunctional adaptors involved in the internalization and signaling of G protein-coupled receptors (GPCRs). They target receptors to clathrin-coated pits (CCPs) through binding with clathrin and AP-2 complex. They also act as transducers of signaling by recruiting c-Src kinase to certain GPCRs. Here we sought to determine whether c-Src regulates the recruitment of AP-2 to ßarrestin and the Angiotensin II (Ang II) type 1 receptor (AT1R) during internalization. We show that the agonist stimulation of native AT1R in vascular smooth muscle cells (VSMCs) induces the formation of an endogenous complex containing c-Src, ßarrestins and AP-2. *In vitro* studies using co-immunoprecipitation experiments and a yeast three-hybrid assay reveal that c-Src stabilizes the agonist-independent association between ßarrestin2 and the ß-subunit of AP-2 independently of the kinase activity of c-Src. However, while c-Src expression promoted the rapid dissociation of AP-2 from both ßarrestin and AT1R following receptor stimulation, a kinase inactive mutant of c-Src failed to induce the dissociation of AP-2 from the agonist-occupied receptor. Thus, the consequence of c-Src in regulating the dissociation of AP-2 from the receptor was also examined on the internalization of AT1R by depleting c-Src in HEK 293 cells using a small interfering RNA strategy. Experiments in c-Src depleted cells reveal that AT1R remained mostly colocalized with AP-2 at the plasma membrane following Ang II stimulation, consistent with the observed delay in receptor internalization. Moreover, co-immunoprecipitation experiments in c-Src depleted HEK 293 cells and VSMCs showed an increased association of AP-2 to the agonist-occupied AT1R and ßarrestin, respectively. Together our results support a role for c-Src in regulating the dissociation of AP-2 from agonist-occupied AT1R and ßarrestin during the clathrin-mediated internalization of receptors, and suggest a novel function for c-Src kinase in the internalization of AT1R.

INTRODUCTION

G protein-coupled receptors (GPCRs) are integral membrane proteins that are responsible for controlling an array of physiological responses such as phototransduction, olfaction, neurotransmission, vascular tone, cardiac output, and pain. The intensity and the duration of a response are dependent on the balance that exists between mechanisms that regulate the coupling of the receptors to their downstream effectors and those that terminate the signaling. Mechanisms that rapidly turn off signaling include desensitization, whereby receptors become refractory to subsequent stimuli, and internalization, where receptors are removed from the plasma membrane (a process also referred to as sequestration or endocytosis) (1, 2). Internalization of GPCRs can lead to the reestablishment of the cellular response by the recycling of receptors to the plasma membrane, or the prevention of further signaling by targeting receptors for degradation.

Homologous desensitization and internalization of receptors involve common and distinct steps. Agonist stimulation of GPCRs promotes the phosphorylation of receptors by G protein-coupled receptor kinases (GRKs), and the recruitment of non-visual arrestin proteins (ßarrestin1 and ßarrestin2) to the phosphorylated receptors (1). Beta-arrestins bind to the agonist-occupied GPCR to prevent second-messenger signaling, and act as adaptors to target desensitized receptors to clathrin-coated vesicles (CCVs) at the plasma membrane (*i.e.* clathrin-coated pits; CCPs) for internalization (3, 4). The involvement of ßarrestins in the internalization of the ß2-adrenergic receptor (ß2AR) and the Angiotensin II (Ang II) type 1 receptor (AT1R) has been demonstrated using different approaches, and more recently by gene disruption and post-transcriptional silencing of ßarrestins (5, 6). In the case of the ß2AR, ßarrestins are believed to link the agonist-occupied receptors to CCPs by interacting through their C-terminal domains with both clathrin and the ß-subunit of the heterotetrameric adaptor AP-2 complex (ß2-adaptin) (7-10). Many studies indicate that AT1R can internalize via CCVs in both heterologous and endogenous systems (11-15) but the underlying mechanisms regulating ßarrestin-mediated endocytosis of AT1R and other GPCRs still remain poorly understood.

In addition to their roles in GPCR desensitization and internalization, recent evidence indicates that ßarrestins can also participate in receptor signaling by functioning as adaptors for the recruitment of signaling molecules to agonist-occupied receptors (16). For instance, ßarrestins have been shown to recruit the non-receptor tyrosine kinase c-Src to the ß2AR (17) and the neurokinin-1 receptor (NK-1) (18). The ßarrestin-mediated recruitment of c-Src to the agonist-occupied ß2AR regulates the function of the endocytic protein dynamin, a GTPase involved in the fission of CCPs from the plasma membrane (8, 17). Given the dual adaptor role for ßarrestins in the internalization and signaling of GPCRs, we hypothesize that c-Src could also regulate the recruitment of other endocytic proteins like AP-2 to the agonist-induced AT1R/ßarrestin complex, and consequently impact the early steps of receptor internalization.

RESULTS

Angiotensin II promotes the formation of an endogenous complex containing c-Src, ßarrestin and AP-2. We have previously shown that the internalization of certain GPCRs, via the clathrin pathway, requires the recruitment of ßarrestin to the receptor, and the interaction of ßarrestin with the heterotetrameric AP-2 complex (via its ß-subunit, *i.e.* ß2-adaptin) (9). More recently, it has been suggested by different groups that ßarrestins act as signaling adaptors for the recruitment of c-Src family kinases to some GPCRs (17-19). In order to determine whether c-Src could participate in the association between ßarrestin and AP-2, we first examined in an endogenous system, whether the agonist stimulation of AT1R promoted the formation of a complex containing c-Src, ßarrestin, and AP-2. Vascular smooth muscle cells (VSMCs) expressing endogenous AT1R (around 100 fmol/mg of total protein as estimated by radioligand binding experiments; data not shown) were serum starved for 12 h and then either left untreated or treated with Ang II for 5 min at 37°C. Cells were then solubilized and endogenous c-Src was immunoprecipitated. Interacting proteins in the complex were identified by Western blot analysis using antibodies against the alpha subunit of AP-2 and ßarrestin (Fig. 1). Results show that in the absence of agonist stimulation, little ßarrestin and no AP-2 complex were detected in the c-Src immunoprecipitates. However, following agonist stimulation of native AT1R, a robust association of both ßarrestin and AP-2 was observed with c-Src. These results show that the agonist stimulation of AT1R in a native system like VSMCs promotes the formation of an endogenous complex including c-Src, ßarrestin, and AP-2.

c-Src increases the association between ßarrestin and ß2-adaptin independently of its kinase activity . The Ang II-mediated formation of a complex containing ßarrestin, AP-2 and c-Src in VSMCs, and the recent demonstration that c-Src and ßarrestin associate with each other when overexpressed in COS-7 cells (20), suggest that c-Src is involved in stabilizing the association between ßarrestin and AP-2. We therefore reasoned that by increasing the expression of c-Src in cells, we could induce the agonist-independent association between ßarrestin and AP-2. To test this

possibility, COS-7 cells were transfected with Flag-tagged ßarrestin2 and increasing amounts of HA-tagged c-Src, and the level of endogenous AP-2 (as assessed by the presence of ß-subunit of the AP-2 complex, *i.e.* ß2-adaptin) in the ßarrestin immunoprecipitates was identified by Western blot analysis (Fig. 2*A*). In the absence of c-Src overexpression (Fig. 2*A*, first lane), no ß2-adaptin was detected in the ßarrestin immuno-complex. However, increasing the expression of c-Src resulted in the increased formation of a ßarrestin/AP-2 complex. We next examined whether this c-Src-mediated GPCR-independent association between ßarrestin2 and AP-2 was dependent on the kinase activity of c-Src. To this end, we overexpressed ßarrestin2-Flag and ß2-adaptin in COS-7 cells with either wild type c-Src or a mutant that mimics the activated form of c-Src (c-Src-Y530F) or the kinase dead c-Src-K298R (Fig. 2*B* and 2*C*), and assessed the ability of the different c-Src constructs to promote the association between ß2-adaptin and ßarrestin. We first determined the relative level of kinase activity of the different c-Src constructs by probing the total lysates from cells expressing wild type and c-Src mutants using an anti-phosphotyrosine antibody (Fig. 2*B*). The expression of wild type c-Src and c-Src-Y530F had similar effects on the pattern of protein phosphorylation, whereas almost no phosphorylation of proteins was detected in cells overexpressing c-Src-K298R, suggesting that at the levels of c-Src and c-Src-Y530F expression, both kinases had a similar activity in cells. Subsequently, these different constructs were overexpressed in COS-7 cells, and the amounts of AP-2 and c-Src associated to the immunopurified ßarrestin2 complexes were analyzed. Although we found that all c-Src constructs were able to associate with ßarrestin2, only wild type c-Src and the K298R mutant promoted the formation of a ternary complex that included ß2-adaptin and ßarrestin (Fig. 2*C*). These results imply that the kinase activity of c-Src is not required to promote the agonist-independent association of AP-2 with ßarrestin2.

To verify that c-Src directly contributed to the formation of a ternary complex with ßarrestin2 and ß2-adaptin, and also to confirm that this complex formation is independent of c-Src kinase activity, we used a modification of the yeast two-hybrid system (yeast three-hybrid). We have previously shown using a similar assay that the

interaction between ßarrestin and ß2-adaptin could be detected (8, 9). We expressed ßarrestin2 fused to the DNA binding domain (DBD) of *GAL4* and ß2-adaptin fused to the activation domain (AD) of *GAL4*, and assessed the effect of c-Src expression on the transactivation of the auxotrophic HIS3 and the reporter lacZ genes (Fig. 3*C*). The expression vectors for ßarrestin2 and the ß2-adaptin were co-transformed into yeast strain PJ69-4a with the empty vector p426-ADH or with the p426-ADH-c-Src mutant (c-Src-G2A,-Y416F,-Y527F). This mutant lacks the myristoylation site, to reduce its membrane association, and is mutated on tyrosines 416 and 527 to prevent tyrosine phosphorylation, and hence the activation of c-Src (21). Yeasts were transformed with the expression vector for ßarrestin2 alone (Fig.3*A*, lane *A*), or with ß2-adaptin (lane *B*), c-Src (lane *C*) or ß2-adaptin and c-Src together (lane *D*). Cells were serially diluted and spotted onto plates lacking leucine, tryptophan, and uracil supplemented or not with histidine (Fig.3*A*, left panel and right panel). As shown here, the overexpression of ßarrestin2 and ß2-adaptin is sufficient to induce yeast growth on selective media lacking histidine (Fig. 3*A* right panel, lane *B*). The expression of c-Src with both ßarrestin2 and ß2-adaptin promoted the faster growth of yeasts (Fig. 3*A* right panel, compare lane *D* to lane *B*), suggesting that c-Src stabilizes the ßarrestin/ß2-adaptin interaction. We also quantified the interaction between ßarrestin2 and ß2-adaptin using a lacZ reporter assay. Our results show that expression of c-Src with ßarrestin2 and ß2-adaptin induced a 2-fold and a 4-fold increase in the ß-galactosidase activity as compared to yeasts expressing ßarrestin and ß2-adaptin, or yeasts expressing ßarrestin and c-Src, respectively (Fig. 3*B, D vs B* and *D vs C*, respectively). These results indicate that the expression of c-Src in yeast stabilizes the ternary complex containing ßarrestin2 and ß2-adaptin. As expected, we did not find that the expression of the c-Src mutant increased the tyrosine phosphorylation of total yeast protein (data not shown). Altogether, these results demonstrate that the interaction between ßarrestin and ß2-adaptin can be stabilized in the presence of c-Src, and support our previous observations that the kinase activity of c-Src is not necessary to induce the agonist-independent formation of the complex.

The c-Src-mediated formation of a ßarrestin/AP-2 complex requires an intact ß2-adaptin binding domain on ßarrestin. We have previously shown that ß2-adaptin binds directly ßarrestin2 through a critical arginine residue (R396) present in the C-terminal domain of ßarrestin (8). This region also contains a motif for the binding of clathrin (LIEF, residues 374-377) (7-9, 22). Therefore, we used different ßarrestin2 mutants impaired in ß2-adaptin binding (ßarr2 R396A), impaired in both clathrin and ß2-adaptin binding (where the hydrophobic residues of LIEF motif were substituted for alanines, ßarr2-R396A,-AAEA) or a truncated form of ßarrestin2 lacking both sites (ßarr2-T372), and assessed the ability of c-Src to promote the agonist-independent binding of ß2-adaptin to the different ßarrestin mutants. COS-7 cells were transfected with either Flag-tagged ßarrestin2 or mutants, with or without HA-c-Src, and the amount of endogenous AP-2 in the ßarrestin immunoprecipitates was determined by Western blot analysis (Fig. 4). As previously observed and shown here, the overexpression of HA-c-Src induced the association of AP-2 with ßarrestin2 (Fig. 4 and Fig. 2). However, c-Src failed to promote the robust association of AP-2 to the different ßarrestin2 mutants as compared to wild type ßarrestin2 (Fig. 4). These results demonstrate that the c-Src-induced formation of a ßarrestin/AP-2 complex requires an intact ß2-adaptin binding site on ßarrestin2 C-terminal domain.

The kinase activity of c-Src is required to promote the dissociation of AP-2 from ßarrestin2 and agonist-occupied AT1R. We have previously shown for some GPCRs internalizing via CCVs, such as the ß2AR that the recruitment of AP-2 to ßarrestin and to the receptor required agonist activation (9). We therefore first verified whether the agonist stimulation of AT1R could induce the recruitment of AP-2 to ßarrestin and the receptor (Fig. 5). COS-7 or HEK 293 cells were used to express Flag-tagged ßarrestin2 and wild type AT1R, or Flag-tagged AT1R alone. Cells were serum-starved and then stimulated with Ang II for different periods of time (Fig. 5*A* and *B*, respectively). ßarrestin2 in COS-7 cells or AT1R in HEK 293 cells was then immunoprecipitated and the amount of endogenous AP-2 in the complexes, as detected by the presence of ß2-adaptin, was determined by Western blot analysis (Fig. 5*A* and *B*, lane 1-3). In unstimulated cells, little association of AP-2 to ßarrestin and AT1R

was observed, consistent with what we observed for other GPCRs (9). Stimulation of receptors induced the recruitment of AP-2 to both ßarrestin and the receptor in a time-dependent manner reaching a maximum after 5 min of agonist treatment (Fig. 5*A* and *B*, lane 1-3). Association of AP-2 with ßarrestin was transient and the complex was lost after 15 min of agonist treatment (data not shown). The time frame of AP-2 recruitment to AT1R in HEK 293 cells (Fig 5*B*) was similar to that observed for the interaction between ßarrestin and AP-2 in COS-7 cells (Fig 5*A*) or in HEK 293 cells (data not shown). Together, these results suggest that AP-2 is recruited to the same AT1R/ßarrestin complex. Indeed, in a different set of experiments, when the agonist-occupied AT1R was immunoprecipitated, both ßarrestin and AP-2 were found in the same receptor complex (data not shown).

To elucidate the mechanism by which c-Src regulates the agonist-mediated formation of this endocytic complex, we next assessed the effect of overexpressing c-Src on the association of AP-2 with ßarrestin and AT1R following receptor stimulation. When c-Src was overexpressed, AP-2 was already detected in the ßarrestin immunoprecipitates in the absence of agonist treatment, and almost amounted to levels of stimulated receptors in the condition where c-Src was not overexpressed (Fig. 5*A*, compare lane 3 with 4). Agonist-stimulation did not promote further recruitment of AP-2 to ßarrestin, but instead induced the rapid dissociation of the complex, which was complete after 2 min of agonist treatment. The overexpression of c-Src also promoted a basal association of AP-2 with AT1R. Similar to the experiment with ßarrestin, Ang II stimulation induced the rapid dissociation of AP-2 from the receptor (Fig 5*B*). AP-2 interaction with AT1R was completely lost after 5 min of Ang II stimulation. Our data indicate that c-Src overexpression promotes the rapid dissociation of AP-2 from agonist-occupied AT1R and ßarrestin complexes.

To determine whether the kinase activity of c-Src is involved in regulating the dissociation of AP-2 from the agonist-stimulated receptor, we overexpressed the kinase inactive c-Src-K298R and assessed the recruitment of AP-2 to the agonist-occupied AT1R. HEK 293 cells were transfected with Flag-AT1R and c-Src-K298R, and the amount of endogenous AP-2 in the receptor immunoprecipitates was determined by Western blot analysis (Fig. 5*C*). The overexpression of c-Src-K298R

induced a basal association of AP-2 with the receptor, similar to that observed with wild type c-Src (compare Fig. 5*C* and 5*B*). However, whereas c-Src induced the rapid dissociation of AP-2 from agonist-occupied receptor (Fig. 5*B*), the stimulation of AT1R in cells expressing c-Src-K298R promoted both an increased and sustained association of AP-2 with the receptor. Altogether, these results indicate that the kinase activity of c-Src promotes the dissociation of AP-2 from ßarrestin and AT1R only when receptors are activated by the ligand.

Depletion of c-Src affects the redistribution of agonist-bound AT1R inside the cell and the dissociation of AP-2 from the receptor and ßarrestin. We next examined the effect of depleting c-Src on the agonist-mediated endocytosis of AT1R using a small interfering RNA strategy (siRNA). HEK 293 cells were transfected with HA-AT1R and siRNA for human c-Src (siRNA-c-Src), and the ability of siRNAs to silence the expression of c-Src was compared to cells transfected with the receptor and either pcDNA3.1 (Mock) or siRNA for GAPDH (siRNA-GAPDH) (Fig. 6*A*). siRNA-c-Src reduced the expression of endogenous c-Src by more than 65 % and 55%, respectively, compared to either mock or the nonsilencing control siRNA-GAPDH. The silencing of c-Src showed significant selectivity and did not affect the expression of ß2-adaptin and AT1R (Fig. 6*A*, and results not shown, respectively). We next investigated using confocal microscopy, the effect of siRNA-c-Src on the early events of AT1R internalization following agonist stimulation. HEK 293 cells were transfected with HA-AT1R and GFP-ß2-adaptin as a marker of AP-2 complex (22, 23) with or without siRNA-c-Src, and cells were labeled at 14°C for receptor detection. The redistribution of labeled-AT1R and GFP-ß2-adaptin was visualized after a 5 min treatment of cells with Ang II at 37°C. In the absence of agonist, AT1R was found at the plasma membrane in punctuate structures, and little colocalization of the receptor with GFP-ß2-adaptin was observed (Fig. 6*B*, upper panel). Agonist treatment of cells for 5 min induced the rapid endocytosis of labeled-AT1R from the plasma membrane into intracellular vesicles with no noticeable redistribution of ß2-adaptin fluorescence. In c-Src depleted cells and in the absence of Ang II stimulation, AT1R was found at the plasma membrane and did not colocalize with GFP-ß2-adaptin even though

qualitatively more ß2-adaptin fluorescence was detected at the plasma membrane. After agonist stimulation of receptors for 5 min, AT1R was found mainly at the plasma membrane and mostly colocalized with GFP-ß2-adaptin (Fig. 6*B*, lower panel). The higher incidence of AT1R colocalization with ß2-adaptin at the plasma membrane in c-Src-depleted cells is consistent with a role of c-Src in regulating the dissociation of AP-2 from the receptor. In order to verify that c-Src depletion affected the dissociation of AP-2 from AT1R, we performed co-immunoprecipitation experiments on cells that were transfected using the same conditions as for the confocal experiments (Fig. 6*C*). Results show that in cells with reduced c-Src expression, a robust increase in the agonist-mediated association of AP-2 with the immunopurified AT1R was observed, as compared to non-depleted cells (mock) and GAPDH-depleted cells. We also assessed the effect of reducing c-Src expression on AT1R internalization by radio-ligand binding assay using ^{125}I-AngII. As shown in Fig. 6*D*, agonist stimulation of HEK 293 cells expressing the AT1R alone promoted the rapid internalization of receptor-ligand complex inside the cell, which reached a maximum after only 5 to 10 min of agonist stimulation. The nonsilencing control siRNA-GAPDH did not affect the rate or the extent to which AT1R internalizes. However, reducing the endogenous expression of c-Src delayed receptor endocytosis, and maximal internalization was reached only after 10 to 15 min of Ang II stimulation (Fig. 6*D*). Internalization was significantly reduced in c-Src-depleted cells as compared to non-depleted cells (AT1R alone) at 2 and 5 min of receptor stimulation (34± 3% *vs* 52± 2%; and 41± 2% *vs* 63± 4%, respectively). The half-time of AT1R internalization (the time to which 50% of receptors were internalized) in c-Src-depleted cells increased 2-fold as compared to non-silenced cells (Fig. 6*D)*. The delay in the initial rate of AT1R internalization observed in c-Src-depleted cells suggests that c-Src is involved in the early events of receptor internalization.

To substantiate the role of c-Src in regulating the association of AP-2 with AT1R/βarrestin complexes, we depleted c-Src expression in an endogenous system like the VSMCs. Cells were first treated with control anti-sense DNA (CTL-1) or anti-sense DNA for c-Src (AS-1), and the amount of c-Src expression was assessed. As shown in figure 7*A*, AS-1 selectively decreased by more than 60% the expression of c-

Src, whereas the CTL-1 had no effect on the expression of the protein. We next performed co-immunoprecipitation experiment to assess the effect of reducing c-Src expression on AP-2 association with endogenous βarrestin following AT1R stimulation. VSMCs transfected with either AS-1 or CTL-1 were left unstimulated or stimulated with Ang II for 5 min, and the level of AP-2 (as assessed by the presence of the ß2-adaptin) in the βarrestin immunoprecipitates was evaluated by Western blot analysis (Fig. 7*B*). Ang II stimulation induced a rapid association of AP-2 with βarrestin. However, the extent to which AP-2 associated with ßarrestin was greater in c-Src depleted VSMCs than in control cells. All together the results support the role of c-Src in regulating the dissociation of AP-2 from AT1R and ßarrestin during the internalization of receptors.

DISCUSSION

Here we show that AP-2 is recruited to ßarrestin and AT1R after receptor stimulation, extending previous studies that AT1R internalizes through the clathrin pathway (11-15). More importantly, we provide evidence that c-Src regulates the binding of AP-2 to ßarrestin and agonist-occupied AT1R. Indeed, we demonstrate that c-Src can form a stable complex with AP-2 and ßarrestin, and that its kinase activity is required to promote the agonist-mediated dissociation of AP-2 from both ßarrestin and AT1R during receptor internalization. In support of these results, is our finding that depleting c-Src expression in cells increases the binding of AP-2 with the agonist stimulated AT1R and ßarrestin, and prevents the rapid redistribution of the agonist-bound receptor inside the cell.

The recruitment of AP-2 to ßarrestin is mediated by the agonist stimulation of GPCRs (9). Interestingly, c-Src overexpression was sufficient to promote the stable association between AP-2 and ßarrestin, and AT1R without inducing receptor endocytosis (results not shown). This effect was independent of the kinase activity of c-Src, but required the presence of an intact ß2-adaptin binding site on ßarrestin. It was recently suggested, based on the crystal structure of ßarrestin that the activation of receptors is necessary to induce favorable conformational changes in ßarrestin required to unveil the ß2-adaptin binding site in the C-terminal domain of ßarrestin (24). Our results suggest that c-Src binding to ßarrestin induces a conformational change in ßarrestin allowing the agonist-independent binding of AP-2 to ßarrestin and AT1R. Interestingly, a mutant that mimics the activated form of c-Src (c-Src-Y530F) was unable to promote the association of AP-2 to ßarrestin. One explanation may be that c-Src-Y530F cannot induce the appropriate conformation in ßarrestin to promote the binding of ß2-adaptin to ßarrestin, even though we found that c-Src-Y530F still associated with ßarrestin. Another possibility is that this c-Src mutant, because of its potentially increased kinase activity, induces the phosphorylation of protein(s) involved in the dissociation of AP-2 from ßarrestin, thus preventing the formation of a ternary complex. This latter possibility raises the question of what proteins c-Src is

targeting? One potential candidate is the ß-subunit of the AP-2 complex itself. It was recently reported that EGFR activation could induce the tyrosine phosphorylation of ß2-adaptin (25). Whether c-Src phosphorylates ß2-adaptin or other endocytic proteins following AT1R stimulation, and to what extent this phosphorylation plays a role in regulating the dissociation of AP-2 from ßarrestin and the receptor remain to be determined.

The recruitment of signaling effectors into CCPs has been shown to affect components of the clathrin coat and to regulate the efficient agonist-dependent endocytosis of receptors. The presence of c-Src into CCPs affects coat proteins such as dynamin and clathrin (17, 26). For instance, EGFR stimulation induces the c-Src-dependent phosphorylation of dynamin and clathrin, which plays a role in EGFR endocytosis (26, 27). For GPCRs such as the ß2AR, the agonist-dependent binding of ßarrestin to receptors induces the formation of a complex containing ßarrestin and c-Src (28). The ßarrestin-mediated recruitment of c-Src to ß2AR brings the kinase in close proximity of dynamin, and regulates the function of dynamin through phosphorylation (17). Our results suggest another function for the ßarrestin-mediated recruitment of c-Src. In this paradigm, the agonist stimulation of AT1R promotes the translocation of ßarrestin to the receptor, and the recruitment of both c-Src and AP-2 to the AT1R (Fig. 8). The binding of c-Src to ßarrestin and AP-2 would then stabilize the endocytic complex, and allow the receptor to be efficiently targeted to the CCP. The Ang II-mediated recruitment of c-Src into the CCP would serve to regulate the dissociation of AP-2 from ßarrestin, and may phosphorylate other proteins of the coat to ensure efficient endocytosis of AT1R. In this regard, it will be of interest to examine whether the stimulation of AT1R triggers the phosphorylation of dynamin and clathrin, and to which extent these c-Src-mediated events contribute in regulating the dissociation of AP-2 from the receptor/ßarrestin complex, and affect the internalization of AT1R and/or other GPCRs that internalize in a ßarrestin- and a clathrin-dependent manner.

Several lines of evidence suggest that for efficient clathrin-mediated internalization, the endocytic proteins that are recruited into CCPs for initiating endocytosis must ultimately dissociate from the internalizing vesicle (29, 30). For instance, the dissociation of AP-2 from its internalized CCV is believed to be necessary to allow the recycling of the clathrin adaptor for further rounds of CCP formation and receptor internalization. Thus, preventing the dissociation of AP-2 from ßarrestin and/or AT1R would presumably affect the internalization of receptors. In support of this is our finding that AP-2 association with AT1R and ßarrestin increases when c-Src expression was reduced in cells, and that under such condition the internalization of receptors was delayed. The accumulation of AP-2 to the receptor at the plasma membrane may ultimately impede the formation of subsequent CCPs, and slow down receptor internalization. Further studies will be necessary to determine the extent to which c-Src participate in the assembly and disassembly of CCPs and the internalization of other GPCRs.

In summary, our results provide a novel function for c-Src in the formation of endocytic complexes during the internalization of AT1R. They also provide another mechanism by which ßarrestin, through the recruitment of signaling effectors like c-Src; can regulate the internalization of receptors through the clathrin pathway.

EXPERIMENTAL PROCEDURES

Materials – Angiotensin II was purchased from Sigma Chemical Co. [^{125}I]-Angiotensin II (1000 Ci/mmol) was obtained from Dr Gaétan Guillemette (Université de Sherbrooke, QC, Canada) and prepared as previously described (31). Antibodies against ß-adaptin, α-adaptin, were from BD Transduction Laboratories, the c-Src SRC2 and HA rabbit antibodies were from Santa Cruz Biotechnology, the mouse anti-HA clone 12CA5 was from Roche, the FLAG antibody was from Sigma-Aldrich, and the c-Src antibody GD11 and the anti-phosphotyrosine 4G10 were from Upstate Biotechnology. The rabbit polyclonal ßarrestin antibody (A1CT) was kindly provided by Dr Marc G. Caron (Duke University, NC).

Plasmids and constructs – The HA-tagged c-Src and the HA-tagged c-Src-Y530F mutant were a gift from Dr William E. Miller (University of Cincinnati, OH). HA-tagged c-Src-K298R mutant was generated by polymerase chain reaction (PCR) using the full-length HA-tagged c-Src in pcDNA3. A PCR fragment was amplified using a forward primer containing the mutation coding for the substitution K298R and a BamHI restriction site, and a reverse primer overlapping the KpnI site in c-Src. The fragment was digested with BamHI and KpnI, and replaced into c-Src cut with the same enzymes. Flag-tagged ßarrestin R396A, and R396A/AAEA were generated by replacing an XhoI/XbaI fragment from the rat ßarrestin2 wild type with a PCR product containing the mutations and the Flag sequence. The truncated Flag-tagged ßarrestin2 (ßarr2-T372) was generated by PCR using a similar cassette replacement strategy to insert the Flag sequence after K372 in ßarrestin. The p426-ADH-c-Src was generated by excising the avian c-Src mutant (c-Src-G2A,-Y416F,-Y527F) from the p413-Gal1 vector (kindly provided by Dr Serge Lemay, McGill University, QC, Canada) with BamHI and SalI, and cloning the fragment into p426-ADH (kindly provided by Dr Bernard Turcotte, McGill University, QC, Canada) using the same restriction sites. Green-fluorescent-tagged ß2-adaptin (GFP-ß2-adaptin) construct was described

elsewhere (9). All constructs were analyzed by DNA sequencing (Service d'analyse et de synthèse d'acides nucléiques, Université Laval, QC, Canada).

Cell Culture and Transfection – Human embryonic kidney cells (HEK 293) were grown in Eagle's minimal essential medium (MEM, Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco) and gentamicin (100 µg/ml, Gibco). HEK 293 cells seeded in a 100 mm dish, at a density of $2.5x10^6$ cells/dish, were transiently transfected using a conventional calcium phosphate co-precipitation method. One to five µg of DNA were mixed in a solution containing 125 mM $CaCl_2$ in HEPES-Buffered Saline (25 mM HEPES pH 7.4, 140 mM NaCl, 0.75 mM Na_2HPO_4) for 5 min, and the mixed solution was then added to cells. Green monkey kidney cells (COS-7) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (v/v) heat-inactivated FBS and gentamicin (100 µg/ml). Transient transfections of COS-7 cells seeded in 60 mm dishes (at a density of $1x10^6$ cells/dish) were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations using a 1:3 ratio of DNA/Lipofectamine in Opti-MEM (Gibco). Twenty-four hours post-transfection, HEK 293 and COS-7 cells were serum-starved for 12h in DMEM before performing the experiments. For vascular smooth muscle cells (VSMCs; kindly provided by Dr Sylvain Meloche at the IRCM, QC, Canada), cells were grown in DMEM low glucose with 10% (v/v) heat-inactivated calf serum (Gibco) and gentamicin (100 µg/ml), and they were serum-starved in DMEM low glucose for 12h before performing the experiments.

Immunoprecipitation and Western blot experiments – HEK 293 or COS-7 cells were left untreated or treated with Ang II (100 nM) at 37°C for the indicated period of time. Following agonist stimulation, the reaction was stopped on ice by washing cells with ice-cold Phosphate-buffered saline (PBS). Cells were then solubilized in P-RIPA buffer (1 ml/100 mm dish) (20 mM TrisHCl pH 7.4, 150 mM NaCl, 1% Nonidet-P40 (v/v), 0.1% deoxycholate (w/v), 1 mM $CaCl_2$) containing 0.1 mM sodium

orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml leupeptin, 25 µg/ml aprotinin, and 1 mM pepstatin A for 30 min at 4°C. Cell lysates were centrifuged for 30 min at 21,000 x *g* and the supernatants were incubated with anti-Flag (30 µg/ml, Sigma) or c-Src antibody (3 µg/ml, clone GD11, Upstate) with 20 µl of a 50% slurry mixture of protein A/G Sepharose beads for 2 h at 4°C. Beads were washed three times with P-RIPA, and denatured in Laemmli buffer (2X) (250 mM TrisHCl pH 6.8, 2% SDS (w/v), 10% Glycerol (v/v), 0.01% Bromophenol Blue (w/v), 5% ß-mercaptoethanol (v/v)). Proteins were separated on a 10% SDS-polyacrylamide gel (PAGE) before being transferred onto nitrocellulose membranes (Amersham Biosciences). Membranes were then blocked with a solution of PBS pH 7.4 containing 0.05% Tween20 (v/v) (PBS-T) supplemented with 10% skimned milk (w/v). Protein detection on membranes was assessed using either anti-c-Src antibody (1 µg/ml, clone GD11; or 0.2 µg/ml, SRC2), Flag antibody (2.5 µg/ml), HA antibody (0.2 µg/ml), phosphotyrosine antibody (0.2 µg/ml, clone 4G10), ß2-adaptin antibody (0.2 µg/ml, ß-adaptin), ß-adaptin antibody (0.25 µg/ml), ßarrestin antibody (diluted 1/5,000; A1CT) in PBS-T containing 1% bovine serum albumin (BSA, fraction V; Sigma) (w/v) and 1% skimned milk (w/v). After 1 h incubation with the different primary antibodies, membranes were washed three times in PBS-T before being incubated at room temperature with horseradish peroxidase (HRP) conjugated goat anti-mouse antibody (diluted 1/10,000; Sigma), or HRP-goat anti-rabbit antibody (diluted 1/10,000; Sigma) for 30 min. Immunoreactivity was visualized by enhanced chemiluminescence according to the manufacter's instructions (SuperSignal, Pierce).

Immunofluorescence Experiments – HEK 293 cells were grown on 22 mm^2 cover slips in 6-well plates (250,000 cells/well), and transfected with GFP-ß2-adaptin (0.5µg) and HA-AT1R (1.5 µg). For AT1R immuno-detection, cells were pre-incubated for 1 h at 14°C with a mouse anti-HA antibody (20 µg/ml, 12CA5), followed by incubation at 14°C for 1 h with a secondary goat anti-mouse antibody conjugated to AlexaFluor 568 (8 µg/ml, Molecular Probes). Labeled cells were stimulated at 37°C with 100 nM Ang II for 5 min, and fixed for 15 min at room temperature with a solution of 4% paraformaldehyde (w/v) in PBS. Cover slips were mounted with GelTol Aqueous

Mounting Medium (IMMUNON). Confocal images of labeled cells were acquired with a Zeiss LSM-510 META laser-scanning microscope using a 60x oil immersion lens. GFP-ß2-adaptin and AlexaFluor 568-labeled receptor fluorescence was visualized using the multi-track mode with dual laser excitation (488 and 543 nm), and emission (BP 505-520 for GFP, and LP 560 for AlexaFluor 568) filter sets. Final figures were collated using Adobe Photoshop 7.0 and Illustrator 10.0 (Adobe, San Jose, CA).

Three-hybrid assay – Yeast two-hybrid MatchMaker System 2 (Clontech) was used with minor modifications to create a three-hybrid assay that allowed the detection of a ternary complex between ßarrestin2, ß2-adaptin and c-Src. Constructs for *GAL4*-DBD ßarrestin2 in pAS2-1 and *GAL4*-AD ß2-adaptin in pACT-2 were described elsewhere (9). PAS2-1-ßarrestin2 and pACT-2-ß2-adaptin vectors were cotransformed with the empty vector p426-ADH or the p426-ADH-c-Src into the yeast strain PJ69-4a using the PEG/lithium acetate method (Clontech). Yeast colonies were grown on medium lacking tryptophan, leucine and uracil for the selection of transformants expressing the bait vector (pAS2-1-ßarrestin2), the prey vectors (pACT-2 or pACT-2-ß2-adaptin), and the bridge vector with or without c-Src (p426-ADH-c-Src or p426-ADH). Protein-protein interaction was assessed using the HIS3 auxotrophic complementation, and a lacZ reporter assay. For the ß-galactosidase assay, three colonies of each transformation were selected and grown on YPD medium at 30°C overnight. The next day, the yeast cultures were diluted to an A_{600} of 0.2 in synthetic dropout medium (SD) lacking tryptophan, leucine and uracil, and grown for another 7 h at 30°C. Cultures were serially diluted at an A_{600} of 0.1, 0.002 and 0.004, and 10 µl of cell suspension were spotted onto medium lacking leucine, tryptophan and uracil (-Leu/-Trp/-Ura) for selection of yeasts expressing the bait, the prey and the bridge vector, respectively, and selected onto medium lacking leucine, tryptophan, uracil and histidine (-Leu/-Trp/-Ura/-His) for the detection of interactions between ßarrestin2 and ß2-adaptin. Histidine complementation was assessed in presence of 2.5 mM 3-amino-1,2,4-triazole (3-AT, Sigma). Beta-galactosidase activity was measured using the ONPG assay (O-

nitrophenyl ß-D-galactopyranoside, Clontech). Briefly, yeasts were grown on a selective medium to an A_{600} of 0.8-1.2, and cells from a 2 ml culture were collected by centrifugation. Pelleted cells were washed with cold Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 100 mM KCl, 1 mM $MgSO_4$, and 30 mM ß-mercaptoethanol, at pH 7.0), and vigorously resuspended in 800 µl of Z-buffer containing 0.1% SDS (w/v) and 0.5% $CHCl_3$ (v/v). Colorimetric reaction was started at 30°C by adding 160 µl of ONPG (4 mg/ml) to permeabilized cells. The reaction was stopped with 400 µl of Na_2CO_3 (1 M) when yellow coloration started to appear. The activity was measured at A_{420} and Miller units were calculated from the equation (Units=1000x (A_{420}/ volume of cells assayed x time of run x A_{600})).

SiRNA and antisense experiments – All double-stranded siRNAs were synthesized using the Silencer™ siRNA kit (Ambion®) according to the manufacturer's instructions. Twenty-one-mer oligonucleotides corresponding to the sense and anti-sense sequences of the human c-Src mRNA were screened for unique sequence in the National Center for Biotechnology Information database by using the BLAST search algorithm (accession number BC011566). The target mRNA identified for c-Src is 5'-AAG CAC UAC AAG AUC CGC AAG -3', and corresponded to the position 607-628 (relative to the start codon) of c-Src. An extension that corresponded to the T7 promoter primer sequence (5'-CCT GTC TCT-3') was added at the 3' end of the sense and anti-sense sequence. Control siRNA sense and anti-sense oligonucleotides for GAPDH were provided in the kit.

For AT1R immunoprecipitation experiments, HEK 293 cells were seeded at a density of 400,000 cells/well in 24-well plates, and transfected with Lipofectamine 2000. Briefly, pcDNA3.1 encoding the HA-AT1R (0.375 µg/well) was added with pcDNA3.1 (0.375 µg/well), siRNA-GAPDH (0.375 µg/well) or siRNA-c-Src (0.375 µg/well) and mixed with Lipofectamine 2000 (using a ratio of DNA/Lipofectamine of 1:2) in Opti-MEM. Complexes of DNA/Lipofectamine were added to cells in MEM without serum, and transfection was carried out for 12 h. The next day, media was replaced with complete MEM medium supplemented with 10% FBS (v/v) and gentamicin (100 µg/ml).

For immunofluorescence experiments, cells were seeded in 6-well plates, at a density of 300,000 cells/well, and transfected with HA-AT1R (0.25 µg/well) and GFP-ß2-adaptin (0.125 µg/well) with or without siRNA-c-Src (0.375 µg/well) using the same DNA/Lipofectamine ratio as described above.

For AT1R internalization experiments, HEK 293 cells were seeded at a density of 70,000 cells/well in 24-well plates, and transfected with Lipofectamine 2000 as described above using the same DNA/Lipofectamine ratio. Briefly, pcDNA3.1 encoding the HA-AT1R (0.125 µg/well) was added with pcDNA3.1 (0.5 µg/well), siRNA-GAPDH (0.5 µg/well) or siRNA-c-Src (0.5 µg/well) and mixed with Lipofectamine 2000 in Opti-MEM. Transfection was carried out for 12h before replacing the medium with complete MEM supplemented with 10% FBS (v/v) and gentamicin (100 µg/ml) and performing the experiment 72 h post-transfection.

For experiments depleting c-Src in VSMCs, an anti-sense oligonucleotide strategy was used. Control scramble phosphorothioate (CTL-1, 5'-GTC TTA GCC GGG ATC CGC TA-3') and anti-sense oligonucleotides complementary to the sequence of the rat c-Src mRNA (AS-1, 5'-TT GCT CTT GTT GCT GCC CAT-3', accession number NM_031977) were designed and synthesized by Alpha DNA (Montreal, QC, Canada). VSMCs were seeded at a density of $2x10^6$ cells in 10 cm dishes and transfected with anti-sense DNAs using Lipofectamine 2000 as described previously. Briefly, c-Src AS-1 (1.2-6.0µg, representing a final concentration of 50-250 nM in 4 ml) or CTL-1 (1.2 –2.4µg; 50-100 nM in 4 ml) were mixed using a ratio of oligonucleotide/Lipofectamine of 1:2 in Opti-MEM. Complexes were added to cells in DMEM (Gibco) without serum and transfection was carried out for 36 h before performing the experiments.

Internalization assay – Receptor internalization was performed as described previously with minor modifications (31). Briefly, HEK 293 cells seeded in 24-well plates were transfected with HA-AT1R with or without siRNAs. Thirty-six 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]-Angiotensin II for the indicated period of time. Incubation was stopped on ice

by rapidly washing the cells three times with either ice-cold PBS to remove the unbound Ang II or ice-cold acid buffer (0.2 N acetic acid pH 3.5, 150 mM NaCl) to remove both the unbound and the cell surface receptor-bound agonist. Cells were then solubilized in 0.5 N NaOH, 0.05% SDS (w/v), and the radioligand content was evaluated by gamma-counting. Percent of receptor internalization was calculated from the ratio of acid-resistant binding over total binding (PBS wash). Data were analyzed by non-linear regression using Prism4 (GraphPad Software).

Data Analysis- Intensity of the signals from Western Blots was determined by densitometric analysis using Alpha Innotech Fluorochem imaging system (Packard Canberra), and was represented as the mean ± S.E.M of at least three independent experiments. For densitometry analysis of Western blot and ß-galactosidase assay, data were analyzed statistically by one-way ANOVA followed by a Bonferroni post-test for multiple comparisons, or by one-tail *t* test for single comparison. Means were considered significantly different when p values were at least below 0.05.

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Fig. 1. Ang II promotes the formation of an endogenous complex containing c-Src, ßarrestin, and AP-2. Vascular smooth muscle cells (VSMCs) were left untreated (-) or treated (+) with Ang II (100 nM) for 5 min at 37°C after serum starvation overnight. Endogenous c-Src was immunoprecipitated (IP) with the GD11 antibody, and proteins in the complex were detected by Western blot using c-Src (SRC2, Santa Cruz Biotechnology), ßarrestins (A1CT), and the α-subunit of AP-2 (BD Transduction Laboratories) antibodies as described in *Experimental Procedures*. Also presented are blots from whole cell extracts (Total, right panel) probed with the same antibodies. Blots are representative of three independent experiments.

Fig.1 Fessart *et al*

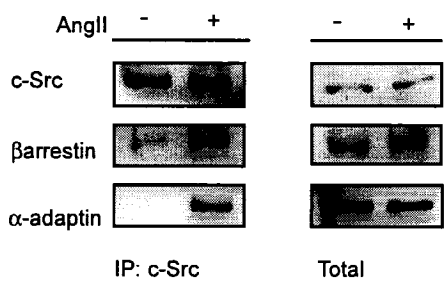


Fig. 2. c-Src overexpression increases the association between ßarrestin and AP-2 independently of the c-Src kinase activity. *A,* COS-7 cells were transiently transfected with Flag-ßarrestin2 and increasing amounts of HA-c-Src. Beta-arrestin was immunoprecipitated (IP) with Flag antibody (Sigma) and the amount of endogenous AP-2, as assessed by the presence of ß2-adaptin in the ßarrestin immunoprecipitates was analyzed by Western blot using a ß2-adaptin (BD Transduction Laboratories) antibody. Whole cell extracts (Total) were also blotted for detecting the level of c-Src expression using the HA (Santa Cruz Biotechnology) and ß2-adaptin (BD Transduction Laboratories) antibodies. *B,* COS-7 cells were transfected with Flag-ßarrestin2, ß2-adaptin and pcDNA3.1 (Mock) and either HA-c-Src, HA-c-Src-Y530F or HA-c-Src-K298R. Whole cell extracts (Total) were analyzed by Western blot (WB) with the anti-phosphotyrosine (P-Tyr) antibody 4G10 (Upstate Biotechnology). *C,* Cells were transfected as in (*B*), and Flag-ßarrestin was immunoprecipitated (IP) with the Flag antibody (Sigma), and complexes were analyzed by Western blot for the detection of ßarrestin, ß2-adaptin and c-Src expression using the antibodies against Flag (Sigma), ß2-adaptin (BD Transduction Laboratories), and HA (Santa Cruz Biotechnology), respectively. Whole cell extracts (Total) were also blotted with anti-HA (Santa Cruz Biotechnology) and anti-ß2-adaptin (BD Transduction Laboratories) antibodies, for detection of the different c-Src constructs and the ß2-adaptin, respectively. Data are representative of at least three independent experiments.

Fig. 2 Fessart *et al*

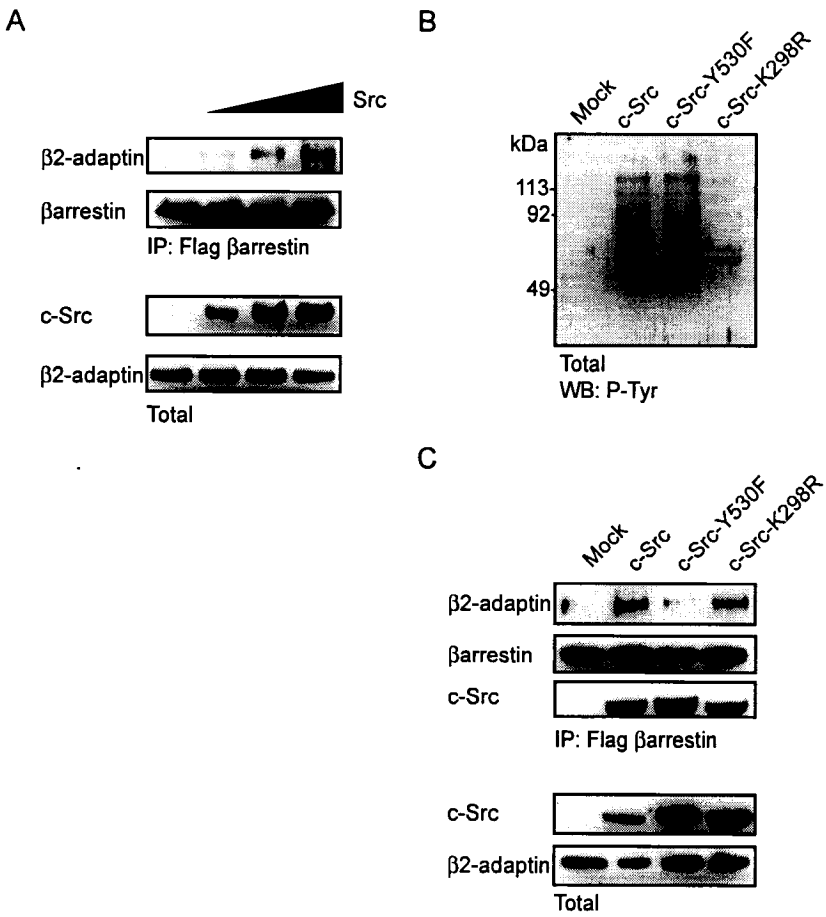


Fig. 3. c-Src increases the interaction between ßarrestin and ß2-adaptin in yeast. *A,* DNA encoding the hybrid protein ßarrestin2 (*GAL4* DBD-ßarr2) was transformed in the yeast strain PJ69-4a alone (lane A), or with the hybrid protein ß2-adaptin (*GAL4* AD ß2-Ad) (lane B), c-Src (lane C), or ß2-adaptin and c-Src together (lane D) as described in *Experimental Procedures*. Transformants were serially diluted (at an A_{600} of 0.1, 0.002, 0.004, respectively) and 10 µl of cell suspension were spotted onto selective medium lacking leucine, tryptophan and uracil (-Leu/-Trp/-Ura), or lacking leucine, tryptophan, uracil and histidine (-Leu/-Trp/-Ura/-His). Protein-protein interaction was assessed for histidine complementation in presence of 2.5 mM 3-AT (*A*) and for ß-galactosidase activity (*B*). Beta-galactosidase activity is expressed as Miller units as described in *Experimental Procedures*, and represents the mean ± S.E.M of three independent transformations in triplicates. *C,* Schematic representation of ternary complex formation between the two hybrid proteins, ßarrestin2 and ß2-adaptin, and the bridge protein c-Src. Results show that the expression of c-Src with the two hybrid proteins (column D) increases the ß-galactosidase activity by 4-fold and 2-fold as compared to conditions when ßarrestin2 and c-Src (column C), or ßarrestin2 and ß2-adaptin (column B) were expressed together. ß2-adaptin expression alone or with c-Src did not increase significantly the ß-galactosidase activity as compared to condition expressing ßarrestin2 and c-Src (data not shown). ** $p<0.01$ and *** $p<0.001$ determined by one-way ANOVA, and was considered highly significant.

Fig. 3 Fessart *et al*

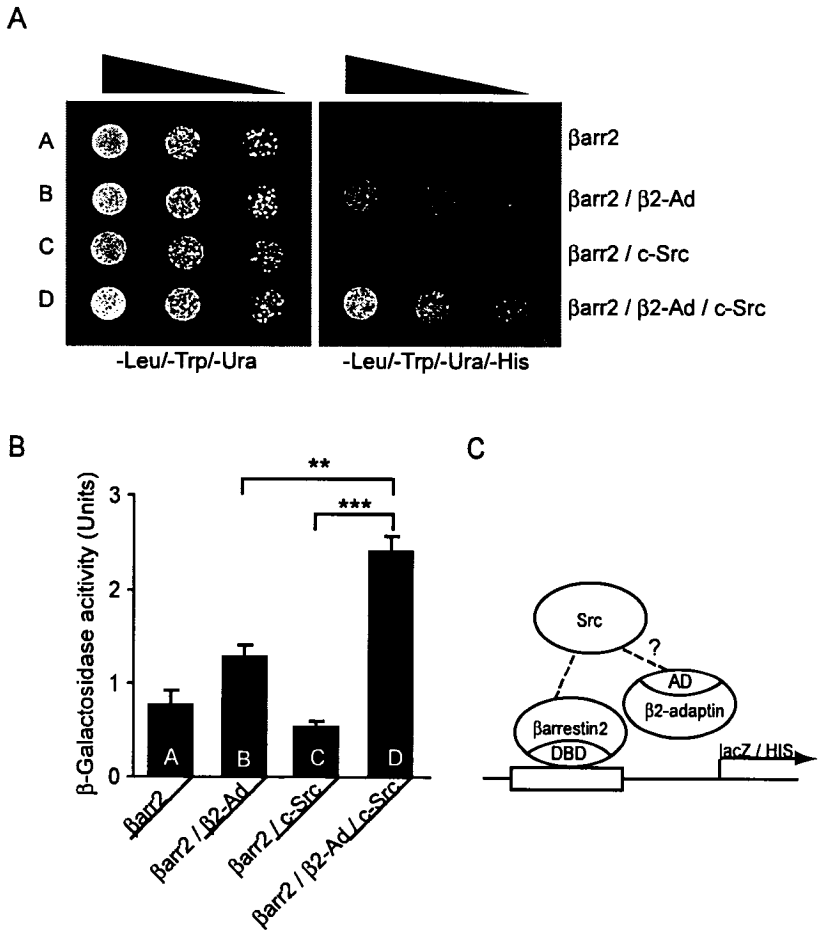


Fig. 4. Effect of c-Src expression on AP-2 association with ß arrestin mutants lacking ß2-adaptin and/or clathrin binding sites. *A-B,* COS-7 cells were transiently transfected with either Flag-ßarrestin2 or Flag-ßarrestin2-R396A, Flag-ßarrestin2-R396A/AAEA, Flag-ßarrestin2-T372, with or without HA-c-Src. ßarrestin2 wild type or mutants were immunoprecipitated with an anti-Flag antibody (Sigma), and the endogenous AP-2 in the immunoprecipitates was detected by Western Blot using ß2-adaptin (BD Transduction Laboratories) antibody. Whole cell extracts (Total) were blotted with anti-HA (Santa Cruz Biotechnology) antibody for c-Src detection, and with the same anti-ß2-adaptin antibody as described above. *B*, Results are expressed as the fold increase association of AP-2 with ßarrestin2 in presence of c-Src (+) as compared to conditions where c-Src was not overexpressed (-), and represent the mean ± S.E.M of at least three independent experiments. * indicates $p<0.05$ and ** $p<0.01$ as compared with wild type ßarrestin determined by one-way ANOVA test.

Fig. 4 Fessart *et al*

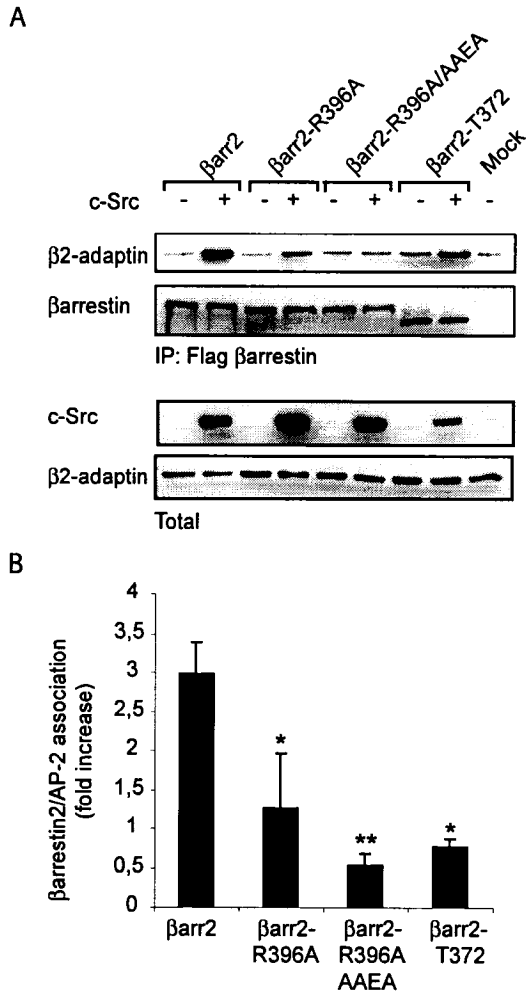


Fig. 5. c-Src promotes the dissociation of AP-2 from the agonist-occupied AT1R and ßarrestin. COS-7 cells were transfected with AT1R and Flag-ßarrestin2 with or without c-Src (*A*), or HEK 293 cells were transfected with Flag-AT1R with or without c-Src and c-Src-K298R (*B-C*). Cells were serum-starved 12 h before being stimulated with Ang II (1 µM) for the indicated time, and ßarrestin2 (*A*) or AT1R (*B-C*) were immunoprecipitated (IP) using an anti-Flag antibody (Sigma). Immunoprecipitates were analyzed by Western blot for the detection of associated endogenous AP-2 using an antibody against ß2-adaptin (BD Transduction Laboratories). Whole cell extracts (Total) were also probed for the detection of c-Src using the GD11 antibody (Upstate) and ßarrestin using the anti-Flag antibody (Sigma), and endogenous AP-2 using the BD Transduction Laboratories antibody. Data represent the relative amount of ß2-adaptin found in AT1R and ßarrestin2 immunoprecipitates following agonist-stimulation of receptors after normalizing for equal amounts of ß2-adaptin present in total proteins, and are the mean ± S.E.M of three independent experiments.

Fig. 5 Fessart *et al*

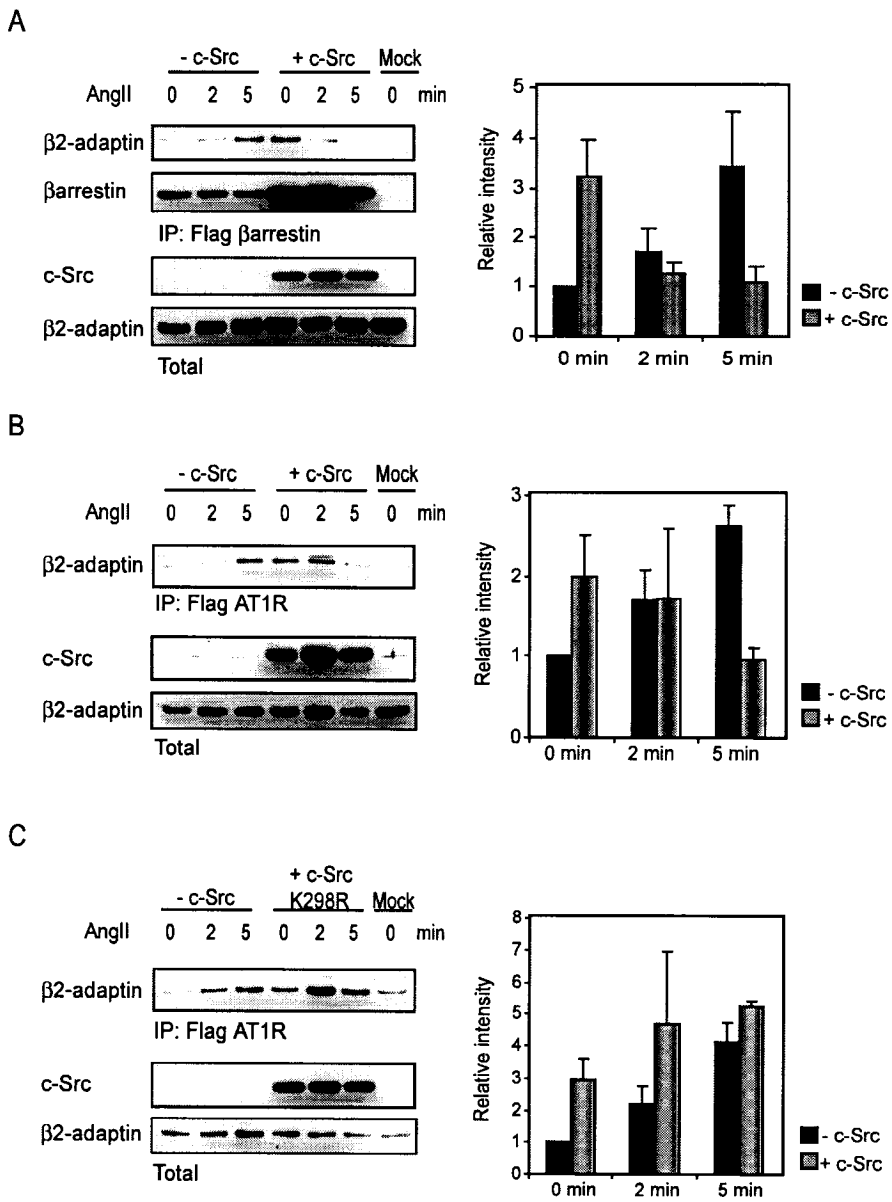


Fig. 6. Depleting c-Src in cells affects the agonist-mediated internalization of AT1R and its association with AP-2. *A,* HEK 293 cells were transiently transfected with HA-AT1R with pcDNA3.1 (Mock), siRNA-GAPDH, or siRNA-c-Src. *A,* Seventy-two hours after transfection, total cell lysates were analyzed by Western blot for the expression of endogenous c-Src using the GD11 (Upstate) and the ß2-adaptin (BD Transduction Laboratories) antibodies. *B,* HEK 293 cells were transfected with HA-AT1R and GFP-ß2-adaptin, and either pcDNA3.1 (*upper panels*) or siRNA-c-Src (*lower panels*). Cells were serum-starved for 12 h before being incubated with anti-HA antibody (12CA5, Roche) and with a secondary goat anti-mouse antibody conjugated to AlexaFluor 568 (Molecular Probes) as described in the *Experimental Procedures.* Cells were then washed, and were either left untreated or treated with Ang II (1 µM) for 5 min at 37°C. The distribution of HA-AT1R and GFP-ß2-adaptin was visualized by confocal microscopy. For each experiment, 25 different fields containing 1 to 2 cells were analyzed. Shown are representative images of receptor immuno-fluorescence (*red*) and GFP-ß2-adaptin fluorescence (*green*) of three independent experiments. Colocalization (*yellow*) of GFP-ß2-adaptin with the receptor is indicated by arrows. *C,* HEK 293 cells were transiently transfected with HA-AT1R with either pcDNA3.1 (Mock), siRNA-c-Src, or siRNA-GAPDH. Cells were serum-starved as previously described before being stimulated with Ang II (1 µM) for 5 min. AT1R was immunoprecipitated (IP) using an anti-HA antibody (Roche), and the immunoprecipitates were analyzed by Western blot for the detection of associated AP-2 using the ß2-adaptin antibody (BD Transduction Laboratories). Whole cell extracts (Total) were also probed for the expression of endogenous c-Src using the GD11 (Upstate) and the ß2-adaptin (BD Transduction Laboratories) antibodies. Blots are representative of two independent experiments. *D,* For receptor internalization, cells were transfected with AT1R alone (■) and with either siRNA-c-Src (Δ) or siRNA-GAPDH (□). Cells were incubated with [^{125}I]-Ang II (0.11 nM) at 37°C for the different period of time, and the percent of receptor internalization was calculated as described in *Experimental Procedures.* Data are the mean ± S.E.M of three to five independent experiments, and were analyzed using Prism4 (GraphPad 4 Software). * indicates $p<0.05$ siRNA-c-Src *vs* AT1R alone determined by an unpaired *t* test.

Fig.6 Fessart *et al*

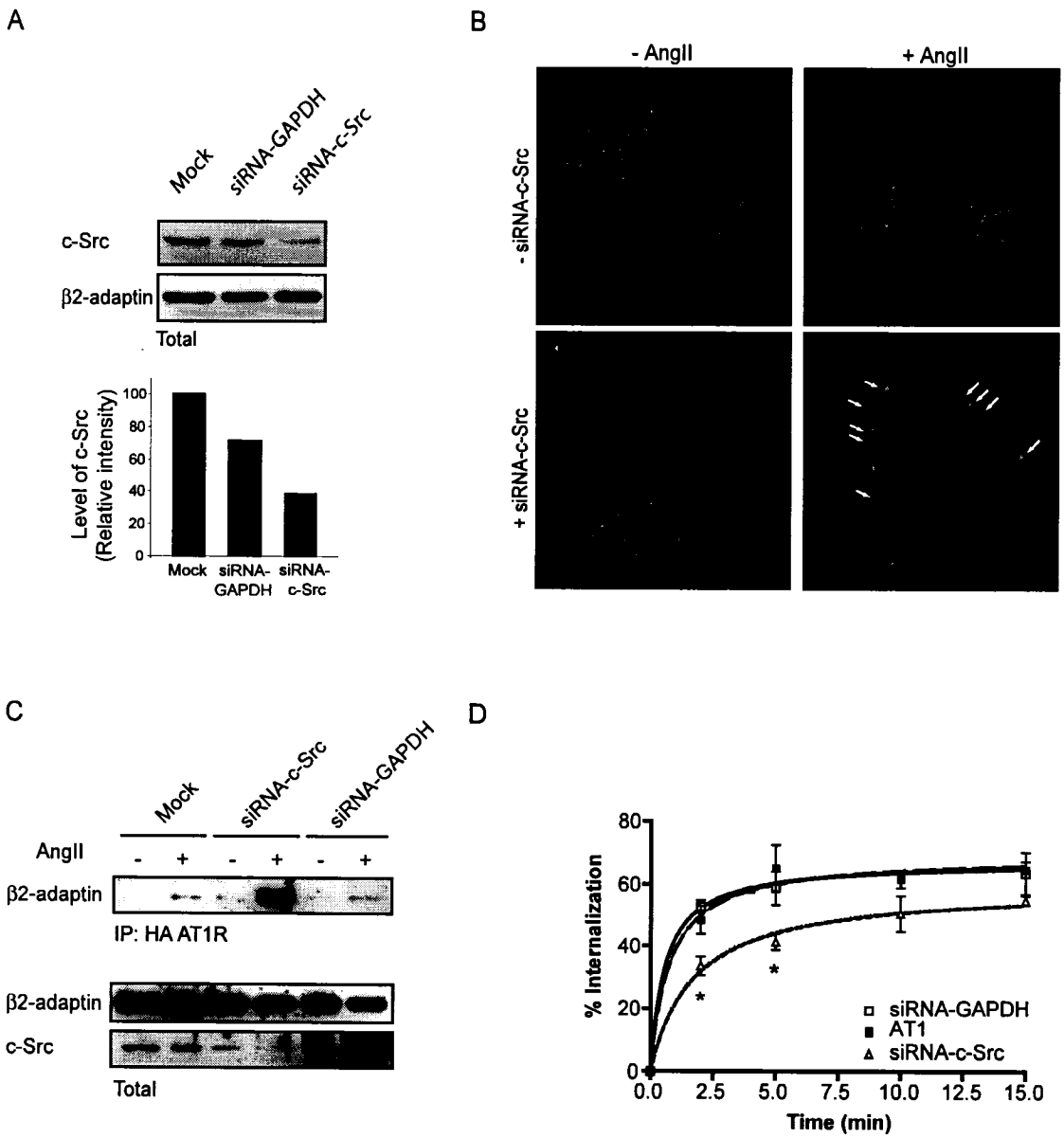


Fig. 7. Depleting c-Src in VSMCs increases the AngII-mediated AP-2 association with ßarrestin *A*, VSMCs cells were transfected or left untransfected (Mock) with either anti-sense oligonucleotides for the rat c-Src (AS-1) or control oligonucleotides (CTL-1). Thirty-six hours after transfection, total cell lysates from VSMCs were analyzed by Western blot for the expression of endogenous c-Src using the GD11 (Upstate) and ß2-adaptin (BD Transduction Laboratories) antibodies. *B*, VSMCs were transfected with oligonucleotides for 36 h in serum-free medium as described in the *Experimental Procedures*. Cells were then stimulated with Ang II (1 µM) for 5 min, and ßarrestin was immunoprecipitated (IP) using the A1CT antibody. Proteins in the immunoprecipitated complexes were analyzed by Western blot for the detection of associated AP-2 using the ß2-adaptin antibody. Whole cell extracts (Total) were also probed for the detection of endogenous c-Src using the GD11 (Upstate), ßarrestin (A1CT) and ß2-adaptin antibodies. Shown in *A* and *B* are representative blots of 4 independent experiments. DATA were quantified, and presented as the relative amount of c-Src found in cells, after normalizing to the total amount of ß2-adaptin (*A*), and the fold increase over basal of the associated ß2-adaptin with ßarrestin (*B*). *, indicates $p<0.05$ AS-1 *vs* CTL-1 determined by one-tail paired *t* test.

Fig. 7 Fessart *et al*

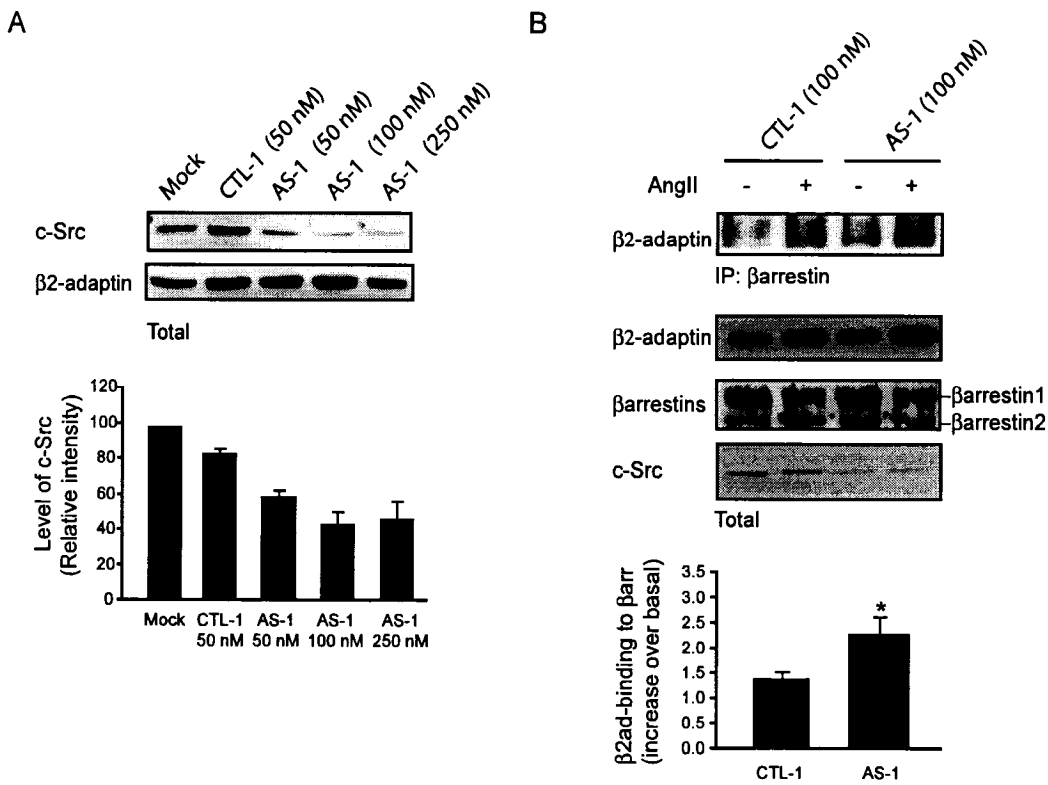
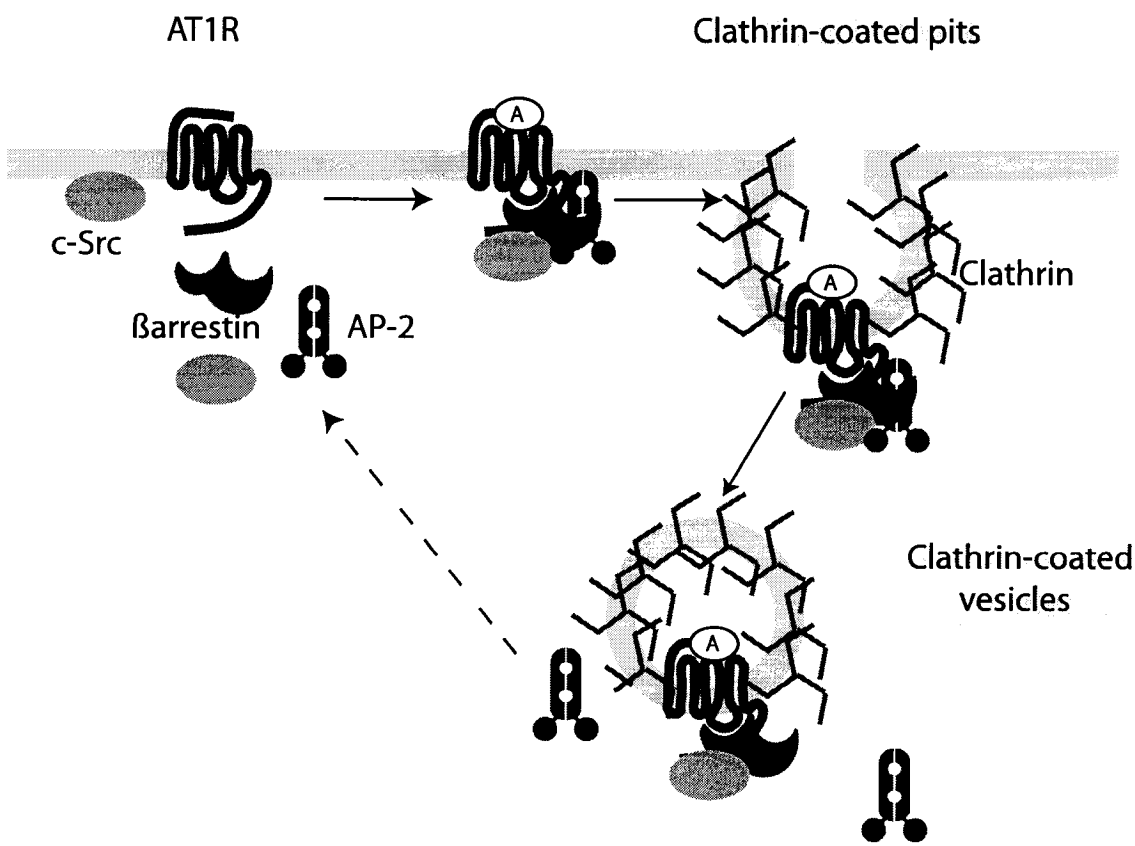


Fig. 8. Proposed model for the role of c-Src in the regulation of the interaction between AP-2, AT1R and ßarrestin during receptor internalization. Agonist stimulation of AT1R promotes the recruitment of a ternary complex containing c-Src, AP-2 and ßarrestin. The complex would then be targeted to CCPs, and the presence of multiple receptor complexes in addition to the recruitment of other endocytic proteins into the CCP would trigger the internalization of the coated vesicle. During AT1R internalization, c-Src could promote the dissociation of AP-2 from the receptor/ßarrestin complex to allow the recycling of AP-2 for further rounds of coat formation and AT1R targeting to CCPs. AT1R, Angiotensin II type 1 receptor; A, agonist.

Fig. 8 Fessart *et al*



CONNECTING TEXT

The following section entitled, ℭ-S rc-mediated phosphorylation of AP-2 regulates its dissociation from ßarrestin During Angiotensin Type 1 Receptor Internalization", characterizes one mechanism regulating the formation of the endocytic complexes during internalization. In section III, our findings demonstrate that following Ang II stimulation of the AT1R, βarrestin forms a complex with the non-receptor tyrosine kinase c-Src and AP-2. The kinase activity of c-Src seems to regulate this endocytic complex (association versus dissociation). This is consistent with the observation reported in the literature showing that β2-adaptin is tyrosine phosphorylated upon EGFR stimulation (297). Taken together, these data suggested that c-Src may phosphorylate AP-2 and thus, might be involved in the formation of endocytic complexes. Therefore, our aims were to determine:

1) whether Angiotensin II mediates c-Src-dependent phosphorylation of AP-2, and if so, where the phosphorylation occurs, and

2) whether phosphorylation may regulate the formation of the βarrestin-scaffold complex.

SECTION IV

C-SRC-MEDIATED PHOSPHORYLATION OF AP-2 REGULATES ITS DISSOCIATION FROM ßARRESTIN DURING ANGIOTENSIN TYPE 1 RECEPTOR INTERNALIZATION.

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ABSTRACT

Apart from their role in G protein-coupled receptor (GPCR) internalization, ßarrestins serve as scaffold proteins for receptor signaling and intracellular trafficking. Recently, we have shown that the stimulation of Angiotensin II type 1 receptor (AT1R), in vascular smooth muscle cells (VSMCs), promoted the formation of a multimeric complex containing endogenous c-Src, ßarrestin and AP-2. Here, we show that stimulation of AT1R in either VSMC or COS-7 cells promotes the tyrosine phosphorylation of ß2-adaptin, a subunit of AP-2 complex. Phosphorylation can be selectively inhibited by a Src family kinase inhibitor PP2 and by the overexpression of a defective c-Src kinase mutant (c-Src-K298R). Moreover, using GST fusion proteins and pull-down assays, we identify a critical tyrosine residue in the ear domain of ß2-adaptin (Y737) as a target for c-Src-mediated phosphorylation, and show that this single phosphotyrosine residue regulates the binding of ßarrestin to ß2-adaptin. Finally, we used a Bioluminescence Resonance Energy Transfer (BRET) assay to investigate, in real time and in living cells, the regulatory role of c-Src toward the interaction between ßarrestin and AP-2. Results show that c-Src dramatically reduced the AngII-promoted interaction between ßarrestin2 and wild-type ß2-adaptin. However, c-Src had only a marginal effect on reducing the interaction between the two proteins when the Y737 in ß2-adaptin was mutated. Taken together, our results support a role for tyrosine phosphorylation of the ß2-adaptin ear domain in the regulation of endocytic complex formation during AT1R internalization.

INTRODUCTION

The G protein-coupled receptor (GPCR) family is intrinsically involved in many physiological functions including autocrine, paracrine and endocrine processes. Following agonist stimulation, the receptor conformation changes, which allow activation of G proteins and second-messenger production. Receptors are then phosphorylated by G protein-coupled receptor kinases (GRKs), which lead to the recruitment of the non-visual arrestin proteins (ßarrestin1 and ßarrestin2) (1), and uncoupling of receptors from their cognate G protein. Consequently ßarrestins, target GPCRs to clathrin-coated vesicles (CCVs) for receptor internalization (2, 3). Clathrin-coated vesicles (CCV) mediate the endocytosis of plasma membrane proteins to the endosomal/lysosomal system (4) and thus, involve cycles of assembly and disassembly of clathrin coat components and their accessory proteins.

Phosphorylation of proteins is usually described as a process to create high-affinity binding sites for recruiting proteins. However, phosphorylation events have been proposed to affect the interactions between proteins involved in endocytosis (5, 6). For example, *in vitro* dephosphorylation of rat brain extracts promotes the assembly of dynamin I, synaptojanin, amphiphysin, clathrin and AP-2 into complexes (5). Another example was showed with the c-Src-mediated phosphorylation of Caveolin-1 (7), suggesting a relationship between tyrosine kinase activity and release of caveolae from the membrane (8). Indeed, expression of either Y14F caveolin-1 or Y597F dynamin-2 abolished albumin endocytosis, indicating c-Src phosphorylation of these residues is required for signaling through caveolae-mediated endocytosis (8). Src family tyrosine kinases, which are activated by stimulation of G protein-coupled receptors (9-11) or tyrosine kinase receptors (12), also phosphorylate tyrosine residues on dynamin (13) important for the internalization of ß2AR and the M1 muscarinic receptor (13, 14). Other studies have showed that both epsin and Eps15 are phosphorylated during mitosis and their phosphorylation inhibits binding to the clathrin adaptor AP-2 (6). Thus, overall these studies suggest that phosphorylation regulates the association and dissociation cycle of the clathrin-based endocytic machinery.

Phosphorylation and dephosphorylation reactions play an important role in regulation of the endocytic machinery but the signaling pathways mediating release of AP-2 from the CCV during GPCR endocytosis are poorly understood. No functional role for AP-2 tyrosine phosphorylation in GPCR endocytosis has been demonstrated yet, therefore our aim was to determine the role of phosphorylation of AP-2 during endocytosis of AT1R. We recently showed that c-Src was also involved in regulating the stability of endocytic complex during receptor internalization. Therefore, we investigated whether c-Src-dependent phosphorylation of AP-2 represents a potential mechanism for controlling the formation of endocytic complexes during the internalization of AT1R. To test our hypothesis, we first determined the extent to which AP-2 is phosphorylated by c-Src following AT1R activation. We further identified the putative tyrosine-phosphorylated residues within ß2-adaptin, one of the major binding partners of ßarrestin. Finally, we investigated the role of the Src-mediated ß2-adaptin phosphorylation in regulating the interaction between AP-2 and ßarrestin. This study illustrates that a general property of proteins involved in endocytosis is to undergo agonist-mediated phosphorylation for disassembly and to assemble in a dephosphorylated state.

RESULTS

Angiotensin II promotes the ßarrestin- and Src-dependent tyrosine phosphorylation of ß2-adaptin. We first assessed whether the agonist-mediated formation of a complex containing ßarrestin, AP-2 and c-Src (15) could lead to the tyrosine phosphorylation of AP-2. We focused on the phosphorylation of the ß-subunit of AP-2, which was shown previously to bind directly to ßarrestin (16). VSMC cells were stimulated for different periods of time with Ang II, and the endogenous ß-subunits of the AP proteins were immunoprecipitated using an antibody recognizing both the ß1-adaptin from AP-1 and the ß2-adaptin from AP-2. The level of tyrosine phosphorylation in the immunopurified ß-adaptin complexes was then analyzed by Western blot using an anti-phosphotyrosine antibody (Fig. 1*A*, *upper panel*). In conditions where dephosphorylation was prevented (using tyrosine phosphatase inhibitors), Ang II induced the phosphorylation of a 105 kDa protein in a time-dependent manner. Densitometric analysis (Fig. 1*A*, *Graph*) revealed that the phosphorylation of the 105 kDa protein increased after 5 min of Ang II stimulation, and reached a maximum after 15 minutes of agonist treatment. To confirm that the ß2-adaptin was a target for tyrosine phosphorylation, COS-7 cells were transfected with AT1R and Flag-tagged ß2-adaptin. The phosphorylation status of the immunopurified ß2-subunit was assessed using the same conditions as for VSMCs. Results show an increase in tyrosine phosphorylation of the ß2-subunit that was perceivable after 5 min of Ang II treatment (Fig. 1*B*, *top panel*), and reached a 4-fold increase after 15 min of agonist stimulation (Fig. 1*B*, *Graph*).

To demonstrate using a different approach that c-Src participates in the tyrosine phosphorylation of ß2-adaptin we first employed pharmacological inhibitors of tyrosine kinase activity. Phosphorylation was assessed following Ang II treatment in COS-7 cells transfected with AT1R and Flag-tagged ß2-adaptin; either treated with DMSO (vehicle), the c-Src kinase inhibitor (PP2), its inactive analogue (PP3), or the epidermal growth factor receptor (EGFR) inhibitor PD158780 (Fig. 1*C*). Results show that PP2 robustly inhibited, by more than 90%, the tyrosine phosphorylation of ß2-adaptin as compared to cells treated with DMSO. PD158780 and PP3 had no

significant inhibitory effects on the Ang II-mediated ß2-adaptin phosphorylation, suggesting that phosphorylation is not dependent on the transactivation of the EGFR.
We next used a dominant negative approach to assess the participation of c-Src in ß2-adaptin phosphorylation. COS-7 cells were transfected with AT1R, Flag-ß2-adaptin, and either full length wild type c-Src or its kinase inactive form, (c-Src-K298R). Cells were then left untreated (-) or treated (+) with Ang II for 5 min, and the level of ß2-adaptin phosphorylation in the immunoprecipitates was evaluated by Western blot (Fig. 1*D*). Results show that upon agonist addition, ß2-adaptin was robustly phosphorylated in cells overexpressing c-Src, whereas overexpressing the inactive kinase prevented the Ang II-mediated-phosphorylation of ß2-adaptin. Taken together, these results provide clear evidence for a role of c-Src in the phosphorylation of ß2-adaptin.

The tyrosine residues 737, 874 and 926 in the C-terminal domain of ß2-adaptin are putative c-Src phosphorylation sites. The Ang II-mediated phosphorylation of ß2-adaptin observed in VSMCs and COS-7 cells, and the recent demonstration that c-Src regulates the dissociation of ß2-adaptin from the AT1R/ßarrestin complex (15) prompted us to investigate whether this phosphorylation process regulates the formation of a complex between ßarrestin and AP-2. To address this possibility, we first sought to identify the putative phosphotyrosine residues within ß2-adaptin. We focussed on the C-terminal region of ß2-adaptin where ßarrestin has been shown to bind (17). The C-terminus of ß2-adaptin is constituted of two distinct interaction domains: the hinge and the ear (Fig. 2*A*). Therefore, we used the ß2-adaptin Hinge +Ear domain (592-937; ß2-ad (H+E)) and ß2-adaptin Ear domain (664-937; ß2-ad (E)), and assessed their ability to be phosphorylated by c-Src *in vitro*. To this end, HEK 293 cells were either transfected with HA-tagged ß2-ad (E) or HA-tagged ß2-ad (H+E), with or without c-Src overexpression. The ß2-adaptin constructs were then immunoprecipitated, and their tyrosine phosphorylation status was analyzed as described previously (Fig. 2*B*). Under conditions where c-Src was not overexpressed in cells (Fig. 2*B*, left panel), little tyrosine phosphorylation of the two ß2-adaptin C-

terminal constructs was observed. However, overexpressing c-Src resulted in a strong increase in the phosphorylation of both ß2-adaptin constructs. As shown in figure 3*A*, the Ear domain of ß2-adaptin contains 9 tyrosine residues in its sequence. Consequently, to determine which tyrosine(s) are the preferred residues for c-Src phosphorylation, we generated a series of GST fusion proteins each one of which contains a different 9 amino-acid region from ß2-adaptin which encompasses the different tyrosine residues (Table I and Fig. 3*A*). GST-fusion proteins were phosphorylated *in vitro* using purified c-Src, and their phosphorylation status was then analyzed by Western blot (Fig. 3*A*, lower panel). Results reveal that only three potential regions 733-741, 869-877 and 921-929 are substrates for c-Src-dependent phosphorylation. To verify that the Y737 and Y874 are phosphorylated, and to determine which tyrosine residue in the 921-929 sequence is phosphorylated, we used a site-directed mutagenesis approach. We first replaced the tyrosine residues by phenylalanines in the GST-733-741 (Y737F), -869-877 (Y874F) and -921-929 (Y926F) fusion peptides, and assessed their phosphorylation *in vitro* (Fig. 3*B*). In accordance with our previous finding (Fig. 3 *A*), an *in vitro* kinase assay reveals that the Y737 and Y874 are sites for c-Src phosphorylation. The substitution of the tyrosine Y926F alone in the 921-929 sequence was sufficient to prevent the phosphorylation of the peptide, suggesting that Y928 and Y931 are not preferred sites for c-Src kinase. Taken together our results suggest that Y737, Y874 and Y926 are all putative c-Src phosphorylation sites.

c-Src phosphorylation of Tyrosine 737 impairs the binding of ß2-adaptin to ßarrestin. We next examined the impact of c-Src-dependent phosphorylation of ß2-adaptin on its association with ßarrestins. To this end, GST-fusion proteins of the ß2-ad (E) were first phosphorylated *in vitro* with c-Src or left unphosphorylated (Fig.4*A*), and incubated with increasing amounts of cells lysates expressing either Flag-tagged ßarrestin 1 or 2. The amounts of ßarrestin associated with GST-fusion proteins were then analyzed by Western Blot (Fig. 4*B*). ßarrestin1 and 2 pull-down with ß2-ad (E) augmented proportionally when increasing amounts of ßarrestins were incubated with

the GST-fusion protein. However, when the ß2-ad (E) was first phosphorylated by c-Src before being incubated with ßarrestins, we observed a substantial reduction in pullo-down of both ßarrestin 1 and 2-associated proteins, as compared to conditions where GST-fusion proteins were left unphosphorylated (Fig 4*B*, compare right lanes with left lanes). These results imply that c-src-dependent phosphorylation of ß2-adaptin decreases its ability to bind ßarrestins.

We next used different GST-ß2-ad (E) fusion proteins in which we substituted individually the 3 putative tyrosine residues identified previously (Y737F, Y874F and Y926F), and determined *in vitro* their relative level of phosphorylation by Western blot before assessing their ability to bind ßarrestins (Fig. 5). Although the three GST-fusion proteins mutants were phosphorylated *in vitro*, we qualitatively observed a reduction in Src-dependent tyrosine phosphorylation of the GST-ß2-ad (E)-Y737F compared to the other two fusion proteins (Fig. 5*A*). The effect of c-Src-mediated tyrosine phosphorylation on the binding of ßarrestin with the three different GST-fusion mutants was next examined (Fig. 5*B*). Results show a clear reduction in the binding of ßarrestin 1 with both Src-phosphorylated GST-ß2-ad (E)-Y874F and -Y926F, similar to what we observed with phosphorylated wild type GST-ß2-ad (E) (Fig. 4). Similar results were also observed with ßarrestin 2 (data no shown). In marked contrast, phosphorylated GST-ß2-ad (E)-Y737F showed no decrease in the binding of ßarrestin 1, suggesting that ß2-ad (E)-Y737F no longer phosphorylated on Tyr737 is not able to dissociate from ßarrestin. Overall, our results demonstrate that Y737 is a critical residue for the c-Src-dependent regulation of ß2-adaptin binding with ßarrestins.

c-Src-mediated regulation of ßarrestin/AP-2 interaction requires the phosphorylation of ß2-adaptin-Y737 in living cells. We next investigated whether c-Src modulates the agonist-induced ß2-adaptin/ßarrestin interaction in living cells using a Bioluminescence Resonance Energy Transfer (BRET) assay. We assessed the transfer of energy between R-luc-ßarrestin2 and the full length YFP-tagged-ß2-adaptin in the absence or presence of c-Src overexpression as previously described (Hamdan *et al.,* submitted manuscript). HEK293 cells stably expressing AT1R-FLAG were

transfected with ßarrestin2-Rluc (the energy donor) and wild type ß2-adaptin-YFP (the energy acceptor), and stimulated with Ang II up to 20 min during which signals were recorded. Results show that Ang II induce a rapid increase in BRET-detected signal that reached a plateau after 5 min of receptor activation (Fig. 6*A*). However, the BRET signal following Ang II stimulation was substantially impaired when c-Src was overexpressed. To further substantiate the effect of c-Src on the interaction between ßarrestin and the full length ß2-adaptin, we used the phosphorylation-impaired ß2-adaptin-Y737F mutant, and compared its association with ßarrestin2 following Ang II stimulation (Fig. 6*B*). HEK 293 cells stably expressing the Flag-tagged AT1R were transfected with R-luc-ßarrestin2 and either the YFP-tagged ß2-adaptin wild type or the Y737F mutant, with or without c-Src overexpression. The BRET signal was measured after challenging the cells with different concentrations of Ang II for 20 minutes. As shown in Figure 6*B*, a dose dependent-increase in BRET reaching similar BRETmax was observed for the ß2-adaptin-YFP and the ß2-adaptin-Y737F-YFP (0.075 ± 0.07 and 0.073 ± 0.07, respectively). In contrast, the overexpression of c-Src decreased the BRETmax by more than 56 % for wild type ß2-adaptin, while for the ß2-adaptin-Y737F mutant the BRETmax slightly decreased by less than 22 % (Fig. 6*C*). These results support our *in vitro* studies showing that Y737F is a critical residue for the c-Src-dependent regulation of ß2-adaptin/ßarrestin interaction in cells.

ß2-adaptin-Y737F mutant delays the early steps of AT1R internalization. We next investigated, using confocal microscopy the ßarrestin/ ß2-adaptin trafficking during the early events of AT1R internalization, and evaluated the impact of the Y737F mutant. HEK 293 cells stably expressing the Flag-AT1R were transfected with RFP-tagged ßarrestin2 and either YFP-tagged-ß2-adaptin wild type or the –Y737F mutant. The redistribution of ßarrestin2-RFP and ß2-adaptin-YFP was visualized after Ang II treatment of cells at 37°C for the indicated time. In the absence of agonist, ßarrestin2 (*red*) was found in the cytosol, and ß2-adaptin-YFP (*yellow*) appears in punctuated structures (Fig. 7*A,* panel D and A respectively). Agonist treatment of cells for 2 min induced the rapid translocation of ßarrestin2 from the cytosol to the plasma membrane, which colocalized with ß2-adaptin at the plasma membrane and also in

endocytic vesicles (Fig. 7*A,* panels B and E). With a longer agonist exposure for 5 minutes, ßarr2-RFP was observed to redistribute from the plasma membrane to endocytic vesicles reflecting clathrin-mediated internalization (Fig. 7*A*, panel F) with some redistribution of ß2-adaptin fluorescence (Fig. 7*A*, panel C). Although we did not observed any major change in the ßarrestin/ß2-adaptin trafficking for the –Y737F mutant (Fig. 7*A*, panels G-L), the trafficking events were delayed. To evaluate the rate ßarrestin/ ß2-adaptin trafficking, we stimulated the cells with AngII at early time points (120s and 300s) and counted the number of cells showing colocalization between ßarrestin/ß2-adaptin at the plasma membrane for the indicated times in 12 different fields (Fig. 7*B*). Maximum colocalization between ßarrestin and ß2-adaptin at the plasma membrane was observed for the ß2-adaptin-Y737F mutant after 2 minutes of agonist stimulation (54% ± 6.4 and only 16.4% ± 1.2 for the wild-type ß2-adaptin). (Fig.7*A-B*, B and E as compared to H and K). Even after 5 minutes of agonist stimulation we still observed some ßarrestin/ß2-adaptin colocalization at the plasma membrane for the ß2-adaptin-Y737F (Fig.7*A-B*, C and F as compared to I and L). The higher impact of the ß2-adaptin-Y737F mutant on βarrestin trafficking is consistent with a role of c-Src-mediated ß2-adaptin phosphorylation in regulating the dissociation of AP-2 during the early events of receptor internalization.

DISCUSSION

Here we demonstrate that c-Src induces the phosphorylation of ß2-adaptin, to regulate the association between ßarrestin and AP-2. We identified the Tyr737 in the ear domain of ß2-adaptin as a critical c-Src phosphorylation site. We provide evidence, in cells, that the phosphorylation of Y737 alone can regulate the profile of agonist-mediated interaction between ßarrestin and AP-2. Our results suggest that the c-Src-mediated phosphorylation of AP-2 represents an important mechanism in controlling the ßarrestin-dependent internalization of receptors.

The involvement of Src family kinases during receptor internalization has been previously described for different GPCRs (10, 13, 18). Here, we show that the ß2-subunit of AP-2 is a target for tyrosine phosphorylation upon AT1R activation in either an endogenous expressing system such as VSMCs or heterologously expressing COS-7 cells. Using pharmacological inhibitors and dominant negative approaches, we selectively inhibit the c-Src-dependent phosphorylation of ß2-adaptin, suggesting a specific role for this kinase in the agonist-mediated phosphorylation of AP-2. The activation of c-Src was not dependent of the transactivation of EGFR. Interestingly, however, it seems to require the binding of c-Src to ßarrestin. Indeed, it was previously shown that blocking the binding of endogenous c-Src to ßarrestin affects beta2-adrenergic receptor internalization (19). This ßarrestin-mediated recruitment of tyrosine kinases and the phosphorylation of key endocytic proteins in the CCV are reminiscent of the role played by ßarrestin as a signalling scaffold in the phosphorylation of dynamin during the internalization of beta2-adrenergic receptor (19).

The identification of Tyr-737 in ß2-adaptin as a major target for c-Src kinase supports the role of tyrosine phosphorylation in the regulation of the interaction between ßarrestins and AP-2. Using BRET to assess the ßarrestin/AP-2 complex formation in live cells, we show that we could reduce the interaction between the two proteins by overexpressing c-Src. Our finding that c-Src still had some minor effect on

the binding between ßarrestin and the Y737F mutant, suggest that other components may participate in regulating this interaction. Alternatively, c-Src may also target other proteins in the complex to control the ßarrestin/AP-2 complex formation.

The phosphorylation of tyrosine residues by Src family kinases is known to be dependent on specific amino acid sequences and to create specific sites for protein-protein interactions (20). In this regard, it is interesting to note that the Y737 is highly conserved in ß2-adaptin from different species, and part of the YMXM consensus motif for binding of SH2 containing proteins (See Table 2) (21). The phosphorylation of Y737 in ß2-adaptin could promote the recruitment of interacting SH2 containing proteins to regulate the interaction between ßarrestin and AP-2. On the other hand, the phosphorylation of this critical residue in ß2-adaptin may promote the necessary change in the ß-subunit conformation to allow the uncoupling of ßarrestin from AP-2. This c-Src-dependent phosphorylation mechanism leading to the dissociation of binding partners was reported for other proteins such as N-cadherin; where the c-Src phosphorylation of N-cadherin was shown to reduce its binding to ß-catenin (22). Tyrosine phosphorylation of Alix by c-Src has also been reported to reduce the interaction of this adaptor protein with Pyk2, and to modulate EGFR internalization (23).

Protein phosphorylation is a well-recognized mechanism for coordinating CCV formation (5), but the actual involvement of tyrosine phosphorylation as a regulatory mechanism for receptor internalization is only now starting to be appreciated. Studies on the ß2-AR revealed that the activation of receptors induces the c-Src-dependent tyrosine phosphorylation of dynamin (13). On the other hand, work on the EGFR reveals that the stimulation of the receptor causes the tyrosine phosphorylation of the clathrin heavy chain, and regulates the internalization of the receptor (12). EGFR activation was also shown to promote the tyrosine phosphorylation of ß2-adaptin (24). This phosphorylation event seems to occur in the N-terminal domain of ß2-adaptin, although its role in receptor internalization remains unclear. The recent findings that c-Src can be recruited in a complex with ßarrestin and AP-2, and that it can

phosphorylate specific proteins of the coat like dynamin and AP-2 itself, is consistent with a signaling role of ßarrestin in regulating internalization of GPCRs (15, 19, 25).

In summary, our findings unveil an unappreciated mechanism to explain how the binding of the clathrin adaptor protein AP-2 to ßarrestin can be regulated during the AT1R internalization. We show that AT1R activation promotes the c-Src-mediated tyrosine phosphorylation of the ß-subunit of AP-2. The functional consequence of ß2-adaptin phosphorylation appears to control the binding of ßarrestin to AP-2. Thus, ßarrestins may act as signalling scaffolds to recruit c-Src, and to phosphorylate AP-2, leading to the dissociation of AP-2 from ßarrestin. This mechanism may represent an important step in controlling the stability of endocytic complexes during the internalization of GPCRs, and also extend the role of c-Src in controlling the phosphorylation status of different proteins of the CCV like the ß2-adaptin during the early steps of receptor internalization.

EXPERIMENTAL PROCEDURES

Materials – Angiotensin II was purchased from Sigma Chemical Co. The inhibitors PP2, PP3, and PD158780 were from Calbiochem-Novabiochem. Antibodies against ß-adaptin and α-adaptin were from BD Transduction Laboratories, the c-Src SRC2 and HA rabbit antibodies were from Santa Cruz Biotechnology, the mouse anti-HA clone 12CA5 was from Roche, the FLAG and the AP-1/2 antibodies were from Sigma-Aldrich, and the c-Src antibody GD11, the anti-phosphotyrosine antibody 4G10 and purified c-Src were from Upstate Biotechnology.

Plasmids and constructs – The wild type human AT1R and the Flag-tagged AT1R, the Flag-tagged ßarrestin1 and ßarrestin2, the Flag-tagged ß2-adaptin, the HA-tagged c-Src, the HA-tagged c-Src-K298R mutant and the wild type c-Src in pcDNA3 were described elsewhere (15, 16, 26). The HA-tagged constructs of the hinge and ear domain (residues 664-937; ß2-ad (H+E)) and ear domain alone of human ß2-adaptin (residues 664-937; ß2-ad (E)), and the GST-fusion protein of the ear domain (GST-ß2-ad (E)) were also described elsewhere (17). Glutathione S-transferase (GST) fusion proteins representing different nanopeptides within the ear domain of ß2-adaptin (see supplementary material, and table 1) and the tyrosine-to-phenylalanine versions (Y737F, Y874F, and Y926F) were generated by annealing a sense and antisense oligonucleotides (41mers) using standard molecular biology procedures. The double-stranded oligonucleotides sequences of the wild type and tyrosine mutants were design to create overhanging BamHI and XhoI sites, and ligated into pGEX-5X2 vector cut with the same restriction enzymes. The tyrosine mutants GST-ß2ad-Y737F, -Y874F were generated by polymerase chain reaction (PCR). Briefly, a first PCR fragment was amplified with a forward primer containing the BamHI restriction site upstream of the Valine 664 in ß2-adaptin and a reverse primer introducing a Kpn I restriction site for ß2-adaptin-Y737F construct or a Pst I restriction site for the ß2-adaptin-Y874F construct. The second PCR fragments were obtained using a forward primer containing the substituted residue and introducing either a silent mutation in position G727 to create a Kpn I site or at position L868 to create a Pst I site, and a reverse

primer overlapping the stop codon and introducing a Xho1 restriction site. These sets of PCR fragments were digested with BamHI/KpnI and Kpn1/XhoI, or BamHI/Pst1 and Pst1/XhoI, and then ligated into pGEX-5X2 previously digested with BamHI and Xho I. To generate the GST-ß2ad-Y926F, a PCR fragment was amplified using a forward primer containing a BamHI restriction site upstream of the V664 and a reverse primer containing the substituted residue Y926F and a Xho I restriction site after the stop codon. The PCR fragment was digested with BamHI and XhoI and cloned into pGEX-5X2 cut with the same enzymes. Flag-tagged ß2-adaptin Y737F was generated by PCR using a forward primer containing the tyrosine substitution and overlapping the existing BspE1 restriction site, and a reverse primer overlapping the existing KpnI site in ß2-adaptin. The PCR fragment was then inserted into Flag-ß2-adaptin wild type previously digested with BspE1 and KpnI restriction enzymes.

The yellow-fluorescent-tagged ß2-adaptin (ß2-adaptin-YFP) was also generated by three-way ligation. Briefly, a PCR fragment was amplified with a forward primer overlapping the existing Cla I restriction site in ß2-adaptin, and a reverse primer removing the stop codon in ß2-adaptin, and introducing a Sal I restriction site. The second fragment was obtained from the digestion of wild type ß2-adaptin in pcDNA3.1 with Nhe I and Cla I restriction enzymes. The two fragments were insert into pEYFP-N1 previously digested with Nhe I and Sal I. To generate the YFP-tagged ß2-adaptin Y737F mutant, a BspE1 and KpnI fragment from the Flag-tagged ß2-adaptin Y737F was ligated into YFP-tagged ß2-adaptin cut with the same restriction site. The *Renilla reniformis* luciferase-tagged-ßarrestin2 (Rluc-ßarrestin2) construct was described elsewhere (Hamdan and Bouvier). All constructs were confirmed by DNA sequencing (Sequencing Service, Genome Quebec Innovation Centre, McGill University, QC, Canada).

Cell Culture and Transfection – Human embryonic kidney cells (HEK 293) were grown in Eagle's minimal essential medium (MEM, Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco) and gentamicin (100 µg/ml, Gibco). HEK 293 cells seeded in 100 mm dishes, at a density of $2.5x10^6$ cells/dish, were transiently transfected using a conventional calcium phosphate co-precipitation

method as previously described (15). One to five µg of DNA were mixed in a solution containing 125 mM $CaCl_2$ in HEPES-Buffered Saline (25 mM HEPES pH 7.4, 140 mM NaCl, 0.75 mM Na_2HPO_4) for 5 min, and the mixed solution was then added to cells. Experiments were performed 36 h after transfection. Green monkey kidney cells (COS-7) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (v/v) heat-inactivated FBS and gentamicin (100 µg/ml). Transient transfections of COS-7 cells seeded in 60 mm dishes (at a density of $1x10^6$ cells/dish) were 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 h after transfection. Vascular smooth muscle cells (VSMCs) were grown in DMEM with 10% (v/v) heat-inactivated calf serum (Gibco) and gentamicin (100 µg/ml). Cells were serum-starved overnight in DMEM before performing the experiments.

Immunoprecipitation and Western blot experiments – HEK 293 or COS-7 cells were left untreated or treated with Ang II (100 nM) at 37°C for the indicated period of time. Following agonist stimulation, the reaction was stopped on ice by washing cells with ice-cold Phosphate-buffered saline (PBS). Cells were then solubilized in P-RIPA buffer (1 ml/100 mm dish) (20 mM TrisHCl pH 7.4, 150 mM NaCl, 1% Nonidet-P40 (v/v), 0.1% deoxycholate (w/v), 1 mM $CaCl_2$), containing 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml leupeptin, 25 µg/ml aprotinin, and 1 mM pepstatin A, for 30 min at 4°C. Cell lysates were centrifuged for 30 min at 21,000 x *g*, and the supernatants were incubated with anti-Flag (30 µg/ml, Sigma) or anti-HA (20 µg/ml, Roche) conjugated beads, or ß-adaptin antibody AP-1/2 (8 µg/ml, AP-1/2, Sigma), with 20 µl of a 50% slurry mixture of protein A/G Sepharose beads for 2 h at 4°C. Beads were washed three times with P-RIPA, denatured in Laemmli buffer (2X) (250 mM Tris-HCl pH 6.8, 2% SDS (w/v), 10% Glycerol (v/v), 0.01% Bromophenol Blue (w/v), 5% ß-mercaptoethanol (v/v)) and incubated for 10 min at 65°C. Proteins were separated on a 10% SDS-polyacrylamide gel (SDS-PAGE) before being transferred onto nitrocellulose membranes (Amersham Biosciences). Membranes were then blocked with a solution of PBS pH 7.4 containing

0.05% Tween20 (v/v) (PBS-T) and 10% dry milk (w/v). Protein detection on membranes was assessed using either anti-c-Src antibody (1 µg/ml, clone GD11; or 0.2 µg/ml, SRC2), Flag antibody (2.5 µg/ml), HA antibody (0.2 µg/ml), phosphotyrosine antibody (0.2 µg/ml, clone 4G10), ß2-adaptin antibody (0.2 µg/ml, ß-adaptin, BD Transduction Laboratories), α-adaptin antibody (0.25 µg/ml). After 1 h incubation with the different primary antibodies, membranes were washed three times before being incubated at room temperature with horseradish peroxidase (HRP) conjugated goat anti-mouse antibody (diluted 1/10,000; Sigma), or HRP-goat anti-rabbit antibody (diluted 1/10,000; Sigma) for 30 min. Immunoreactivity was visualized by enhanced chemiluminescence according to the manufacter's instructions (SuperSignal, Pierce).

For phosphoprotein detection experiments, COS-7 cells or VSMCs were serum-starved overnight, and the next day incubated for 30 min at 37°C in DMEM buffered with 20 mM HEPES pH 7.4. Cells were then pretreated with 20 mM pervanadate for 10 min before adding Ang II (1 µM) for the indicated period of time. Stimulation was stopped on ice with cold PBS, and cells were solubilized for 30 min at 4°C 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 inhibitors (0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml leupeptine, 25 µg/ml aprotinin, 1 mM pepstatin A). Lysates were then centrifuged at 21,000 x *g* for 30 min, and the supernatants were incubated for 1 h at 4°C with the anti-Flag conjugated beads (30 µg/ml, Sigma), or with the ß-adaptin antibody AP-1/2 (8 µg/ml, Sigma) with 20 µl of a 50% slurry mixture of protein A/G Sepharose beads (Roche) for 2 h at 4°C. Beads were then washed three times in TGH buffer, denatured in Laemmli buffer (2X) and incubated for 10 min at 65°C. Western blots were performed as described above.

GST Fusion Protein Expression and Pull Down assay – For GST generation, the pGEX-5X2 vector bearing the different constructs were transformed into *Escherichia coli* BL21 cells and grown in LB medium with Ampicillin (100 µg/ml) at 37°C overnight. The next day, cell cultures were diluted to an OD_{600} of 0.2 in LB with Ampicillin and grown until an OD_{600} between 0.5 and 0.6. Protein production was then induced using 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3 h at

room temperature. Cells were then pelleted, washed once with PBS and resuspended in cold PBS containing 2 mM EDTA, 4 mM PMSF, 1% Triton X-100 (v/v) supplemented with 1 mg/ml lysozyme and incubated 30 min on ice. Cells were then sonicated and centrifuged at 21 000x*g* for 30 min. Glutathione-Sepharose 4B beads were added to the supernatant and agitated for 1h at 4°C. Beads were then washed three times with PBS. Protein concentration was determined using a DC protein assay kit (Bio-Rad) and the integrity of the GST fusion proteins was analyzed by SDS-PAGE and Coomassie blue staining.

In vitro Src kinase assays – For Src kinase in vitro phosphorylation assays, GST-fusion proteins were first washed twice with the kinase reaction buffer (100 mM Tris-HCl pH 7.2, 25 mM Manganese Chloride, 125 mM Magnesium Chloride, 2 mM EDTA), before being resuspended in 50 µl of the same kinase buffer supplemented with 10 mM ATP, 0.25 mM sodium orthovanadate, 2 mM DTT and 1U of purified c-Src. Proteins were incubated for 20 min at 30°C, and the reaction was stopped either washing the beads with cold TGH buffer, or by adding an equal amount of Laemmli buffer (2X) and incubated for 10 min at 65°C. The phosphorylation status of the proteins was analyzed by Western Blot using a phosphotyrosine antibody as previously described. To assess the effect of ß2-adaptin tyrosine phosphorylation on ßarrestin binding, c-Src from the kinase reaction was first remove by washing the beads 3 times with cold TGH buffer containing inhibitors. Similarly the unphosphorylated control GST-fusion proteins were washed with 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-tagged ßarrestin1 or Flag-tagged ßarrestin2 for 1h at 4°C. Beads were spun and washed three times with cold TGH buffer before being re-suspended in Laemmli buffer (2X), and then incubated for 10 min at 65°C. Proteins were resolved by electrophoresis on a 10% SDS-PAGE gel and analyzed by Western blot for ßarrestin detection or stained with Coomassie Blue for GST-protein analysis.

Monitoring interaction between ßarrestin-Rluc and ß2-adaptin *by BRET*^{1}- HEK293 cells stably expressing Flag-tagged AT1R were co-transfected in 6-well plates with 1 µg of ß2-adaptin-YFP and 10 - 60 ng of ßarrestin2-Rluc, in the presence or absence of 1µg of c-Src plasmid. Approximately 18 hrs later, cells were detached with trypsin, diluted in 2 ml culture medium, and seeded (~ 50,000 cells /well) into 96-well plates (white wall, clear bottom) tissue culture plates that had been treated with poly-D-lysine. After 18 hrs, the medium was replaced with 90 µl of PBS containing 0.5 $MgCl_2$ and incubated with or without various concentrations of the agonist for the indicated time at room temperature. To measure the BRET^1 signal, the transparent bottom of the 96-well plate was covered with a white back-tape adhesive (Perkin Elmer) and the BRET^1 substrate for Rluc, coelenterazine-*h* (nanolight), was added to all the wells (5 µM final concentration), followed by BRET^1 measurement on 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 EYFP light emissions, respectively. In the case of kinetic measurements, coelenterazine-*h* was added simultaneously with the agonist followed by BRET kinetic measurements. The BRET^1 signal was calculated as a ratio of the light emitted by EYFP (530 ± 20 nm) to the light emitted by Rluc (480 ± 20 nm) upon adding the coelenterazine-*h* substrate. Agonist promoted BRET^1 was calculated by subtracting the BRET^1 ratio obtained in the absence of agonist addition from the BRET^1 signal obtained in the presence of an agonist.

Immunofluorescence Experiments – HEK 293 cells stably expressing the AT1-Flag were grown on 22 mm^2 cover slips in 6-well plates (250,000 cells/well), and transfected with YFP-ß2-adaptin (0.5µg) and RFP-ßarrestin2 (0.5 µg). Cells were stimulated at 37°C with 100 nM Ang II for the indicated time, and fixed for 15 min at room temperature with a solution of 4% paraformaldehyde (w/v) in PBS. Cover slips were mounted with GelTol Aqueous Mounting Medium (IMMUNON). Confocal images of labeled cells were acquired with a Zeiss LSM-510 META laser-scanning microscope using a 60x oil immersion lens. YFP-ß2-adaptin and RFP- ßarrestin2 fluorescence was visualized using the multi-track mode with dual laser excitation (514

and 543 nm), and emission (BP 530-600 for YFP, and LP 560 for AlexaFluor 568) filter sets. Final figures were collated using Adobe Photoshop 7.0 and Illustrator 10.0 (Adobe, San Jose, CA).

Data Analysis- Intensity of the signals from Western Blots was determined by densitometric analysis using the MetaMorph Software (Universal Imaging Corporation), and are represented as the mean ± S.E.M of at least three experiments. For densitometry analysis of Western blot data, were analyzed statistically by one-way ANOVA followed by a Bonferroni test for multiple comparisons. Means were considered significantly different when p values were at least below 0.05.

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FIGURE LEGENDS

Fig. 1. Ang II induces tyrosine phosphorylation of ß2-adaptin. *A,* VSMCs were serum-starved overnight and then pretreated with pervanadate for 10 min before being stimulated with Ang II (100 nM) for the indicated time. The endogenous ß-adaptins were immunoprecipitated using an anti-ß-adaptin antibody, and the immunoprecipitates were analyzed by Western blot (WB) using the anti-phosphotyrosine 4G10 (upper panel), and the anti-ß-adaptin (bottom panel) antibodies. *B-C,* COS-7 cells were transiently transfected with HA-AT1R and Flag-ß2-adaptin. Twenty-four hours after transfection cells were serum-starved overnight, and then pretreated with pervanadate for 10 min before being stimulated with Ang II (100 nM) for the indicated time. Flag-ß2-adaptin were immunoprecipitated (IP) from cell lysates using an anti-Flag antibody, and the immunoprecipitates were analyzed as described in (*A*). *C,* COS-7 cells were first treated as described above, and then incubated for 30 min with either the vehicle (DMSO) or the c-Src inhibitor (PP2, 4 µM) or the EGFR inhibitor (PD158780, 50 nM) or the less active form of PP2 (PP3, 4 µM), before adding Ang II (100 nM) for 15 min. Densitometry analysis was carried out as described in *Experimental Procedures*, and data are presented as the mean ± S.E.M of three independent experiments. They represent the relative amount of ß-adaptin phosphorylated as compared to non-stimulated cells (*A, B*), or the percent of ß2-adaptin phosphorylation as compared to vehicle (*C*), after normalizing for equal amount of ß-adaptin present in the immunoprecipitates. *D,* COS-7 cells were transiently transfected with HA-AT1R, Flag-ß2-adaptin and either c-Src wild type or c-Src-K298R (kinase defective mutant). Twenty-four hours after transfection cells were serum starved overnight, and then pretreated as described in *A-C*, before being stimulated for 5 min with Ang II (100 nM). Detection of the ß2-adaptin in the immunoprecipitate and its phosphorylation status were performed as described in *A-B*. ** indicates $p<0.01$ *vs* DMSO determined by one-way ANOVA, and was considered significant.

Fig. 1 Fessart et al

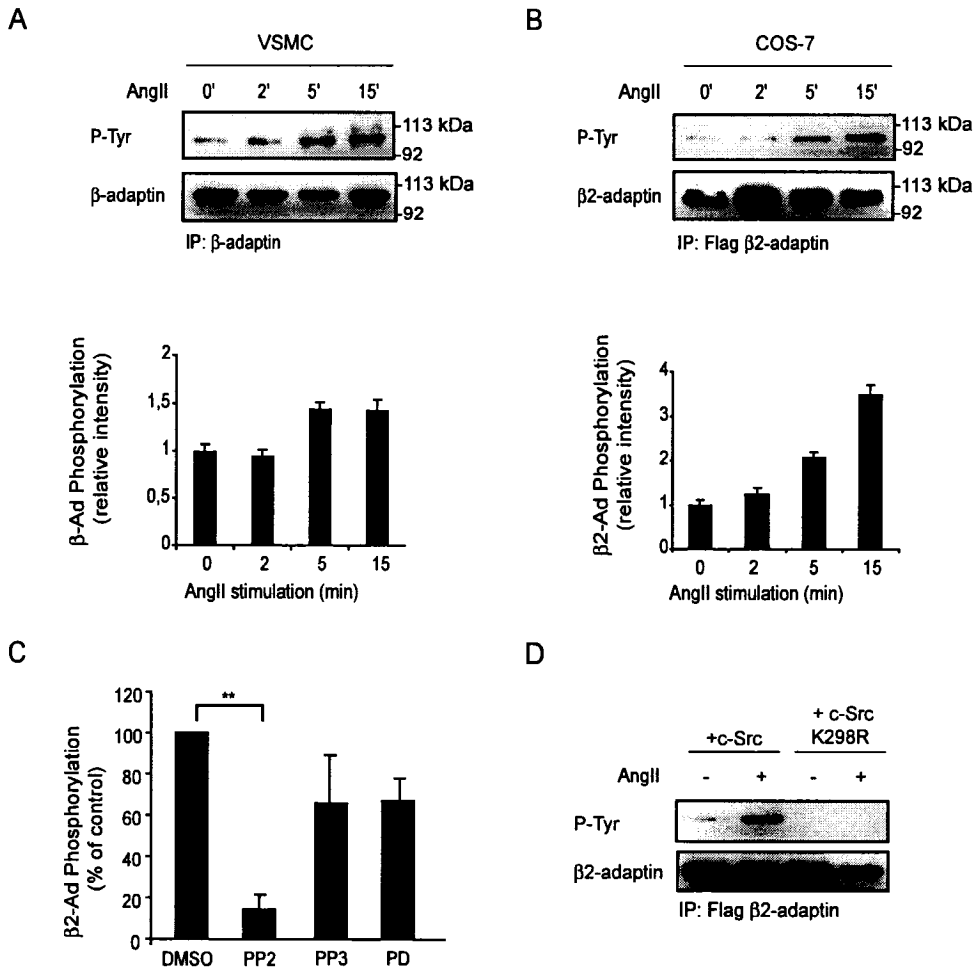

Fig. 2. Tyrosine Phosphorylation of the ear domain of β2-adaptin. *A,* Representation of the different domains of ß2-adaptin with the nine putative tyrosine phosphorylation sites between the 664-937 amino acid residues. *B*, HEK 293 cells were transfected with the empty vector pcDNA3.1 (Mock) or HA-ß2-adaptin constructs of the Ear (ß2-ad (E)) or the Hinge + Ear domains of ß2-adaptin (ß2-ad (H+E)), with or without c-Src. HA-tagged ß2-adaptin constructs were immunoprecipitated (IP) using the anti-HA conjugated beads antibody, and their tyrosine phosphorylation status was detected by Western Blot (WB) using an antibody against phosphotyrosine residues (P-Tyr). Data are representative of at least three independent experiments.

Fig. 2 Fessart *et al*

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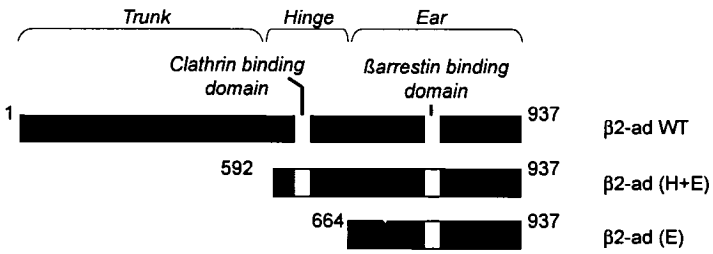

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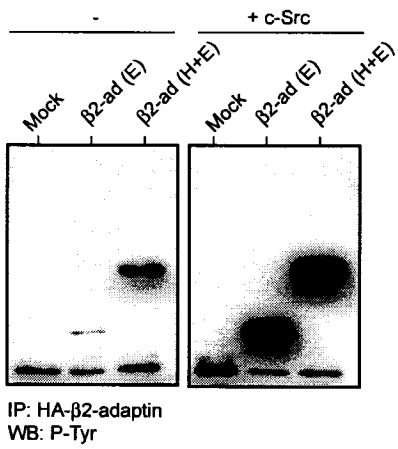

Fig. 3. ß2-adaptin Tyrosines 737, 874 and 926 are sufficient for phosphorylation of ß2-adaptin by c-Src. *A,* Recombinant fusion proteins of 9 amino-acids encoding various portions of the Ear domain of ß2-adaptin (residues 704-712, 733-741, 811-819, 869-877, 884-892, 907-915, 921-929 and 927-933; detailed in Table I) or GST alone were subjected to *in vitro* phosphorylation by purified c-Src kinase. The amounts of GST and GST- ß2-adaptin fusion proteins were assessed by Ponceau Red. c-Src-phosphorylated proteins were detected by Western Blot (WB) using an antibody against phosphotyrosine residues (P-Tyr). *B, In vitro* phosphorylation by c-Src of GST-fusion proteins containing residues 733-741, 869-877, 921-929 and point-mutation mutants where the tyrosine (Y) was replaced by a phenylalanine (F) residue to obtain Y737F , Y874F and Y926F mutants, respectively. The amounts of GST and GST- ß2-adaptin fusion proteins were assessed by Ponceau Red. c-Src-phosphorylated proteins were detected by WB using an antibody against phosphotyrosine residues (P-Tyr). Note that the Y737F, Y874F and Y926F mutants do not undergo phosphorylation. Data are representative of at least three independent experiments.

Fig. 3 Fessart *et al*

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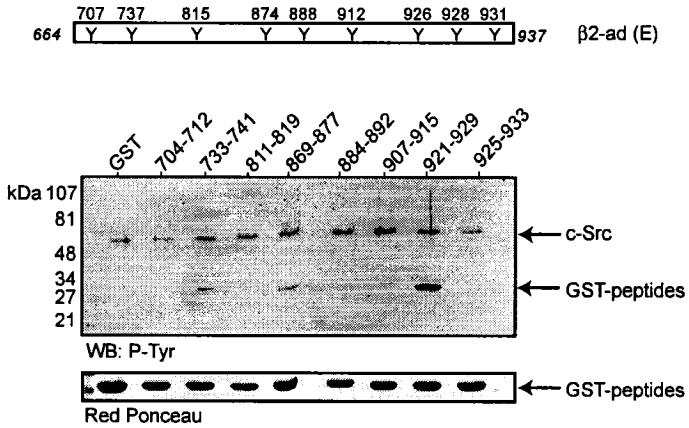

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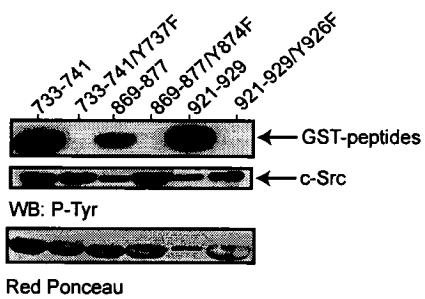

Fig. 4. c-Src phosphorylation of ß2-adaptin reduces its association with ßarrestin 1,2. *A,* GST-fusion proteins of the Ear domain of ß2-adaptin (ß2-ad (E)) were phosphorylated *in vitro* with purified c-Src (+) or left unphosphorylated (-).The amounts of GST- ß2-adaptin fusion proteins was assessed by Ponceau Red and c-Src-phosphorylated proteins were detected by Western Blot (WB) using an antibody against phosphotyrosine residues (P-Tyr). *B,* After removing c-Src from the reaction, the unphosphorylated (-Tyr) or phosphorylated GST-fusion proteins (+Tyr) were incubated with increasing amounts of Flag-tagged ßarrestin 1 or 2. The amount of ßarrestin associated with the GST-fusion proteins was determined by WB using an anti-Flag antibody. Whole cell extracts (Total, right panels) were also blotted for detecting the level of Flag-ßarrestin 1 or 2 expression using the anti-Flag antibody. The amounts of GST- ß2-adaptin fusion proteins were assessed by Ponceau Red. Data are representative of at least three independent experiments.

Fig. 4 Fessart *et al*

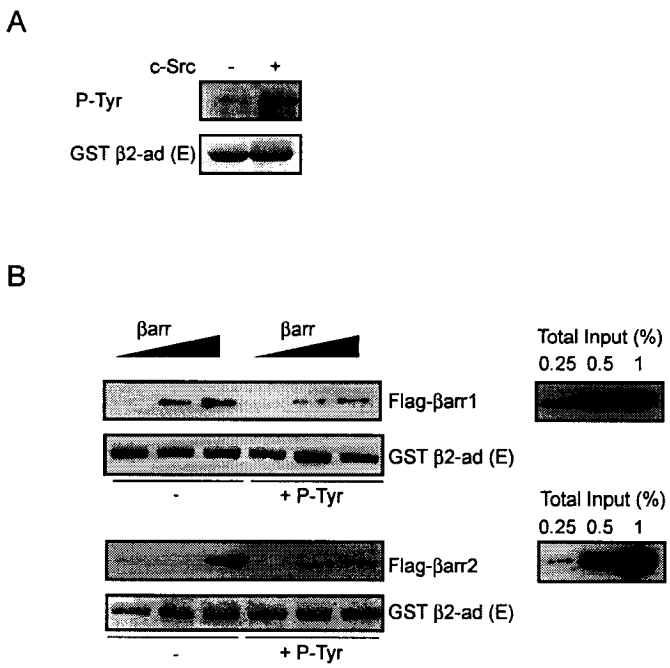

Fig. 5. ß2-adaptin Y737F mutant abrogates the c-Src-induced dissociation of ßarrestin 1. *A,* GST-fusion proteins of the Ear domain of ß2-adaptin single mutants (ß2-ad (E) Y737F, ß2-ad (E) Y874F and ß2-ad (E) Y926F) were phosphorylated *in vitro* with purified c-Src (+) or left unphosphorylated (-). The amount of GST- ß2-adaptin fusion proteins was assessed by Ponceau Red and c-Src-phosphorylated proteins were detected by Western Blot (WB) using an antibody against phosphotyrosine residues (P-Tyr). *B,* After removing c-Src from the reaction, the unphosphorylated (-Tyr) or phosphorylated GST-fusion proteins (+Tyr) were incubated with increasing amounts of Flag-tagged ßarrestin1. The amounts of ßarrestin associated with the GST-fusion proteins were determined by WB using the anti-Flag and GST antibodies respectively. *C,* Densitometry analysis was carried out as described in *Experimental Procedures*, and data are presented as the mean ± S.E.M of three independent experiments. They represent the relative amount of ßarrestin associated to GST-ß2-adaptin after normalizing for equal amount of GST-ß-adaptin present in the pull down. Data are representative of three independent experiments.

Fig. 5 Fessart *et al*

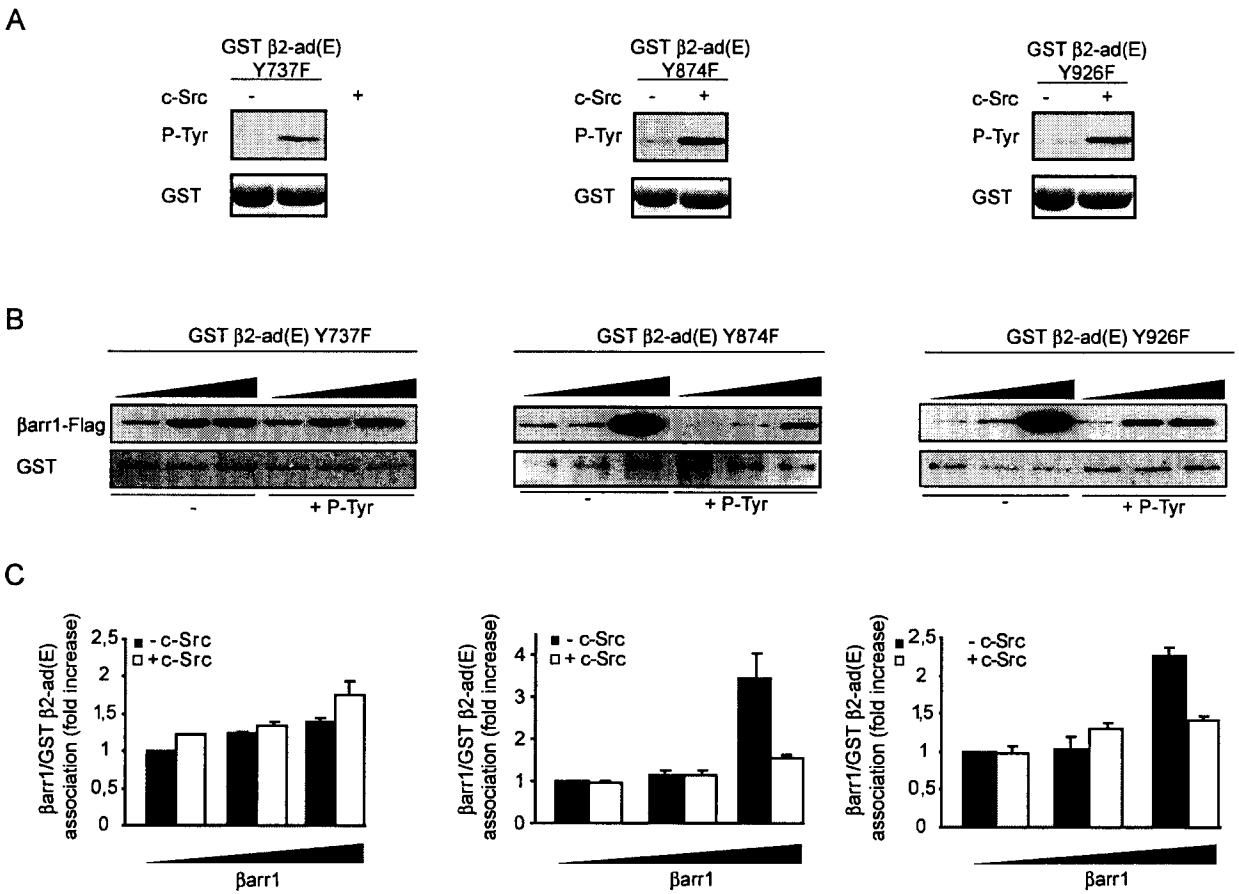

Fig. 6. Agonist-promoted interaction between ß2-adaptin and ßarrestin2 as measured by BRET. *A,* Association kinetics between ß2-adaptin-EYFP and ß-arrestin2-Rluc. HEK293 cells stably expressing Flag-AT1R were co-transfected with ßarrestin2-Rluc and ß2-adaptin-YFP in the absence (-) or presence of c-Src (+) and $BRET^1$ measurements were done as described in *Experimental Procedures*. Real-time $BRET^1$ measurements were taken at regular intervals for the indicated time period directly after the addition of coelenterazine-h and receptor stimulation with Ang II. *B,* Dose-dependent agonist-promoted BRET. HEK293 cells stably expressing Flag-AT1R were co-transfected with ßarrestin2-Rluc and either ß2-adaptin-YFP wild type or -Y737F mutant, in absence (-) or presence of c-Src (+). AT1R was stimulated with increasing concentrations of Ang II for ~ 20 min at room temperature followed by $BRET^1$ measurements. *C,* Agonist-promoted BRETmax between either ß2-adaptin-Y737F-YFP or wilt type ß2-adaptin-YFP and ß-arrestin2-Rluc. HEK293 cells stably expressing Flag-AT1R were co-transfected with ßarrestin2-Rluc and ß2-adaptin in absence (-) or presence of c-Src (+) and $BRET^1$ measurements were done as described in *Experimental Procedures*. AT1R was stimulated as described in *B*. Data are pools of 3-5 independent experiments.

Fig. 6 Fessart *et al*

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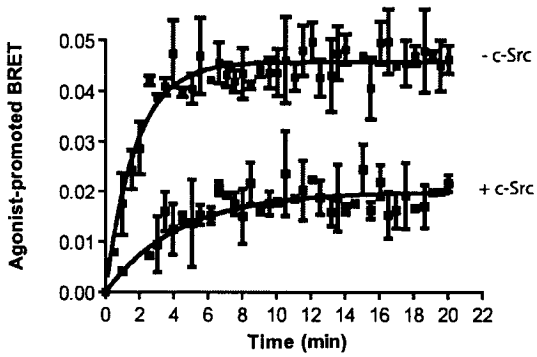

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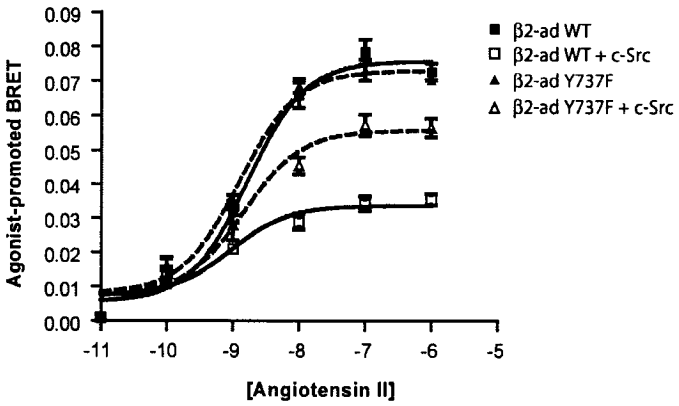

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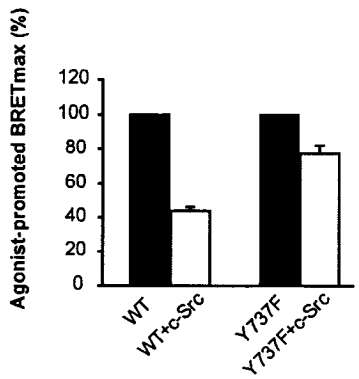

Fig. 7. Effect of the Y737F on the ßarrestin/ß2-adaptin trafficking upon AngII stimulation in cells. *A,* HEK 293 cells expressing the Flag-tagged AT1R were transfected with RFP-ßarrestin2 and either YFP-ß2-adaptin or –Y737F mutant. Cells were either left untreated or treated with Ang II (1 µM) at 37°C for the indicated times. The distribution of RFP-ßarrestin2 (upper panels D-F) and YFP-ß2-adaptin (lower panels A-C) was compared with the RFP-ßarrestin2 (J-L) in presence of the YFP-ß2-adaptin-Y737F mutant (G-I) and the fluorescence was visualized by Confocal microscopy. Shown are representative images of three independent experiments. *B,* Representation of the percentage of cells where a colocalization at the plasma membrane between ßarrestin and ß2-adaptin was observed. Randomly 12 different fields per time point were analyzed, with a total of about 30 to 50 cells per condition in 3 independent experiments. ** $p<0.01$ determined by one-way ANOVA, and was considered highly significant.

Fig.7 Fessart et al

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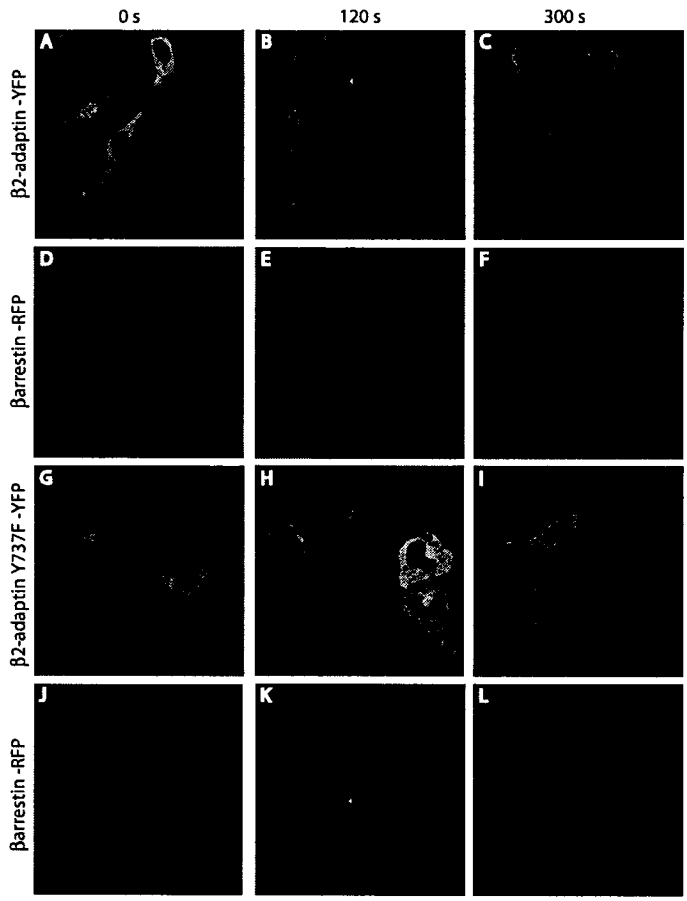

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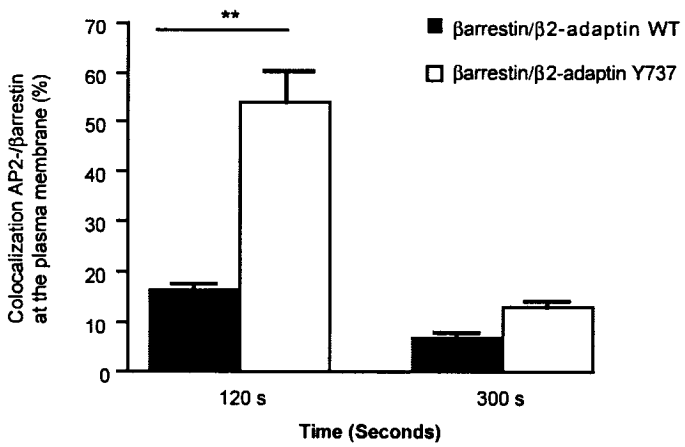

Table 1. *Sequences of the GST fusion proteins of 10 amino-acids region in β2-adaptin*

664VGOSFIPSSVPATFAPSPTPAVVSSGLNDLFELSTGIGMAPGG**Y**VAPKAVWLPAV
KAKGLEISGTFTHRQGHI**Y**MEMNFTNKALQHMTDFAIQFNKNSFGVIPSTPLAIHT
PLMPNQSIDVSLPLNTLGPVMKMEPLNNLQVAVKNNIDVF**Y**FSCLIPLNVLFVEDG
KMERQVFLATWKDIPNENELQFQIKECHLNADTVSSKLQNNNV**Y**TIAKRNVEGQ
DML**Y**QSLKLTNGIWILAELRIQPGNPN**Y**TLSLKCRAPEVSQ**Y**I**Y**QV**Y**DSILKN

β2-adaptin residues	Sequence
704-712	PGGY^{707}VAPKA
733-741	QGHIY^{737}MEMN
811-819	IDVFY^{815}FSCL
869-877	QNNNVY^{874}TIA
884-892	QDMLY^{888}QSLK
907-915	PGNPNY^{912}TLS
921-929	PEVSQY^{926}IY^{928}Q
925-933	QY^{926}IY^{928}QVY^{931}DS

Table 2. *The Ear region of β2-adaptin (664-937) is highly conserved among the species.*

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Mus musculus              VGQSFIPSSVPATFAPSPTPAVVSSGLNDLFELSTGIGMAPGGYVAPKAVWLPAVKAKGLEISGTFTHRQ GHIYMEMNFTNKALQHMTDFAIQFNKNSFG
Homo sapiens1             VGQSFIPSSVPATFAPSPTPAVVSSGLNDLFELSTGIGMAPGGYVAPKAVWLPAVKAKGLEISGTFTHRQ GHIYMEMNFTNKALQHMTDFAIQFNKNSFG
Gallus gallus             VGQTYIPSSVPATFAPSPTPAVVSSGLNDLFELSSGIGMAPGGYVAPKSVWLPAVKAKGLEISGTFSHRQGHIYMEMNFTNKALQHMTDFAIQFNKNSFG
Rattus norvegicus           GQSFIPSSVPATFAPSPTPAVVSSGLNDLFELSTGIGMAPGGYVAPKAVWLPAVKAKGLEISGTFTHRQGHIYMEMNFTNKALQHMTDFAIQFNKNSFG
Xenopus laevis            VGQTFIPSSVPATFAPSPTPAVVSSGLNDLFELSSGIGMAPGGYVAPKAVWLPAVKAKGLEISGTFSHRQ SHIYMDMTFTNKALQHMTDFAIQFNKNSFG
Danio rerio (zebrafish)   VGQNFIPSSVPNTFAPSPTPAALSSGLNDLFELSTGMATATGGYVAAKTVWLPAVKAKGLEISGTFSRRQGMYMDMTFTNKALQHMTDFAIQFNKNSFG

Mus musculus              VIPSTPLAIHTPLMPNQSIDVSLPLNTLGPVMKMEPLNNLQVAVKNNIDVFYFSCLIPLNVLFVEDGKMERQVFLATWKDIPNENELQFQIKECHLNADTVS
Homo sapiens1             VIPSTPLAIHTPLMPNQSIDVSLPLNTLGPVMKMEPLNNLQVAVKNNIDVFYFSCLIPLNVLFVEDGKMERQVFLATWKDIPNENELQFQIKECHLNADTVS
Gallus gallus             VIPSTPLAIHTPLMPNQSIDVSLPLNTLGPVMKMEPLNNLQVAVKNNIDVFYFSCLIPLNVLFVEDGKMERQVFLATWKDIPNENELQFQIKDCHLNADTVS
Rattus norvegicus         VIPSTPLAIHTPLMSNQSIDVSLPLNTLGPLKMEPLNNLQVAVKNNIDVFYFSCLIPLNVLFVEDGKMERQVFLATWKDIPNENELQFQIKECHLNADTVS
Xenopus laevis            VIPSAPLAIHTPLMPNQSIEI SLPLNTLGPVMKMEPLNNLQVAVKNNIDVFYFSCLIPLHVLFVEDGKMERQVFLATWKDIPNENELQFQIKDCHLNADTVS
Danio rerio (zebrafish)   VIPTTPLPVHTPLMPSQSIDISLPLNTIGPVMKMDPLNNLQVAVKNNIDVFYFSTLIPLNVFFVEDGKMERQVFLATWKDIPNENELQYQIKDCHLNADTVS

Mus musculus              SKLQNNNVYTIAKRNVEGQDMLYQSLKLTNGIWILAELRIQPGNPNYTLSLKCRAPEVSQYI YQVYDSILKN
Homo sapiens1             SKLQNNNVYTIAKRNVEGQDMLYQSLKLTNGIWILAELRIQPGNPNYTLSLKCRAPEVSQYI YQVYDSILKN
Gallus gallus             SKLQNNNVYTIAKRNVEGQDMLYQSLKLTNGIWILAELRIQPGNPNYTLSLKCRAPEVSQYI YQAYDAILKN
Rattus norvegicus         SKLQNNNVYTIAKRNVEGQDMLYQSLKLTNGIWILAELRIQPGNPNYTLSLKCRAPEVSQYI YQVYDSILKN
Xenopus laevis            SKLQNNNVYTIAKRNVEGQDMLYQSLKLTNGIWILAELRIQPGNPNYTLSLKCRAPEVCNYVYQVYDSILKN
Danio rerio (zebrafish)   GKLQSNNVYTIAKRNVEGQDMLYQSLKLTNGIWILAELRIQPGNPNYTLSLKCRAPEVSQYVYQMYDATLKN
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CONNECTING TEXT

The aim of this thesis was to describe the intermolecular mechanisms regulating the early events of GPCR internalization with one particular receptor, the Angiotensin II type 1 receptor. This Section V serves to address the aforementioned results and compare them to the actual literature. Proposed future directions pertaining to some specific points will also be discussed.

SECTION V

Discussion and Conclusions

In general, in receptor-mediated endocytosis, the agonist (A) is bound to receptors at the cell surface (see Figure 1, step 1). Once activated, receptors associate with a network of regulatory proteins (such as GRK and βarrestin) via their cytoplasmic domains. These proteins, in turn, directly or indirectly bind to clathrin, which forms a cage-like curved pit (Clathrin-coated pit, CCP) at the plasma membrane (step 2). As the coat continues to grow, the pit deepens until it pinches off as a closed vesicle (Clathrin-coated vesicles, CCV) (step 3). Following internalization, the clathrin coat disassembles (step 4) and the vesicle fuses with other newly uncoated vesicles or with previously internalized membranes to form early endosomes. In the early endosome, some agonists are released from their receptors and these endosomes go back to the plasma membrane, recycling GPCRs to the cell surface (step 5). Some receptors, however, interact with a second set of cytosolic proteins that retain them, creating a multivesicular endosome. This internal vesicle will ultimately be delivered to lysosomes for degradation (step 6). Even though several groups have studied GPCR internalization via clathrin-coated vesicles, a clear picture of all the proteins involved in the mechanisms that regulate GPCR internalization still remains elusive. The work accomplished in this thesis describes some of the intermolecular mechanisms regulating the early events of AT1R internalization leading to the formation of CCPs. First, βarrestins play a well-established role in the termination of G protein coupling and receptor signalling. GPCR phosphorylation by GRK and βarrestin binding are central to the processes of homologous desensitization, sequestration, recycling and down regulation of most mammalian GPCRs. Moreover, βarrestins are also now being accepted as GPCR signal transducers. The initial evidence suggesting that βarrestins function as signal transducers was the observation that βarrestin 1 and βarrestin 2 can bind directly to Src and recruit it to agonist-occupied GPCRs (96, 97, 171). Furthermore, stimulation of β2 adrenergic receptors results in co-localization of the receptor with both βarrestins and Src in CCPs (97). Similar results have been observed with other GPCRs, where βarrestins are involved in recruiting Src to the neurokinin NK-1 receptor (262), and to the CXCR-1 chemokine receptor (111). Our findings reveal that, another receptor, the AT1R (171) also induced the association of c-Src with βarrestin. In addition to Src, we found that βarrestin recruits and binds the

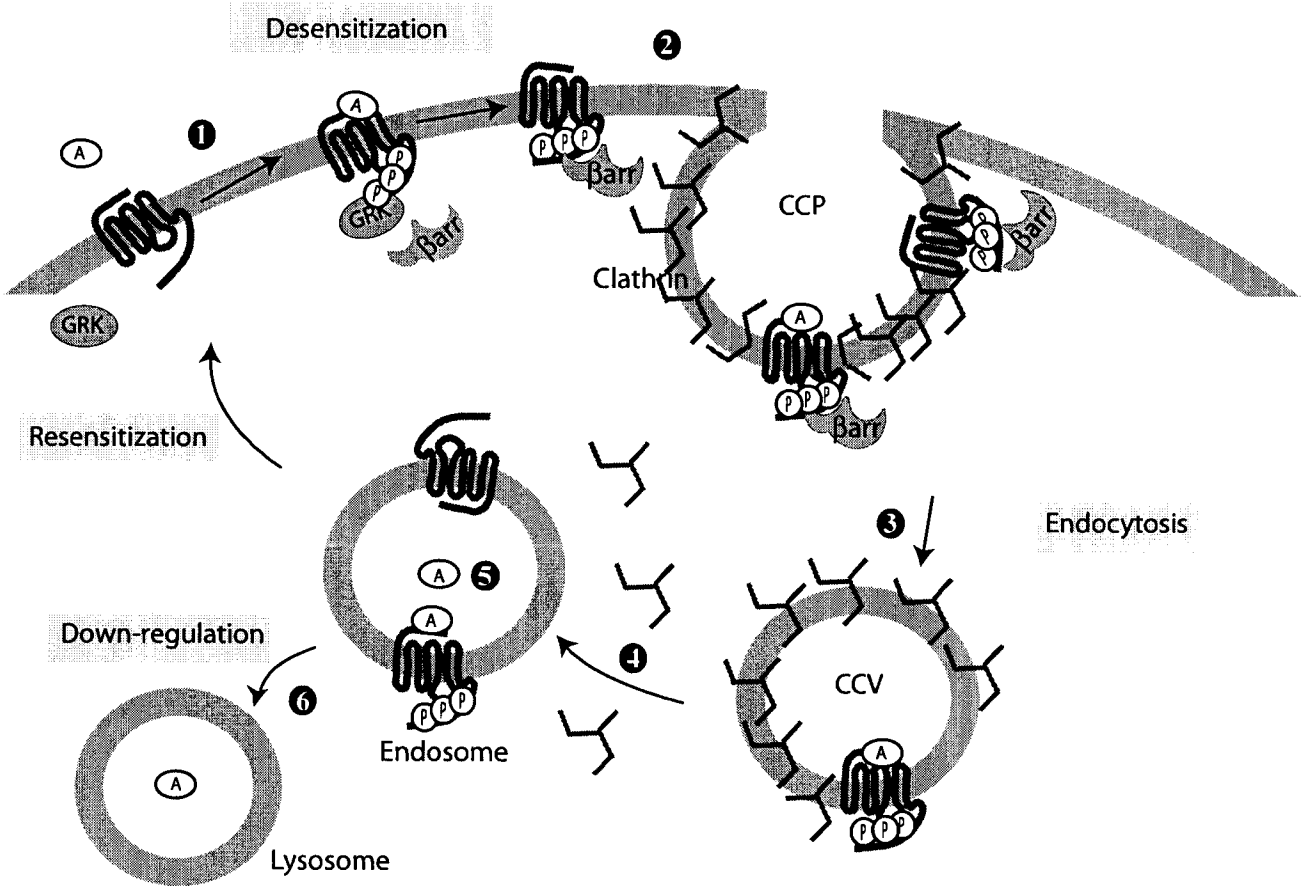


Figure 1. *Model for βarrestin-mediated internalization of GPCR via clathrin-coated pits*

clathrin adaptor complex AP-2, via its beta-subunit (β2-adaptin) following AT1R activation. This complex appears to be regulated by c-Src protein kinase activity and thus modulates the speed and the rate of AT1R internalization. Even if we can not exclude that blocking c-Src protein kinase activity also inhibits other important phosphorylation events (*e.g.* phosphorylation of dynamin), it appears clearly that c-Src plays an important role in receptor endocytosis. Indeed, using siRNA we observed that reducing c-Src expression not only affected receptor internalization but also increased AP-2 association with the receptor and trapped AP-2 at the plasma membrane. Overall, these results suggest that the ternary complex c-Src/AP-2/βarrestin is involved in the early events of AT1R internalization: CCP formation.

The formation of βarrestin/Src complexes with desensitized-GPCRs appears to initiate a 'second wave' of GPCR signalling. Indeed, several proteins involved with GPCR desensitization, endocytosis, and trafficking are also Src substrates such as dynamin. Dynamin was found to be tyrosine phosphorylated following activation of β2-AR (170) and M1 muscarinic receptor internalization (303). Src phosphorylation of dynamin increases both dynamin self-assembly and GTPase activity, and was reported to be required for epidermal growth factor (EGF)-mediated EGF receptor internalization (304). GRK2 appears to be another important substrate for Src: Src has been reported to phosphorylate GRK2 following activation of either the β2-AR or the CXCR4 receptor (69, 177). Our results provide evidence for a new substrate phosphorylated by c-Src: the beta-subunit of AP-2, β2-adaptin.

While the mechanisms involved in regulating the clathrin-mediated endocytic pathway are not fully understood, accumulating evidence suggests that phosphorylation cycles may be a key step (300, 305). Indeed, phosphorylation of the clathrin heavy chain (175) and the large and medium subunits of AP-2 (306, 307) have been demonstrated to be important to the clathrin-mediated internalization of growth factors and nutrients, respectively. However, the phosphorylation of endocytic factors is not limited to the major coat components. Many others constituents of the endocytic machinery including dynamin1, amphiphysin 1 and 2, synaptojanin, AP180, epsin and eps15, are

phosphorylated. Although it is clear that phosphorylation plays an important role in clathrin-mediated internalization, a more detailed understanding of the endocytic regulatory mechanisms will require the identification of the respective protein kinases that target these endocytic components. Multiple protein kinases of clathrin-coated vesicles have been defined, for example α-adaptin-associated protein kinase-1 (AAK1) phosphorylates the μ2 subunit of AP-2 (158), Src protein kinase phosphorylates clathrin (175) and herein the β2-subunit of AP-2. While β2-adaptin appears to be a good substrate for c-Src, it was necessary to address the function in clathrin-mediated internalization. Previous observations suggest that the poly-L-lysine dependent phosphorylation of AP-2 prevents its recruitment to clathrin cages (306). Thus, it is reasonable to believe that phosphorylation may regulate the ability of β2-adaptin to interact with other endocytic proteins, such as βarrestin. However, we can not exclude the fact that β2-adaptin phosphorylation may also influence AP-2 localization. Previous studies have established that β2-adaptin is phosphorylated *in vivo* by a staurosporine-sensitive protein kinase whose function is balanced by the constitutive activity of protein phosphatase 2A (PP2A) (307). They also observed that treatment of cells with agents that block PP2A function perturb AP-2 localization at the plasma membrane and disrupt transferrin internalization. Thus, as suggested by Slepnev *et al.*(300), it is possible that one potential mechanism to regulate the cycles of coat protein assembly and disassembly is reversible phosphorylation.

It remains unclear how Src is activated during AT1R stimulation. To be able to answer to this question we performed a preliminary study. Recently, the stimulation of a mutant AT1R (Tyr^{302}Ala or Y302A) with Angiotensin II or a wild-type receptor with an Ang II analogue ([Sar^1,Val^5, $DPhe^8$]AngII) was shown to block the activation of classical G protein but still lead to βarrestin2 recruitment and ERK1/2 activation (308), suggesting that internalization by itself can promote intracellular signalling. In order to determine if the c-Src-mediated effect on AP-2 phosphorylation was G protein-dependent we used a similar approach with an AT1R mutant, where the tyrosine 302 was replaced by alanine (Tyr^{302}Ala or Y302A) (Figure 2A). This mutation was first described as inhibiting G protein signalling without affecting GPCR internalization (287). We confirmed the effect of this mutant by measuring the AngII-induced calcium

responses, since the G protein-mediated actions of Ang II are typically transduced by G_q (leading to the generation of IP_3 and DAG, and the increase of Ca^{2+} in cells). When HEK293 cells expressing the human AT1R were stimulated with Ang II (100nM), calcium levels, as assessed by confocal microscopy with Fluo-3AM dye, did not increase over time as compared to cells expressing the wild-type receptor (Figure 2B). These results are consistent with the previous finding by Laporte *et al.*,(287) showing that the mutation Y302A abolishes G protein coupling to the AT1R.

We further observed that βarrestin 2 can still interact with Ang II-stimulated Y302A receptors and [Sar^1,Val^5, $DPhe^8$]Ang II-bound to AT1R, using confocal microscopy. When βarrestin2-GFP was coexpressed with the wild-type AT1R (AT1R WT) or the mutant Y302A (AT1R-Y302A), as shown previously, Ang II induced the translocation of βarrestin 2-GFP to endocytic vesicles in cells expressing AT1R receptors (Fig. 3A) (121). Similarly, [Sar^1,Val^5, D-Phe^8]Ang II also induced the recruitment of βarrestin2-GFP to the plasma membrane and then into vesicles in the cells (Figure 3B), indicating that [Sar1,Val5, D-Phe8]Ang II-bound to AT1R can recruit βarrestin 2 and promote endocytosis in HEK293 cells. These results are consistent with a previous report that the mutant Y302A receptor undergoes internalization following Ang II stimulation (287) and that [Sar1,Val5, D-Phe8]AngII can induce AT1 receptor internalization (309). Under these conditions, stimulation of the wild-type AT1R with [Sar1,Val5, D-Phe8]Ang II or the Y302A receptor with Ang II, despite their inability to activate G proteins, led to a robust and transient phosphorylation of the β2 subunit of the AP-2 complex (Figure 4A and B respectively). Also, AP-2 was found to be recruited to the AT1R following [Sar1,Val5, D-Phe8]AngII stimulation (Fig. 4B bottom panel). The finding that both the wild-type AT1R stimulated with [Sar1,Val5, D-Phe8]AngII and the Y302A mutant receptor stimulated with Ang II recruited AP-2 and are phosphorylated on a tyrosine residue in its beta-subunit in the absence of detectable G protein signalling led us to conclude that c-Src contributes to the AT1R-induced AP-2 phosphorylation independently of G protein activation. These results are consistent with recent findings where c-Src activation was shown to be independent of G protein signalling upon AT1R stimulation (295).

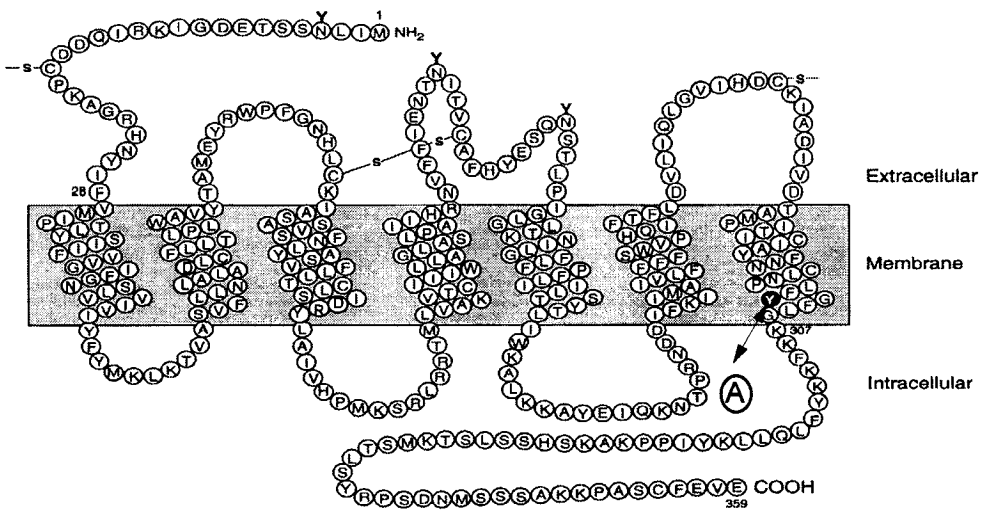
Altogether, these results indicate that Ang II-c-Src-mediated β2-adaptin phosphorylation is G protein-signalling independent and suggest that it is being mediated by βarrestin/c-Src. Using the catalytic domain of c-Src, SH1 (active form) and SH1-KD (inactive form) (Figure 5A), which retained a strong catalytic activity and good βarrestin binding, we showed that following Ang II stimulation, the overexpression of SH1 potentiates the AngII-mediated β2-adaptin phosphorylation (Figure 5B, SH1). Strikingly, when SH1-KD was overexpressed, the β2-adaptin tyrosine phosphorylation was dramatically reduced (Figure 5B, SH1-KD) confirming the involvment of c-Src/βarrestin complex in the β2-adaptin phosphorylation following AT1R activation. Figure 6 represents a model based on our findings in this regard. Following receptor stimulation, GRK phosphorylates the C-tail of the receptor allowing the recruitment of βarrestin and c-Src. βarrestin serves as an adaptor to recruit components of the endocytic machinery such as clathrin and AP-2, and then c-Src phosphorylates β2-adaptin on tyrosine residues to induce its dissociation from βarrestin. Here we show that a c-Src mutant (SH1-KD) that retains the βarrestin1 binding domain but is catalytically inactive can block the AngII-mediated β2-adaptin phosphorylation. This finding implies that βarrestin-mediated recruitment of c-Src is crucial for localizing the tyrosine protein kinase activity in close proximity with the β2-adaptin.

In addition to dynamin, we have now identified another target for c-Src during receptor-mediated endocytosis: the beta subunit of AP-2. Overall, these results highlight the important role of βarrestin in recruiting c-Src to the agonist-occupied receptor prior to internalization. Furthermore, our results suggest that the receptor can exist in more than one 'active' conformation. Thus, the receptor conformation induced by [Sar1,Val5, D-Phe8]AngII in the wild-type receptor, or by the Ang II for Y302A mutant receptor, which can stimulate the c-Src-mediated AP-2 phosphorylation, is not able to activate G proteins. The demonstration of such functionally distinct active conformations, potentially coupled to distinct signalling pathways, gives credibility to the development of novel therapeutics that might target only one of several such conformations.

Fig. 2. Calcium responses of Fluo-3 AM on transfected HEK 293 cells. *A,* Schematic representation of the amino acid sequence of the human AT1R. Mutation of the human AT1R was made by replacing Tyr^{302} with alanine (AT1R-Y302A), as previously described by Laporte *et al.(287). B,* Time response of calcium release in HEK293 cells after Ang II stimulation in living cells. HEK293 cells were grown on a coverslip and transfected with the wild-type AT1R (AT1R-WT, top panels) or AT1R-Y302A (bottom panels). Before the experiment, cells were washed 3 times with a Tyrode solution (5 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1.9 mM CaCl2, 5.6 mM glucose, buffered to pH 7.4 with Tris base, and supplemented with 0.1% BSA). Cells were then incubated for 45 min at room temperature in the dark, and then washed twice with Tyrode-BSA buffer, and twice in Tyrode buffer alone. Cells were then treated with Ang II (1 μM) at 37°C for the indicated times. Fluorescence was visualized by confocal microscopy with the following settings: 488 nm excitation and filter set at LP 505. Figures represent the calcium dye distribution as assessed by fluorescence intensity. The colour scale represents pseudocolor intensity levels of Fluo-3 dye. Shown are representative images of three independent experiments.

Fig. 2 Fessart *et al*

A



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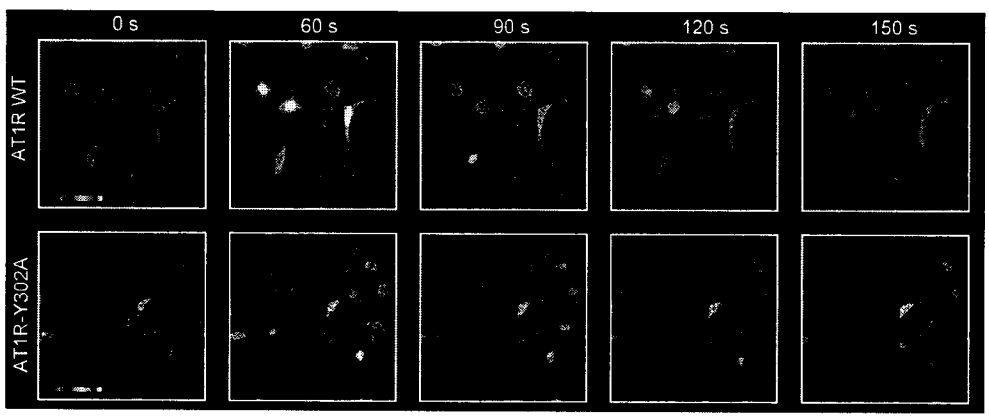

Fig. 3. Agonist-mediated βarrestin2-translocation assay in HEK 293 cells. *A,* HEK 293 cells were transiently transfected with AT1R (top panels) or AT1R-Y302A (bottom panels) in presence of GFP-tagged βarrestin2. Cells were then either left untreated or treated with Ang II (1 μM) for indicated time at 37°C. The distribution of GFP-βarrestin was visualized by confocal microscopy. For each experiment, 25 different fields containing 1 to 2 cells were analyzed. Shown are representative images of GFP- βarrestin fluorescence of three independent experiments. *B,* HEK 293 cells were transiently transfected with AT1R in the presence of GFP-βarrestin2. Cells were then either left untreated or treated with Ang II (1 μM) or [Sar1,Val5, D-Phe8]AngII (10 μM) for the indicated time. The distribution of GFP-βarrestin was visualized by confocal microscopy as described in *A*.

Fig. 3 Fessart *et al*

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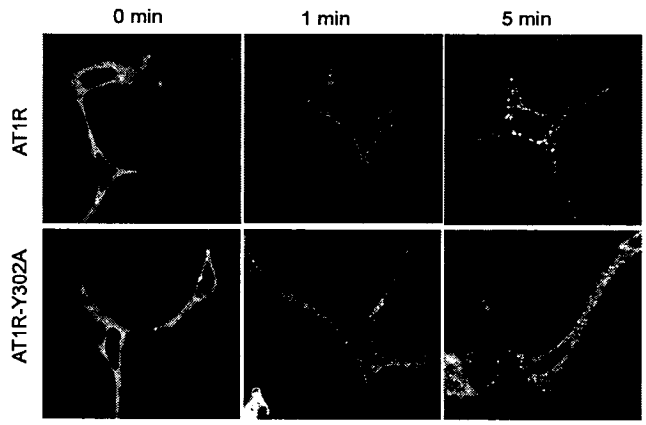

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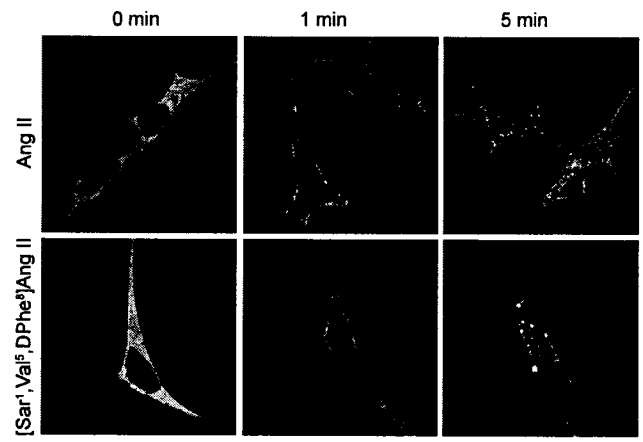

Fig. 4. Agonist-mediated β2-adaptin phosphorylation independently of G protein signalling. *A,* HEK 293 cells were transfected with AT1R-Y02A and Flag-β2-adaptin or pcDNA3.1 (Mock). Twenty-four hours after transfection cells were serum-starved overnight, and then pretreated with pervanadate for 10 min before being stimulated with Ang II (100 nM) for the indicated time. Flag-β2-adaptin were immunoprecipitated (IP) from cell lysates using an anti-Flag antibody and immunoprecipitates were analyzed by Western blot (WB) using the anti-phosphotyrosine 4G10 (upper panel), and the anti-β-adaptin (bottom panel) antibodies. *B,* HEK 293 cells were transiently transfected with HA-AT1R and Flag-β2-adaptin. Twenty-four hours after transfection cells were serum-starved overnight, and then pretreated with pervanadate for 10 min before being stimulated with [Sar1,Val5, D-Phe8]AngII (10 μM) for the indicated time. Flag-ß2-adaptin or HA-AT1 were immunoprecipitated (IP). The β2-adaptin immunoprecipitates were analyzed as described in (*A*), (*top panels*). The AT1R immunoprecipitates were analyzed for the detection of associated-β2-adaptin using an antibody against β2-adaptin. Whole cell extracts (Total) were also probed for the detection of β2-adaptin using the β2-adaptin antibody, (*bottom panels).*

Fig. 4 Fessart *et al*

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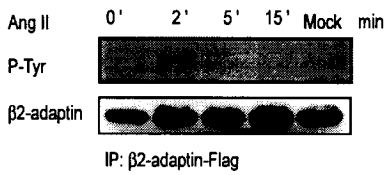

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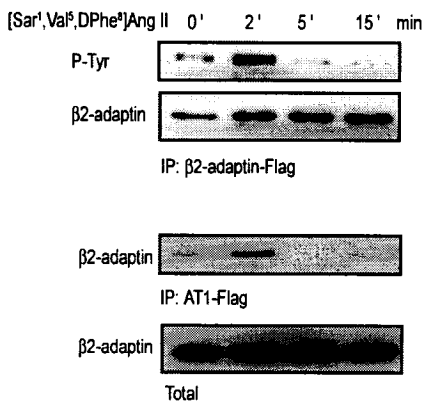
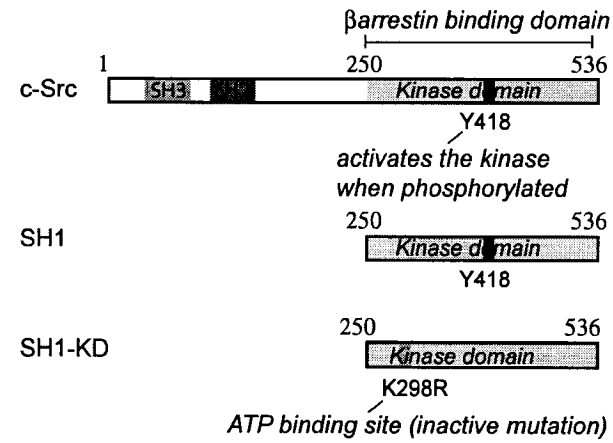


Fig. 5. βarrestin/c-Src-mediated β2-adaptin phosphorylation following AT1R stimulation. *A,* Representation of the non receptor tyrosine protein kinase c-Src and its different constructs. SH1 correspond to the small region containing the protein kinase activity and SH1KD to the same region with an inactive kinase (KD) (96). *B,* HEK 293 cells were transfected with AT1R, Flag-β2-adaptin and either pcDNA3.1 (Mock), HA-tagged SH1 or HA-tagged SH1KD. Twenty-four hours after transfection cells were serum-starved overnight, and then pretreated with pervanadate for 10 min before being left untreated or stimulated with Ang II (100 nM) for 5 min at 37°C. Flag-β2-adaptin were immunoprecipitated (IP) from cell lysates using an anti-Flag antibody. Immunoprecipitates were analyzed by Western blot (WB) using the anti-phosphotyrosine 4G10 (upper panel), and the anti-β-adaptin (bottom panel) antibodies. Whole cell extracts (Total) were also probed for the detection of HA-SH1 and HA-SH1KD using an anti-HA antibody.

Fig. 5 Fessart *et al*

A



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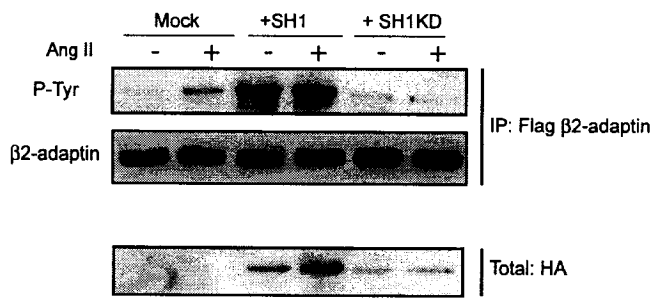

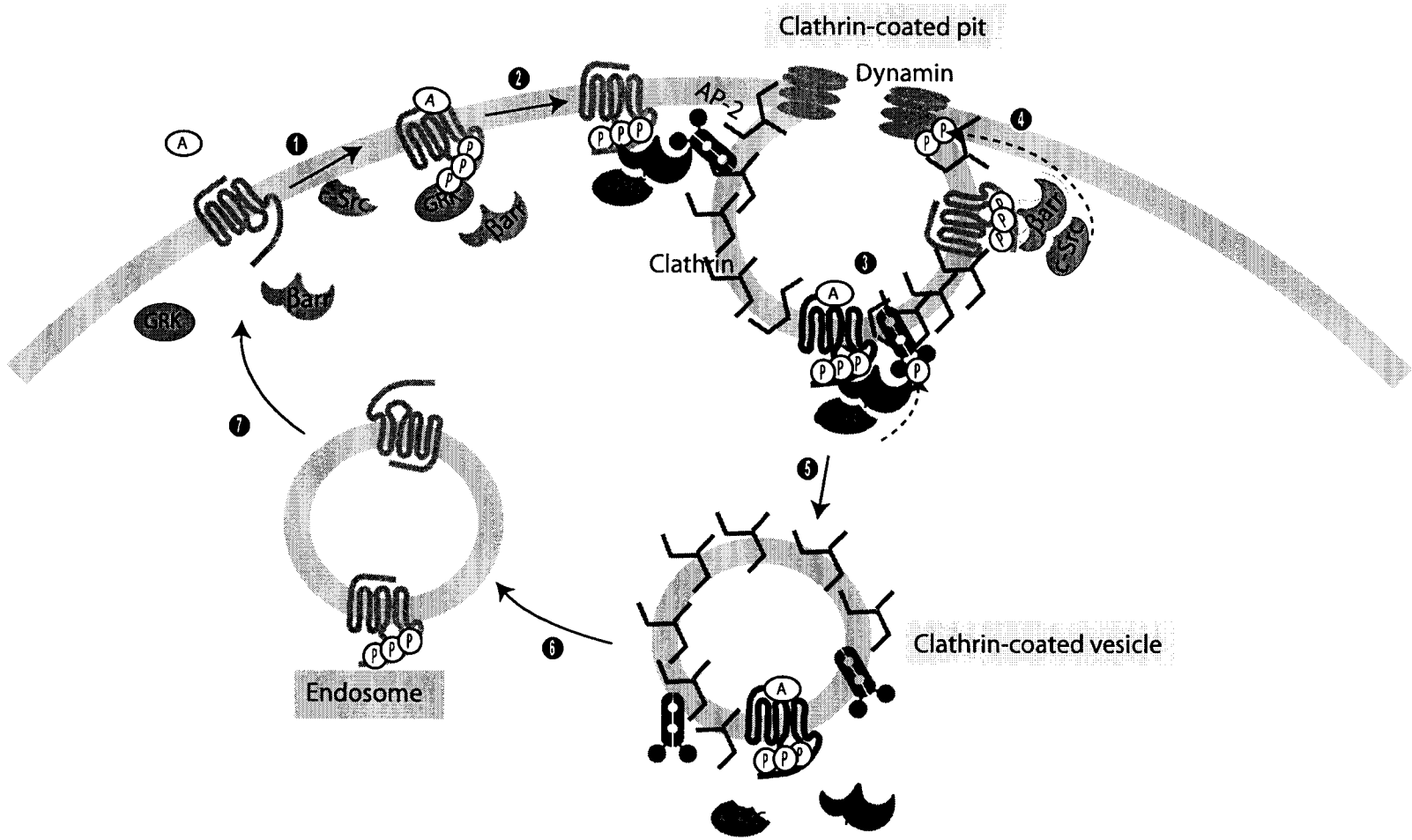

Figure 6. Model for βarrestin-mediated internalization of AT1R via clathrin-coated pits

Upon agonist binding, receptors are phosphorylated by GRKs leading to the recruitment of βarrestins (1). Beta-arrestins, through their interaction with clathrin and AP-2, target the receptor/arrestin complexes to clathrin coated pits (2). Beta-arrestins also bind c-Src (2 and 3) which was shown to phosphorylate dynamin (4), a GTPase that regulates the pinching off from the cell surface of clathrin coated-pits (5). Src also phosphorylates β2-adaptin to promote its dissociation from βarrestin. Once clathrin coated vesicles are formed, βarrestin and c-Src dissociate from AT1R and the receptor is then internalized into endosomes (6) where it is dephosphorylated before returning to the cell surface (7). Colors represent the new elementS added to the proposed model in the literature explained at the begining of the thesis (Section I).

The recent use of siRNA against the α and μ2 subunits has shown that the AP-2 complex is required for the formation of most of plasma membrane-associated CCPs (163, 164). However, it remains unclear where the dissociation of AP-2 that we observe following its phosphorylation occurs in CCP or CCV? Recent studies have contributed some elements that address this point. Using a GFP-tagged clathrin light-chain in living cells, various groups showed that clathrin spots at the plasma membrane surface can be categorized into four groups. The largest group (approximately 80%) is static during the period of observation, new spots can be observed to appear or form, approximately 2% of spots move laterally in the plane of the membrane and approximately 15% of spots disappear. Those observed disappearing into the cell have been suggested to represent nascent CCVs entering the cytosol (310, 311). Furthermore, other markers for the endocytic compartment, such as dynamin, have been observed disappearing at the same time, place, and rate as the clathrin spots (311). Recently, a GFP-tagged α-adaptin was used as an AP-2 marker in living cells. Surprisingly, the fluorescence intensity of clathrin and the AP-2 complex were comparable in static spots but the AP-2 complex was absent from disappearing clathrin spots (310). These unexpected findings suggest that the AP-2 complex is absent from forming CCVs and proposes a new model for CCP/CCV formation. In this model, the AP-2 complex would be involved in the formation of stable platforms for clathrin assembly and cargo selection. This model diverges from the previous ones that suggest that each formed CCP is progressively transformed into one nascent CCV (162), where AP-2 is present all along the CCV formation process. These models suggest that AP-2 plays its adaptor function beginning with the early steps of CCP assembly and then is incorporated in the vesicle. Finally, in the case of cargo, the fact that activated GPCR/β-arrestin complexes are recruited to static pre-existing clathrin spots (182, 183) suggests that at least in this case, they can correspond to pre-existing platforms for cargo selection. In conclusion, there is a clear distinction between the AP-2 content in static versus disappearing clathrin spots. These disappearing spots contain endocytic cargo but have reduced AP-2 relative to static plasma membrane domains. These observations suggested that the AP-2 complex was excluded from the vast majority of nascent endocytic vesicles, and led us to hypothesize that the dissociation of

phosphorylated-AP-2 that we observed, occurs in CCPs before the CCVs formation. However this point still remains to be determined.

What is the functional significance of the role for βarrestin-dependent scaffolding complexes in AT1R internalization? One possibility is that the βarrestin scaffolding complex could serve as a molecular switch for activation of proliferative or apoptotic pathways. In some cell types, the βarrestin/Src complex formation is involved in GPCR-mediated activation of the ERK1/2 cascade (97, 114). Indeed, studies on the β2AR, suggest that the formation of a receptor/βarrestin/Src complex directs activation and nuclear translocation of ERK1/2, leading to proliferative effects (312). In the case of NK1, formation of a βarrestin-containing complex allows nuclear translocation of ERK1/2, proliferation, and protection from apoptosis (262). In other cell types, βarrestin binds c-Src, which is believed to be required for CXCL8/IL-8-mediated chemotaxis of neutrophils (313). This chemotactic effect of the scaffold βarrestin with ERK1/2 was also observed for the PAR2 receptor (314). In support of these findings, lymphocytes from βarrestin 2 knockout mice show significantly reduced CXCR4-mediated migration (315). However, some results have demonstrated that activation of numerous GPCRs in the absence of βarrestin expression leads to cellular apoptosis (316). Thus, the appropriate regulation of GPCRs by βarrestins seems not to be only essential for cell signalling but also for cell survival.

Future Perspectives

The present study characterized the mechanisms regulating the formation of endocytic complexes during AT1R internalization. First, the protein kinase c-Src is not only recruited to the endocytic complexes in an agonist-dependent fashion, but also regulates their formation. One proposed mechanism is that c-Src phosphorylates the β2-adaptin and thus induces its dissociation from βarrestin. Although we were able to identify one of the tyrosine residues phosphorylated, we can not favor a common mechanism. However, based on the hypothesis that all GPCRs share a common mechanism of activation and βarrestin-mediated desensitization, it will be of interest to assess whether the β2-adaptin phosphorylation is a common mechanism for GPCRs and for other receptor types, such as the tyrosine kinase receptors (EGFR). In this respect, the development of an antibody directed against the critical phosphotyrosine residue will allow us to determine whether this residue on AP-2 is critical for the internalization of other GPCRs.

Protein phosphorylation and dephosphorylation are closely related to signalling proteins and can directly mediate protein-protein interactions. Thus, protein tyrosine phosphatases, which dephosphorylate signalling molecules, play an important role in transducing signals and controlling cellular behaviour. Evidence has shown that SHP2 could be recruited in an agonist-dependent fashion to GPCRs (317). Furthermore, βarrestin 2 was recently characterized as a signalling intermediate through a kinase/phosphatase scaffold for Akt/PP2A involved in the expression of dopamine-associated behaviours (318). Arrestin acts as a positive mediator of dopaminergic synaptic transmission. Thus, regarding the role of c-Src in GPCRs internalization, we can speculate that tyrosine phosphatase could also play a role. Indeed, SHP2 has been reported to be recruited to the AT1R (319). It will be interesting to assess whether SHP2, or even other phosphatases, regulate GPCR endocytosis.

In what could be qualified as "challenging times", much attention will probably be foccussed on deciphering the real-time dynamics of the spatio-temporal movement of the βarrestin/β2-adaptin/Src complex in living cells in order to advance our

understanding of the dynamic of β2-adaptin, and others proteins in CCPs or CCVs. The development of fluorescent resonance energy transfer (FRET) and a Src reporter (320) will offer the unique opportunity to monitor in intact living cells Src protein protein kinase activity and, finally, to determine whether Src is activated at the plasma membrane or inside vesicles. These sophisticated techniques could better define how the organization and distribution of the endocytic complex formation are regulated during GPCR internalization. What is the temporal recruitment of endocytic proteins during receptor internalization? Where are the complexes dissociated? The elegant demonstration of the *in vivo* kinetics during receptor activation recently reported for β2-AR by BRET is a good example of the innovative breakthroughs that biophysical techniques bring to the field of GPCR internalization (321). The ability to probe for three proteins in very close proximity, in the future, will give further insight into how these complexes are regulated.

The other angle of the story that would be particularly interesting is to explore the *in vivo* roles of β2-adaptin dissociation. In others words, how and why do these complexes dissociate? Close examination of AP-1 (322), and β-arrestin (323) shows that an effective way of regulating compartmental specificity could be dephosphorylation or phosphorylation by phosphatases and kinases restricted to defined membrane locales. These focal posttranslational modifications could modulate the avidity between network members, promoting assembly (and disassembly) of sorting lattices only at the appropriate location and time. For example, it has just been reported that Ark1p/Prk1p-mediated phosphorylation of a *S. cerevisiae* endocytic, eps15-like accessory factor governs the post budding inactivation of Arp2/3-dependent actin polymerization, preparing the transport vesicle for subsequent fusion (324). Except for AP-1, the role of phosphorylation in controlling clathrin-coated vesicle formation at the cell surface is relatively unexplored. How endocytic phosphorylation cycles are regulated is an important question for the future. Further studies on regulatory inputs and control mechanisms are sure to provide important insight into the events controlling receptor endocytosis.

An alternative explanation for why c-Src phosphorylates β2-adaptin is that the c-Src-mediated phosphorylation of β2-adaptin could also promote the recruitment of new interacting proteins with an SH2 domain, which is in accordance with the crystal structure of β2-adaptin, in which theTyr^{737} could be accessible (153) (Figure 7).

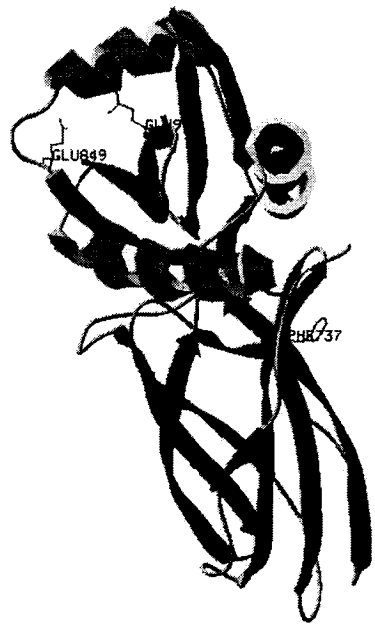

Figure 7. *Structure of the ear domain of β2-adaptin. Structure of β2-adaptin (residues 664-937) is shown as ribbon, and the position of the glutamate 849 (Glu^{849}), the glutamate 902 (Glu^{902}) and the tyrosine 737 mutated for phenylalanine (Phe^{737}) residues are deduced from coordinates of the crystal structure of β2-adaptin (deposited at the Protein Data Bank, identification code 1E42). The structure and the position of different residues were visualized using Swiss-PDB Viewer (Glaxo Wellcome Experimental Research).*

Nevertheless, proof of this model will require crystallization of the phosphorylated AP-2 complex. Since the crystal structure was obtained for β2-adaptin (153), it will be important in future studies to examine the crystal structure of wild-type β2-adaptin as compared to phosphorylated β2-adaptin. A structural approach will be useful in demonstrating the ability of phosphorylated β2-adaptin to bind, or not, to endocytic proteins. It is not unlikely that a protein with an SH2 domain would make an

excellent binding partner for c-Src phosphorylated tyrosine residue within β2-adaptin. One potential candidate is phosphatidylinositol 3-kinase, a key enzyme for the generation of phospholipids involved in clathrin-mediated trafficking (325). In searching the database for specific consensus sequences for phosphorylated β2-adaptin, it appears that PI3K could be a good candidate. Recent evidence suggests that phosphorylated YMXM motifs play a role in mediating specific interactions with SH2 domains. In particular, the SH2 domains of PI3K bind to autophosphorylated YMXM or YXAM motifs in the platelet-derived growth factor receptor (326-328) and colony-stimulating factor 1 receptor (329), as well as in IRS-1 itself (330). This is consistent with previous studies reporting that PI3K is required for β2-AR endocytosis (331). In parallel, to verify and identify new proteins interacting with phosphorylated β2-adaptin, a proteomics approach would inform us of the partners recruited during AT1R endocytosis.

In conclusion, originally thought only to be important in GPCR desensitization, βarrestins are now known to be involved in receptor endocytosis, adaptor and kinase scaffolding, intracellular trafficking, and most recently, to be required for receptor recycling. At present, little is known about the physiological significance of these novel βarrestin-dependent signalling mechanisms. Recently, using a genomic approach to silence predicted human protein kinases by RNA interference (RNAi), Pelkmans *et al.* (332) evaluated the effect on two endocytic routes: clathrin- and caveolae/raft-mediated endocytosis. Their analysis demonstrates that signalling functions such as those controlling cell adhesion, growth and proliferation, are inducible by the machinery of endocytosis. Of the 590 human kinases screened (including lipid, protein, carbohydrate and hypothetical protein kinases), they identified some protein kinases previously implicated in endocytosis (AAK1, Lck, c-Src, GSK3β, PKC isoforms and CK2). They also assigned a prominent role in endocytosis to a number of regulators of the actin and tubulin cytoskeleton, as well as mitogenic pathways, cell growth, proliferation and apoptosis. This promising screen highlights the complexity in the regulation of the clathrin-mediated endocytosis and might explain the differences observed between receptors.

SECTION VI

Contributions to Original Research

I- In the work reported in Section III, the endogenous formation of a ternary endocytic complex containing AP-2, βarrestin and c-Src during AT1R internalization was characterized by co-immunoprecipitation experiments. In addition, *in vitro* studies identified the binding region important for these interactions, and also whether the protein kinase was activated inside the complex by yeast-three hybrid and co-immunoprecipitation assays. In addition, in an agonist-dependent fashion, the protein kinase activity of c-Src involved in the dissociation of the endocytic complex in cells was demonstrated by co-immunoprecipitation. These interactions were confirmed with two approaches by depleting c-Src expression with siRNA or phosphorotioates antisense. Moreover, the impact of reducing c-Src expression on receptor internalization was measured by radioligand binding assay and confocal microscopy. It was shown that AP-2 and the receptor are trapped at the plasma membrane in c-Src-depleted cells, and this also reduced the speed and rate of AT1R internalization observed by radioligand binding assay.

II- In the work reported in Section IV, it was shown that the beta-subunit of AP-2 was tyrosine phosphorylated upon Ang II stimulation by co-immunoprecipitation experiments. In addition, using different inactive c-Src protein kinase constructs and co-immunoprecipitation experiments, the c-Src protein kinase was identified as responsible for β2-adaptin phosphorylation. The tyrosine residue was identified using GST-peptides of a small region of β2-adaptin and *in vitro* kinase assays. By directed mutagenesis, the potential tyrosine residues were mutated and verified by *in vitro* kinase assays in order to assess their phosphorylation status. In addition, the ability of phosphorylated β2-adaptin mutants, to bind βarrestin with an *in vitro* phosphorylation following GST-pull down assays was determined. In living cells, the role of the tyrosine phosphorylation of β2-adaptin onto βarrestin association was confirmed by BRET assay. Finally, in order to observe the dynamics of these interactions, confocal experiments were performed to observe both βarrestin and β2-adaptin upon Ang II stimulation.

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APPENDICES

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c-Src Regulates Clathrin Adapter Protein 2 Interaction with β-Arrestin and the Angiotensin II Type 1 Receptor during Clathrin-Mediated Internalization

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β-Arrestins are multifunctional adapters involved in the internalization and signaling of G protein-coupled receptors (GPCRs). They target receptors to clathrin-coated pits (CCPs) through binding with clathrin and clathrin adapter 2 (AP-2) complex. They also act as transducers of signaling by recruiting c-Src kinase to certain GPCRs. Here we sought to determine whether c-Src regulates the recruitment of AP-2 to β-arrestin and the angiotensin II (Ang II) type 1 receptor (AT1R) during internalization. We show that the agonist stimulation of native AT1R in vascular smooth muscle cells (VSMCs) induces the formation of an endogenous complex containing c-Src, β-arrestins and AP-2. *In vitro* studies using coimmunoprecipitation experiments and a yeast three-hybrid assay reveal that c-Src stabilizes the agonist-independent association between β-arrestin2 and the β-subunit of AP-2 independently of the kinase activity of c-Src. However, although c-Src expression promoted the rapid dissociation of AP-2 from both β-arrestin and AT1R after receptor stimulation, a kinase-inactive mutant of c-Src failed to induce the dissociation of AP-2 from the agonist-occupied receptor. Thus, the consequence of c-Src in regulating the dissociation of AP-2 from the receptor was also examined on the internalization of AT1R by depleting c-Src in human embryonic kidney (HEK) 293 cells using a small interfering RNA strategy. Experiments in c-Src depleted cells reveal that AT1R remained mostly colocalized with AP-2 at the plasma membrane after Ang II stimulation, consistent with the observed delay in receptor internalization. Moreover, coimmunoprecipitation experiments in c-Src depleted HEK 293 cells and VSMCs showed an increased association of AP-2 to the agonist-occupied AT1R and β-arrestin, respectively. Together, our results support a role for c-Src in regulating the dissociation of AP-2 from agonist-occupied AT1R and β-arrestin during the clathrin-mediated internalization of receptors and suggest a novel function for c-Src kinase in the internalization of AT1R. (*Molecular Endocrinology* 19: 491–503, 2005)

G PROTEIN-COUPLED RECEPTORS (GPCRs) are integral membrane proteins that are responsible for controlling an array of physiological responses such as phototransduction, olfaction, neurotransmission, vascular tone, cardiac output, and pain. The intensity and the duration of a response are dependent on the balance that exists between mechanisms that regulate the coupling of the receptors to their downstream effectors and those that terminate the signaling. Mechanisms that rapidly turn off signaling include desensitization, whereby receptors become refractory to subsequent stimuli, and internalization, where receptors are removed from the plasma membrane (a process also referred to as sequestration or endocytosis) (1, 2). Internalization of GPCRs can lead to the reestablishment of the cellular response by the recycling of receptors to the plasma membrane, or the prevention of further signaling by targeting receptors for degradation.

Homologous desensitization and internalization of receptors involve common and distinct steps. Agonist stimulation of GPCRs promotes the phosphorylation of receptors by GPCR kinases (GRKs), and the recruitment of nonvisual arrestin proteins (β-arrestin1 and β-arrestin2) to the phosphorylated receptors (1). β-Arrestins bind to the agonist-occupied GPCR to prevent second-messenger signaling and act as adapters to target desensitized receptors to clathrin-coated vesicles (CCVs) at the plasma membrane (*i.e.* clathrin-

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Abbreviations: AD, Activation domain; AP-1/2, clathrin adapter protein 1 and 2; Ang II, angiotensin II; AS-1, antisense DNA for c-Src; AT1R, angiotensin II type 1 receptor; β2AR, β2-adrenergic receptor; CCP, clathrin-coated pit; CCV, clathrin-coated vesicle; CTL-1, control antisense DNA; DBD, DNA binding domain; EGFR, epidermal growth factor receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HA, hemagglutinin; HEK, human embryonic kidney; siRNA, small interfering RNA; SDS, sodium dodecyl sulfate; VSMC, vascular smooth muscle cell.

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coated pits; CCPs) for internalization (3, 4). The involvement of β-arrestins in the internalization of the β2-adrenergic receptor (β2AR) and the angiotensin II (Ang II) type 1 receptor (AT1R) has been demonstrated using different approaches, and more recently by gene disruption and posttranscriptional silencing of β-arrestins (5, 6). In the case of the β2AR, β-arrestins are believed to link the agonist-occupied receptors to CCPs by interacting through their C-terminal domains with both clathrin and the β-subunit of the heterotetrameric adapter AP-2 complex (β2-adaptin) (7–10). Many studies indicate that AT1R can internalize via CCVs in both heterologous and endogenous systems (11–15), but the underlying mechanisms regulating β-arrestin-mediated endocytosis of AT1R and other GPCRs still remain poorly understood.

In addition to their roles in GPCR desensitization and internalization, recent evidence indicates that β-arrestins can also participate in receptor signaling by functioning as adapters for the recruitment of signaling molecules to agonist-occupied receptors (16). For instance, β-arrestins have been shown to recruit the nonreceptor tyrosine kinase c-Src to the β2AR (17) and the neurokinin-1 receptor (18). The β-arrestin-mediated recruitment of c-Src to the agonist-occupied β2AR regulates the function of the endocytic protein dynamin, a GTPase involved in the fission of CCPs from the plasma membrane (8, 17). Given the dual adapter role for β-arrestins in the internalization and signaling of GPCRs, we hypothesize that c-Src could also regulate the recruitment of other endocytic proteins like AP-2 to the agonist-induced AT1R/β-arrestin complex, and consequently impact the early steps of receptor internalization.

RESULTS

Ang II Promotes the Formation of an Endogenous Complex Containing c-Src, β-Arrestin, and AP-2

We have previously shown that the internalization of certain GPCRs, via the clathrin pathway, requires the recruitment of β-arrestin to the receptor, and the interaction of β-arrestin with the heterotetrameric AP-2 complex (via its β-subunit, *i.e.* β2-adaptin) (9). More recently, it has been suggested by different groups that β-arrestins act as signaling adapters for the recruitment of c-Src family kinases to some GPCRs (17–19). To determine whether c-Src could participate in the association between β-arrestin and AP-2, we first examined in an endogenous system, whether the agonist stimulation of AT1R promoted the formation of a complex containing c-Src, β-arrestin, and AP-2. Vascular smooth muscle cells (VSMCs) expressing endogenous AT1R (around 100 fmol/mg of total protein as estimated by radioligand binding experiments; data not shown) were serum starved for 12 h and then either left untreated or treated with Ang II for 5 min at 37 C. Cells were then solubilized and endogenous c-Src was immunoprecipitated. Interacting proteins in the complex were identified by Western blot analysis using antibodies against the α-subunit of AP-2 and β-arrestin (Fig. 1). Results show that, in the absence of agonist stimulation, little β-arrestin and no AP-2 complex were detected in the c-Src immunoprecipitates. However, after agonist stimulation of native AT1R, a robust association of both β-arrestin and AP-2 was observed with c-Src. These results show that the agonist stimulation of AT1R in a native system like VSMCs promotes the formation of an endogenous complex including c-Src, β-arrestin, and AP-2.

c-Src Increases the Association between β-Arrestin and β2-Adaptin Independently of Its Kinase Activity

The Ang II-mediated formation of a complex containing β-arrestin, AP-2, and c-Src in VSMCs, and the recent demonstration that c-Src and β-arrestin associate with each other when overexpressed in COS-7 cells (20), suggest that c-Src is involved in stabilizing the association between β-arrestin and AP-2. We therefore reasoned that, by increasing the expression of c-Src in cells, we could induce the agonist-independent association between β-arrestin and AP-2. To test this possibility, COS-7 cells were transfected with Flag-tagged β-arrestin2 and increasing amounts of hemagglutinin (HA)-tagged c-Src, and the level of endogenous AP-2 (as assessed by the presence of β-subunit of the AP-2 complex, *i.e.* β2-adaptin) in the β-arrestin immunoprecipitates was identified by Western blot analysis (Fig. 2A). In the absence of c-Src overexpression (Fig. 2A, lane 1), no β2-adaptin was detected in the β-arrestin immunocomplex. However, increasing the expression of c-Src resulted in the increased formation of a β-arrestin/AP-2 complex. We next examined whether this c-Src-mediated GPCR-independent association between β-arrestin2 and AP-2 was dependent on the kinase activity of c-Src.

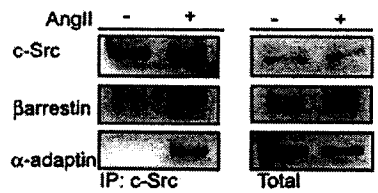

Fig. 1. Ang II Promotes the Formation of an Endogenous Complex Containing c-Src, β-Arrestin, and AP-2

VSMCs were left untreated (−) or treated (+) with Ang II (100 nM) for 5 min at 37 C after serum starvation overnight. Endogenous c-Src was immunoprecipitated (IP) with the GD11 antibody, and proteins in the complex were detected by Western blot using c-Src (SRC2, Santa Cruz Biotechnology), β-arrestins (A1CT), and the α-subunit of AP-2 (BD Transduction Laboratories) antibodies as described in *Materials and Methods*. Also presented are blots from whole cell extracts (Total, *right panel*) probed with the same antibodies. Blots are representative of three independent experiments.

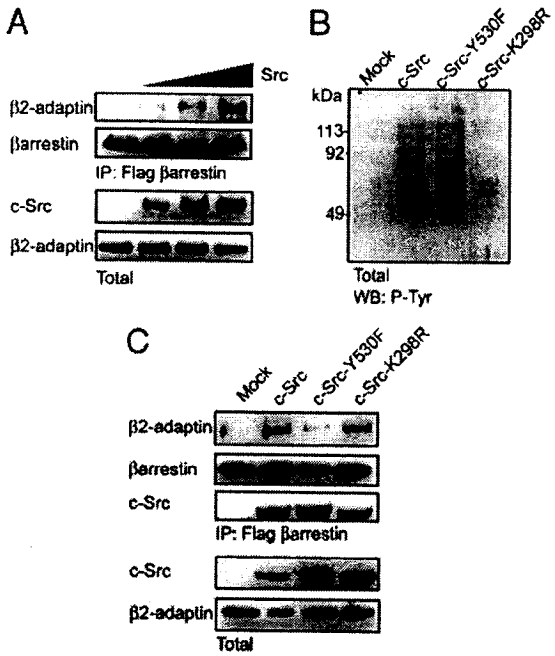

Fig. 2. c-Src Overexpression Increases the Association between β-Arrestin and AP-2 Independently of the c-Src Kinase Activity

A, COS-7 cells were transiently transfected with Flag-β-arrestin2 and increasing amounts of HA-c-Src. β-Arrestin was immunoprecipitated (IP) with Flag antibody (Sigma) and the amount of endogenous AP-2, as assessed by the presence of β2-adaptin in the β-arrestin immunoprecipitates was analyzed by Western blot using a β2-adaptin (BD Transduction Laboratories) antibody. Whole cell extracts (Total) were also blotted for detecting the level of c-Src expression using the HA (Santa Cruz Biotechnology) and β2-adaptin (BD Transduction Laboratories) antibodies. B, COS-7 cells were transfected with Flag-β-arrestin2, β2-adaptin and pcDNA3.1 (Mock) and either HA-c-Src, HA-c-Src-Y530F or HA-c-Src-K298R. Whole cell extracts (Total) were analyzed by Western blot (WB) with the antiphosphotyrosine (P-Tyr) antibody 4G10 (Upstate Biotechnology). C, Cells were transfected as in (B), and Flag-β-arrestin was IP with the Flag antibody (Sigma), and complexes were analyzed by Western blot for the detection of β-arrestin, β2-adaptin, and c-Src expression using the antibodies against Flag (Sigma), β2-adaptin (BD Transduction Laboratories), and HA (Santa Cruz Biotechnology), respectively. Whole cell extracts (Total) were also blotted with anti-HA (Santa Cruz Biotechnology) and anti-β2-adaptin (BD Transduction Laboratories) antibodies, for detection of the different c-Src constructs and the β2-adaptin, respectively. Data are representative of at least three independent experiments.

To this end, we overexpressed β-arrestin2-Flag and β2-adaptin in COS-7 cells with either wild-type c-Src or a mutant that mimics the activated form of c-Src (c-Src-Y530F) or the kinase dead c-Src-K298R (Fig. 2, B and C), and assessed the ability of the different c-Src constructs to promote the association between β2-adaptin and β-arrestin. We first determined the relative level of kinase activity of the different c-Src constructs by probing the total lysates from cells expressing wild-type and c-Src mutants using an anti-phosphotyrosine antibody (Fig. 2B). The expression of wild-type c-Src and c-Src-Y530F had similar effects on the pattern of protein phosphorylation, whereas almost no phosphorylation of proteins was detected in cells overexpressing c-Src-K298R, suggesting that at the levels of c-Src and c-Src-Y530F expression, both kinases had a similar activity in cells. Subsequently, these different constructs were overexpressed in COS-7 cells, and the amounts of AP-2 and c-Src associated to the immunopurified β-arrestin2 complexes were analyzed. Although we found that all c-Src constructs were able to associate with β-arrestin2, only wild type c-Src and the K298R mutant promoted the formation of a ternary complex that included β2-adaptin and β-arrestin (Fig. 2C). These results imply that the kinase activity of c-Src is not required to promote the agonist-independent association of AP-2 with β-arrestin2.

To verify that c-Src directly contributed to the formation of a ternary complex with β-arrestin2 and β2-adaptin, and also to confirm that this complex formation is independent of c-Src kinase activity, we used a modification of the yeast two-hybrid system (yeast three-hybrid). We have previously shown using a similar assay that the interaction between β-arrestin2 and β2-adaptin could be detected (8, 9). We expressed β-arrestin2 fused to the DNA binding domain (DBD) of *GAL4* and β2-adaptin fused to the activation domain (AD) of *GAL4*, and assessed the effect of c-Src expression on the transactivation of the auxotrophic HIS3 and the reporter lacZ genes (Fig. 3C). The expression vectors for β-arrestin2 and the β2-adaptin were cotransformed into yeast strain PJ69–4a with the empty vector p426-ADH or with the p426-ADH-c-Src mutant (c-Src-G2A, -Y416F, -Y527F). This mutant lacks the myristoylation site, to reduce its membrane association, and is mutated on tyrosines 416 and 527 to prevent tyrosine phosphorylation, and hence the activation of c-Src (21). Yeasts were transformed with the expression vector for β-arrestin2 alone (Fig. 3A, lane A), or with β2-adaptin (lane B), c-Src (lane C) or β2-adaptin and c-Src together (lane D). Cells were serially diluted and spotted onto plates lacking leucine, tryptophan, and uracil supplemented or not with histidine (Fig. 3A, *left* and *right panels*). As shown here, the overexpression of β-arrestin2 and β2-adaptin is sufficient to induce yeast growth on selective media lacking histidine (Fig. 3A, *right panel*, lane B). The expression of c-Src with both β-arrestin2 and β2-adaptin promoted the faster growth of yeasts (Fig. 3A, *right panel*, compare lanes D and B), suggesting that c-Src stabilizes the β-arrestin/β2-adaptin interaction. We also quantified the interaction between β-arrestin2 and β2-adaptin using a lacZ reporter assay. Our results show that expression of c-Src with β-arrestin2 and β2-adaptin induced a 2- and 4-fold increase in the β-galactosidase activity as compared with yeasts expressing β-arrestin and β2-adaptin, or yeasts express-

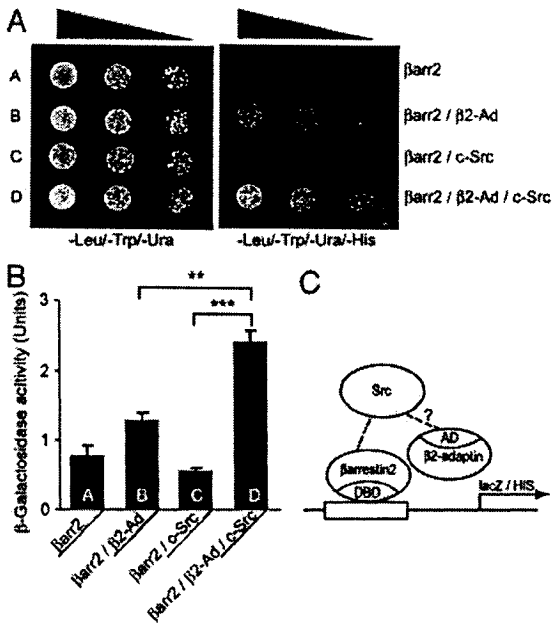

Fig. 3. c-Src Increases the Interaction between β-Arrestin and β2-Adaptin in Yeast

A, DNA encoding the hybrid protein β-arrestin2 (*GAL4* DBD-βarr2) was transformed in the yeast strain PJ69–4a alone (lane A), or with the hybrid protein β2-adaptin (*GAL4* AD β2-Ad) (lane B), c-Src (lane C), or β2-adaptin and c-Src together (lane D) as described in *Materials and Methods*. Transformants were serially diluted (at an A_{600} of 0.1, 0.002, 0.004, respectively) and 10 μl of cell suspension were spotted onto selective medium lacking leucine, tryptophan and uracil (−Leu/−Trp/−Ura), or lacking leucine, tryptophan, uracil and histidine (−Leu/−Trp/−Ura/−His). Protein-protein interaction was assessed for histidine complementation in presence of 2.5 mM 3-AT (A) and for β-galactosidase activity (B). β-Galactosidase activity is expressed as Miller units as described in *Materials and Methods*, and represents the mean ± SEM of three independent transformations in triplicates. C, Schematic representation of ternary complex formation between the two hybrid proteins, β-arrestin2 and β2-adaptin, and the bridge protein c-Src. Results show that the expression of c-Src with the two-hybrid proteins (column D) increases the β-galactosidase activity by 4- and 2-fold as compared with conditions when β-arrestin2 and c-Src (column C), or β-arrestin2 and β2-adaptin (column B) were expressed together. β2-Adaptin expression alone or with c-Src did not increase significantly the β-galactosidase activity as compared with conditions expressing β-arrestin2 and c-Src (data not shown). **, $P < 0.01$; ***, $P < 0.001$ determined by one-way ANOVA, and was considered highly significant.

ing β-arrestin and c-Src, respectively (Fig. 3B, D *vs.* B and D *vs.* C, respectively). These results indicate that the expression of c-Src in yeast stabilizes the ternary complex containing β-arrestin2 and β2-adaptin. As expected, we did not find that the expression of the c-Src mutant increased the tyrosine phosphorylation of total yeast protein (data not shown). Altogether, these results demonstrate that the interaction between β-arrestin and β2-adaptin can be stabilized in the presence of c-Src, and support our previous observations that the kinase activity of c-Src is not necessary to induce the agonist-independent formation of the complex.

The c-Src-Mediated Formation of a β-Arrestin/AP-2 Complex Requires an Intact β2-Adaptin Binding Domain on β-Arrestin

We have previously shown that β2-adaptin binds directly β-arrestin2 through a critical arginine residue (R396) present in the C-terminal domain of β-arrestin (8). This region also contains a motif for the binding of clathrin (LIEF, residues 374–377) (7–9, 22). Therefore, we used different β-arrestin2 mutants impaired in β2-adaptin binding (βarr2 R396A), impaired in both clathrin and β2-adaptin binding (where the hydrophobic residues of LIEF motif were substituted for alanines, βarr2-R396A,-AAEA) or a truncated form of β-arrestin2 lacking both sites (βarr2-T372), and assessed the ability of c-Src to promote the agonist-independent binding of β2-adaptin to the different β-arrestin mutants. COS-7 cells were transfected with either Flag-tagged β-arrestin2 or mutants, with or without HA-c-Src, and the amount of endogenous AP-2 in the β-arrestin immunoprecipitates was determined by Western blot analysis (Fig. 4). As previously observed and shown here, the overexpression of HA-c-Src induced the association of AP-2 with β-arrestin2 (Figs. 2 and 4). However, c-Src failed to promote the robust association of AP-2 to the different β-arrestin2 mutants as compared with wild-type β-arrestin2 (Fig. 4). These results demonstrate that the c-Src-induced formation of a β-arrestin/AP-2 complex requires an intact β2-adaptin binding site on β-arrestin2 C-terminal domain.

The Kinase Activity of c-Src Is Required to Promote the Dissociation of AP-2 from β-Arrestin2 and Agonist-Occupied AT1R

We have previously shown for some GPCRs internalizing via CCVs, such as the β2AR that the recruitment of AP-2 to β-arrestin and to the receptor required agonist activation (9). We therefore first verified whether the agonist stimulation of AT1R could induce the recruitment of AP-2 to β-arrestin and the receptor (Fig. 5). COS-7 or human embryonic kidney (HEK) 293 cells were used to express Flag-tagged β-arrestin2 and wild-type AT1R, or Flag-tagged AT1R alone. Cells were serum starved and then stimulated with Ang II for different periods of time (Fig. 5, A and B, respectively). β-Arrestin2 in COS-7 cells or AT1R in HEK 293 cells was then immunoprecipitated and the amount of endogenous AP-2 in the complexes, as detected by the presence of β2-adaptin, was determined by Western blot analysis (Fig. 5, A and B, lane 1–3). In unstimulated cells, little association of AP-2 to β-arrestin and AT1R was observed, consistent with what we observed for other GPCRs (9). Stimulation of receptors induced the

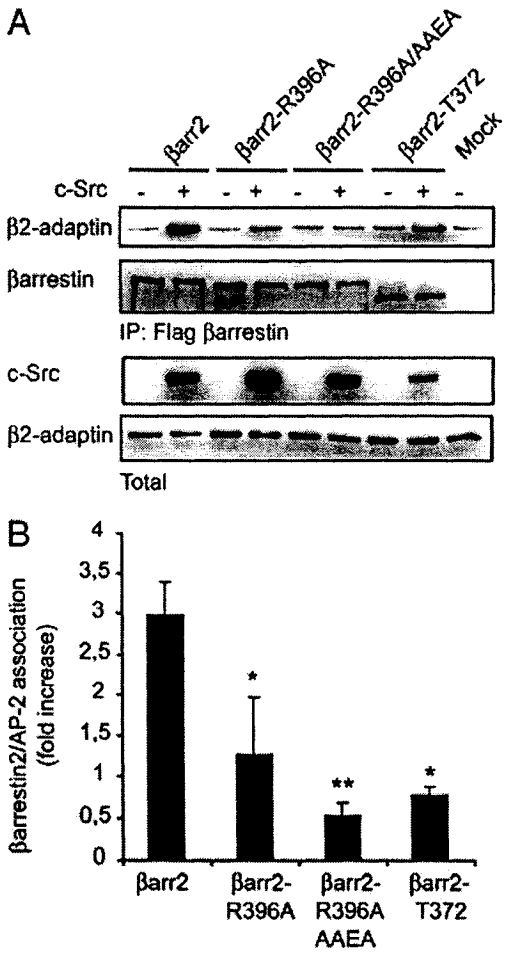

Fig. 4. Effect of c-Src expression on AP-2 Association with β-Arrestin Mutants Lacking β2-Adaptin and/or Clathrin Binding Sites

A and B, COS-7 cells were transiently transfected with either Flag-β-arrestin2 or Flag-β-arrestin2-R396A, Flag-β-arrestin2-R396A/AAEA, Flag-β-arrestin2-T372, with or without HA-c-Src. β-Arrestin2 wild type or mutants were immunoprecipitated with an anti-Flag antibody (Sigma), and the endogenous AP-2 in the immunoprecipitates was detected by Western blot using β2-adaptin (BD Transduction Laboratories) antibody. Whole cell extracts (Total) were blotted with anti-HA (Santa Cruz Biotechnology) antibody for c-Src detection, and with the same anti-β2-adaptin antibody as described above. B, Results are expressed as the fold increase association of AP-2 with β-arrestin2 in presence of c-Src (+) as compared with conditions where c-Src was not overexpressed (−), and represent the mean ± SEM of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$ as compared with wild-type β-arrestin determined by one-way ANOVA test.

recruitment of AP-2 to both β-arrestin and the receptor in a time-dependent manner reaching a maximum after 5 min of agonist treatment (Fig. 5, A and B, lanes 1–3). Association of AP-2 with β-arrestin was transient and the complex was lost after 15 min of agonist treatment (data not shown). The time frame of AP-2 recruitment to AT1R in HEK 293 cells (Fig. 5B) was similar to that observed for the interaction between β-arrestin and AP-2 in COS-7 cells (Fig. 5A) or in HEK 293 cells (data not shown). Together, these results suggest that AP-2 is recruited to the same AT1R/β-arrestin complex. Indeed, in a different set of experiments, when the agonist-occupied AT1R was immunoprecipitated, both β-arrestin and AP-2 were found in the same receptor complex (data not shown).

To elucidate the mechanism by which c-Src regulates the agonist-mediated formation of this endocytic complex, we next assessed the effect of overexpressing c-Src on the association of AP-2 with β-arrestin and AT1R after receptor stimulation. When c-Src was overexpressed, AP-2 was already detected in the β-arrestin immunoprecipitates in the absence of agonist treatment, and almost amounted to levels of stimulated receptors in the condition where c-Src was not overexpressed (Fig. 5A, compare lanes 3 and 4). Agonist stimulation did not promote further recruitment of AP-2 to β-arrestin but instead induced the rapid dissociation of the complex, which was complete after 5 min of agonist treatment. The overexpression of c-Src also promoted a basal association of AP-2 with AT1R. Similar to the experiment with β-arrestin, Ang II stimulation induced the rapid dissociation of AP-2 from the receptor (Fig. 5B). AP-2 interaction with AT1R was completely lost after 5 min of Ang II stimulation. Our data indicate that c-Src overexpression promotes the rapid dissociation of AP-2 from agonist-occupied AT1R and β-arrestin complexes.

To determine whether the kinase activity of c-Src is involved in regulating the dissociation of AP-2 from the agonist-stimulated receptor, we overexpressed the kinase inactive c-Src-K298R and assessed the recruitment of AP-2 to the agonist-occupied AT1R. HEK 293 cells were transfected with Flag-AT1R and c-Src-K298R, and the amount of endogenous AP-2 in the receptor immunoprecipitates was determined by Western blot analysis (Fig. 5C). The overexpression of c-Src-K298R induced a basal association of AP-2 with the receptor, similar to that observed with wild-type c-Src (compare Fig. 5, C and B). However, whereas c-Src induced the rapid dissociation of AP-2 from agonist-occupied receptor (Fig. 5B), the stimulation of AT1R in cells expressing c-Src-K298R promoted both an increased and sustained association of AP-2 with the receptor. Altogether, these results indicate that the kinase activity of c-Src promotes the dissociation of AP-2 from β-arrestin and AT1R only when receptors are activated by the ligand.

Depletion of c-Src Affects the Redistribution of Agonist-Bound AT1R Inside the Cell and the Dissociation of AP-2 from the Receptor and β-Arrestin

We next examined the effect of depleting c-Src on the agonist-mediated endocytosis of AT1R using a small interfering RNA (siRNA) strategy. HEK 293 cells were

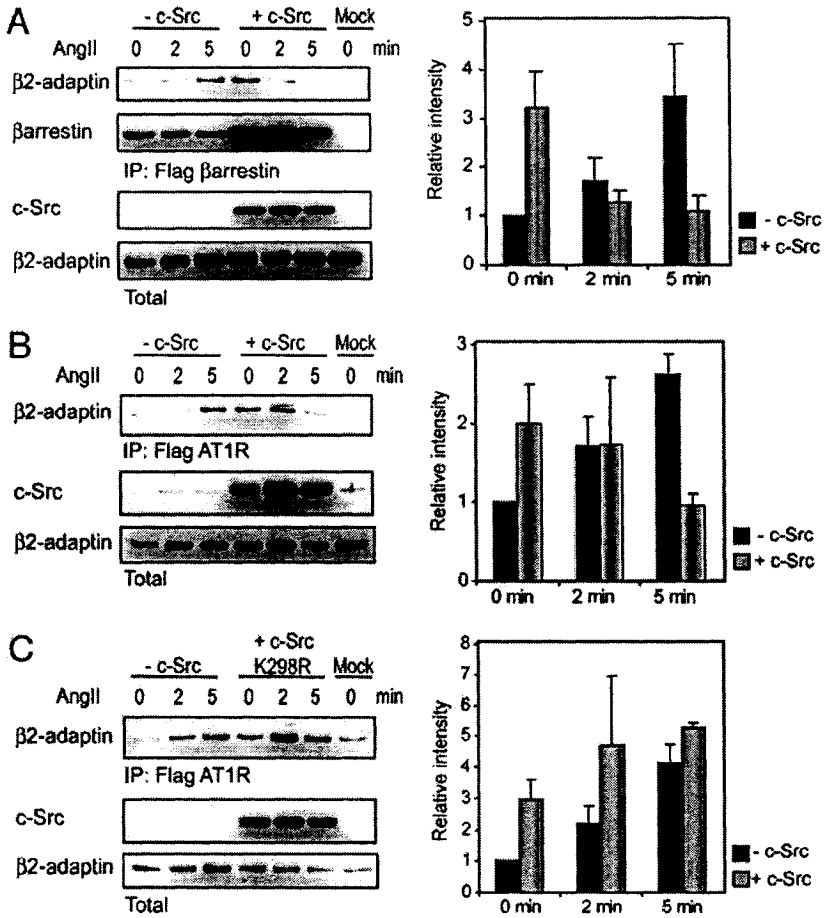

Fig. 5. c-Src Promotes the Dissociation of AP-2 from the Agonist-Occupied AT1R and β-Arrestin

COS-7 cells were transfected with AT1R and Flag-β-arrestin2 with or without c-Src (A), or HEK 293 cells were transfected with Flag-AT1R with or without c-Src and c-Src-K298R (B and C). Cells were serum-starved 12 h before being stimulated with Ang II (1 μM) for the indicated time, and β-arrestin2 (A) or AT1R (B and C) were immunoprecipitated (IP) using an anti-Flag antibody (Sigma). Immunoprecipitates were analyzed by Western blot for the detection of associated endogenous AP-2 using an antibody against β2-adaptin (BD Transduction Laboratories). Whole cell extracts (Total) were also probed for the detection of c-Src using the GD11 antibody (Upstate) and endogenous AP-2 using the BD Transduction Laboratories antibody. Data represent the relative amount of β2-adaptin found in AT1R and β-arrestin2 immunoprecipitates after agonist-stimulation of receptors after normalizing for equal amounts of β2-adaptin present in total proteins, and are the mean ± SEM of three independent experiments.

transfected with HA-AT1R and siRNA for human c-Src (siRNA-c-Src), and the ability of siRNAs to silence the expression of c-Src was compared with cells transfected with the receptor and either pcDNA3.1 (Mock) or siRNA for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (siRNA-GAPDH) (Fig. 6A). siRNA-c-Src reduced the expression of endogenous c-Src by more than 65% and 55%, respectively, compared with either mock or the nonsilencing control siRNA-GAPDH. The silencing of c-Src showed significant selectivity and did not affect the expression of β2-adaptin and AT1R (Fig. 6A, and results not shown, respectively). We next investigated using confocal microscopy, the effect of siRNA-c-Src on the early events of AT1R internalization after agonist stimulation. HEK 293 cells were transfected with HA-AT1R and green fluorescent protein (GFP)-β2-adaptin as a marker of AP-2 complex (22, 23) with or without siRNA-c-Src, and cells were labeled at 14 C for receptor detection. The redistribution of labeled-AT1R and GFP-β2-adaptin was visualized after a 5 min treatment of cells with Ang II at 37 C. In the absence of agonist, AT1R was found at the plasma membrane in punctuate structures, and little colocalization of the receptor with GFP-β2-adaptin was observed (Fig. 6B, *upper left panel*). Agonist treatment of cells for 5 min induced the rapid endocytosis of labeled-AT1R from the plasma membrane into intracellular vesicles with no noticeable redistribution of β2-adaptin fluorescence. In c-Src-depleted cells and in the absence of Ang II stimulation, AT1R was found at the plasma membrane and did not colocalize with GFP-β2-adaptin even though qualitatively more β2-adaptin fluorescence was detected at the plasma membrane. After agonist stimulation of receptors for 5

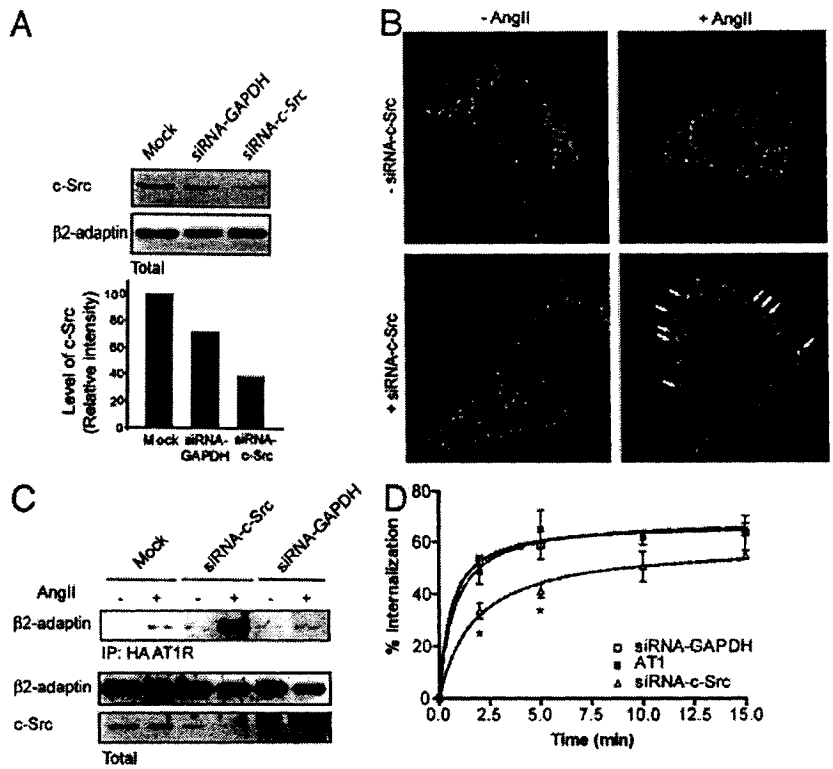

Fig. 6. Depleting c-Src in Cells Affects the Agonist-Mediated Internalization of AT1R and Its Association with AP-2

A, HEK 293 cells were transiently transfected with HA-AT1R with pcDNA3.1 (Mock), siRNA-GAPDH, or siRNA-c-Src. Seventy-two hours after transfection, total cell lysates were analyzed by Western blot for the expression of endogenous c-Src using the GD11 (Upstate) and the β2-adaptin (BD Transduction Laboratories) antibodies. B, HEK 293 cells were transfected with HA-AT1R and GFP-β2-adaptin, and either pcDNA3.1 (*upper panels*) or siRNA-c-Src (*lower panels*). Cells were serum starved for 12 h before being incubated with anti-HA antibody (12CA5, Roche) and with a secondary goat antimouse antibody conjugated to AlexaFluor 568 (Molecular Probes) as described in *Materials and Methods*. Cells were then washed, and were either left untreated or treated with Ang II (1 μM) for 5 min at 37 C. The distribution of HA-AT1R and GFP-β2-adaptin was visualized by confocal microscopy. For each experiment, 25 different fields containing one to two cells were analyzed. Shown are representative images of receptor immunofluorescence (*red*) and GFP-β2-adaptin fluorescence (*green*) of three independent experiments. Colocalization (*yellow*) of GFP-β2-adaptin with the receptor is indicated by *arrows*. C, HEK 293 cells were transiently transfected with HA-AT1R with either pcDNA3.1 (Mock), siRNA-c-Src, or siRNA-GAPDH. Cells were serum-starved as previously described before being stimulated with Ang II (1 μM) for 5 min. AT1R was immunoprecipitated (IP) using an anti-HA antibody (Roche), and the immunoprecipitates were analyzed by Western blot for the detection of associated AP-2 using the β2-adaptin antibody (BD Transduction Laboratories). Whole cell extracts (Total) were also probed for the expression of endogenous c-Src using the GD11 (Upstate) and the β2-adaptin (BD Transduction Laboratories) antibodies. Blots are representative of two independent experiments. D, For receptor internalization, cells were transfected with AT1R alone (■) and with either siRNA-c-Src (Δ) or siRNA-GAPDH (□). Cells were incubated with [^{125}I]-Ang II (0.11 nM) at 37 C for the different period of time, and the percent of receptor internalization was calculated as described in *Materials and Methods*. Data are the mean ± SEM of three to five independent experiments and were analyzed using Prism4 (GraphPad 4 Software). *, $P < 0.05$ siRNA-c-Src *vs.* AT1R alone determined by an unpaired *t* test.

min, AT1R was found mainly at the plasma membrane and mostly colocalized with GFP-β2-adaptin (Fig. 6B, *lower right panel*). The higher incidence of AT1R colocalization with β2-adaptin at the plasma membrane in c-Src-depleted cells is consistent with a role of c-Src in regulating the dissociation of AP-2 from the receptor. To verify that c-Src depletion affected the dissociation of AP-2 from AT1R, we performed coimmunoprecipitation experiments on cells that were transfected using the same conditions as for the confocal experiments (Fig. 6C). Results show that in cells with reduced c-Src expression, a robust increase in the agonist-mediated association of AP-2 with the immunopurified AT1R was observed, as compared with nondepleted cells (mock) and GAPDH-depleted cells. We also assessed the effect of reducing c-Src expression on AT1R internalization by radio-ligand binding assay using ^{125}I-AngII. As shown in Fig. 6D, agonist stimulation of HEK 293 cells expressing the AT1R alone promoted the rapid internalization of receptor-ligand complex inside the cell, which reached a maximum after only 5–10 min of agonist stimulation. The nonsilencing control siRNA-GAPDH did not affect the rate or the extent to which AT1R

internalizes. However, reducing the endogenous expression of c-Src delayed receptor endocytosis, and maximal internalization was reached only after 10–15 min of Ang II stimulation (Fig. 6D). Internalization was significantly reduced in c-Src-depleted cells as compared with nondepleted cells (AT1R alone) at 2 and 5 min of receptor stimulation (34 ± 3% *vs.* 52 ± 2%; and 41 ± 2% *vs.* 63 ± 4%, respectively). The half-time of AT1R internalization (the time to which 50% of receptors were internalized) in c-Src-depleted cells increased 2-fold as compared with nonsilenced cells (Fig. 6D). The delay in the initial rate of AT1R internalization observed in c-Src-depleted cells suggests that c-Src is involved in the early events of receptor internalization.

To substantiate the role of c-Src in regulating the association of AP-2 with AT1R/β-arrestin complexes, we depleted c-Src expression in an endogenous system like the VSMCs. Cells were first treated with control antisense DNA (CTL-1) or antisense DNA for c-Src (AS-1), and the amount of c-Src expression was assessed. As shown in Fig. 7A, AS-1 selectively decreased by more than 60% the expression of c-Src, whereas the CTL-1 had no effect on the expression of the protein. We next performed coimmunoprecipitation experiment to assess the effect of reducing c-Src expression on AP-2 association with endogenous β-arrestin after AT1R stimulation. VSMCs transfected with either AS-1 or CTL-1 were left unstimulated or stimulated with Ang II for 5 min, and the level of AP-2 (as assessed by the presence of the β2-adaptin) in the β-arrestin immunoprecipitates was evaluated by Western blot analysis (Fig. 7B). Ang II stimulation induced a rapid association of AP-2 with β-arrestin. However, the extent to which AP-2 associated with β-arrestin was greater in c-Src-depleted VSMCs than in control cells. All together, these results support the role of c-Src in regulating the dissociation of AP-2 from AT1R and β-arrestin during the internalization of receptors.

DISCUSSION

Here we show that AP-2 is recruited to β-arrestin and AT1R after receptor stimulation, extending previous studies that AT1R internalizes through the clathrin pathway (11–15). More importantly, we provide evidence that c-Src regulates the binding of AP-2 to

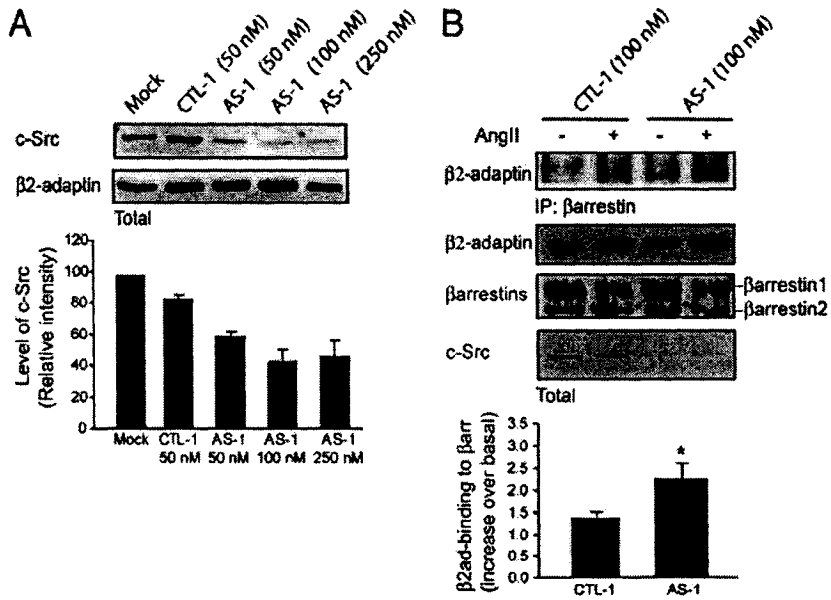

Fig. 7. Depleting c-Src in VSMCs Increases the AngII-Mediated AP-2 Association with β-Arrestin

A, VSMCs cells were transfected or left untransfected (Mock) with either antisense oligonucleotides for the rat c-Src (AS-1) or control oligonucleotides (CTL-1). Thirty-six hours after transfection, total cell lysates from VSMCs were analyzed by Western blot for the expression of endogenous c-Src using the GD11 (Upstate) and β2-adaptin (BD Transduction Laboratories) antibodies. B, VSMCs were transfected with oligonucleotides for 36 h in serum-free medium as described in *Materials and Methods*. Cells were then stimulated with Ang II (1 μM) for 5 min, and β-arrestin was immunoprecipitated (IP) using the A1CT antibody. Proteins in the immunoprecipitated complexes were analyzed by Western blot for the detection of associated AP-2 using the β2-adaptin antibody. Whole cell extracts (Total) were also probed for the detection of endogenous c-Src using the GD11 (Upstate), β-arrestin (A1CT) and β2-adaptin antibodies. Shown in A and B are representative blots of four independent experiments. Data were quantified and presented as the relative amount of c-Src found in cells after normalizing to the total amount of β2-adaptin (A), and the fold increase over basal of the associated β2-adaptin with β-arrestin (B). *, $P < 0.05$ AS-1 *vs.* CTL-1 as determined by one-tail paired *t* test.

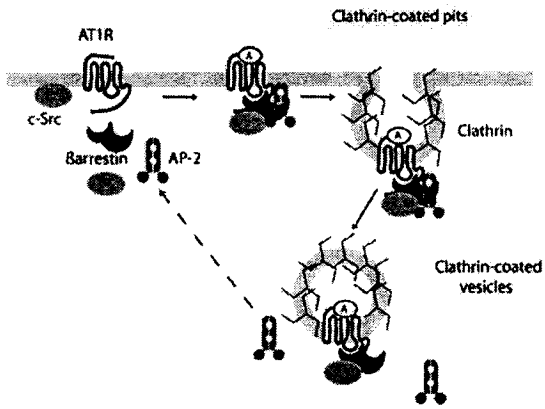

Fig. 8. Proposed Model for the Role of c-Src in the Regulation of the Interaction between AP-2, AT1R, and β-Arrestin during Receptor Internalization

Agonist stimulation of AT1R promotes the recruitment of a ternary complex containing c-Src, AP-2 and β-arrestin. The complex would then be targeted to CCPs, and the presence of multiple receptor complexes in addition to the recruitment of other endocytic proteins into the CCP would trigger the internalization of the coated vesicle. During AT1R internalization, c-Src could promote the dissociation of AP-2 from the receptor/β-arrestin complex to allow the recycling of AP-2 for further rounds of coat formation and AT1R targeting to CCPs. AT1R, Ang II type 1 receptor; A, agonist.

β-arrestin and agonist-occupied AT1R. Indeed, we demonstrate that c-Src can form a stable complex with AP-2 and β-arrestin, and that its kinase activity is required to promote the agonist-mediated dissociation of AP-2 from both β-arrestin and AT1R during receptor internalization. In support of these results is our finding that depleting c-Src expression in cells increases the binding of AP-2 with the agonist-stimulated AT1R and β-arrestin and prevents the rapid redistribution of the agonist-bound receptor inside the cell.

The recruitment of AP-2 to β-arrestin is mediated by the agonist stimulation of GPCRs (9). Interestingly, c-Src overexpression was sufficient to promote the stable association between AP-2 and β-arrestin, and AT1R without inducing receptor endocytosis (results not shown). This effect was independent of the kinase activity of c-Src but required the presence of an intact β2-adaptin binding site on β-arrestin. It was recently suggested, based on the crystal structure of β-arrestin, that the activation of receptors is necessary to induce favorable conformational changes in β-arrestin required to unveil the β2-adaptin binding site in the C-terminal domain of β-arrestin (24). Our results suggest that c-Src binding to β-arrestin induces a conformational change in β-arrestin allowing the agonist-independent binding of AP-2 to β-arrestin and AT1R. Interestingly, a mutant that mimics the activated form of c-Src (c-Src-Y530F) was unable to promote the association of AP-2 to β-arrestin. One explanation may be that c-Src-Y530F cannot induce the appropriate conformation in β-arrestin to promote the binding of β2-adaptin to β-arrestin, even though we found that c-Src-Y530F still associated with β-arrestin. Another possibility is that this c-Src mutant, because of its potentially increased kinase activity, induces the phosphorylation of protein(s) involved in the dissociation of AP-2 from β-arrestin, thus preventing the formation of a ternary complex. This latter possibility raises the question of what proteins c-Src is targeting? One potential candidate is the β-subunit of the AP-2 complex itself. It was recently reported that EGFR activation could induce the tyrosine phosphorylation of β2-adaptin (25). Whether c-Src phosphorylates β2-adaptin or other endocytic proteins after AT1R stimulation, and to what extent this phosphorylation plays a role in regulating the dissociation of AP-2 from β-arrestin and the receptor remain to be determined.

The recruitment of signaling effectors into CCPs has been shown to affect components of the clathrin coat and to regulate the efficient agonist-dependent endocytosis of receptors. The presence of c-Src into CCPs affects coat proteins such as dynamin and clathrin (17, 26). For instance, EGFR stimulation induces the c-Src-dependent phosphorylation of dynamin and clathrin, which plays a role in EGFR endocytosis (26, 27). For GPCRs such as the β2AR, the agonist-dependent binding of β-arrestin to receptors induces the formation of a complex containing β-arrestin and c-Src (28). The β-arrestin-mediated recruitment of c-Src to β2AR brings the kinase in close proximity of dynamin, and regulates the function of dynamin through phosphorylation (17). Our results suggest another function for the β-arrestin-mediated recruitment of c-Src. In this paradigm, the agonist stimulation of AT1R promotes the translocation of β-arrestin to the receptor, and the recruitment of both c-Src and AP-2 to the AT1R (Fig. 8). The binding of c-Src to β-arrestin and AP-2 would then stabilize the endocytic complex and allow the receptor to be efficiently targeted to the CCP. The Ang II-mediated recruitment of c-Src into the CCP would serve to regulate the dissociation of AP-2 from β-arrestin and may phosphorylate other proteins of the coat to ensure efficient endocytosis of AT1R. In this regard, it will be of interest to examine whether the stimulation of AT1R triggers the phosphorylation of dynamin and clathrin, and to which extent these c-Src-mediated events contribute in regulating the dissociation of AP-2 from the receptor/β-arrestin complex, and affect the internalization of AT1R and/or other GPCRs that internalize in a β-arrestin- and a clathrin-dependent manner.

Several lines of evidence suggest that for efficient clathrin-mediated internalization, the endocytic proteins that are recruited into CCPs for initiating endocytosis must ultimately dissociate from the internalizing vesicle (29, 30). For instance, the dissociation of AP-2 from its internalized CCV is believed to be necessary to allow the recycling of the clathrin adapter for further rounds of CCP formation and receptor internalization. Thus, preventing the dissociation of AP-2

from β-arrestin and/or AT1R would presumably affect the internalization of receptors. In support of this is our finding that AP-2 association with AT1R and β-arrestin increases when c-Src expression was reduced in cells, and that under such condition the internalization of receptors was delayed. The accumulation of AP-2 to the receptor at the plasma membrane may ultimately impede the formation of subsequent CCPs, and slow down receptor internalization. Further studies will be necessary to determine the extent to which c-Src participate in the assembly and disassembly of CCPs and the internalization of other GPCRs.

In summary, our results provide a novel function for c-Src in the formation of endocytic complexes during the internalization of AT1R. They also provide another mechanism by which β-arrestin, through the recruitment of signaling effectors like c-Src, can regulate the internalization of receptors through the clathrin pathway.

MATERIALS AND METHODS

Materials

Ang II was purchased from Sigma (St. Louis, MO) [^{125}I]-Ang II (1000 Ci/mmol) was obtained from Dr. Gaétan Guillemette (Université de Sherbrooke, Québec, Canada) and prepared as previously described (31). Antibodies against β-adaptin and α-adaptin were from BD Transduction Laboratories (BD Biosciences, Palo Alto, CA), the c-Src (SRC2) and HA rabbit antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), the mouse anti-HA clone 12CA5 was from Roche Molecular Biochemicals (Indianapolis, IN), the FLAG antibody was from Sigma, and the c-Src antibody GD11 and the antiphosphotyrosine 4G10 were from Upstate Biotechnology (Lake Placid, NY). The rabbit polyclonal β-arrestin antibody (A1CT) was kindly provided by Dr. Marc G. Caron (Duke University, Durham, NC).

Plasmids and Constructs

The HA-tagged c-Src and the HA-tagged c-Src-Y530F mutant were a gift from Dr. William E. Miller (University of Cincinnati, Cincinnati, OH). HA-tagged c-Src-K298R mutant was generated by PCR using the full-length HA-tagged c-Src in pcDNA3. A PCR fragment was amplified using a forward primer containing the mutation coding for the substitution K298R and a *Bam*HI restriction site, and a reverse primer overlapping the *Kpn*I site in c-Src. The fragment was digested with *Bam*HI and *Kpn*I, and replaced into c-Src cut with the same enzymes. Flag-tagged β-arrestin R396A, and R396A/AAEA were generated by replacing an *Xho*I/*Xba*I fragment from the rat β-arrestin2 wild type with a PCR product containing the mutations and the Flag sequence. The truncated Flag-tagged β-arrestin2 (βarr2-T372) was generated by PCR using a similar cassette replacement strategy to insert the Flag sequence after K372 in β-arrestin. The p426-ADH-c-Src was generated by excising the avian c-Src mutant (c-Src-G2A,-Y416F,-Y527F) from the p413-Gal1 vector (kindly provided by Dr. Serge Lemay, McGill University, Québec, Canada) with *Bam*HI and *Sal*I, and cloning the fragment into p426-ADH (kindly provided by Dr. Bernard Turcotte, McGill University) using the same restriction sites. Green-fluorescent-tagged β2-adaptin (GFP-β2-adaptin) construct was described elsewhere (9). All constructs were analyzed by DNA sequencing (Service d'analyze et de synthèse d'acides nucléiques, Université Laval, Québec, Canada).

Cell Culture and Transfection

Human embryonic kidney cells (HEK 293) were grown in Eagle's MEM (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS, Invitrogen Life Technologies) and gentamicin (100 μg/ml, Invitrogen Life Technologies). HEK 293 cells seeded in a 100-mm dish, at a density of 2.5×10^6 cells/dish, were transiently transfected using a conventional calcium phosphate coprecipitation method. One to 5 μg of DNA were mixed in a solution containing 125 mM $CaCl_2$ in HEPES-buffered saline [25 mM HEPES (pH 7.4), 140 mM NaCl, 0.75 mM Na_2HPO_4] for 5 min, and the mixed solution was then added to cells. Green monkey kidney cells (COS-7) were grown in DMEM (Invitrogen Life Technologies) supplemented with 10% (vol/vol) heat-inactivated FBS and gentamicin (100 μg/ml). Transient transfections of COS-7 cells seeded in 60-mm dishes (at a density of 1×10^6 cells/dish) were performed using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's recommendations using a 1:3 ratio of DNA/Lipofectamine in Opti-MEM (Invitrogen Life Technologies). Twenty-four hours post transfection, HEK 293 and COS-7 cells were serum starved for 12 h in DMEM before performing the experiments. For vascular smooth muscle cells (VSMCs; kindly provided by Dr. Sylvain Meloche at the Université de Montréal, Québec, Canada), cells were grown in DMEM low glucose with 10% (vol/vol) heat-inactivated calf serum (Invitrogen Life Technologies) and gentamicin (100 μg/ml), and they were serum-starved in DMEM low glucose for 12 h before performing the experiments.

Immunoprecipitation and Western Blot Experiments

HEK 293 or COS-7 cells were left untreated or treated with Ang II (100 nM) at 37 C for the indicated period of time. After agonist stimulation, the reaction was stopped on ice by washing cells with ice-cold PBS. Cells were then solubilized in P-RIPA buffer (1 ml/100 mm dish) [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40 (vol/vol), 0.1% deoxycholate (wt/vol), 1 mM $CaCl_2$] containing 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptine, 25 μg/ml aprotinin, and 1 mM pepstatin A for 30 min at 4 C. Cell lysates were centrifuged for 30 min at 21,000 × *g* and the supernatants were incubated with anti-Flag (30 μg/ml, Sigma) or c-Src antibody (3 μg/ml, clone GD11, Upstate) with 20 μl of a 50% slurry mixture of protein A/G Sepharose beads for 2 h at 4 C. Beads were washed three times with P-RIPA, and denatured in Laemmli buffer (2×) [250 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS) (wt/vol), 10% glycerol (vol/vol), 0.01% Bromophenol Blue (wt/vol), 5% β-mercaptoethanol (vol/vol)]. Proteins were separated on a 10% SDS-PAGE before being transferred onto nitrocellulose membranes (Amersham Biosciences, Arlington Heights, IL). Membranes were then blocked with a solution of PBS (pH 7.4) containing 0.05% Tween 20 (vol/vol) (PBS-T) supplemented with 10% skin milk (wt/vol). Protein detection on membranes was assessed using either anti-c-Src antibody (1 μg/ml, clone GD11; or 0.2 μg/ml, SRC2), Flag antibody (2.5 μg/ml), HA antibody (0.2 μg/ml), phosphotyrosine antibody (0.2 μg/ml, clone 4G10), β2-adaptin antibody (0.2 μg/ml, β-adaptin), α-adaptin antibody (0.25 μg/ml), β-arrestin antibody (diluted 1/5,000; A1CT) in PBS-T containing 1% BSA (fraction V; Sigma) (wt/vol) and 1% skin milk (wt/vol). After 1 h incubation with the different primary antibodies, membranes were washed three times in PBS-T before being incubated at room temperature with horseradish peroxidase-conjugated goat antimouse antibody (diluted 1/10,000; Sigma), or horseradish peroxidase-goat antirabbit antibody (diluted 1/10,000; Sigma) for 30 min. Immunoreactivity was visualized by en-

hanced chemiluminescence according to the manufacturer's instructions (SuperSignal, Pierce, Rockford, IL).

Immunofluorescence Experiments

HEK 293 cells were grown on 22-mm^2 coverslips in six-well plates (250,000 cells/well), and transfected with GFP-β2-adaptin (0.5 μg) and HA-AT1R (1.5 μg). For AT1R immunodetection, cells were preincubated for 1 h at 14 C with a mouse anti-HA antibody (20 μg/ml, 12CA5), followed by incubation at 14 C for 1 h with a secondary goat antimouse antibody conjugated to AlexaFluor 568 (8 μg/ml, Molecular Probes, Eugene, OR). Labeled cells were stimulated at 37 C with 100 nM Ang II for 5 min, and fixed for 15 min at room temperature with a solution of 4% paraformaldehyde (wt/vol) in PBS. Coverslips were mounted with GelTol Aqueous Mounting Medium (IMMUNON, Thermo Shandon, Pittsburgh, PA). Confocal images of labeled cells were acquired with a Zeiss LSM-510 META laser-scanning microscope (Carl Zeiss, Jena, Germany) using a 60× oil immersion lens. GFP-β2-adaptin and AlexaFluor 568-labeled receptor fluorescence was visualized using the multitrack mode with dual laser excitation (488 and 543 nm), and emission (BP 505–520 for GFP, and LP 560 for AlexaFluor 568) filter sets. Final figures were collated using Adobe Photoshop 7.0 and Illustrator 10.0 (Adobe, San Jose, CA).

Three-Hybrid Assay

Yeast two-hybrid MatchMaker System 2 (CLONTECH, BD Biosciences), was used with minor modifications to create a three-hybrid assay that allowed the detection of a ternary complex between β-arrestin2, β2-adaptin and c-Src. Constructs for *GAL4*-DBD β-arrestin2 in pAS2-1 and *GAL4*-AD β2-adaptin in pACT-2 were described elsewhere (9). PAS2-1-β-arrestin2 and pACT-2-β2-adaptin vectors were cotransformed with the empty vector p426-ADH or the p426-ADH-c-Src into the yeast strain PJ69-4a using the poly(ethylene-glycol) (PEG)/lithium acetate method (CLONTECH). Yeast colonies were grown on medium lacking tryptophan, leucine, and uracil for the selection of transformants expressing the bait vector (pAS2-1-β-arrestin2), the prey vectors (pACT-2 or pACT-2-β2-adaptin), and the bridge vector with or without c-Src (p426-ADH-c-Src or p426-ADH). Protein-protein interaction was assessed using the HIS3 auxotrophic complementation, and a lacZ reporter assay. For the β-galactosidase assay, three colonies of each transformation were selected and grown on yeast extract peptone dextrose (YPD) medium at 30 C overnight. The next day, the yeast cultures were diluted to an A_{600} of 0.2 in synthetic dropout medium lacking tryptophan, leucine, and uracil, and grown for another 7 h at 30 C. Cultures were serially diluted at an A_{600} of 0.1, 0.002, and 0.004, and 10 μl of cell suspension were spotted onto medium lacking leucine, tryptophan, and uracil (−Leu/−Trp/−Ura) for selection of yeasts expressing the bait, the prey and the bridge vector, respectively, and selected onto medium lacking leucine, tryptophan, uracil, and histidine (−Leu/−Trp/−Ura/−His) for the detection of interactions between β-arrestin2 and β2-adaptin. Histidine complementation was assessed in presence of 2.5 mM 3-amino-1,2,4-triazole (3-AT, Sigma). β-Galactosidase activity was measured using the ONPG assay (O-nitrophenyl β-D-galactopyranoside, CLONTECH). Briefly, yeasts were grown on a selective medium to an A_{600} of 0.8–1.2, and cells from a 2-ml culture were collected by centrifugation. Pelleted cells were washed with cold Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 100 mM KCl, 1 mM $MgSO_4$, and 30 mM β-mercaptoethanol, at pH 7.0), and vigorously resuspended in 800 μl of Z-buffer containing 0.1% SDS (wt/vol) and 0.5% $CHCl_3$ (vol/vol). Colorimetric reaction was started at 30 C by adding 160 μl of ONPG (4 mg/ml) to permeabilized cells. The reaction was stopped with 400 μl of Na_2CO_3 (1 M) when yellow coloration started to appear. The activity was measured at A_{420} and Miller units were calculated from the equation [Units = 1000 × (A_{420}/ volume of cells assayed × time of run × A_{600})].

SiRNA and Antisense Experiments

All double-stranded siRNAs were synthesized using the Silencer siRNA kit (Ambion, Austin, TX) according to the manufacturer's instructions. Twenty-one-oligomer corresponding to the sense and antisense sequences of the human c-Src mRNA were screened for unique sequence in the National Center for Biotechnology Information database by using the basic local alignment and search tool algorithm (accession no. BC011566). The target mRNA identified for c-Src is 5′-AAG CAC UAC AAG AUC CGC AAG-3′, and corresponded to the position 607–628 (relative to the start codon) of c-Src. An extension that corresponded to the T7 promoter primer sequence (5′-CCT GTC TCT-3′) was added at the 3′ end of the sense and antisense sequence. Control siRNA sense and antisense oligonucleotides for GAPDH were provided in the kit.

For AT1R immunoprecipitation experiments, HEK 293 cells were seeded at a density of 400,000 cells/well in six-well plates, and transfected with Lipofectamine 2000. Briefly, pcDNA3.1 encoding the HA-AT1R (0.375 μg/well) was added with pcDNA3.1 (0.375 μg/well), siRNA-GAPDH (0.375 μg/well) or siRNA-c-Src (0.375 μg/well) and mixed with Lipofectamine 2000 (using a ratio of DNA/Lipofectamine of 1:2) in Opti-MEM. Complexes of DNA/Lipofectamine were added to cells in MEM without serum, and transfection was carried out for 12 h. The next day, media was replaced with complete MEM medium supplemented with 10% FBS (vol/vol) and gentamicin (100 μg/ml).

For immunofluorescence experiments, cells were seeded in six-well plates, at a density of 300,000 cells/well, and transfected with HA-AT1R (0.25 μg/well) and GFP-β2-adaptin (0.125 μg/well) with or without siRNA-c-Src (0.375 μg/well) using the same DNA/Lipofectamine ratio as described above.

For AT1R internalization experiments, HEK 293 cells were seeded at a density of 70,000 cells/well in 24-well plates, and transfected with Lipofectamine 2000 as described above using the same DNA/Lipofectamine ratio. Briefly, pcDNA3.1 encoding the HA-AT1R (0.125 μg/well) was added with pcDNA3.1 (0.5 μg/well), siRNA-GAPDH (0.5 μg/well) or siRNA-c-Src (0.5 μg/well) and mixed with Lipofectamine 2000 in Opti-MEM. Transfection was carried out for 12 h before replacing the medium with complete MEM supplemented with 10% FBS (vol/vol) and gentamicin (100 μg/ml) and performing the experiment 72 h post transfection.

For experiments depleting c-Src in VSMCs, an antisense oligonucleotide strategy was used. Control scramble phosphorothioate (CTL-1, 5′-GTC TTA GCC GGG ATC CGC TA-3′) and antisense oligonucleotides complementary to the sequence of the rat c-Src mRNA (AS-1, 5′-TT GCT CTT GTT GCT GCC CAT-3′, accession number NM_031977) were designed and synthesized by Alpha DNA (Montreal, Québec, Canada). VSMCs were seeded at a density of 2 × 10^6 cells in 10-cm dishes and transfected with antisense DNAs using Lipofectamine 2000 as described previously. Briefly, c-Src AS-1 (1.2–6.0 μg, representing a final concentration of 50–250 nM in 4 ml) or CTL-1 (1.2–2.4 μg; 50–100 nM in 4 ml) were mixed using a ratio of oligonucleotide/Lipofectamine of 1:2 in Opti-MEM. Complexes were added to cells in DMEM (Invitrogen Life Technologies) without serum and transfection was carried out for 36 h before performing the experiments.

Internalization Assay

Receptor internalization was performed as described previously with minor modifications (31). Briefly, HEK 293 cells

seeded in 24-well plates were transfected with HA-AT1R with or without siRNAs. Thirty-six 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 (wt/vol), 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 three times with either ice-cold PBS to remove the unbound Ang II or ice-cold acid buffer [0.2 N acetic acid (pH 3.5), 150 mM NaCl] to remove both the unbound and the cell surface receptor-bound agonist. Cells were then solubilized in 0.5N NaOH, 0.05% SDS (wt/vol), 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 Alpha Innotech Fluorochem imaging system (Packard Canberra, Montréal, Québec, Canada) and was represented as the mean ± SEM of at least three independent experiments. For densitometry analysis of Western blot and β-galactosidase assay, data were analyzed statistically by one-way ANOVA followed by a Bonferroni posttest for multiple comparisons, or by one-tail *t* test for single comparison. Means were considered significantly different when *P* values were at least below 0.05.

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