G Protein-coupled Receptor Kinase 2–mediated Phosphorylation of Ezrin Is Required for G Protein-coupled Receptor–dependent Reorganization of the Actin Cytoskeleton

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INTRODUCTION

G protein-coupled receptor (GPCR) kinase 2 (GRK2) phosphorylates and desensitizes activated G protein-coupled receptors (GPCRs). Here, we identify ezrin as a novel non-GPCR substrate of GRK2. GRK2 phosphorylates glutathione S-transferase (GST)-ezrin, but not an ezrin fusion protein lacking threonine 567 (T567), in vitro. These results suggest that T567, the regulatory phosphorylation site responsible for maintaining ezrin in its active conformation, represents the principle site of GRK2-mediated phosphorylation. Two lines of evidence indicate that GRK2-mediated ezrin-radixin-moesin (ERM) phosphorylation serves to link GPCR activation to cytoskeletal reorganization. First, in Hep2 cells muscarinic M1 receptor (M1MR) activation causes membrane ruffling. This ruffling response is ERM dependent and is accompanied by ERM phosphorylation. Inhibition of GRK2, but not rho kinase or protein kinase C, prevents ERM phosphorylation and membrane ruffling. Second, agonist-induced internalization of the β2-adrenergic receptor (β2AR) and M1MR is accompanied by ERM phosphorylation and localization of phosphorylated ERM to receptor-containing endocytic vesicles. The colocalization of internalized β2AR and phosphorylated ERM is not dependent on Na+/H+ exchanger regulatory factor binding to the β2AR. Inhibition of ezrin function impedes β2AR internalization, further linking GPCR activation, GRK activity, and ezrin function. Overall, our results suggest that GRK2 serves not only to attenuate but also to transduce GPCR-mediated signals.

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Abbreviations used: ACh, acetylcholine; β2AR, β2-adrenergic receptor; cGMP, cyclic guanosine monophosphate; ERM, ezrin-radixin-moesin; FERM, four-point-one, ERM (FERM) domain that interacts with both plasma membrane-associated proteins and phosphatidylinositol 4,5-bisphosphate (PIP2) as well as a carboxy-terminal actin binding region. In the cytosol, ERM

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proteins adopt an inactive conformation wherein intramolecular or intermolecular interactions between the amino and carboxy termini mask the membrane and actin binding sites (Gary and Bretscher, 1995). Activation of ERK proteins, which results in the unmasking of ligand binding sites, requires PIP2 binding to the FERM domain and subsequent phosphorylation of a carboxy-terminal threonine (T567 in ezrin) (Matsui et al., 1998; Fievet et al., 2004; Chambers and Bretscher, 2005). Rho kinase and protein kinase C (PKC) have been shown to mediate ERK activation via T567 phosphorylation both in vitro and in vivo (Matsui et al., 1998; Simons et al., 1998).

In this report, we show that ezrin is a substrate of GRK2 in vitro and in cultured mammalian cells. Furthermore, GRK2-mediated ERK phosphorylation is shown to be required for M1MR-dependent membrane ruffle formation in Hep2 cells and agonist-induced internalization of the β2AR in human embryonic kidney 293 (HEK293) cells. Together, these results suggest a signaling function for GRK2, linking GPCR activation with GPCR-dependent reorganization of the actin cytoskeleton.

MATERIALS AND METHODS

Cell Culture
HEK293 cells stably expressing His6-tagged β2AR (HEK293 cells, a generous gift of Dr. Stefano Marullo, Institut Cochin, Paris, France) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum (FCS), 100 IU penicillin, and 100 μg/ml streptomycin (all from Sigma-Aldrich, St. Louis, MO) and 2 mg/ml Geneticin (Invitrogen, Paisley, United Kingdom) at 37°C, 5% CO2, 80%, Hep2, and HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% FCS, penicillin, and streptomycin at 37°C, 5% CO2.

cDNA Constructs
The plasmids for pGEX-2T-ezrin, pGEX-2T-ezrin-β, pGEX-2T-ezrin-βt, and pGEX-2T-ezrin-βx were generously provided by Dr. Stéphane Marullo, Institut Curie, Paris, France. pCB6-Ezrin-VSVG, pCB6-Ezrin-Nter-VSVG, and pCB6-Ezrin-Cter-VSVG (WT-ezrin, N- and ezrin, and C-ezrin; Algrain et al., 1993) were generously provided by Dr. Monique Arpin (Institut Curie, Paris, France). GRK2(45-178)-GF and D110A-GRK2(45-178)-GF (GRK2-RH and GRK2-MRF1; Sterne-Marr et al., 2003) were generously provided by Dr. Rachel Sterne-Marr (Loudonville, New York). pEGBβARK-CT (GRK2-CT; Pierce et al., 2001), pBIC12I-βARK1 and pBIC12I-βARK1-K220 (GRK2 and GRK2βARK2, Freedman et al., 1995), pSV2-neo-FLAG-βARK1 and pSV2-neo-FLAG-βARK2 (Liu et al., 1998b), pPK3-HA-M1MR and pPK3-HA-M2MR (Claign et al., 2000) were generously provided by Prof. Robert Lefkowitz (Howard Hughes Medical Institute, Durham, NC). To generate a glutathione S-transferase (GST)-tagged ezrin construct with threonine-567 mutated to alanine (T567A), the T567A-ezrin cDNA was amplified from pcB6-Ezrin-T567A-VSVG (Dr. Monique Arpin, Institut Curie) by using PCR. The PCR primers were designed so that the resulting T567A-ezrin cDNA had the following sequence: 5′-ACA TCT CTC CAA CAA CAC CCA CGC CCA GGA GAG GGT GAA GAC GAA GGT GAA CAA CAG CAG CAG CAC CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA
RESULTS

Identification of Ezrin as a GRK2 Substrate

In an attempt to identify non-GPCR substrates for GRK2, HEK293 cells transfected with the β2AR or the β2AR and GRK2 were labeled with [32P]orthophosphate and left untreated, or alternatively, treated with isoproterenol, a β-adrenergic receptor agonist. Cells were subsequently lysed and [32P]-labeled proteins subjected to two-dimensional gel electrophoresis and autoradiography. Proteins whose [32P] content increased upon expression of GRK2 in an agonist-dependent manner were excised and identified using matrix-assisted laser desorption ionization/time of flight mass spectrometry. One potential GRK2 substrate identified in this manner was the actin binding protein ezrin (our unpublished data).

To determine whether indeed ezrin serves as a GRK2 substrate, GST-ezrin was expressed in, and purified from, E. coli and subject to in vitro phosphorylation by purified GRK2. The full-length ezrin construct (1–586) was efficiently phosphorylated by GRK2 in the presence of Gβγ and lipid vesicles consisting of phosphatidylincholine and PIP2 (Figure 1, 1–586). GRK2 contains a carboxy-terminal pleckstrin homology (PH) domain that binds PIP2 and Gβγ in a coordinated manner to promote membrane localization of the kinase (Pitcher et al., 1995). PIP2 additionally binds the amino terminus of ezrin; this interaction is required for phosphorylation of T567 and leads to ezrin activation (Niggli et al., 1995; Fievet et al., 2004). The PIP2 and Gβγ dependence of GRK2-mediated ezrin phosphorylation thus presumably reflects the colocalization of both kinase and substrate to the surface of lipid vesicles and PIP2-dependent changes in ezrin conformation.

GST-ezrin constructs with different domain deletions were used to identify the region of ezrin that contains the GRK2 phosphorylation site. A GST fusion protein encompassing residues 310–586 of ezrin was also a substrate of GRK2, localizing the GRK2 phosphorylation site to the carboxy terminus of the protein (Figure 1, top). Addition of Gβγ- and PIP2-containing lipid vesicles inhibited GRK2-mediated phosphorylation of this construct (Figure 1). These results are potentially consistent with the PIP2 and Gβγ dependence observed for GRK2-mediated phosphorylation of full-length ezrin. GST-310-586 lacks the autoinhibitory amino-terminal PIP2 binding domain of ezrin. Phosphorylation sites encompassed within this carboxy-terminal domain would thus be predicted to be accessible in the absence of PIP2. Because in the presence of Gβγ GRK2 binds PIP2-containing vesicles (Pitcher et al., 1996), their addition may sequester GRK2 away from its non-PIP2 binding GST-310-586 substrate. An ezrin construct lacking the carboxy terminus (1–533) was poorly phosphorylated by GRK2, both in the presence and absence of Gβγ and lipid (Figure 1, top).

These results indicate that GRK2 phosphorylates the carboxy terminus of ezrin, between residues 533 and 586. This region includes T567, phosphorylation of which stabilizes the activated form of ezrin (Matsui et al., 1998). To assess whether GRK2-mediated ezrin phosphorylation occurs at this regulatory phosphorylation site, the ability of a mutant ezrin fusion protein, in which T567 is mutated to alanine (T567A), to serve as a GRK2 substrate was examined. GST-T567A is a poor GRK2 substrate in vitro in either the presence or absence of PIP2 and Gβγ (Figure 1, bottom), suggesting that T567 represents the principle site of GRK2-mediated ezrin phosphorylation. Based on these observations, it is tempting to speculate that GRK2 may, like rho kinase and PKC, play a role in modulating the ability of ezrin to cross-link plasma membrane proteins to actin. Notably, the Gβγ dependence of GRK2-mediated phosphorylation of full-length ezrin suggests that in a cellular context this phosphorylation event may be dependent upon GPCR activation.

Ezrin and GRK2 Are Required for M1MR-mediated Membrane Ruffling

It has previously been shown that ERM proteins are involved in the biogenesis of plasma membrane structures, such as microvilli and membrane ruffles, which are formed after changes in the underlying actin cytoskeleton (Bretscher, 1989; Crepaldi et al., 1997; Bonilha et al., 1999; Yonemura et al., 1999). We developed a model system by using Hep2 cells overexpressing HA-tagged M1MR to investigate the potential functional significance of Gβγ-dependent GRK2-mediated ezrin phosphorylation in GPCR-mediated cytoskeletal reorganization. A 5-min agonist (ACh) treatment of Hep2 cells expressing M1MR caused rapid and transient membrane ruffles to which endogenous ezrin was localized (Figure 2A, left). Under these conditions, 87 ± 9.7% of the M1MR-transfected cells ruffled. Using an antibody (297S) that specifically recognizes pERM (Matsui et al., 1998;
Hayashi et al., 1999), it was observed that the cellular levels of pERM increase after agonist stimulation and that it is activated ERM proteins that are localized to ruffles (Figure 2A, right). These results demonstrate that M1MR activation promotes ERM phosphorylation and suggest that activated ERM may play a role in ruffle formation.

To investigate a potential functional role for ezrin in M1MR-mediated membrane ruffling, Hep2 cells were co-transfected with the M1MR and ezrin (WT-ezrin) or the amino-terminal FERM domain of ezrin (N-ezrin). Expression of the FERM domain of ezrin has been shown to inhibit ezrin function in a number of model systems (Crepaldi et al., 1997; Amieva et al., 1999). Expression of full-length ezrin has no effect on agonist-dependent M1MR-mediated ruffling and the expressed protein, like the endogenous, localizes to ruffles (WT-ezrin; Figure 2B). M1MR/ezrin cotransfected cells ruffled after a 5-min Ach treatment (90.9/11006 90%, Figure 3B). In contrast, only 31.5/11006 8% of Hep2 cells cotransfected with M1MR and the FERM domain of ezrin (N-ezrin) exhibited ruffling under identical conditions (N-ezrin; Figures 2B and 3B). These results are consistent with an obligate role for activated, phosphorylated ERM proteins in M1MR-mediated membrane ruffle formation in our system.

Because ezrin is a GRK2 substrate in vitro, we investigated a potential role for GRK2-mediated phosphorylation events in this model of cytoskeletal reorganization. Hep2 cells were cotransfected with M1MR and either the carboxy terminus of GRK2 (GRK2-CT), the catalytically inactive mutant kinase (K220R), the amino-terminal regulator of G protein signaling homology (RH) domain of GRK2 (GRK2-RH), or a mutant RH domain of GRK2 (GRK2-mRH). Both GRK2-CT and GRK2K220R contain the Gβγ/PIP₂ binding PH domain of GRK2, and their expression inhibits agonist-dependent translocation of endogenous GRK2 to activated GPCRs (Daaka et al., 1997). The green fluorescent protein (GFP)-tagged RH domain of GRK2 binds activated Gαq and is recruited to the plasma membrane in a Gαq-dependent manner (Sterne-Marr et al., 2003). The M1MR is Gαq coupled; expression of GRK2-RH would thus be anticipated to inhibit the translocation of endogenous GRK2 to the activated M1MR. Expression of GRK2-CT, GRK2K220R, or GRK2-RH should therefore inhibit receptor-activated GRK2-mediated phosphorylation events. A mutant GFP-tagged GRK2 RH domain construct, GRK2-mRH, which fails to bind activated Gαq and so would be predicted to have no effect on the agonist-induced translocation of endogenous GRK2, was used as a control in these experiments. Cells coexpressing M1MR and GRK2-CT or M1MR and GRK2K220R show diminished membrane ruffling (by ~90 and 89%, respectively, compared with M1MR alone) after ACh treatment (Figure 3, A and B). Similarly, expression of the RH domain of GRK2 inhibited M1MR-dependent ruffling by ~81% (Figure 3, A and B). In marked contrast, the mutant RH domain of GRK2, GRK2-mRH, had no inhibitory effect on ruffling. These results imply that, in addition to
ezrin, active GRK2 is required for the formation of membrane ruffles in Hep2 cells expressing M1MR.

**GRK2 Activity Is Required for ERM Activation in Hep2 Cells**
To examine the relationship between GRK2 activity and ERM phosphorylation in M1MR-mediated membrane ruffling, Hep2 cells were transfected with M1MR alone, or were cotransfected with M1MR and GRK2-RH or GRK2-mRH. Transfected cells were agonist treated, fixed, and M1MR, the RH domain of GRK2, and endogenous pERM were visualized (Figure 4). In cells expressing M1MR, or coexpressing M1MR and the mutant RH domain of GRK2, pERM was observed after agonist treatment and was localized to the membrane ruffles.

**Figure 3.** M1MR-mediated membrane ruffling is GRK2 dependent. (A) Hep2 cells were transfected with M1MR or cotransfected with M1MR and either the carboxy terminus of GRK2 (M1MR/GRK2-CT), catalytically inactive GRK2 (M1MR/K220R), the RH domain of GRK2 (M1MR/GRK2-RH), or a mutant RH domain (M1MR/GRK2-mRH). Forty-eight hours after transfection, the cells were treated with 100 μM ACh for 5 min (ACh), and expressed proteins were visualized by immunofluorescence. (B) The effect of different ezrin and GRK2 constructs on M1MR-mediated ruffling. Approximately 100 appropriately transfected cells were scored as ruffling or nonruffling per experiment. Data indicate the mean number of ruffling cells. Error bars represent SD of mean data collected from multiple experiments (n ≥ 3). ***p ≤ 0.001.
ensuing ruffles (Figure 4). In cells coexpressing M1MR and GRK2-RH, however, no agonist-dependent ruffling or increase in pERM staining was detected (Figure 4). The fact that endogenous GRK2 activity is required for the detection of active ERM species in the cell suggests that it is GRK2 that is responsible for the phosphorylation of ERM proteins. Furthermore, because the antibody used recognizes specifically phospho-ezrin (T567), -radixin (T564), or -moesin (T558), GRK2 is implicated as the kinase responsible for phosphorylating ERM proteins at this functionally relevant site.

M1MR-mediated Membrane Ruffling in Hep2 Cells Is Not Rho Kinase or PKC Dependent

Previous studies have shown that ezrin can be phosphorylated at T567 by PKC and rho kinase (Matsui et al., 1998; Simons et al., 1998; Ng et al., 2001). We examined whether these kinases were responsible for the phosphorylation of ERM proteins in our model system. Hep2 cells overexpressing M1MR were left untreated or pretreated with the rho kinase inhibitor Y27632 or the PKC inhibitor GF109203X before agonist stimulation (Figure 5A). M1MR and pERM was subsequently visualized after TCA fixation of treated cells. Neither rho kinase inhibition nor PKC inhibition prevented ruffle formation after agonist treatment and neither inhibited ERM phosphorylation or localization of pERM to the ruffles (Figure 5A, M1MR and pERM). Phalloidin staining of paraformaldehyde-fixed coverslips treated in an identical manner revealed similar agonist-dependent changes in the actin cytoskeleton in either the presence or absence of inhibitors (Figure 5A, Phalloidin). Both inhibitors, did however, significantly inhibit EGF-mediated ruffling and ERM phosphorylation (Figure 5B). Thus, inhibition of GRK2 specifically inhibits agonist-dependent M1MR-mediated ERM phosphorylation and ERM-dependent membrane ruffling in this Hep2 cell model system.

pERM Colocalizes with Internalized β2AR and M1MR

Because we have shown that ezrin is a substrate of GRK2 with a potential role in cytoskeletal reorganization, we looked to see whether GRK2-mediated ERM phosphorylation was involved in receptor endocytosis, a process that has previously been shown to be actin dependent (da Costa et al., 2003). In HEK293 cells stably expressing His₆-tagged β₂AR (HEK₂H9252), agonist (isoproterenol) treatment for 15 min induced receptor internalization (Figure 6A). This was concomitant with an increase in endogenous pERM levels, which colocalize with β2AR at internal vesicles (Figure 6A, ISO enlarged). This phenomenon is not restricted to β₂AR or HEK293 cells. In Hep2 cells expressing M1MR, prolonged ACh treatment (20 min) resulted in receptor endocytosis, which occurred after transient membrane ruffling (Figure 6B). Agonist treatment of M1MR-transfected Hep2 cells was associated with increased endogenous pERM levels, and, in these cells also, active ERM colocalizes with internalized receptor (Figure 6B, ACh enlarged). In marked contrast, in Hep2 cells transfected with the HA-tagged M2 muscarinic receptor (M2MR), internalization but not ERM phosphorylation is detected after agonist stimulation (Figure 6C). In HEK293 cells, M2MR has been shown to internalize in a β-arrestin-, and therefore GRK-, independent manner (Pals-Rylaarsdam et al., 1995; Vogler et al., 1999; Claing et al., 2000). These results support our contention that GRK2 is responsible for GPCR-dependent ERM phosphorylation and suggest that pERM localization to receptor-containing endocytic

Figure 4. GRK2 is required for M1MR-mediated ERM phosphorylation. Hep2 cells were transfected with M1MR, M1MR, and GRK2-RH or M1MR and GRK2-mRH and incubated for 48 h. The cells were subsequently treated with ACh for 5 min (ACh), and M1MR, GRK2-RH, and endogenous pERM were detected by immunofluorescence. A rabbit anti-pERM antibody (Chemicon International) was used to detect pERM, as described in Materials and Methods. T indicates CDNA constructs transfected. Bars, 20 μm.

T:  M1MR  M1MR  M1MR
     GRK2-RH GRK2-mRH

Ezrin Is a Novel Substrate of GRK2

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Figure 5. M1MR-mediated ruffling in Hep2 is not rho kinase or PKC dependent. (A) Forty-eight hours after transfection, Hep2 cells overexpressing M1MR were treated with either the rho kinase inhibitor (Y27632, 10 μM) or the PKC inhibitor (GF109203X, 20 μM) for 1 h. Cells were then subject to agonist (100 μM ACh, 5 min) before TCA fixation (ACh). M1MR and endogenous pERM were visualized by immunofluorescence. A rabbit anti-pERM antibody (Chemicon International) was used. In parallel, a second set of identically treated coverslips were paraformaldehyde fixed and stained with phalloidin and examined for M1MR expression. The phalloidin staining from this set of coverslips is shown (Phalloidin). (B) M1MR transfected Hep2 cells were left untreated or treated with rho kinase or PKC inhibitors, as described in A. After preincubation with kinase inhibitors, cells were treated with EGF (200 ng/ml, 5 min; Sigma-Aldrich), fixed, and the EGF receptor (EGFR) and pERM were visualized by immunofluorescence as described in Materials and Methods. Bars, 20 μm.
vesicles may occur specifically with receptors that internalize via a GRK- and β-arrestin-dependent pathway.

**Ezrin Is Required for β₂AR Endocytosis**

Because pERM colocalize with internalized β₂AR, we sought to examine whether activated ezrin played a role in regulating the agonist-dependent trafficking of this receptor. Mock-transfected HEKβ² cells were subject to 30 min of agonist treatment followed immediately by 30 min of antagonist (alprenolol) treatment. Samples were removed before treatment (untreated), after agonist treatment (ISO [30 min]), and after agonist removal and subsequent antagonist treatment (alprenolol, ALP [30 min]). Plasma membrane-located β₂AR was labeled with an anti-His₉₉ monoclonal antibody and a fluorescently labeled secondary antibody and quantified by flow cytometry analysis. Results were compared with those of HEKβ² cells expressing a dominant negative dynamin construct (dynamin K44A) or the dominant negative N-ezrin construct.

**Figure 6.** pERM proteins colocalize with internalized β₂AR and M1MR. (A) HEK293 cells stably expressing β₂AR were treated with 50 μM ISO for 20 min. β₂AR and endogenous pERM were detected using immunofluorescence. (B and C) Hep2 cells were transfected with M1MR (B) and M2MR (C) and incubated for 48 h. The cells were then subject to 100 μM ACh for 20 min (ACh), and receptor and endogenous pERM were visualized by immunofluorescence. 297S, a rat anti-pERM antibody, was used for visualization of endogenous pERM. T indicates cDNA constructs transfected. D indicates proteins detected. Bars, 20 μm.
After agonist treatment of mock-transfected cells, levels of β₂AR at the plasma membrane decreased by ~32% (Figure 7, ISO [30 min]). Removal of agonist and treatment with antagonist resulted in the delivery of both internalized and newly synthesized receptor to the plasma membrane (Figure 7, ALP [30 min]). In cells transfected with dynamin K44A, which is known to inhibit endocytosis (van der Bieke et al., 1993), <5% of total β₂AR at the cell surface was internalized. Similarly, expression of N-ezrin inhibited β₂AR internalization such that only 10% of cell surface receptor was endocytosed after isoproterenol treatment (Figure 7). Neither dynamin K44A nor N-ezrin affected delivery of β₂AR to the plasma membrane after removal of agonist. Thus, it seems that not only does phosphorylated, active ezrin colocalize with internalized β₂AR but it is also required for agonist-induced β₂AR endocytosis.

**Colocalization of pERM with β₂AR Does Not Require NHERF**

Previous studies have shown that the Na⁺/H⁺ exchanger regulatory factor (NHERF) can bind, in an agonist-dependent manner, to the carboxy terminus of the β₂AR via its PSD-95, Discs-large, ZO-1 (PDZ) domain (Hall et al., 1998b) and to the FERM domain of ERM proteins (Reczek et al., 1997; Reczek and Bretscher, 1998). We investigated whether NHERF was responsible for the localization of pERM with internalized β₂AR. HEK293 cells were transiently transfected with either wild-type β₂AR or a mutant β₂AR that cannot bind NHERF (β₂ARL413A). Cells were subject to 15 min of isoproterenol treatment, fixed, and permeabilized. Receptor and endogenous pERM were visualized by immunofluorescence. A rat anti-pERM antibody (2976) was used for pERM staining. T indicates cDNA constructs transfected. D indicates proteins detected. Bars, 20 μm.

**DISCUSSION**

We have identified ezrin as a novel substrate of GRK2 and as a downstream effector of GPCR signaling. Our data increase the list of known nonreceptor GRK2 substrates, which already includes tubulin, phosducin, ribosomal protein P2, synucleins, the inhibitory γ subunit of the type 6 retinal cGMP phosphodiesterase, and the β-subunit of the epithelial Na⁺ channel (Carman et al., 1998; Haga et al., 1998; Pitcher et al., 1998b; Pronin et al., 2000; Ruiz-Gomez et al., 2000; Wan et al., 2001; Freeman et al., 2002; Dinudom et al., 2004). These findings also corroborate the hypothesis that GRK2 may have additional functions within the cell, besides phosphorylation of agonist-occupied GPCRs.

In this report, the ability of GRK2 to phosphorylate ezrin was initially discovered in cells and subsequently confirmed...
in vitro. Notably, GRK2-mediated phosphorylation of full-length ezrin is PIP2 and Gβγ-dependent, consistent with previous findings that show PIP2-mediated changes in ezrin conformation are required for phosphorylation at T567 (Nakamura et al., 1999; Fievet et al., 2004). The PIP2 and Gβγ dependence of GRK2-mediated phosphorylation additionally suggests that this phosphorylation event might occur in an agonist- and GPCR-dependent manner.

The GRK2 phosphorylation site within ezrin was localized to a carboxy-terminal region encompassing T567, and indeed, a mutant ezrin construct in which T567 is mutated to alanine fails to serve as a GRK2 substrate. The phosphorylation of T567 has been shown to be important for maintaining ezrin in its active conformation such that the plasma membrane and actin binding domains are exposed (Pearson et al., 2000). Our results suggest that in the presence of PIP2 and Gβγ GRK2-mediated ezrin phosphorylation may facilitate ezrin activation.

Stimulation of Hep2 cells overexpressing M1MR and of HEK293 cells stably expressing β2AR leads to an increase in endogenous ERM specifically phosphorylated at T567 (ezrin), T564 (radixin), or T558 (moesin). In the Hep2 cell system, brief exposure to agonist (5 min) causes rapid and transient membrane ruffling that requires both active ezrin and GRK2. Cytoskeletal reorganization and the association of ezrin with plasma membrane and actin filaments have previously been found to be regulated by the Rho-family of small GTPases (Okamoto et al., 1999; Nakamura et al., 2000; Aspenstrom et al., 2004) and to involve phosphorylation of ezrin by either rho kinase or PKC, depending on the cellular context (Matsui et al., 1998; Simons et al., 1998). In this study, we have shown that in Hep2 cells expressing M1MR, ACh treatment leads to membrane ruffling and ERM activation even in the presence of the rho kinase inhibitor Y27632 or the PKC inhibitor GF109203X. That GRK2, but not rho kinase or PKC, inhibition ablates the M1MR-mediated ruffling response suggests that GRK2 phosphorylation facilitates ERM activation and is, at least in part, responsible for the GPCR- and ERM-dependent membrane ruffling observed in Hep2 cells.

Other GPCRs such as CCR2 (Jones et al., 2003), the sphingosine-1-phosphate receptors 1 (S1P1) and 2 (S1P2) (Sugi et al., 2004), the proteinase-activated receptor type 2 (PAR-2) (Ge et al., 2003), and CXCR4 (D’Apuzzo et al., 1997) induce membrane ruffling and cell migration on agonist activation. Although little is known about the interaction of S1P1–S1P2, and PAR-2 with GRKs, CCR2 and CXCR4 have been shown to undergo GRK2-dependent endocytosis after ligand binding (Aragay et al., 1998; Orsini et al., 2000). Furthermore, neutrophils and splenocytes derived from GRK6-deficient mice have altered chemotactic responses (Fong et al., 2002; Kavelaars et al., 2003; Vroon et al., 2004), indicating that GRK6 may be involved in regulating the cytoskeletal reorganization required for cell migration. In conjunction with our results, these data suggest that GRKs may have a general role in regulation of the actin cytoskeleton in processes such as cell motility. It would be interesting to investigate whether ezrin, or other ERM proteins, serve as substrates for other GRK family members, which would indicate a common pathway for actin filament modulation downstream of activated GPCRs.

Notably, not all GPCRs induce agonist-dependent ruffling when overexpressed in Hep2 cells. Indeed, the β2AR, which would be anticipated to recruit GRK2 in an agonist-dependent manner, fails to induce a ruffling phenotype. Agonist occupancy of the β2AR does, however, promote ezrin phosphorylation in HEK293 cells (Figure 8). These results suggest that GRK2-mediated ezrin phosphorylation, although required, is not sufficient to induce the cytoskeletal changes needed for ruffling. The additional factors required for GPCR-dependent membrane ruffling in Hep2 cells are currently a subject of investigation.

Many studies have demonstrated that the actin cytoskeleton has a role in clathrin-mediated endocytosis. Because GRK2 activity initiates GPCR desensitization and down-modulation and can also phosphorylate ezrin, a membrane-actin cross-linker, we investigated whether ezrin plays a functional role in GPCR internalization. In HEK293 cells stably expressing β2AR and Hep2 cells expressing M1MR, prolonged agonist stimulation causes receptor endocytosis paralleled by the colocalization of pERM with receptor at internalized vesicles. Flow cytometry analysis to quantify surface β2AR after isoproterenol treatment demonstrates that expression of the dominant negative N-erm construct inhibits receptor down-modulation to a similar extent as dynamin K44A expression. Dynamin is essential for clathrin-mediated endocytosis: dynamin K44A has a dominant negative effect that disrupts this process (van der Bliek et al., 1993).

Although our data suggest that ezrin may also have an important role in receptor internalization, this role may be limited to GPCRs that internalize in a GRK2- and β-arrestin-dependent manner. M2MR endocytosis is not correlated with ERM activation, and pERM is not associated with internalized M2 receptor. In HEK293 cells, M2MR has been shown to undergo endocytosis in a β-arrestin–dependent manner and does not seem to internalize via typical clathrin- or caveolin-dependent pathways in HeLa or HEK293 (Pals-Rylaarsdam et al., 1995; Vogler et al., 1999; Claing et al., 2000; Roseberry and Hoose, 2001; Delaney et al., 2002). In contrast, both β2AR and M1MR are sequestered via β-arrestin-, and therefore GRK-, dependent pathway in these cells (Claing et al., 2000). These results suggest that activated ezrin may play an obligate role in the internalization of a subset of GPCRs.

Much is known about GPCR internalization and the different mechanisms involved. Rapid sequestration of receptors such as β2AR is characterized by GRK phosphorylation leading to β-arrestin binding and endocytosis via clathrin-coated pits; other GPCRs may internalize via caveolae or in a GRK-independent manner (von Zastrow, 2003; Chini and Parenti, 2004). Our results suggest that a requirement for activated ERM may be an additional distinguishing feature of these endocytic pathways and may correlate with GRK dependence.

Non-G-protein–coupled receptors also may use an ezrin- and clathrin-dependent endocytosis pathway. The association of the LDL receptor with ezrin has been shown to be necessary for its clathrin-mediated endocytosis (Smith et al., 2004). pERM proteins colocalize with low-density lipoprotein (LDL) particles at the plasma membrane, and although active ERM proteins do not associate with internalized LDL, GFP-N-erm significantly inhibited LDL uptake (Smith et al., 2004).

Further investigation is required to establish how ezrin associates with GPCRs after agonist occupation. The ERM-binding protein NHERF is known to bind to the carboxy termini of certain receptors, including the β2AR, and has been shown to be involved in sorting β2AR between the recycling endosome and lysosome after endocytosis (Cao et al., 1999). NHERF also has been shown to regulate the trafficking of the human κ opioid receptor and the parathyroid hormone receptor (Li et al., 2002; Sneddon et al., 2003). Our data indicate that NHERF is not required to link ERM to a receptor after stimulation because pERM colocalize with
both a mutant β₂AR that is unable to bind NHERF and to M1MR, which does not contain the carboxy terminal motif recognized by NHERF (Hall et al., 1998a). It is possible that, like the integral membrane glycoprotein podocalyxin, β₂AR may be able to bind ERM both directly and via NHERF (Schneider et al., 2004). A recent study showed that Arf6 colocalizes with ezrin at the plasma membrane (Macia et al., 2004). Arf6 is thought to regulate vesicle formation in endocytosis and interacts with β-arrestin, suggesting that β-arrestin may be responsible for the recruitment of Arf6 (Claing et al., 2001), and hence possibly ezrin, to agonist-occupied GPCRs. It remains to be determined whether the requirement for activated ERM proteins in β₂AR internalization reflects a direct, an indirect, or is independent of an association between these two proteins.

In conclusion, we present evidence to suggest that a non-receptor substrate of GRK2 has an effector function after GPCR activation. We show that ezrin is a novel substrate of GRK2, that ERM proteins are activated after receptor stimulation, and that GRK2-mediated ERM phosphorylation is required for GPCR-mediated cytoskeletal rearrangements as well as internalization of a subset of receptors. Recently β-arrestins, key regulators of GPCR signaling, have been shown to have additional roles as scaffold and adaptor proteins in pathways downstream of GPCRs (Ahn et al., 2003). Similarly, it is becoming clear that GRK2, with its growing list of nonreceptor substrates, has a more diverse role within the cell than previously appreciated.

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