THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 49, Issue of December 5, pp. 31051–31057, 1997
© 1997 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Clathrin-mediated Endocytosis of the β-Adrenergic Receptor Is Regulated by Phosphorylation/Dephosphorylation of β-Arrestin1*

(Received for publication, August 8, 1997, and in revised form, September 24, 1997)

Fang-Tsyr Lin, Kathleen M. Krueger, Humphrey E. Kendall, Yehia Daaka, Zoey L. Fredericks, Julie A. Pitcher, and Robert J. Lefkowitz‡

From the Howard Hughes Medical Institute, Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

β-Arrestins serve a dual regulatory role in the life cycle of G protein-coupled receptors such as the β_2-adrenergic receptor. First, they mediate rapid desensitization by binding to G protein-coupled receptor kinase-phosphorylated receptors. Second, they target the receptors for internalization into endosomal vesicles, wherein receptor dephosphorylation and resensitization occur. Here we report that phosphorylation of a carboxyl-terminal serine (Ser-412) in β-arrestin1 regulates its endocytotic but not its desensitization function. Cytoplasmic β-arrestin1 is constitutively phosphorylated and is recruited to the plasma membrane by agonist stimulation of the receptors. At the plasma membrane, β-arrestin1 is rapidly dephosphorylated, a process that is required for its clathrin binding and receptor endocytosis but not for its receptor binding and desensitization. Once internalized, β-arrestin1 is rephosphorylated. Thus, as with the classical endocytic adaptor protein complex AP2, β-arrestin1 functions as a clathrin adaptor in receptor endocytosis which is regulated by dephosphorylation at the plasma membrane.

Endocytosis of many cell-surface receptors including those for epidermal growth factor, insulin, and transferrin is mediated by classical clathrin-coated vesicle mechanisms (1). G protein-coupled receptors such as the β_2-adrenergic receptor, M1 muscarinic cholinergic receptor, LH/HCG receptor, gastrin releasing peptide receptor, and others also utilize this pathway (2–5). In the case of tyrosine kinase receptors, endocytosis involves clustering of the receptors in coated pits formed by the recruitment and assembly of clathrin and associated molecules such as the AP2 complex and dynamin on the plasma membrane (6, 7). The heterotetrameric AP2 complex is a structural component of clathrin-coated pits on the plasma membrane that triggers the assembly of clathrin cages (8–10). It serves as an adaptor linking receptors to the structure of clathrin cages.

In the case of G protein-coupled β_2-adrenergic receptors, recent *in vitro* evidence has suggested that β-arrestins may play a role in linking the receptors to clathrin-coated pits (11–13). β-Arrestins were originally discovered in the context of homologous or agonist-specific desensitization of β_2-adrenergic receptors (14, 15). Following phosphorylation of the agonist-occupied receptors by β-adrenergic receptor kinase, β-arrestins bind to the receptors, thereby interdicting signal transduction to heterotrimeric G proteins (16). The arrestin family includes visual arrestin, β-arrestin1 (arrestin2), β-arrestin2 (arrestin3), and other splicing variants (17). Arrestin functions specifically in inactivation of rhodopsin (18, 19), whereas β-arrestin1 and β-arrestin2 exhibit similar functions in desensitization of non-visual G protein-coupled receptors (15).

When β-arrestin1 or -2 are overexpressed in cells, not only is desensitization of β_2-adrenergic receptors augmented, but their sequestration or internalization is promoted as well (20). Moreover, a "dominant negative" mutant of β-arrestin1 (V53D) impairs receptor endocytosis (20). β-Arrestin1 and -2 have been shown *in vitro* to bind with high affinity to clathrin cages, whereas visual arrestin does not, and β-arrestin/arrestin chimeras defective in either receptor or clathrin binding do not support agonist-dependent internalization of the β_2-adrenergic receptor (11–13).

Despite these data suggesting a role for β-arrestin1 in clathrin-mediated β_2-adrenergic receptor internalization, its role as an adaptor linking the receptors to clathrin cages has not been demonstrated in cells. Nor is anything known of how the putative association of β-arrestins and clathrin-coated pits on the plasma membrane might be regulated. Here we demonstrate that agonist-promoted recruitment and dephosphorylation of β-arrestin1 on the plasma membrane transforms it into a clathrin adaptor and thus controls the process of receptor endocytosis.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Site-specific Mutagenesis—A 1.26-kb[1] *Eco*RV fragment containing the entire β-arrestin1 coding sequences was digested from pVL1393/βarr1[2] and subcloned into the *Sma*I site of pKK223-3, a bacterial expression vector (Pharmacia Biotech Inc.). Then an oligonucleotide 5′-CATCACCATCACCATCAC-3′, encoding six histidine residues, was engineered at the end of the carboxyl-terminal coding sequences by the polymerase chain reaction (PCR) and designated pKK/βarr1–6xHis. This PCR product was verified by DNA sequencing. To express His-tagged β-arrestin1 in mammalian cells, a 0.5-kb 5′ *Eco*RI fragment of β-arrestin1 from pCMV5/βarr1 (15) was ligated to a 0.8-kb 3′ *Eco*RI/*Sal*I fragment of β-arrestin1–6xHis from pKK/βarr1–6xHis and was then inserted into the *Eco*RI/*Sal*I sites of pCMV5 to create pCMV5/βarr1–6xHis.

The β-arrestin1–6xHis coding sequences, removed from pKK/βarr1–6xHis by *Kpn*I and *Hin*dIII digestions, were subcloned into pBluescript KS(+). This plasmid was then linearized at the *Xho*I or *Xba*I site of the polylinkers as a template for mutagenesis by recombination PCR (21). The nucleotides TCT, encoding serine at amino acid 412, were replaced with GCT or GAT which mutates the serine to alanine or aspartic acid. The PCR

* This work was supported by the Howard Hughes Medical Institute and Grant HL16037 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Howard Hughes Medical Institute, Depts. of Medicine and Biochemistry, Duke University Medical Center, Box 3821, Durham, NC 27710. Tel.: 919-684-2974; Fax: 919-684-8875.

[1] The abbreviations used are: kb, kilobase pair; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; β_2-AR, β_2-adrenergic receptors; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

[2] H. Attramadal and R. J. Lefkowitz, unpublished data.

products were verified by DNA sequencing. To express mutant β-arrestin1, the 0.3-kb 3′ *Sma*I fragment of pCMV5/βarr1–6xHis was replaced with a corresponding fragment containing the mutated site and designated pCMV5/(S412A)βarr1–6xHis or pCMV5/(S412D)βarr1–6xHis.

Transfection and Metabolic Labeling—The plasmids of interest were transfected into HEK 293 cells by calcium phosphate co-precipitation. Stable cell lines were generated by co-transfecting empty vector or different β-arrestin1–6xHis expression plasmids with pSV_2Neo at a 10:1 ratio and were then selected with 400 μg/ml G418 for 2–3 weeks. Overexpression of wild-type or mutant β-arrestin1 was determined by Western blot analysis using an antibody specific to β-arrestin1 (15) and was visualized by enhanced chemiluminescence assay (ECL, Amersham Corp.).

For metabolic labeling, HEK 293 cells stably transfected with pCMV5/βarr1–6xHis were starved in phosphate-free Dulbecco's modified Eagle's medium (Life Technologies, Inc.) for 30 min, labeled for 2 h in the same medium containing [^{32}P]orthophosphate (1 mCi/ml), and harvested for β-arrestin1 purification. The stoichiometry of ^{32}P-labeled β-arrestin1 was determined as described (22).

Subcellular Fractionation—Cells incubated with or without 10 μM (−)-isoproterenol were washed with ice-cold phosphate-buffered saline (PBS), incubated with 250 μg/ml concanavalin A in PBS on ice for 20 min, scraped into 0.25 M sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, and disrupted by Dounce homogenization. Differential centrifugation was carried out as described (23). Nuclei and unbroken cells were removed by centrifugation at 1000 × *g* for 10 min. A crude plasma membrane fraction was precipitated by centrifugation of the supernatant at 3000 × *g* for 15 min. Then centrifugation of the resulting supernatant at 300,000 × *g* for 30 min gave rise to a pellet composed of the vesicles and other subcellular organelles as well as a supernatant containing cytosol. The cytosol was dialyzed in 1 × binding buffer (20 mM Tris, pH 7.9, 5 mM imidazole, and 0.5 M NaCl) containing 50 mM NaF, 10 mM sodium pyrophosphate, and a mixture of protease inhibitors for β-arrestin1 purification.

Purification of β-arrestin1 by Nickel Affinity and Heparin-Sepharose Chromatography—The cell pellets were lysed in 1 × binding buffer containing a mixture of protease inhibitors and 0.2% Nonidet P-40. To purify phospho-β-arrestin1, 50 mM NaF and 10 mM sodium pyrophosphate were also added to inhibit phosphatase activity. β-Arrestin1 was purified by Nickel Affinity Chromatography following the manufacturer's protocol (Novagen) except that following washing with 20 mM Tris, pH 7.9, containing 30 mM imidazole and 0.5 M NaCl, it was eluted with 100 mM imidazole in the same buffer.

To purify β-arrestin1 to the highest homogeneity, the 100 mM imidazole eluate was dialyzed in 25 mM Tris, pH 7.4, 5 mM EDTA, and 0.2 M NaCl and applied to heparin-Sepharose as described (24). β-Arrestin1 was purified with a linear gradient of NaCl from 0.2 to 1 M (a regular procedure) or directly eluted with 1 M NaCl (as shown in Fig. 3) and was desalted in 20 mM Tris, pH 7.4, and 2 mM EDTA. All fractions were analyzed by SDS-PAGE. The purity of β-arrestin1 was determined by either Coomassie Blue staining or silver staining (Bio-Rad) of the gel.

Phosphoamino Acid Analysis and Purification of Tryptic-digested Peptides by Reverse-phase HPLC—Phosphorylated β-arrestin1 was purified by nickel affinity chromatography, electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon PVDF, Millipore), and then eluted (25). Proteins were hydrolyzed in 6 N HCl for 1 h at 110 °C. The hydrolysates were lyophilized, combined with phosphoamino acid standards, and fractionated by one-dimensional thin layer electrophoresis as described (26). Phosphoamino acid standards were stained with ninhydrin, and radiolabeled phosphoamino acids were detected by autoradiography.

Proteins transferred to PVDF membranes were digested *in situ* with modified sequencing-grade trypsin (Boehringer Mannheim) and purified by reverse-phase HPLC as described (27). Radiolabeled peptides were collected for amino acid sequencing using an Applied Biosystems model 477A protein sequencer with an in-line 120A PTH-analyzer (Protein Chemistry Core Facility, Baylor College of Medicine).

Receptor Binding and Desensitization Assays—HEK 293 cells were transiently co-transfected with pcDNA1/FLAG-β_2AR (28) and either empty vector (mock) or plasmid encoding β-arrestin1 (wild-type or β-arrestin1 mutated at Ser-412). Two days after transfection, cells were incubated with or without 10 μM (−)-isoproterenol in PBS for 5 min and then dithiobis(succinimidyl propionate) (Pierce) was added for cross-linking as described (29). Cells were scraped into the lysis buffer (1% Triton X-100, 10% glycerol, 50 mM HEPES, pH 7.5, 10 mM NaCl, 1 mM EDTA, and 1 mM EGTA) with 10 mM sodium pyrophosphate, 50 mM sodium fluoride, and a mixture of protease inhibitors and briefly sonicated. Insoluble proteins were pelleted by centrifugation at 14,000 rpm for 10 min. The expression levels of wild-type or mutant β-arrestin1 were determined by Western blot analysis using 5% of the lysates from a 10-cm plate. For the rest of the samples, the FLAG-tagged β_2-adrenergic receptor was immunoprecipitated with the M2 antibody directed aganist the FLAG epitope (Kodak). Then the immunoprecipitates were subjected to SDS-PAGE, and Western blot analysis was performed using the antibody specific to β-arrestin1.

For desensitization assays, the cells described above were incubated in serum-free medium either alone (control cells) or in the presence of 10 μM (−)-isoproterenol for 5 min at 37 °C (desensitized cells). After washing with cold PBS on ice, cells were scraped in lysis buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 1 μM microcystin, and a mixture of protease inhibitors), homogenized, and centrifuged at 500 × *g* for 10 min to pellet nuclei and debris. Crude membranes were isolated by centrifugation of the supernatant at 40,000 × *g* for 30 min and resuspended in 75 mM Tris, pH 7.5, 2 mM EDTA, 15 mM $MgCl_2$, and protease inhibitors. Cyclase assays were performed as described (30). Samples containing 10 μg of membrane protein, 30 mM Tris, pH 7.5, 0.8 mM EDTA, 6 mM $MgCl_2$, 120 μM [^{3}H]ATP (50 μCi/ml), 100 μM cAMP, 53 μM GTP, 2.7 mM phosphoenolpyruvate, 20 units/ml myokinase, 4 units/ml pyruvate kinase, and either 10 μM forskolin or 10 μM (−)-isoproterenol were incubated at 37 °C for 25 min. Assays were terminated by placing the samples on ice and adding 1 ml of 0.3 mM ATP and 0.3 mM [^{14}C]cAMP. The amount of cAMP generated was determined (31).

Agonist-promoted Sequestration of β_2-Adrenergic Receptors—Transiently transfected HEK 293 cells, as described above, were aliquoted into 6-well dishes. Two days after transfection, they were incubated with or without 10 μM (−)-isoproterenol in 0.1 mM ascorbic acid for 30 min followed by sequestration assays as described (32). In brief, cells were incubated at 14 °C for 3 h with [^{125}I]cyanopindolol either alone (to define total receptors) or in the presence of CGP-12177 (a hydrophilic ligand binding to cell-surface receptors only) or in the presence of propranolol (to define nonspecific binding). The agonist-promoted receptor sequestration was determined as the percentage of radiolabeled binding not competed by CGP-12177 measured with agonist exposure minus that without agonist treatment.

Co-immunoprecipitation of β-Arrestin1 and Clathrin—One 150-mm plate of stable HEK 293 cells transfected with pCMV5 empty vector or β-arrestin1 (wild-type, S412A, or S412D) were homogenized and subjected to subcellular fractionation. After removing nuclei and unbroken cells by centrifugation at 1000 × *g* for 10 min, all of the membrane fractions were pelleted by centrifugation of the supernatant at 300,000 × *g* for 30 min and dissolved in lysis buffer (same as that used in receptor binding described above). β-Arrestin1 was immunoprecipitated using an antibody directed against β-arrestin1 (15), and the proteins were resolved by SDS-PAGE. Western blot analysis was performed using a monoclonal antibody specific to the heavy chain of clathrin (ICN).

RESULTS AND DISCUSSION

Constitutive Phosphorylation of β-Arrestin1 in Unstimulated Cells—To investigate the functional role of β-arrestin1 in β_2-adrenergic receptor endocytosis, we established a line of human embryonic kidney (HEK) 293 cells stably overexpressing β-arrestin1, tagged at its carboxyl terminus with hexahistidine. Purification of the β-arrestin1 to >95% homogeneity could be achieved by a two-step procedure as follows: a first step on a nickel-chelating column, followed by a heparin-Sepharose column (Fig. 1, *A* and *B*).

To determine if cellular β-arrestin1 is a phosphoprotein, HEK 293 cells overexpressing β-arrestin1 were metabolically labeled with $^{32}P_i$, and then β-arrestin1 was purified from whole cell extracts by nickel affinity chromatography and was subjected to SDS-PAGE. As shown in Fig. 2, the 50-kDa β-arrestin1, which was not present in the mock transfectant, was clearly phosphorylated. The stoichiometry of phospho-β-arrestin1 from whole cell extracts was ~0.84 mol of P_i/mol of protein, suggesting a potential physiological relevance of this modification.

These results are consistent with our previous observations that the immunoprecipitated native β-arrestin1 from overexpressing COS-7 cells is a phosphoprotein.[2] Moreover, we have determined that the function of the hexahistidine-tagged β-arrestin1 is equivalent to that of native β-arrestin1 as assayed

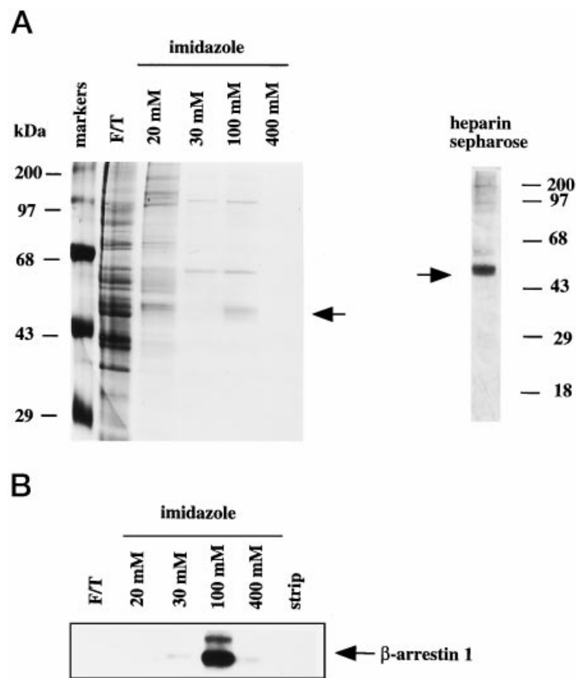
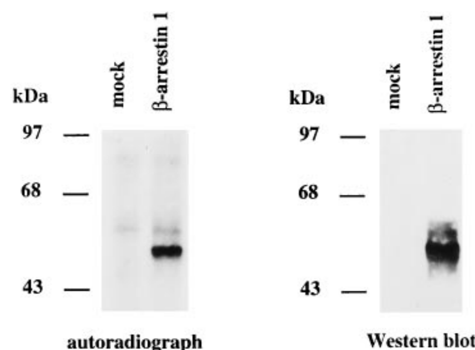

FIG. 1. **Purification of β-arrestin1 from HEK 293 cells stably overexpressing His-tagged β-arrestin1.** His-tagged β-arrestin1, stably expressed in HEK 293 cells, was purified by nickel affinity chromatography. Proteins were eluted with increasing concentrations of imidazole from 5 to 100 mM and fractionated by 12% SDS-PAGE. *Left panel* of *A* shows Coomassie Blue staining of the gel. *B* is its immunoblot using an antibody specific to β-arrestin1. The 100 mM imidazole eluate was further dialyzed in 25 mM Tris, pH 7.4, 5 mM EDTA, and 0.2 M NaCl and applied to a heparin-Sepharose column. β-Arrestin1 was eluted with a linear gradient of NaCl from 0.2 to 1 M. The pooled protein was subjected to SDS-PAGE. *Right panel* of *A* is the silver staining of the gel. The *arrows* indicate the position of β-arrestin1. *F/T*, flow-through. *Strip*, the fraction stripped with 20 mM Tris, pH 7.9, 0.1 M EDTA, and 0.5 M NaCl.

FIG. 2. **Cellular phosphorylation of β-arrestin1.** HEK 293 cells, stably transfected with pCMV5 vector alone (mock), or pCMV5/βarr1–6xHis, were metabolically labeled with [^{32}P]orthophosphate for 90 min followed by purification through nickel affinity chromatography as described in Fig. 1. The 100 mM imidazole eluate was fractionated by SDS-PAGE. After transfer to Immobilon PVDF membranes, autoradiography was performed as shown in the *left panel*. The same blot was then subjected to Western blot analysis using an antibody specific to β-arrestin1 and visualized by chemiluminescence assay as shown in the *right panel*.

by the binding to β_2-adrenergic receptors and the promotion of receptor desensitization and internalization (see below). Accordingly, since it is much easier to purify, the tagged β-arrestin1 was used for all subsequent studies.

Agonist Stimulation Promotes Dephosphorylation of β-Arrestin1—To study the potential effects of agonist stimulation on the phosphorylation status of β-arrestin1, HEK 293 cells overexpressing β-arrestin1 were labeled with $^{32}P_i$ and subsequently

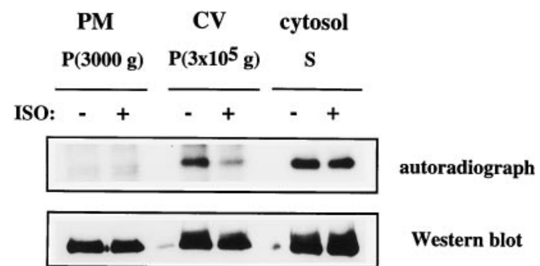

FIG. 3. **The effect of agonist stimulation on phosphorylation of β-arrestin1.** HEK 293 cells stably overexpressing His-tagged β-arrestin1 were transiently transfected with an expression vector for the β_2-adrenergic receptor. After metabolic labeling with [^{32}P]orthophosphate for 90 min, the cells were incubated with or without 10 μM (−)-isoproterenol for 5 min and subjected to subcellular fractionation. Lysates devoid of nuclei and unbroken cells were sequentially centrifuged at 3000 × *g* and 300,000 × *g* to obtain two pellets (*P*) and the supernatant (*S*), *i.e.* plasma membrane (*PM*), crude vesicles (*CV*), and the cytosol, respectively. Lysates from each fraction were subjected to the two-step purification as described under "Experimental Procedures." Similar amounts of purified β-arrestin1 were fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and detected by autoradiography as shown in the *upper panel*. The same blot was then subjected to Western blot analysis using the antibody specific to β-arrestin1 as shown in the *lower panel*.

incubated either alone or with the β-adrenergic agonist isoproterenol for 10 min. β-Arrestin1 was then purified from whole cell extracts by nickel affinity chromatography. Under these conditions, a modest 20% reduction of β-arrestin1 phosphorylation was observed (data not shown).

As the bulk of β-arrestin1 is cytosolic (~70%, data not shown), we thought this agonist-induced dephosphorylation event might be limited to a particular subcellular location. Therefore, the phosphorylation status of β-arrestin1 isolated from several different cellular fractions was examined. After metabolic labeling and treatment with or without isoproterenol for 5 min, cells were fractionated by differential centrifugation into low speed "plasma membrane," high speed "crude vesicle," and supernatant "cytosol" fractions (see "Experimental Procedures"). β-Arrestin1 was purified from each of these fractions by the two-step procedure shown in Fig. 1. The results are shown in Fig. 3, where similar amounts of β-arrestin1 were loaded in each lane. β-Arrestin1 isolated from the plasma membrane was almost completely dephosphorylated, and hence no agonist effect could be observed. Cytosolic β-arrestin1 was significantly phosphorylated (1.02 mol P_i/mol protein) and showed a slight decrease after agonist treatment. The most pronounced agonist-induced dephosphorylation of β-arrestin1 was observed in the crude vesicle fraction, which showed a 50% decrease in phosphorylation after a 5-min isoproterenol treatment of the cells.

If the plasma membrane is the site of β-arrestin1 dephosphorylation, then an obvious explanation for the agonist-promoted decrease of β-arrestin1 phosphorylation in crude vesicles is an agonist-promoted transit of dephosphorylated β-arrestin1 from plasma membrane to the internalized vesicles. This seemed a reasonable hypothesis since isoproterenol stimulates the association of β-arrestins with plasma membrane β_2-adrenergic receptors, as judged by receptor desensitization as well as receptor translocation from plasma membrane to a vesicular fraction. To confirm this notion, we treated cells with isoproterenol for 5 min, isolated plasma membrane and vesicle fractions, and assessed their β-arrestin1 content by Western blot. As shown in Fig. 4, both fractions showed an agonist-promoted increase in β-arrestin1. Taken together, these data suggest that agonist stimulation of β_2-adrenergic receptors promotes translocation of β-arrestin1 to the plasma membrane, which is the site of its dephosphorylation.

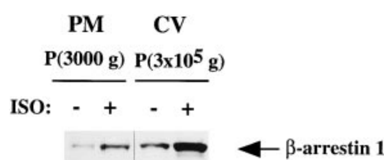

FIG. 4. **Translocation of β-arrestin1 by agonist stimulation.** HEK 293 cells transiently co-transfected with plasmids encoding β_2-AR and β-arrestin1 were treated with or without 10 μM (−)-isoproterenol for 5 min. After harvesting, cells were subjected to subcellular fractionation as described in Fig. 3. The 3000 × *g* (plasma membrane, *PM*) and 300,000 × *g* (crude vesicles, *CV*) pellets (*P*) were dissolved in lysis buffer. Equal volumes of each fraction were subjected to SDS-PAGE and Western blot analysis using an antibody specific to β-arrestin1.

β-Arrestin1 Is Phosphorylated at the Most Carboxyl-terminal Serine Residue—To identify the physiologically relevant sites of β-arrestin1 phosphorylation, we performed phosphoamino acid analysis on phosphorylated β-arrestin1 purified from HEK 293 cells overexpressing β-arrestin1. As shown in Fig. 5*A*, phosphate was incorporated almost exclusively on serine.

Trypsin-digested phospho-β-arrestin1 was then analyzed by reverse-phase HPLC, followed by protein sequencing of the two major phosphopeptides (Fig. 5*B*). These results revealed that Ser-412, which is located seven residues from the carboxyl terminus, is the only phosphorylation site. Interestingly, this serine is only present in β-arrestin1 and not in other arrestin family members.

To confirm the assignment of Ser-412 as the physiologically relevant site of β-arrestin1 phosphorylation, we prepared a point mutation in which Ser-412 was substituted with alanine. S412A β-arrestin1 was expressed in HEK 293 cells at levels similar to wild-type (Fig. 6). However, this single mutation virtually eliminated phosphorylation of β-arrestin1 (Fig. 6). Further confirming this conclusion was the absence, by tryptic phosphopeptide mapping of S412A β-arrestin1, of the two partial tryptic digestion products shown in Fig. 5*B* (data not shown).

Receptor Binding of β-Arrestin1 and Desensitization of β_2-Adrenergic Receptors Are Not Affected by Ser-412 Mutation—To investigate how phosphorylation regulates the activities of β-arrestin1 within the cell, we utilized two mutant β-arrestin1 expression vectors where Ser-412 was replaced with Ala or Asp. The S412A mutant should simulate the unphosphorylated form of β-arrestin1, whereas S412D would be predicted to mimic its phosphorylated form. We then set out to investigate the biological properties of the wild-type and mutant proteins expressed in 293 cells. As shown in Fig. 7*A* (*upper panel*), the three proteins were expressed at similar levels.

We first investigated the ability of each β-arrestin1 to interact with the agonist-occupied receptors. Cells expressing β_2-adrenergic receptor and either wild-type or mutant β-arrestin1 were treated with isoproterenol for 5 min and subjected to reversible cross-linking with Dithiobis(succinimidyl propionate). The receptors were immunoprecipitated with an antibody directed at the FLAG epitope (see "Experimental Procedures"). After SDS-PAGE, co-immunoprecipitating β-arrestin1 was visualized by immunoblot. As shown in Fig. 7*A* (*lower panel*), isoproterenol treatment of cells strikingly increased β-arrestin/receptor interaction. However, neither mutation of Ser-412 altered receptor/β-arrestin1 interaction. The failure of these mutations to alter receptor binding of β-arrestin1 is consistent with previous observations indicating that the carboxyl domain of β-arrestins is not involved in this interaction (33).

We next examined the ability of different β-arrestin1 proteins to desensitize the receptors. Previously it has been demonstrated that overexpression of β-arrestin1 enhances desensitization of G protein-coupled receptors (16). As shown in Fig. 7*B*, when expressed at equal levels, wild-type β-arrestin1 and its two mutants, S412A and S412D, equally augmented desensitization of the β_2-adrenergic receptor. In view of the apparently unchanged receptor-binding ability of the two mutants (Fig. 7*A*), this finding is not surprising.

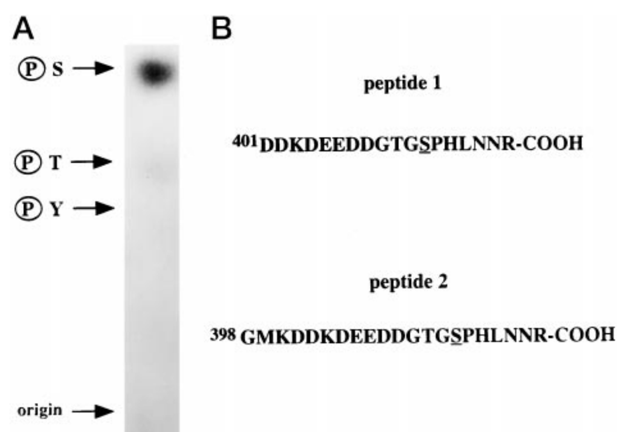

FIG. 5. **Cellular β-arrestin1 is predominantly phosphorylated at amino acid 412, the most carboxyl-terminal serine residue.** *A*, phosphoamino acid analysis of β-arrestin1. ^{32}P-labeled β-arrestin1 was purified from HEK 293 cells, resolved by SDS-PAGE, and transferred to Immobilon PVDF membranes. The ^{32}P-labeled β-arrestin1 band was cut out for one-dimensional phosphoamino acid analysis as described under "Experimental Procedures." The positions of phosphorylated serine, threonine, and tyrosine standards are marked with *arrows*. *B*, sequences of tryptic β-arrestin1 phosphopeptides purified by reverse-phase HPLC. The ^{32}P-labeled β-arrestin1 band was cut out from Immobilon PVDF membranes and was digested with trypsin. The digested peptides were separated by reverse-phase HPLC as described (27). Only two peaks showed significant phospholabeling (data not shown). These two phosphopeptides (~15 pmol each) were sequenced using an Applied Biosystems model 477A protein sequencer. The phosphorylated serine is *underlined*.

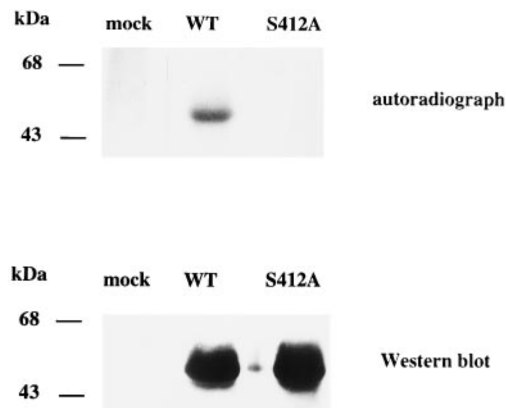

FIG. 6. **Mutation of serine 412 to alanine eliminates phosphorylation of β-arrestin1 in HEK 293 cells.** HEK 293 cells overexpressing wild-type or S412A β-arrestin1 were metabolically labeled with [^{32}P]orthophosphate. β-Arrestin1 from the whole cell extracts was purified by nickel affinity chromatography, separated by SDS-PAGE, and transferred to nitrocellulose membranes. After development by autoradiography (*upper panel*), this membrane was subjected to Western blot analysis using the antibody specific to β-arrestin1 (*lower panel*).

Ser-412 Phosphorylation Regulates Agonist-promoted Internalization of β_2-Adrenergic Receptors—As noted earlier β-arrestins appear to be bifunctional molecules, binding to and desensitizing receptors on the one hand and targeting them for internalization on the other (20). Accordingly, we next assessed the effects of phosphorylation of β-arrestin1 on its ability to promote receptor sequestration. These data are shown in Fig. 8. Overexpression of wild-type β-arrestin1 significantly in-

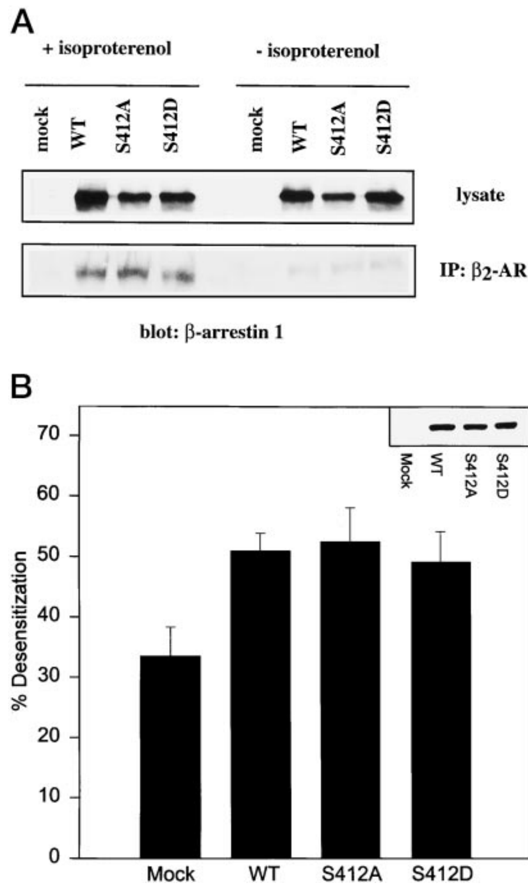

FIG. 7. **Receptor binding of β-arrestin1 and desensitization of β_2-adrenergic receptors are not affected by Ser-412 mutation.** *A*, HEK 293 cells, transiently expressing FLAG-tagged β_2-AR, and either a control vector (*mock*), or wild-type β-arrestin1 (*WT*), or β-arrestin1 mutated at residue 412 (*S412A* and *S412D*), were incubated either alone or in the presence of (−)-isoproterenol for 5 min followed by the addition of dithiobis(succinimidyl propionate) for cross-linking. The *upper panel* is an immunoblot of β-arrestin1 representing 5% of the whole cell extracts. From the rest of the samples, β_2-AR was immunoprecipitated with an antibody specific to the FLAG epitope. After SDS-PAGE, co-immunoprecipitated β-arrestin1 was detected by Western blot analysis using its specific antibody as shown in the *lower panel*. *B*, after isoproterenol treatment for 5 min, cells (as shown above) were harvested, and membranes were prepared from control and desensitized cells. The amount of adenylyl cyclase stimulation observed in the presence of (−)-isoproterenol was determined and expressed as a percent loss of adenylyl cyclase response (% desensitization = 100 × (cyclase response in control cells-cyclase response in desensitized cells)/cyclase response in control cells), as described (43). The result shown here is representative of three independent experiments. The *error bars* represent the standard deviation of quadruplicates in this experiment. The expression levels of wild-type and mutant β-arrestin1 shown at the *right upper corner* are determined by Western blot analysis.

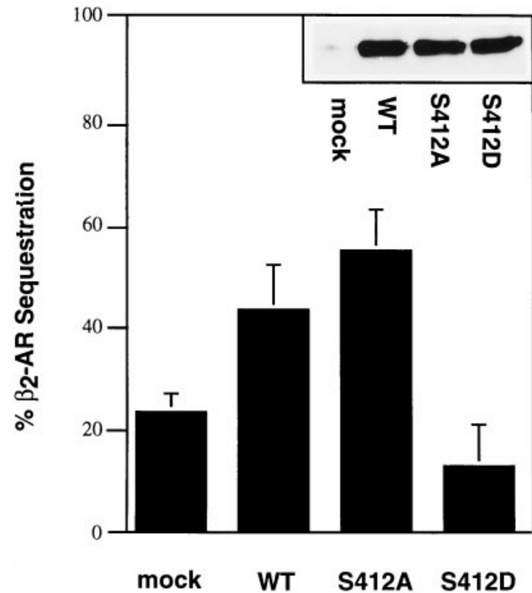

FIG. 8. **Phosphorylation of β-arrestin1 at Ser-412 regulates sequestration of the β_2-adrenergic receptor.** The β_2-adrenergic receptor expression vector was transiently transfected into HEK 293 cells with either pCMV5 empty vector (*mock*), or one of the β-arrestin1 expression plasmids (*wild-type, S412A,* and *S412D*). Cells were incubated with or without (−)-isoproterenol for 30 min before harvesting. Sequestration assay was carried out with ^{125}I-cyanopindolol in the presence or absence of CGP-12177 or propranolol as described under "Experimental Procedures." The agonist-promoted receptor sequestration was determined as the percentage of radiolabeled binding not competed by CGP-12177 measured with agonist exposure minus that without agonist treatment. The result shown is the mean ± S.E. of four independent experiments done in triplicate. For all transfection conditions, $p < 0.05$ compared with control values. At the *right upper corner* is a representative Western blot showing the expressional levels of β-arrestin1.

creased β_2-adrenergic receptor sequestration, causing a doubling in the percentage of surface receptors internalized after a 30-min incubation with isoproterenol. Interestingly, the S412A mutant, which cannot be phosphorylated, enhanced receptor internalization to an even greater extent. In contrast, the S412D mutant, which would be predicted to mimic the phosphorylated form of β-arrestin1, failed to increase receptor sequestration and in fact acted as a dominant negative mutant and significantly reduced it. These data are consistent with the notion that dephosphorylation of β-arrestin1 is required for internalization of the β_2-adrenergic receptor.

Ser-412 Phosphorylation Regulates the Ability of β-Arrestin1 to Serve as a Clathrin Adaptor—Previously published data (11) have indicated that β-arrestins bind to clathrin cages *in vitro* and that the carboxyl-terminal 77 amino acid residues of β-arrestins are important for this interaction. Alanine scanning mutagenesis further localized the clathrin-binding domain to residues 371–379 of β-arrestin2 (12). It was also shown, in intact cells, that β-arrestin1 and clathrin co-localize by immunofluorescent microscopy after isoproterenol stimulation. Since Ser-412 is located within the carboxyl domain of β-arrestin1 previously shown to be involved in its *in vitro* binding to clathrin cages, we sought to investigate the potential regulatory role of Ser-412 phosphorylation in determining the ability of β-arrestin1 to serve as a clathrin adaptor in intact cells.

A 300,000 × *g* particulate fraction from HEK 293 cells stably expressing wild-type or mutant β-arrestin1 at equivalent levels (Fig. 9, *upper panel*) was subjected to immunoprecipitation with anti-β-arrestin1 antibodies. After SDS-PAGE, the immunoprecipitates were blotted for clathrin heavy chain (Fig. 9, *lower panel*). Clathrin was detected only in the immunoprecipitated complex of S412A β-arrestin1. The failure to detect specific clathrin binding to the wild-type β-arrestin1 likely reflects the fact that it is already highly phosphorylated. S412D β-arrestin1, which mimics phosphorylated β-arrestin1, also did not co-immunoprecipitate with clathrin heavy chain.

A Model for β-Arrestin1 Function—Our data suggest the following model for the regulation of β_2-adrenergic receptor desensitization and internalization by β-arrestin1. Prior to agonist stimulation, the bulk of β-arrestin1 is cytosolic and is a phosphoprotein. The phosphate is present almost exclusively on Ser-412. The nature of the kinase(s) that is responsible for this phosphorylation is currently unknown. Following agonist stimulation, β-arrestin1 is translocated to the plasma membrane where it binds tightly to agonist-occupied receptors that have been phosphorylated by a G protein-coupled receptor ki-

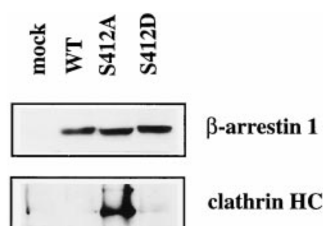

FIG. 9. **Phosphorylation of β-arrestin1 at Ser-412 regulates its interaction with clathrin.** One 150-mm plate of HEK 293 cells stably transfected with pCMV5 vector (*mock*) or one of the β-arrestin1 expression plasmids (*wild-type, S412A,* and *S412D*) was subjected to subcellular fractionation. After removing nuclei and unbroken cells, all of the membrane fractions were pelleted by centrifugation at 300,000 × *g* and dissolved in lysis buffer (see "Experimental Procedures"). Five percent of total proteins were fractionated by SDS-PAGE and subjected to Western blot analysis using an antibody specific to β-arrestin1, which is shown in the *upper panel*. From the rest of the samples, equal amounts of proteins were immunoprecipitated with anti-β-arrestin1 antibody, fractionated by SDS-PAGE, and subjected to Western blot analysis using an antibody specific to the heavy chain (*HC*) of clathrin as shown in the *lower panel*.

nase such as β-adrenergic receptor kinase. In association with its movement to the plasma membrane, β-arrestin1 is dephosphorylated by an as yet unknown phosphatase.

It is not presently clear whether β-arrestin1 dephosphorylation precedes or follows its receptor binding. Even in the absence of receptor stimulation, plasma-membrane β-arrestin1 is dephosphorylated (Fig. 3). It is known that β-arrestin1 can bind to β_2-adrenergic receptors even in the absence of agonist, albeit with lower affinity (Fig. 7*A*). Moreover, the concentrations of overexpressed β_2-adrenergic receptors in our experiments are likely high enough to promote just such agonist-independent binding of β-arrestin1 to the receptors. On the other hand, as shown in Fig. 7*A*, phosphorylation of Ser-412 does not seem to regulate receptor binding of β-arrestin1. Thus, it is not necessary for the dephosphorylation to precede β-arrestin1 binding to the receptors. It seems plausible that such receptor binding of β-arrestin1 positions it in proximity to the relevant phosphatase or alters its conformation such that it becomes a substrate for the phosphatase. Since receptor binding of β-arrestin1 does not appear to require its dephosphorylation, it is not surprising that receptor desensitization is also unaffected by the phosphorylation status of β-arrestin1 (Fig. 7*B*).

Once β-arrestin1 has bound to the β_2-adrenergic receptor and the receptors have become functionally uncoupled from G proteins, they move to clathrin-coated pits and become internalized. As shown here, the dephosphorylated form of β-arrestin1 seems to uniquely function as a clathrin adaptor targeting the desensitized receptors for internalization by this pathway. In fact, S412D β-arrestin1, which mimics the phosphorylated form of β-arrestin1, actually acts as a dominant negative mutant with respect to receptor internalization (Fig. 8). As dephosphorylated β-arrestin1 moves into the internalized vesicular fraction of the cells along with the receptors, the overall phosphorylation status of β-arrestin1 in this cellular fraction transiently drops. Once internalized, the receptors arrive in late endosomes and become dephosphorylated by the G protein-coupled receptor phosphatase (34). Other studies have shown that the low endosomal pH uniquely induces a conformational change of the receptor which is then subjected to dephosphorylation (35). Subsequently the receptors recycle to the cell surface by incompletely characterized mechanisms. The exact time at which β-arrestin1 falls off the receptor (presumably prior to receptor dephosphorylation) and the cellular locus of β-arrestin1 rephosphorylation are not yet known.

Why should β-arrestin1 dephosphorylation be necessary for its clathrin interaction? Previous *in vitro* studies have revealed that the carboxyl terminus of β-arrestin1 interacts with clathrin (11). Presumably dephosphorylation of the very carboxyl-terminal Ser-412 alters the conformation and/or charge of this region of the β-arrestin1 molecule so that the acidic carboxyl terminus can bind to clathrin cages. It is of further interest that Ser-412 is not present in the other members of the arrestin family. Thus, they must be regulated either by phosphorylation at other sites or by totally different mechanisms.

Analogies between β-Arrestin1 and the AP2 Complex—β-Arrestin1 seems to function as an adaptor linking activated β_2-adrenergic receptors, and presumably other G protein-coupled receptors, to clathrin cages. Recently, comparisons have been made between the clathrin adaptor functions of β-arrestins and the AP2 complex (13). A number of distinctions were drawn between these two molecules. First, the AP2 complex is a large multi-subunit entity containing four distinct proteins, whereas β-arrestins are monomeric. Second, AP2 was shown to bind to clathrin at two distinct sites, whereas β-arrestins bind a single site (13, 36, 37). Finally, unlike the AP2 complex, which supports clathrin-coat assembly *in vitro*, β-arrestins do not. However, it should be pointed out that β-arrestins might recruit additional as yet unrecognized proteins into their complexes with clathrin.

However, there are clear analogies between β-arrestins and AP2 as well. These include the fact that both β-arrestins and AP2 bind to clathrin and to the receptors. In the case of the AP2 complex, these receptors include the low density lipoprotein receptor, epidermal growth factor receptor, asialoglycoprotein receptor, and mannose 6-phosphate receptor (38–41). Another analogy, revealed by our study, is that only dephosphorylated β-arrestin1 can bind to clathrin, just as *in vitro* clathrin binding assays have shown that only dephosphorylated AP2 can bind to clathrin (42). In addition, the β_2 subunit of the cytosolic AP2 complex is phosphorylated on serine residues, whereas membrane-bound AP2 is unphosphorylated (42). These data suggest that adaptor phosphorylation may generally regulate adaptor-clathrin interaction on the plasma membrane. This mechanism would provide a potential means for bringing such endocytic processes under the control of receptor-mediated signaling pathways.

Acknowledgments—We thank Dr. Richard Cook for amino acid and phosphopeptide sequencing; Drs. Randy Hall and Neil J. Freedman for helpful discussions; and Mary Holben and Donna Addison for secretarial assistance.

REFERENCES

1. Pearse, B. M. F., and Robinson, M. S. (1990) *Annu. Rev. Cell Biol.* **6,** 151–171
2. von Zastrow, M., and Kobilka, B. K. (1992) *J. Biol. Chem.* **267,** 3530–3538
3. Tolbert, L. M., and Lameh, J. (1996) *J. Biol. Chem.* **271,** 17335–17342
4. Ghinea, N., Hai, M. T. V., Groyer-Picard, M.-T., Houllie, A., Schoevaert, D., and Milgrom, E. (1992) *J. Cell Biol.* **118,** 1347–1358
5. Grady, E. F., Slice, L. W., Brant, W. O., Walsh, J. H., Payan, D. G., and Bunnett, N. W. (1995) *J. Biol. Chem.* **270,** 4603–4611
6. Robinson, M. S. (1994) *Curr. Opin. Cell Biol.* **6,** 538–544
7. Damke, H. (1996) *FEBS Lett.* **389,** 48–51
8. Zaremba, S., and Keen, J. H. (1983) *J. Cell Biol.* **97,** 1339–1347
9. Heuser, J. E., and Keen, J. (1988) *J. Cell Biol.* **57,** 315–344
10. Mahaffey, D. T., Peeler, J. S., Brodsky, F. M., and Anderson, R. G. W. (1990) *J. Biol. Chem.* **265,** 16514–16520
11. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) *Nature* **383,** 447–450
12. Krupnick, J. G., Goodman, O. B., Jr., Keen, J. H., and Benovic, J. L. (1997) *J. Biol. Chem.* **272,** 15011–15016
13. Goodman, O. B., Jr., Krupnick, J. G., Gurevich, V. V., Benovic, J. L., and Keen, J. H. (1997) *J. Biol. Chem.* **272,** 15017–15022
14. Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1990) *Science* **248,** 1547–1550
15. Attramadal, H., Arriza, J. L., Aoki, C., Dawson, T. M., Codina, J., Kwatra, M. M., Snyder, S. H., Caron, M. G., and Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267,** 17882–17890
16. Pippig, S., Andexinger, S., Daniel, K., Puzicha, M., Caron, M. G., Lefkowitz R. J., and Lohse, M. (1993) *J. Biol. Chem.* **268,** 3201–3208
17. Sterne-Marr, R., and Benovic, J. L. (1995) *Vitam. Horm.* **51,** 193–234
18. Benett, N., and Sitaramayya, A. (1988) *Biochemistry* **27,** 1710–1715

19. Dolph, P. J., Ranganathan, R., Colley, N. J., Hardy, R. W., Socolich, M., and Zuker, C. S. (1993) *Science* **260,** 1910–1916
20. Ferguson, S. S. G., Downey, W. E., III, Colapietro, A.-M., Barak, L. S., Menard, L., and Caron, M. G. (1996) *Science* **271,** 363–366
21. Jones, D. H., and Winistorfer, S. C. (1992) *BioTechniques* **12,** 528–520
22. Sefton, B. M. (1991) *Methods Enzymol.* **201,** 245–251
23. Graham, J. M. (1992) *Cell Biology labfax*, (Dealtry, G. B., and Rickwood, D., eds) Academic Press, Oxford, UK
24. Sohlemann, P., Hekman, M., Puzicha, M., Buchen, C., and Lohse, M. (1995) *Eur. J. Biochem.* **232,** 464–472
25. Kamps, M. P., and Sefton, B. M. (1989) *Anal. Biochem.* **176,** 22–27
26. Hirano, A. A., Greengard, P., and Huganir, R. L. (1988) *J. Neurochem.* **50,** 1447–1455
27. Fredericks, Z. L., Pitcher, J. A., and Lefkowitz, R. J. (1996) *J. Biol. Chem.* **271,** 13796–13803
28. Oppermann, M., Diverse-Pierluissi, M., Drazner, M. H., Dyer, S. L., Freedman, N. J., Peppel, K. C., and Lefkowitz, R. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7649–7654
29. Freedman, N. J., Ament, A. S. Oppermann, M., Stoffel, R. H., Exum, S. T., and Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272,** 17734–17743
30. Lohse, M. J., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. (1990) *J. Biol. Chem.* **265,** 3202–3211
31. Salomon, Y., Londos, C., and Rodbell, M. (1974) *Anal. Biochem.* **58,** 541–548
32. Hausdorff, W. P., Bouvier, M., O'Dowd, B. F., Irons, G. P., Caron, M. G., and Lefkowitz, R. J. (1989) *J. Biol. Chem.* **264,** 12657–12665
33. Gurevich, V. V., Dion, S. B., Onorato, J. J., Ptasienski, J., Kim, C. M., Sterne-Marr, R., Hosey, M. M., and Benovic, J. L. (1995) *J. Biol. Chem.* **270,** 720–731
34. Pitcher, J. A., Payne, E. S., Csortos, C., Depaoli-Roach, A. A., and Lefkowitz, R. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8343–8347
35. Krueger, K. M., Daaka, Y., Pitcher, J. A., and Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272,** 5–8
36. Gallusser, A., and Kirchhausen, T. (1993) *EMBO J.* **12,** 5237–5244
37. Goodman O. B., Jr., and Keen, J. H. (1995) *J. Biol. Chem.* **270,** 23768–23773
38. Pearse, B. M. F. (1988) *EMBO J.* **7,** 3331–3336
39. Sorkin, A., and Carpenter, G. (1993) *Science* **261,** 612–615
40. Beltzer, J. P., and Spiess, M. (1991) *EMBO J.* **10,** 3735–3742
41. Glickman, J. N., Conibear, E., and Pearse, B. M. F. (1989) *EMBO J.* **8,** 1041–1047
42. Wilde, A., and Brodsky, F. M. (1996) *J. Cell Biol.* **135,** 635–645
43. Yu, S. S., Lefkowitz, R. J., and Hausdorff, W. P. (1993) *J. Biol. Chem.* **268,** 337–341