Members of the G Protein-Coupled Receptor Kinase Family That Phosphorylate the β_2-Adrenergic Receptor Facilitate Sequestration†

Luc Ménard,‡,§ Stephen S. G. Ferguson,‡,§ Larry S. Barak,‡ Lucie Bertrand,|| Richard T. Premont,⊥ Anne-Marie Colapietro,|| Robert J. Lefkowitz,⊥ and Marc G. Caron*,‡

Howard Hughes Medical Institute Laboratories and Departments of Cell Biology, Medicine, and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Received December 15, 1995; Revised Manuscript Received February 9, 1996⊗

ABSTRACT: We recently reported that a β_2-adrenergic receptor (β_2AR) mutant, Y326A, defective in its ability to sequester in response to agonist stimulation was a poor substrate for G protein-coupled receptor kinase (GRK)-mediated phosphorylation; however, its ability to be phosphorylated and sequestered could be restored by overexpressing GRK2 [Ferguson *et al*. (1995) *J. Biol. Chem. 270*, 24782]. In the present report, we tested the ability of each of the known GRKs (GRK1−6) to phosphorylate and rescue the sequestration of the Y326A mutant in HEK-293 cells. We demonstrate that in addition to GRK2, GRK3−6 can phosphorylate the Y326A mutant and rescue its sequestration; however, GRK1 was totally ineffective in rescuing either the phosphorylation or the sequestration of the mutant receptor. We found that the agonist-dependent rescue of Y326A mutant phosphorylation by GRK2, -3, and -5 was associated with the agonist-dependent rescue of sequestration. In contrast, overexpression of GRK4 and -6 led mainly to agonist-independent phosphorylation of the Y326A mutant accompanied by increased basal receptor sequestration. Our results demonstrate that phosphorylation *per se*, but not the interaction with a specific GRK, is required to facilitate β_2AR sequestration.

The regulation of the β_2-adrenergic receptor (β_2AR) has been extensively studied. Agonist activation of β_2AR signaling through Gs is followed closely by the inactivation of receptor responsiveness, primarily as the consequence of rapid phosphorylation (Dohlman *et al*., 1991; Hausdorff *et al*., 1990, 1992; Schwinn *et al*., 1992). Two families of kinases have been identified as contributing to this process: the second messenger-activated kinases (e.g., cAMP-dependent protein kinase and protein kinase C) and the G protein-coupled receptor kinases (GRKs) (Dohlman *et al*., 1991; Schwinn *et al*., 1992; Lefkowitz, 1993). Six members have thus far been identified in the GRK family (GRK1−6) (Inglese *et al*., 1993; Premont *et al*., 1995).

In the systems studied, the phosphorylation of a particular receptor by a GRK requires agonist-occupancy of the receptor, whereas second messenger-activated kinases phosphorylate either the agonist-bound or the agonist-free forms of the receptor (Hausdorff *et al*., 1990; Inglese *et al*., 1993). The exact mechanisms by which GRKs are activated are as yet unknown. However, the translocation of these cytosolic proteins to membranes appears to involve a variety of different mechanisms. These mechanisms include farnesylation (GRK1, rhodopsin kinase) or palmitoylation (GRK4[1] and -6) of cysteine residues; polybasic domains in the carboxyl-terminal tail (GRK5); or interaction with the $G_{\beta\gamma}$ subunits of heterotrimeric G proteins and phospholipid moieties via a pleckstrin homology domain in the carboxyl termini of both GRK2 and GRK3 (βARK1 and -2) (Inglese *et al*., 1992, 1993; Pitcher *et al*., 1992, 1995; Stoffel *et al*., 1994).

In addition to receptor phosphorylation, agonist stimulation promotes the removal of cell surface receptors to an intracellular compartment, probably endosomes (Sibley *et al*., 1986; von Zastrow & Kobilka, 1992; Roettger *et al*., 1995), by a process referred to as sequestration. Although sequestration of G protein-coupled receptors away from the cell surface might contribute to the desensitization of G protein-coupled receptor responsiveness, the primary role of β_2AR sequestration appears to be the reestablishment of receptor responsiveness (i.e., resensitization), since inhibition of sequestration does not prevent receptor desensitization but does inhibit receptor resensitization (Lohse *et al*., 1990; Yu *et al*., 1993; Barak *et al*., 1994).

Early studies of the β_2AR sequestration using phosphorylation site-deficient β_2AR mutants suggested that phosphorylation was not required for normal sequestration (Hausdorff *et al*., 1989). However, we recently reported that the sequestration-defective β_2AR mutant, Y326A, was a poor substrate for GRKs and that GRK2 overexpression could restore the phosphorylation and sequestration of this receptor mutant (Ferguson *et al*., 1995). This observation, as well as the role reported for GRK2 in facilitating m2 muscarinic acetylcholine receptor sequestration (Tsuga *et al*., 1994), clearly demonstrates a previously unappreciated role for GRK-mediated phosphorylation in G protein-coupled receptor sequestration.

† This work was supported by NIH Grant NS 19576 and by a Bristol Myers Squibb Unrestricted Grant Award to M.G.C. S.S.G.F. is the recipient of a fellowship from The Medical Research Council of Canada. L.S.B. is the recipient of a fellowship from the Howard Hughes Medical Institute.

* To whom correspondence should be addressed at the Duke University Medical Center, Box 3287, Durham, NC 27710. Telephone: (919) 684-5433. Fax: (919) 681-8641.

‡ Department of Cell Biology, Duke University Medical Center.

§ These authors contributed equally to this work.

|| Howard Hughes Medical Institute Laboratories.

⊥ Department of Biochemistry, Duke University Medical Center.

⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1996.

[1] R. T. Premont and R. J. Lefkowitz, unpublished results.

0006-2960/96/0435-4155$12.00/0

In the present paper, we test whether this newly described role for GRK-mediated phosphorylation in facilitating G protein-coupled receptor sequestration is a property unique to GRK2 or whether other members of this kinase family share this ability to rescue the sequestration of the Y326A mutant.

MATERIALS AND METHODS

Materials

[^{125}I]Pindolol and [$^{32}PO_4$]phosphoric acid (HCl free) were purchased from Dupont NEN. Isoproterenol, pepstatin A, and bovine serum albumin were purchased from Sigma. CGP-12177 was purchased from Research Biochemical International. Protein A−Sepharose 4B was obtained from Pharmacia. Human embryonic kidney cells (HEK-293 cells) were obtained from American Type Culture Collection. Minimal essential media and Hank's balance salt solution were purchased from Life Technologies. Hot Tub DNA polymerase and the ECL Western blotting system were obtained from Amersham. Nitrocellulose membranes and Tween 20 were purchased from BioRad. All other chemicals were reagent grade.

Methods

Generation of β_2AR and GRK Mutants. The β_2AR and Y326A mutants were epitope-tagged at their amino terminus with the 12CA5 peptide sequence as previously described (Barak *et al.*, 1994).

Subcloning and Expression Vectors. β_2AR, Y326A, and GRK2 cDNAs were in pcDNA1-Amp. GRK1, GRK5, and GRK6 cDNAs were in pCMV5. GRK4α cDNA was in pRK5 (Premont *et al.*, 1994). GRK3 cDNA was in pcDNA1.

Cell Transfection. The β_2AR and Y326A mutants together with the appropriate GRK were transfected using coprecipitation with calcium phosphate (Cullen, 1987). The total amount of DNA used per transfection was 10 μg per 100 mm dish. The level of receptor expression throughout this study was 0.5−1.5 pmol/mg and 0.5−4 pmol/mg total cellular protein in the sequestration and phosphorylation assays, respectively. Cells were seeded at a density of 2.5 million per 100 mm dish 1 day prior to transfection. They were incubated with the DNAs for 16−18 h, medium was removed, and fresh medium was added for 6−8 h. Cells were trypsinized, seeded in 6 well plates, and used the following day.

Sequestration Assay. The fraction of sequestered receptor was determined using CGP-12177 (150 nM final) and [^{125}I]-pindolol (350−550 pM) and 10 μM propranolol or by flow cytometry analysis as previously described (Hausdorff *et al.*, 1989; Barak *et al.*, 1994).

Whole Cell Phosphorylation. HEK-293 cells were seeded 1 day after transfection at a density of 0.6−1.5 million cells per 25 mm well. Cells were labeled with [$^{32}PO_4$]phosphoric acid (50−100 μCi/ml) for 1−2 h in serum-free and phosphate-free media. Duplicate wells were stimulated with or without 10 μM (−)-isoproterenol (in 500 μM ascorbate, final concentration) for 15 min. The incubation was stopped by rapid removal of the media and 2−3 rapid washes with 3 mL of ice-cold phosphate-buffered saline. Cells were lysed in 400 μL/25 mm well of RIPA buffer [50 mM Tris, pH 8.0 at 20 °C, 150 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, 10 mM NaF, 10 mM sodium pyrophosphate, 0.2 mM phenylmethanesulfonyl fluoride, and 1 μg/mL pepstatin A]. Duplicate wells were pooled and centrifuged twice for 15 min at 436000*g*. The soluble fraction was precleared for 30−60 min with 100 μL of a 20% protein A suspension in RIPA buffer containing 2% (w/v) bovine serum albumin. Receptors in each sample were immunoprecipitated at 4 °C for 1−2 h with 8−12 μg of 12CA5 monoclonal antibody and 100 μL of a 20% suspension of protein A−Sepharose. The immunoprecipitate was collected by centrifugation and washed 3−4 times with RIPA buffer. The immunoprecipitate was eluted from the Sepharose by heating the sample at 65 °C for 15 min in sample buffer containing 5% sodium dodecyl sulfate (Laemmli, 1970). The amount of sample buffer was adjusted for each sample to account for the amount of receptor in the different transfections (−/+ GRK) and the amount of soluble protein recovered after solubilization for each sample. Proteins were resolved on 12.5% polyacrylamide gels. Phosphorylation levels were determined using a PhosphorImager (Molecular Dynamics) and ImageQuant software. The phosphorylation level in each experiment was normalized to the agonist-stimulated level obtained with the β_2AR receptor alone.

Protein Determination. Protein levels were determined using the BioRad Dc protein assay with bovine serum albumin as the standard.

Immunoblotting. The expression level for each GRK was determined by immunoblotting using specific antibodies. The generation of anti-GRK1, -2, -3, -4, -5, and -6 polyclonal antisera has been previously described (Ariza *et al.*, 1992; Inglese *et al.*, 1992a; Stoffel *et al.*, 1994; Premont *et al.*, 1994). An equivalent protein amount from the different transfections was electrophoresed on a 12.5% polyacrylamide gel and transferred onto a nitrocellulose membrane using a Millipore semi-dry transfer system. The membranes were incubated at 4 °C overnight in phosphate-buffered saline containing 3% (w/v) BSA. Primary antibodies were diluted in PBS−BSA containing 0.05% Tween 20 and incubated for 1−2 h at room temperature. The membrane was washed 3 times with PBS−0.1% Tween 20 and incubated with HRP-conjugated secondary antibody (ECL Amersham) in PBS−BSA−0.05% Tween 20 for 1 h at room temperature. The membrane was washed 3 or 4 times with PBS−0.1% Tween 20, and the immunoreactivity was detected using the ECL detection system (Amersham).

RESULTS

Effect of Different GRKs on the Sequestration of the β_2AR and the Y326A Mutant. In initial experiments, we tested the ability of each of the known GRKs to rescue the sequestration defect of the Y326A mutant. To do this, HEK-293 cells were transfected to express either the wild-type β_2AR or the Y326A mutant along with overexpressed GRK1−6. Under these conditions, all the GRKs were overexpressed as shown in Figure 1. Results showing the effects of overexpression of the different GRKs on the sequestration of the β_2AR and the Y326A mutant are summarized in Figure 2. Under the conditions tested, none of the kinases, when overexpressed, adversely affected the ability of the wild-type β_2AR to sequester (Figure 2A). However, like GRK2 (Ferguson *et al.*, 1995), GRK3−6, but not GRK1, exhibited the capacity

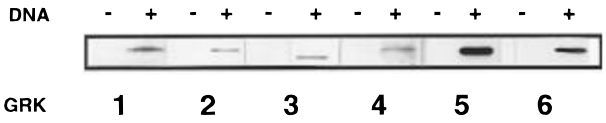

FIGURE 1: Protein expression level of the different GRKs. Cells used for the sequestration experiments that were cotransfected with adrenergic receptors (β_2AR or Y326A) alone (−) or with 1 μg of plasmid encoding for GRK1−6 (+) were used to determine the expression level of each GRK. Twenty-five micrograms of total cellular protein was resolved on SDS−PAGE. The proteins were transferred onto nitrocellulose, and the expression of each GRK was determined using specific antibody to each GRK and HRP-conjugated secondary antibodies using the ECL system (see Methods). Exposures varied from 1 to 10 s. Cotransfection of either receptor did not affect the expression of the different GRKs.

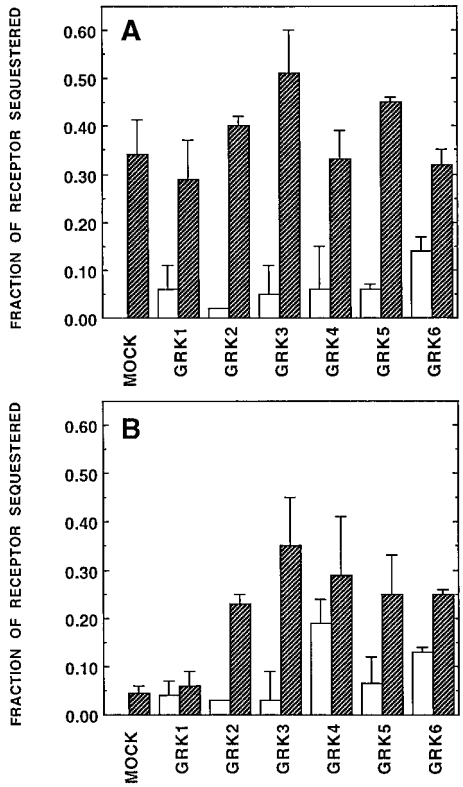

FIGURE 2: Effect of the different GRKs on the sequestration of β_2AR and Y326A. Cells were transfected with β_2AR (panel A) and Y326A (panel B) without (mock) or with 1 μg of the different GRKs. Sequestration was determined using [^{125}I]pindolol and CGP-12177 as described under Materials and Methods. Sequestration is expressed as the value above basal (− isoproterenol) for the receptor alone. The values are the mean ± standard deviation of 3−5 experiments, except mock-transfected experiments (18 experiments). Open bars, no isoproterenol; hatched bars, +isoproterenol.

to rescue the sequestration of the Y326A mutant to levels comparable to that observed for the wild-type β_2AR following agonist stimulation (Figure 2B). However, the mechanism by which this was achieved appeared dependent upon the particular GRK coexpressed with the sequestration-defective Y326A mutant. The rescue of Y326A mutant sequestration by GRK2, -3, and -5 was agonist-dependent, and in the absence of agonist stimulation, the number of intracellular receptors was not altered by overexpressing these kinases. In contrast, overexpression of GRK4 and -6, particularly GRK4, increased the proportion of mutant receptors internalized, in the absence of agonist stimulation. GRK1 overexpression had no effect on the sequestration of the Y326A mutant.

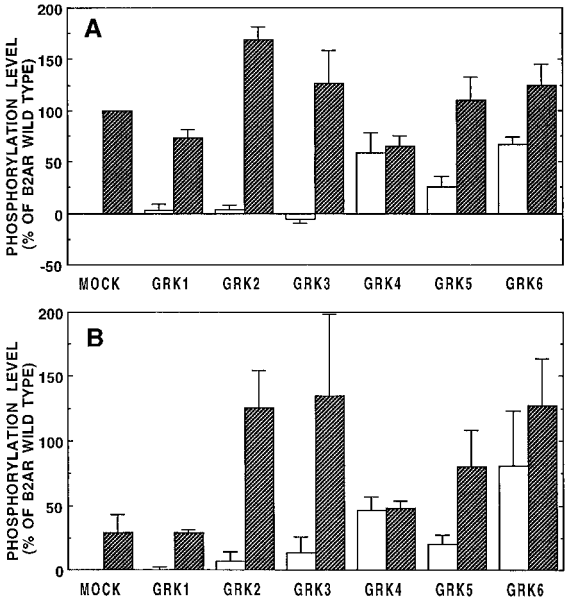

FIGURE 3: Effect of the different GRKs on the phosphorylation of β_2AR and Y326A. Cells were transfected with β_2AR (panel A) and Y326A (panel B) without (mock) or with 1 μg of the different GRKs. Cells were metabolically labeled with [$^{32}PO_4$], the receptors were immunoprecipitated and resolved on polyacrylamide gels, and the radioactivity migrating at the position of the glycosylated receptor (molecular mass 50−80 kDa) was quantitated using a PhosphorImager. Data for the β_2AR and Y326A mutant were normalized to the increase in radioactivity above basal obtained with β_2AR without any additional kinases [(3.6 ± 1.0)-fold above basal, 100%]. The results are the mean ± standard deviation of 3−5 experiments. Open bars, no isoproterenol; hatched bars, +isoproterenol.

Effect of the GRKs on β_2AR and Y326A Mutant Phosphorylation. The rescue of the Y326A mutant sequestration phenotype by GRK2 was associated with the reversal of its phosphorylation deficit (Ferguson *et al*., 1995). Therefore, we tested whether the pattern of rescued sequestration by each of the other members of the GRK family of kinases was reflected in their relative ability to rescue the Y326A mutant phosphorylation. As reported previously (Ferguson *et al*., 1995), the Y326A mutant served as a poor substrate for agonist-induced phosphorylation as it was phosphorylated to ∼25% of the extent of the wild-type β_2AR (Figure 3). GRK1 overexpression, consistent with its inability to affect Y326A mutant sequestration, was unable to rescue agonist-induced phosphorylation. In contrast, similar to GRK2, overexpression of GRK3 and -5 resulted in the rescue of agonist-induced phosphorylation of the Y326A mutant to levels comparable to that observed for the wild-type β_2AR (Figure 3A,B). However, while some agonist-dependent phosphorylation of the Y326A mutant could be observed following cotransfection with GRK6, the primary consequence of GRK6 overexpression was to increase the basal level of receptor phosphorylation for both the wild-type and mutant β_2ARs. GRK4 differed from the other kinases in that only agonist-independent phosphorylation of the wild-type β_2AR and Y326A mutant was observed following its overexpression.

Effect of GRK4 on the Basal Sequestration of Wild-Type β_2AR and Y326A Mutant. Radioligand binding experiments indicated that one of the primary effects of GRK4 overexpression was an increase in the proportion of constitutively recycling Y326A mutants (Figure 2B). However, radioligand

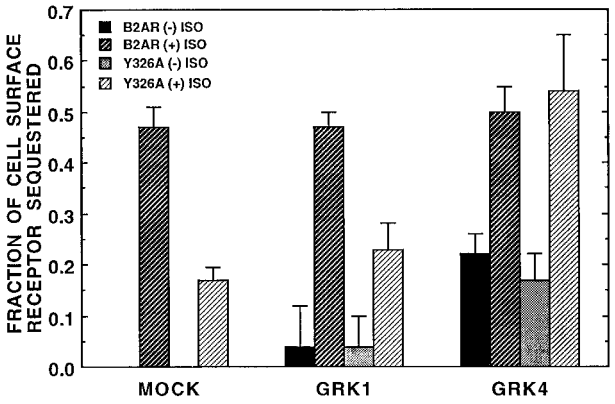

FIGURE 4: Effect of GRK1 and GRK4 on basal sequestration of β_2AR and Y326A. Cells were transfected with β_2AR and Y326A without (mock) or with 1 μg of GRK1 or GRK4. Cells were prelabeled with 12CA5 antibody for 30 min on ice, washed, and stimulated with 100 μM ascorbate $\pm$10 μM isoproterenol (final concentrations) for 10 min at 37 °C. Cell surface receptor expression was measured by flow cytometry as described under Methods. Values were normalized to paired samples that were kept on ice. The data represent the mean $\pm$ standard deviation of 3 experiments. (−) ISO, −isoproterenol; (+) ISO, +isoproterenol.

binding in the presence of the hydrophilic ligand CGP-12177 cannot distinguish between a static or dynamic population of internalizing receptors. Therefore, to test whether GRK4 overexpression stimulates increased basal recycling of both wild-type and mutant β_2ARs in the absence of agonist stimulation, the following experiment was performed. 12CA5 epitope-tagged wild-type and mutant β_2ARs were prelabeled with anti-12CA5 monoclonal antibody, and the subsequent loss of cell surface receptors over a 10 min period, in the absence or presence of agonist-stimulation, was measured by flow cytometry. GRK1 was used as a negative control in these experiments, as it had minimal effects on the sequestration of both receptors. As shown in Figure 4, while GRK1 had no effect on the sequestration of each receptor, overexpression of GRK4 not only resulted in the rescue of agonist-promoted Y326A mutant sequestration, when measured by flow-cytometry, but also induced an increased mobilization (4−5-fold) of cell surface receptor to an internalized pool even in the absence of agonist stimulation.

DISCUSSION

In the present experiments, we examined whether each of the members of the GRK family, in addition to GRK2, could rescue the phosphorylation and sequestration of the Y326A mutant. In doing so, we found that rescued agonist-promoted Y326A sequestration by phosphorylation is not a property unique to GRK2 but is shared by other members of the GRK family. Nonetheless, the capacity of each of the GRKs to rescue Y326A mutant sequestration was dependent upon their ability to phosphorylate the receptor mutant, indicating kinase subtype specificity for the β_2AR.

The relative ability of each of the GRKs to rescue Y326A mutant sequestration correlated with their capacity to phosphorylate the receptor mutant. Of all of the GRKs tested, only GRK1 was unable to rescue either Y326A mutant sequestration or phosphorylation. In contrast, each of the other kinases influenced the extent of both the sequestration and phosphorylation of the Y326A mutant. However, the mechanism by which this was achieved differed depending upon the kinase tested. Two distinct mechanisms were observed: (i) agonist-dependent phosphorylation associated with agonist-dependent rescue of sequestration (GRK2, -3, and -5); and (ii) agonist-independent phosphorylation leading to both increased basal recycling (sequestration) of mutant receptors and rescued agonist-promoted sequestration (GRK4 and -6) as measured by both binding and flow cytometry.

Previously, we proposed that GRK-mediated phosphorylation, while able to rescue the sequestration of the Y326A mutant, was not the primary determinant regulating β_2AR sequestration but that it facilitated the interaction of some other cellular component with the receptor (Ferguson *et al*., 1995). We have identified this component as β-arrestin. β-Arrestin overexpression rescues Y326A sequestration in the absence of receptor phosphorylation, and this property of β-arrestin is potentiated by GRK-mediated phosphorylation (Ferguson *et al*., 1996). *In vitro* experiments have demonstrated that β-arrestins bind effectively to phosphorylated−unactivated β_2AR and that receptor−β-arrestin binding is facilitated further by agonist activation (Gurevich *et al*., 1995). These observations suggest that the agonist-independent receptor phosphorylation described here for GRK4 and -6 might promote the interaction of endogenous β-arrestins with the receptor, which in turn mediate receptor sequestration in the absence of agonist activation.

The observed differences in the ability of the various GRKs to mediate agonist-dependent or -independent phosphorylation of the wild-type and mutant β_2ARs might be linked to proposed differences in the mechanism by which each kinase is targeted to the plasma membrane. GRK2 and -3 are thought to be translocated to the plasma membrane in response to agonist stimulation as a consequence of their interaction with the $G_{\beta\gamma}$ subunits of heterotrimeric G proteins via a pleckstrin homology domain in their carboxyl termini (Koch *et al*., 1993; Touhara *et al*., 1994; Pitcher *et al*., 1995). The liberation of the $G_{\beta\gamma}$ subunits from G_α occurs as a consequence of agonist stimulation and the subsequent coupling of the β_2AR to G_s (Gilman, 1987; Birnbaumer *et al*., 1990). Therefore, the translocation of GRK2 and -3 to the membrane might be considered a signal-dependent event, and the resulting receptor phosphorylation would be primarily agonist-dependent (Haga & Haga, 1992; Pitcher *et al*., 1992). In contrast, membrane targeting of each of the other kinases involves post-translational modifications that promote lipid association, such as farnesylation (GRK1), palmitoylation (GRK4 and -6), or a polybasic carboxyl tail (GRK5), and would not necessarily be signal-dependent (Inglese *et al*., 1993; Casey, 1995). Isomerization of the β_2AR from its low- to high-affinity state (R to R*) can occur spontaneously (independent of agonist stimulation) (Samana *et al*., 1993; Bond *et al*., 1995). This change in affinity might serve not only to trigger changes in receptor conformation that promote coupling but also to influence the relative ability of GRKs to interact with and phosphorylate the receptor (Barak *et al*., 1995). As a consequence, overexpression of GRKs that are constitutively targeted to the plasma membrane might effectively interact with and phosphorylate receptors spontaneously isomerizing to R* in the absence of agonist stimulation. The β_2AR serves as a better substrate for agonist-independent phosphorylation by a palmitoylated rather than nonpalmitoylated kinase (Stoffel *et al*., 1994), and the fact that palmitoylation is dynamically regulated (Casey *et al*., 1995) might suggest that, in addition to targeting GRK4 and -6 to the plasma membrane, palmitoy-

lation may also regulate their enzymatic activity. Alternatively, palmitoylation may be a more effective membrane targeting signal than a carboxyl tail polybasic domain. In either case, this might explain why GRK4 and -6, but not GRK5, substantially increased receptor phosphorylation in the absence of agonist stimulation.

The β_2AR has been demonstrated *in vitro* to be an effective substrate for phosphorylation by GRK2, -3, and -5, but a relatively poor substrate for GRK1, -4, and -6 (Benovic *et al*., 1986, 1987, 1991; Benovic & Gomez, 1993; Kunapuli *et al*., 1994a; Pei *et al*., 1994). Figure 2B illustrates the limitations of heterologous cell expression systems for the study of receptor−GRK specificity, as overexpression of kinases that effectively phosphorylate the β_2AR receptor *in vitro* contributes little to the level of receptor phosphorylation already achieved with endogenously expressed kinases. On the other hand, the Y326A mutant provides a unique tool for addressing these questions, at least with respect to the β_2AR. Under control conditions, the Y326A mutant does not serve as an effective substrate for GRK-mediated phosphorylation (Ferguson *et al*., 1995), and as a consequence, the mutant receptor might be expected to provide a more sensitive assay for the specificity of kinase/receptor interactions. This was indeed the case, as overexpression of GRK2, -3 and -5 effectively rescued the phosphorylation phenotype of the Y326A mutant, whereas GRK1 and -4 were less effective. The ability of each of the GRKs to rescue the β_2AR−Y326A mutant phosphorylation recapitulated their ability to phosphorylate the β_2AR *in vitro*, except that the β_2AR may be a better substrate for GRK6 than previously envisaged (Palczewski & Benovic, 1991; Benovic & Gomez, 1993; Kunapuli *et al*., 1994; Pei *et al*., 1994). However, the effectiveness of GRK1 and -4 to affect the phosphorylation and the sequestration of the Y326A mutant could also be attributable, at least in part, to different expression levels for these kinases compared to the other kinases. This was found not to be the case since the use of 10 times the amount of DNA, which resulted in a marked increase in the expression level of these two GRKs, did not further increase the phosphorylation and/or the sequestration of the Y326A mutant. GRK4 was more effective at rescuing the sequestration of the Y326A mutant than at rescuing its phosphorylation (Figures 2−4). The increase in agonist-independent sequestration seen with this kinase offers an explanation for this discrepancy, since constitutively sequestering receptors would be expected to be constitutively dephosphorylated (Sibley *et al*., 1986).

In summary, we have shown that receptor phosphorylation by GRK2−6 leads to increased receptor sequestration and that the agonist-dependency of this phenomenon was best preserved when GRK2, -3, and -5 were overexpressed. These results support the idea first put forward by Sibley *et al*. (1986), that receptor phosphorylation not only plays a primary role in receptor desensitization but also may act as the initial signal leading to receptor sequestration. However, GRK-mediated phosphorylation does not appear to be absolutely required for receptor sequestration since phosphorylation-deficient mutants sequester either normally as seen in CHW cells (Hausdorff *et al*., 1989) or partially as seen in HEK-293 cells (Ferguson *et al*., 1995, 1996) or CHO cells (Barak and Caron, unpublished results). We have recently shown in HEK-293 cells that the role of phosphorylation is probably to promote the interaction of the receptor with β-arrestins (Ferguson *et al*., 1996). However, at high expression levels, β-arrestin could function independently of phosphorylation and rescue the sequestration of the phosphorylation-deficient β_2AR mutant (Ferguson *et al*., 1996). These results suggest that under normal conditions, both β-arrestin and GRKs are required for β_2AR receptor sequestration. However, since we are using receptor mutants to study the role of phosphorylation and accessory protein in sequestration, the possibility always exists that phosphorylation is not required for the sequestration of the native β_2AR. The different behavior of β_2AR mutants in various cell types (e.g., CHW vs HEK-293 cells) might suggest that different levels of GRK and/or β-arrestins are present in those cells, or that different cells could use alternative pathways for receptor sequestration, i.e., coated pits vs caveolae (Raposo *et al*., 1989; von Zastrow & Kobilka, 1992). The ultimate proof for a role of GRK phosphorylation in β_2AR receptor sequestration would be achieved by using GRK-deficient cells generated by gene disruption. Experiments are currently under way to examine these different possibilities.

REFERENCES

Ariza, J. L., Dawson, T. M., Simerly, R. B., Martin, L. J., Caron, M. G., Snyder, S. H., & Lefkowitz, R. J. (1992) *J. Neurosci. 12*, 4045−4055.

Barak, L. S., Tiberi, M., Freedman, N. J., Kwatra, M. M., Lefkowitz, R. J., & Caron, M. G. (1994) *J. Biol. Chem. 269*, 2790−2795.

Barak, L. S., Ménard, L., Ferguson, S. S. G., Colapietro, A. M., & Caron, M. G. (1995) *Biochemistry 34*, 15407−15414.

Benovic, J. L., & Gomez, J. (1993) *J. Biol. Chem. 268*, 19521−19527.

Benovic, J. L., Mayor, F., Jr., Somers, R. L., Caron, M. G., & Lefkowitz, R. J. (1986) *Nature 322*, 869−872.

Benovic, J. L., Mayor, F., Jr., Staniszewski, C., Lefkowitz, R. J., & Caron, M. G. (1987) *J. Biol. Chem. 262*, 9026−9032.

Benovic, J. L., Onorato, J. J., Arriza, J. L., Stone, W. C., Lohse, M., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., Caron, M. G., & Lefkowitz, R. J. (1991) *J. Biol. Chem. 266*, 14939−14946.

Birnbaumer, L., Abramowitz, J., & Brown, A. M. (1990) *Biochim. Biophys. Acta 1031*, 163−224.

Bond, R. A., Leff, P., Johnson, T. D., Milano, C. A., Rockman, H. A., McMinn, T. R., Apparsundaram, S., Hyek, M. F., Kenakin, T. P., Allen, L. F., & Lefkowitz, R. J. (1995) *Nature 374*, 272−276.

Casey, P. J. (1995) *Science 268*, 221−225.

Cheung, A. H., Sigal, I. S., Dixon, R. A., & Strader, C. D. (1989) *Mol. Pharmacol. 34*, 132−138.

Cullen, B. (1987) *Methods Enzymol. 152*, 684−704.

Dohlman, H. G., Thorner, J., Caron, M. G., & Lefkowitz, R. J. (1991) *Annu. Rev. Biochem. 60*, 653−688.

Ferguson, S. S. G., Ménard, L., Barak, L., Koch, W. J., Colapietro, A.-M., & Caron, M. G. (1995) *J. Biol. Chem. 270*, 24782−24789.

Ferguson, S. S. G., Downey, W. E., III, Colapietro, A.-M., Barak, L. S., Ménard, L., & Caron, M. G. (1996) *Science 271*, 363−366.

Gilman, A. (1987) *Annu. Rev. Biochem. 56*, 615−649.

Gurevich, V. V., Dion, S. B., Onorato, J. J., Ptasienski, J., Kim, C. M., Sterne-Marr, R., Hosey, M. M., & Benovic, J. L. (1995) *J. Biol. Chem. 270*, 720−731.

Haga, K., & Haga, T. (1992) *J. Biol. Chem. 267*, 2222−2227.

Hausdorff, W. P., Bouvier, M., O'Dowd, B. F., Irons, G. P., Caron, M. G., & Lefkowitz, R. J. (1989) *J. Biol. Chem. 264*, 12657−12665.

Hausdorff, W. P., Caron, M. G., & Lefkowitz, R. J. (1990) *FASEB J. 4*, 2881−2889.

Hausdorff, W. P., Campbell, P. T., Otrowski, J., Yu, S. S., Caron, M. G., & Lefkowitz, R. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2979−2983.

Hausdorff, W. P., Sung, J., Caron, M. G., & Lefkowitz, R. J. (1992) *Asian Pacific J. Pharmacol. 7*, 149−158.
Inglese, J., Glickman, J. F., Lorenz, W., Caron, M. G., & Lefkowitz, R. J. (1992) *J. Biol. Chem. 267*, 1422−1425.
Inglese, J., Freedman, N. J., Koch, W. J., & Lefkowitz, R. J. (1993) *J. Biol. Chem. 268*, 23735−23738.
Koch, W. J., Inglese, J., Stone, W. C., & Lefkowitz, R. J. (1993) *J. Biol. Chem. 268*, 8256−8260.
Kunapuli, P., Onorato, J. J., Hosey, M. M., & Benovic, J. L. (1994) *J. Biol. Chem. 269*, 1099−1105.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Lefkowitz, R. J. (1993) *Cell 74*, 409−412.
Lohse, M. J., Benovic, J. L., Caron, M. G., & Lefkowitz, R. J. (1990) *J. Biol. Chem. 265*, 3202−3209.
Palczewski, K., & Benovic, J. L. (1991) *Trends Biol. Sci. 16*, 387−391.
Pei, G., Tiberi, M., Caron, M. G., & Lefkowitz, R. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3633−3636.
Pitcher, J. A., Inglese, J., Higgins, J. B., Arriza, J. L., Casey, P. J., Kim, C., Benovic, J. L., Kwatra, M. M., Caron, M. G., & Lefkowitz, R. J. (1992) *Science 257*, 1264−1267.
Pitcher, J. A., Touhara, K., Payne, E. S., & Lefkowitz, R. J. (1995) *J. Biol. Chem. 270*, 11707−11710.
Premont, R. T., Koch, W. J., Inglese, J., & Lefkowitz, R. J. (1994) *J. Biol. Chem. 269*, 6832−6841.
Premont, R. T., Inglese, J., & Lefkowitz, R. J. (1995) *FASEB J. 9*, 175−182.
Raposo, G., Dunia, I., Delavier-Klutchko, C., Kaveri, S., Strosberg, A. D., & Benedetti, E. L. (1989) *Eur. J. Cell Biol. 50*, 340−352.
Roettger, B. F., Rentsch, R. U., Pinon, D., Holicky, E., Hadac, E., Larkin, J. M., & Miller, L. J. (1995) *J. Cell Biol. 128*, 1029−1041.
Samama, P., Cotecchia, S., Costa, T., & Lefkowitz, R. J. (1993) *J. Biol. Chem. 268*, 4625−4636.
Schwinn, D. A., Caron, M. G., & Lefkowitz, R. J. (1992) in *The heart and cardiovascular system* (Fozzard, H. A., Ed.) pp 1657−1684, Raven Press, New York.
Sibley, D. R., Strasser, R. H., Benovic, J. L., Daniel, K., & Lefkowitz, R. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9408−9412.
Stoffel, R. H., Randall, R. R., Premont, R. T., Lefkowitz, R. J., & Inglese, J. (1994) *J. Biol. Chem. 269*, 27791−27794.
Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G., & Lefkowitz, R. J. (1994) *J. Biol. Chem. 269*, 10217−10220.
Tsuga, H., Kameyama, K., Haga, T., Kurose, H., & Nagao, T. (1994) *J. Biol. Chem. 269*, 32522−32527.
von Zastrow, M., & Kobilka, B. K. (1992) *J. Biol. Chem. 267*, 3530−3538.
Yu, S. S., Lefkowitz, R. J., & Hausdorff, W. P. (1993) *J. Biol. Chem. 268*, 337−341.

BI952961+