G Protein-coupled Receptors Mediate Two Functionally Distinct Pathways of Tyrosine Phosphorylation in Rat 1a Fibroblasts

Shc PHOSPHORYLATION AND RECEPTOR ENDOCYTOSIS CORRELATE WITH ACTIVATION OF Erk KINASES

(Received for publication, July 15, 1997, and in revised form, September 25, 1997)

Louis M. Luttrell*, Yehia Daaka, Gregory J. Della Rocca‡, and Robert J. Lefkowitz¶

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

The regulation of gene expression by many types of cell surface receptor involves the Ras-dependent activation of Erk kinases. Receptors as structurally diverse as growth factor receptors that possess intrinsic ligand-stimulated tyrosine kinase activity (RTKs),1 integrins, which mediate cell surface adhesion, and seven transmembrane-spanning GPCRs have been shown to mediate Ras-dependent growth regulatory signals.

The mechanisms whereby such distinct receptors regulate Ras activity are diverse. In each case, however, Ras activation is preceded by the recruitment of Ras guanine nucleotide exchange factors (GEFs) into a plasma membrane-associated signaling complex, where they activate Ras by catalyzing Ras-GTP exchange. Ras-GEF recruitment is the consequence of receptor-mediated stimulation of tyrosine protein kinases, which phosphorylate adapter proteins to create tyrosine phosphoprotein scaffolds on the plasma membrane. The individual components of the complex, including Ras-GEFs and other catalytic signaling proteins, are recruited via specific interactions between phosphotyrosine residues and modular phosphotyrosine-recognition domains within the component proteins, such as SH2 and PTB domains (1, 2).

Both the tyrosine kinases activated and the protein scaffolds employed vary between receptor and cell type. RTKs, such as the receptors for epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), undergo dimerization and transphosphorylation following ligand binding. Transphosphorylation creates docking sites, on the receptor itself, for the components of the Ras activation complex. The Ras-GEF, mSos, which is constitutively associated with the SH2 domain-containing adapter protein Grb2, associates either directly with the tyrosine phosphorylated RTK, or with other receptor-associated docking proteins, such as Shc (3). Interestingly, RTK-mediated signaling to Erk kinases may also involve endocytic trafficking since inhibition of clathrin-mediated endocytosis has recently been shown to impair rapid EGF-stimulated activation of Erk kinases (4).

In contrast to the RTK paradigm, α and β integrins are single transmembrane-spanning proteins that lack intrinsic enzymatic activity. Engagement of extracellular matrix proteins results in formation of αβ integrin heterodimers, which nucleate the formation of multiprotein focal adhesion complexes containing both cytoskeletal elements and catalytic signaling proteins. Part of this complex, the focal adhesion kinase p125Fak, undergoes tyrosine autophosphorylation after binding β integrin and provides a docking site for several signaling proteins, including Grb2 (5).
As they also lack intrinsic tyrosine kinase activity, the mechanisms whereby GPCRs regulate Ras activity are not well understood. Many GPCRs have been shown to activate both Src family nonreceptor tyrosine kinases (6–10) and FAK (11, 12). In addition, some GPCRs stimulate tyrosine phosphorylation of the EGF (13), PDGF (14), or insulin-like growth factor 1 (IGF1) (15) receptor tyrosine kinases via a ligand-independent “activation” mechanism (13) that may involve Src-mediated phosphorylation (16). As with the tyrosine kinases, the identity of the putative scaffolding proteins for GPCR-induced assembly of the Ras activation complex are also unclear. In COS-7 cells, GPCR-mediated Erk kinase activation correlates with tyrosine phosphorylation of Shc and its rapid association with Ras-GEF activity (17), c-Src (10), and EGF receptor (16). However in neuronal cells, GPCR-induced activation of the FAK family kinase, Pyk2, is apparently sufficient to mediate calcium-dependent activation of Ras and Erk kinases (18).

Since tyrosine-phosphorylated Shc and FAK each contain potential SH2-binding sites for Grb2, either, or both, might function as docking proteins for the membrane recruitment of the Grb2-mSos complex following GPCR stimulation. In this paper, we have investigated the role of FAK and Shc in the activation of Erk kinases by endogenous GPCRs and RTKs in Rat 1a fibroblasts. While both proteins undergo tyrosine phosphorylation and bind Grb2 in response to agonist stimulation, we find that only Shc phosphorylation correlates with the rapid activation of Erk kinases by these receptors. Further, both GPCR- and RTK-mediated Erk activation are sensitive to inhibitors of receptor internalization, suggesting that both tyrosine phosphorylation and clathrin-mediated endocytosis are required for signal transduction between the plasma membrane and cytoplasmic components of the Erk activation cascade.

**Experimental Procedures**

**Cell Culture and Transfection**—Rat 1a fibroblasts and HEK-293 cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum and 100 μg/ml gentamicin at 37 °C in a humidified 5% CO2 atmosphere. Transient transfection of HEK-293 cells with FLAGTM epitope-tagged
gp125FAK and Shc and Activation of Erk 1/2 in Rat 1a Fibroblasts—To determine the effects of GPCR and RTK stimulation on the tyrosine phosphorylation state of FAK and Shc, we employed endogenously expressed receptors for LPA, α-thrombin, bombesin, and EGF. In most cells, LPA and α-thrombin receptors mediate both pertussis toxin-sensitive and -insensitive signals (24), indicating coupling to both Gi/o family and Gq/11 family heterotrimeric G proteins. In contrast, bombesin receptors mediate predominantly pertussis toxin-insensitive mitogenic signals (25).

As shown in Fig. 1, stimulation of quiescent, serum-starved Rat 1a fibroblasts with LPA, the thrombin agonist peptide SFLLRN, bombesin, or EGF resulted in similar 4- to 5-fold increases in tyrosine phosphorylation of FAK (Fig. 1, A and C), LPA-, bombesin-, and EGF-stimulated tyrosine phosphorylation of FAK followed a similar time course as with maximal phosphorylation occurring within 2 min of stimulation and persisting for at least 10 min (Fig. 1B). Theadapter protein Grb2 coprecipitated with FAK from lysates of LPA-, SFLLRN-, bombesin-, and EGF-stimulated cells prepared under nondenaturing conditions (Fig. 1, A and D). The amount of coprecipitated Grb2 varied in proportion to the extent of FAK tyrosine phosphorylation (Amersham) and quantified by scanning laser densitometry.

**GST-Grb2 Fusion Proteins**—The cDNA encoding the full-length Grb2-glutathione S-transferase (GST) fusion protein (20) was generously provided by A. R. Saltiel. GST fusion proteins were prepared as glutathione-Sepharose conjugates as described previously (21). For the determination of Grb2 binding, appropriately stimulated Rat 1a cells were lysed in RIPa buffer containing 5 mM diithiothreitol, sonicated, clarified by centrifugation, precleared with 10 μg/ml GST-Sepharose for 1 h, and incubated with 10 μg/ml GST fusion protein-Sepharose for 2 h at 4 °C. After incubation, fusion protein complexes were washed twice with ice-cold RIPa buffer and once with PBS, denatured in Laemmli sample buffer, and resolved by SDS-PAGE. Precipitated proteins were detected by protein immunoblotting as described.

**Erk 1/2 Phosphorylation**—For the determination of Erk 1/2 phosphorylation, monolayers of serum-starved Rat 1a fibroblasts in 6-well culture plates were stimulated as described, washed once with ice-cold PBS, and solubilized by the direct addition of Laemmli sample buffer. Aliquots of cell lysate (approximately 30 μg of protein/lane) were resolved by SDS-PAGE, and Erk 1/2 phosphorylation was detected by protein immunoblotting using 1:20,000 dilution of rabbit polyclonal phospho-MAP kinase-specific IgG (Promega) with alkaline phosphatase-conjugated goat anti-rabbit IgG (Amersham) as secondary antibody. Quantitation of Erk 1/2 phosphorylation was performed using a Storm PhosphorImager (Molecular Dynamics). After quantitation of Erk 1/2 phosphorylation, nitrocellulose membranes were stripped of immunoglobulin and reprobed using rabbit polyclonal anti-Erk 2 IgG to confirm equal loading of Erk 2 protein.

**Intracellular Potassium Depletion and β2 Adrenergic Receptor Endocytosis**—Depletion of intracellular potassium was performed as described previously (22). Briefly, confluent monolayers of Rat 1 or HEK-293 cells in 6-well culture plates were washed twice with potassium-free buffer (140 mM NaCl, 20 mM HEPES, pH 7.4, 1 mM CaCl2, 1 mM MgCl2, 1 gm/liter dextrose) prior to incubation in hypotonic medium (potassium-free buffer/water (1:1 v/v)) for 10 min. Following hypotonic shock, monolayers were washed three times with potassium-free buffer or DMEM supplemented with 20 mM HEPES, pH 7.4 (control cells), for 30 min prior to stimulation. The effects of inhibitors of clathrin-mediated endocytosis on agonist-induced sequestration of β2 adrenergic receptors was determined by immunofluorescence flow cytometry as described previously (23). HEK-293 cells expressing FLAGTM epitope-tagged β2 adrenergic receptors at 300–400 fmol/mg of whole cell protein were exposed to 10 μM isoprenaline for 30 min at 37 °C prior to antibody staining using a 1:500 dilution of M2 anti-FLAG antibody (Kodak Scientific Imaging Systems), with a 1:250 dilution of fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (Jackson Laboratories) as secondary antibody. Receptor sequestration was defined as the fraction of total cell surface receptors that are removed from the plasma membrane following agonist treatment.

**Results**

**GPCRs and RTKs Induce Tyrosine Phosphorylation of p125FAK and Shc and Activation of Erk 1/2 in Rat 1a Fibroblasts**—To determine the effects of GPCR and RTK stimulation on the tyrosine phosphorylation state of FAK and Shc, we employed endogenously expressed receptors for LPA, α-thrombin, bombesin, and EGF. In most cells, LPA and α-thrombin receptors mediate both pertussis toxin-sensitive and -insensitive signals (24), indicating coupling to both Gi/o family and Gq/11 family heterotrimeric G proteins. In contrast, bombesin receptors mediate predominantly pertussis toxin-insensitive mitogenic signals (25).

As shown in Fig. 1, stimulation of quiescent, serum-starved Rat 1a fibroblasts with LPA, the thrombin agonist peptide SFLLRN, bombesin, or EGF resulted in similar 4- to 5-fold increases in tyrosine phosphorylation of FAK (Fig. 1, A and C). LPA-, bombesin-, and EGF-stimulated tyrosine phosphorylation of FAK followed a similar time course as with maximal phosphorylation occurring within 2 min of stimulation and persisting for at least 10 min (Fig. 1B). The adapter protein Grb2 coprecipitated with FAK from lysates of LPA-, SFLLRN-, bombesin-, and EGF-stimulated cells prepared under nondenaturing conditions (Fig. 1, A and D). The amount of coprecipitated Grb2 varied in proportion to the extent of FAK tyrosine phosphorylation.
phosphorylation, indicating that stimulation of each receptor led to the formation of a FAK-Grb2 complex.

As shown in Fig. 2, LPA, α-thrombin, bombesin, and EGF receptors differed significantly in their ability to stimulate tyrosine phosphorylation of Shc. EGF receptor stimulation resulted in an 8- to 10-fold increase in tyrosine phosphorylation of Shc. EGF receptor stimulation resulted in an 8- to 10-fold increase, which peaked at 5 min and declined thereafter. The α-thrombin receptor mediated a 3-fold response. Bombesin receptor stimulation resulted in a less than 2-fold increase in Erk 1/2 phosphorylation. This pattern of responsiveness is clearly distinct from the effects of these agonists on FAK tyrosine phosphorylation and FAK-Grb2 complex formation but parallels agonist-induced Shc tyrosine phosphorylation and Shc-Grb2 complex formation.

Tyrosine Phosphorylation of Shc, but Not FAK, Correlates with GPCR- and RTK-mediated Erk 1/2 Activation—As shown in Fig. 4, tyrosine phosphorylation of FAK and Shc in Rat 1a fibroblasts may also be distinguished on the basis of calcium dependence. Exposure of serum-starved cells to the calcium ionophore, A23187, was sufficient to induce detectable tyrosine phosphorylation of FAK but failed to increase either Shc phosphorylation content or Erk 1/2 phosphorylation. Stimulation of cells with phorbol ester modestly increased both FAK and Shc phosphorylation and caused weak Erk 1/2 activation. However, phorbol ester treatment induced less Erk 1/2 activation than either LPA or α-thrombin receptor stimulation, suggesting that the activation of protein kinase C is not sufficient to account for the GPCR effects. Neither LPA nor α-thrombin receptor-stimulated Erk 1/2 activation was sensitive to inhibition by bisindolylmaleimide I, a potent inhibitor of protein kinase C (data not shown).

To further examine the relationship between tyrosine phosphorylation of FAK and Shc, and the rapid activation of Erk 1/2, we studied GPCR- and RTK-mediated signaling in cells following treatment with pertussis toxin or cytochalasin D. Pertussis toxin catalyzes the selective ADP-ribosylation and inactivation of Gi/o proteins. Previous studies have shown that LPA-stimulated Erk 1/2 activation (12, 26, 27), but not FAK phosphorylation (12), is pertussis toxin-sensitive in Rat 1a fibroblasts. The α-thrombin receptor stimulated FAK tyrosine phosphorylation in a time-dependent manner that was maximal within 5 min of stimulation. The LPA receptor mediated a 6-fold increase, which peaked at 5 min and declined thereafter. The α-thrombin receptor mediated a 3-fold response. Bombesin receptor stimulation resulted in a less than 2-fold increase in Erk 1/2 phosphorylation. This pattern of responsiveness is clearly distinct from the effects of these agonists on FAK tyrosine phosphorylation and FAK-Grb2 complex formation but parallels agonist-induced Shc tyrosine phosphorylation and Shc-Grb2 complex formation.

Tyrosine Phosphorylation of Shc, but Not FAK, Correlates with GPCR- and RTK-mediated Erk 1/2 Activation—As shown in Fig. 2, LPA, α-thrombin, bombesin, and EGF receptor stimulation resulted in an 8- to 10-fold increase in tyrosine phosphorylation of Shc. EGF receptor stimulation resulted in an 8- to 10-fold increase, which peaked at 5 min and declined thereafter. The α-thrombin receptor mediated a 3-fold response. Bombesin receptor stimulation resulted in a less than 2-fold increase in Erk 1/2 phosphorylation. This pattern of responsiveness is clearly distinct from the effects of these agonists on FAK tyrosine phosphorylation and FAK-Grb2 complex formation but parallels agonist-induced Shc tyrosine phosphorylation and Shc-Grb2 complex formation.

Tyrosine Phosphorylation of Shc, but Not FAK, Correlates with GPCR- and RTK-mediated Erk 1/2 Activation—As shown in Fig. 2, LPA, α-thrombin, bombesin, and EGF receptor stimulation resulted in an 8- to 10-fold increase in tyrosine phosphorylation of Shc. EGF receptor stimulation resulted in an 8- to 10-fold increase, which peaked at 5 min and declined thereafter. The α-thrombin receptor mediated a 3-fold response. Bombesin receptor stimulation resulted in a less than 2-fold increase in Erk 1/2 phosphorylation. This pattern of responsiveness is clearly distinct from the effects of these agonists on FAK tyrosine phosphorylation and FAK-Grb2 complex formation but parallels agonist-induced Shc tyrosine phosphorylation and Shc-Grb2 complex formation.

Tyrosine Phosphorylation of Shc, but Not FAK, Correlates with GPCR- and RTK-mediated Erk 1/2 Activation—As shown in Fig. 2, LPA, α-thrombin, bombesin, and EGF receptor stimulation resulted in an 8- to 10-fold increase in tyrosine phosphorylation of Shc. EGF receptor stimulation resulted in an 8- to 10-fold increase, which peaked at 5 min and declined thereafter. The α-thrombin receptor mediated a 3-fold response. Bombesin receptor stimulation resulted in a less than 2-fold increase in Erk 1/2 phosphorylation. This pattern of responsiveness is clearly distinct from the effects of these agonists on FAK tyrosine phosphorylation and FAK-Grb2 complex formation but parallels agonist-induced Shc tyrosine phosphorylation and Shc-Grb2 complex formation.

Tyrosine Phosphorylation of Shc, but Not FAK, Correlates with GPCR- and RTK-mediated Erk 1/2 Activation—As shown in Fig. 2, LPA, α-thrombin, bombesin, and EGF receptor stimulation resulted in an 8- to 10-fold increase in tyrosine phosphorylation of Shc. EGF receptor stimulation resulted in an 8- to 10-fold increase, which peaked at 5 min and declined thereafter. The α-thrombin receptor mediated a 3-fold response. Bombesin receptor stimulation resulted in a less than 2-fold increase in Erk 1/2 phosphorylation. This pattern of responsiveness is clearly distinct from the effects of these agonists on FAK tyrosine phosphorylation and FAK-Grb2 complex formation but parallels agonist-induced Shc tyrosine phosphorylation and Shc-Grb2 complex formation.
fibroblasts. As shown in Fig. 5, pertussis toxin treatment modestly enhanced basal tyrosine phosphorylation of FAK, with no effect on LPA-, SFLLRN-, bombesin-, or EGF receptor-stimulated tyrosine phosphorylation (Fig. 5A). Basal tyrosine phosphorylation of Shc was modestly, but significantly, increased by pertussis toxin treatment. In pertussis toxin-treated cells, stimulation with LPA, SFLLRN, or bombesin failed to produce any further increase in Shc tyrosine phosphorylation. Serum-starved cells were stimulated for 3 min with LPA, SFLLRN, bombesin, EGF, or vehicle (NS). Stimulated monolayers were lysed in RIPA buffer, and Shc was immunoprecipitated as described. After immunoblotting for phosphotyrosine (upper panel), nitrocellulose filters were stripped of immunoglobulin and reprobed for coprecipitated Grb2 (lower panel). Immunoprecipitation of equal amounts of Shc protein from nonstimulated and stimulated cells was confirmed by immunoblotting with anti-Shc IgG (data not shown). B, association of Shc and GST-Grb2 fusion proteins following LPA, SFLLRN, bombesin, and EGF receptor stimulation. Shc was immunoprecipitated from serum-starved cells, and coprecipitated Grb2 was detected by immunoblotting using anti-Grb2 IgG. C, time course of LPA-, SFLLRN-, and EGF-stimulated Shc tyrosine phosphorylation. Serum-starved cells were stimulated for the indicated times with LPA, SFLLRN, or EGF, and Shc tyrosine phosphorylation was determined. Data were normalized as fold increase in phosphotyrosine content, assigning the Shc phosphotyrosine content in nonstimulated cells a value of 1. Data shown represent the means ± S.E. for three separate experiments. D, quantitation of LPA-, SFLLRN-, bombesin-, and EGF-stimulated Shc tyrosine phosphorylation. Basal and 3-min agonist-stimulated Shc phosphotyrosine content was determined and normalized as described. Data shown represent the means ± S.E. for three separate experiments. E, quantitation of LPA-, SFLLRN-, bombesin-, and EGF-stimulated Shc-Grb2 complex formation. Immunoblots of basal and 3-min agonist-stimulated Shc immunoprecipitates were probed for coprecipitated Grb2 and normalized as described. Data shown represent the means ± S.E. for three separate experiments.

Collectively, these data suggest that GPCRs mediate at least two, distinctly regulated, sets of tyrosine phosphorylation events in Rat 1a fibroblasts. Tyrosine phosphorylation of FAK is mediated by pertussis toxin-insensitive G proteins, can be mimicked by calcium ionophore, and is inhibited by cytochalasin D. In contrast, tyrosine phosphorylation of Shc is inhibited by pertussis toxin treatment, is not induced by calcium ionophore, and is insensitive to cytochalasin D. In each case, the rapid stimulation of Erk 1/2 by GPCRs correlates with tyrosine phosphorylation of Shc, but not with that of FAK.

Since both FAK and Shc recruit Grb2 in response to GPCR stimulation, the lack of correlation between FAK phosphorylation and GPCR-mediated Erk 1/2 activation suggests that the locus of Grb2-mSos binding must play an important role in determining the nature of the resulting signal. Activated FAK localizes to focal adhesions, points of integrin-dependent cell attachment to the extracellular matrix (5). Following EGF stimulation, Shc binds directly to the EGF receptor and is probably targeted, with the receptor, to clathrin-coated vesicles (28). The locus of Shc binding following GPCR stimulation is...
FIG. 3. GPCR- and RTK-stimulated Erk 1/2 phosphorylation in Rat 1a fibroblasts. A, immunoblot of Erk 1/2 phosphorylation following stimulation of endogenous GPCRs and EGFR receptor. Serum-starved cells were stimulated for 5 min with LPA, SFLLRN, bombesin, EGF, or vehicle (NS). Stimulated monolayers were lysed in Laemmli sample buffer, and Erk 1/2 phosphorylation was determined using rabbit polyclonal phospho-specific MAP kinase IgG as described. Loading of equal amounts of Erk 2 protein from nonstimulated and stimulated cells was confirmed by immunoblotting with anti-Erk 2 IgG (data not shown). B, time course of LPA-, SFLLRN-, and EGF-stimulated Erk 1/2 phosphorylation. Serum-starved cells were stimulated for the indicated times with LPA, SFLLRN, or EGF, and Erk 1/2 phosphorylation was determined. Data were normalized as -fold increase in Erk 1/2 phosphorylation, assigning the phosphorylated Erk 1/2 signal in nonstimulated cells a value of 1. Data shown represent the means ± S.E. for three separate experiments. C, quantitation of LPA-, SFLLRN-bombesin-, and EGF-stimulated Erk 1/2 phosphorylation. Basal and 5-min agonist-stimulated Erk 1/2 phosphorylation were determined and normalized as described. Data shown represent the means ± S.E. for three separate experiments.

unclear. Modest GPCR-induced increases in EGF receptor (13, 16), p185ErbB (13), and PDGF receptor (14) phosphorylation have been reported, suggesting that RTKs may function as scaffolds for the GPCR-induced Ras activation complex. Indeed, binding of Shc and Grb2 to tyrosine phosphorylated EGFR receptor can be detected following LPA stimulation of COS-7 cells (16).

Inhibition of Clathrin-mediated Receptor Endocytosis Impairs Both GPCR- and RTK-mediated Erk 1/2 Activation—Recently, some aspects of EGFR receptor signaling, including rapid EGF-stimulated activation of Erk kinases, were found to be impaired in HeLa cells overexpressing a dominant negative mutant of dynamin (4). Since dynamin is required for clathrin-coated vesicle formation, these data suggest that RTK-mediated signaling to Erk kinases may involve endocytic trafficking. In addition, a requirement for endocytosis of nerve growth factor and its receptor TrkA has been shown for nerve growth factor-mediated activation of CRE-binding protein in the nuclei of cultured rat sympathetic neurons (29). To determine whether the requirement for clathrin-mediated endocytosis extended to GPCR-mediated signals, we studied the effects of inhibitors of GPCR-receptor internalization on GPCR-mediated activation of Erk 1/2 in Rat 1a fibroblasts.

The lectin concanavalin A binds to cell surface glycoproteins and impairs their mobility within the membrane bilayer. Concanavalin A has been shown to inhibit the endocytosis of β-adrenergic receptors without affecting the initial events in GPCR-signaling, including ligand-binding, G protein-activation, and generation of small molecule second messengers (30, 31). In HEK-293 cells, concanavalin A treatment inhibited 80–90% of agonist-induced sequestration of transiently expressed epitope-tagged β2 adrenergic receptors, with no effect on isoproterenol-stimulated cAMP generation or LPA-stimulated production of inositol phosphates. As shown in Fig. 7, incubation of Rat 1a fibroblasts with concanavalin A had no effect on LPA-, SFLLRN-, or bombesin-induced tyrosine phosphorylation of FAK, suggesting that this process depends only upon G protein activation. Since ligand-induced EGFR receptor dimerization is the initial step in EGFR receptor activation (32), concanavalin A treatment did inhibit EGFR-stimulated FAK phosphorylation, as expected (Fig. 7A). In contrast to the lack of effect on GPCR-mediated FAK phosphorylation, concanavalin A treatment markedly inhibited LPA-, SFLLRN-, and EGF-stimulated tyrosine phosphorylation of Shc (Fig. 7B). Identical inhibition of Shc phosphorylation by concanavalin A treatment was observed in LPA-stimulated HEK-293 cells (data not shown), indicating that G protein activation is insufficient to account for GPCR-mediated Shc phosphorylation. Rather, these data suggest that Shc phosphorylation in response to GPCR-activation requires that the receptors retain mobility within the lipid bilayer. Both GPCR- and RTK-mediated phosphorylation of Erk 1/2, like Shc phosphorylation, is markedly inhibited by concanavalin A treatment in both Rat 1 and HEK-293 cells (data not shown). Since many GPCRs are targeted to clathrin-coated pits prior to receptor internalization, these data are consistent with a role for endocytosis in GPCR-mediated activation of Erk kinases.

To further examine the role of endocytosis in GPCR-mediated Erk 1/2 activation, we determined the effects of three chemically distinct inhibitors of clathrin-mediated endocytosis: hypertonic medium (31, 33), intracellular potassium depletion (22, 34), and monodansylcadaverine treatment (35). Exposure to hypertonic medium and intracellular potassium depletion have been shown to induce the loss of plasma membrane-associated clathrin lattices and disappearance of clathrin-coated pits (34). Inhibition of receptor endocytosis by monodansylcadaverine may result from stabilization of clathrin-coated vesicles (35). In HEK-293 cells, incubation in hypertonic medium resulted in greater than 90% inhibition of agonist-induced sequestration of β2 adrenergic receptors. Intracellular potassium depletion and monodansylcadaverine treatment were less effective, inhibiting β2 adrenergic receptor sequestration by 40–60%. As shown in Fig. 8, all three agents significantly inhibited LPA-, SFLLRN-, and EGF-mediated Erk 1/2 phosphorylation. Neither hypertonic medium, intracellular potassium depletion, nor monodansylcadaverine treatment inhibited the weak phosphor-ester-induced signal. Thus, these data support the hypothesis that the signaling cascade which mediates Ras-dependent activation of Erk kinases by both RTKs and GPCRs involves an additional step that is blocked by the inhibition of endocytic trafficking.

DISCUSSION

The data presented here demonstrate that GPCR-induced tyrosine phosphorylation of FAK and Shc are independently regulated in Rat 1a fibroblasts. Not only are FAK and Shc tyrosine phosphorylation regulated by different G protein pools, but depolymerization of the actin cytoskeleton using cytochalasin D, which blocks agonist-induced FAK phosphorylation, has no effect on agonist-induced tyrosine phosphorylation of Shc. Further, the inhibition of Shc, but not FAK, phosphorylation by concanavalin A suggests that GPCR-induced Shc phosphorylation involves a distinct mechanism that is dependent upon the mobility of membrane glycoproteins within the lipid bilayer. Since GPCR-stimulated Erk 1/2 activation in these cells correlates with Shc phosphorylation, we propose that the tyrosine phosphorylation events which regulate Shc
phosphorylation are required for Gi-coupled receptor-mediated Erk 1/2 activation in these cells. GPCR-induced FAK-Grb2 association, which should recruit Ras-GEF activity into the focal adhesion complex via a pertussis toxin-insensitive mechanism, apparently does not contribute to the initial rapid activation of Erk 1/2.

Clear heterogeneity exists in the mechanisms of Ras activation employed by GPCRs in different cell types. In rat vascular smooth muscle (36) and NIH-3T3 cells (37), the Gq/11-coupled receptors for angiotensin II and prostaglandin F2α, respectively, have been shown to mediate pertussis toxin-insensitive activation of Ras. These signals are sensitive to inhibitors of tyrosine protein kinases and independent of protein kinase C activity (36, 37) but may be calcium-dependent (36). In cells of neuronal origin, LPA stimulates pertussis toxin-sensitive and bradykinin stimulates pertussis toxin-insensitive activation of the calcium-dependent FAK family tyrosine kinase, Pyk2 (18). Pyk2 activation correlates with tyrosine phosphorylation of the Shc adapter protein and activation of Erk kinases (38). We have obtained similar results in HEK-293 cells, where the Gq/11-coupled α1B and Gq-coupled α2A adrenergic receptors mediate Ras-dependent Erk 1/2 activation via a pathway that involves phospholipase C, intracellular calcium, and tyrosine phosphorylation but not protein kinase C (39).

In other cell types, Ras-dependent activation of Erk kinases by GPCRs is clearly independent of both protein kinase C and intracellular calcium. In Rat 1a fibroblasts, LPA receptor-mediated hydrolysis of inositol phospholipids and release of intracellular calcium (40) are pertussis toxin-insensitive, whereas activation of Ras (12, 26) and Erk kinases is pertussis toxin-sensitive (12, 26, 27). Similar results have been obtained with α2A adrenergic receptors in Rat 1a (41) and COS-7 cells (10, 17). The effects of both LPA and α2A adrenergic receptors on Ras-GTP loading and Erk kinase activation are apparently mediated by Gβγ subunits derived from pertussis toxin-sensitive G proteins (17, 26), working via unknown effectors.

Recruitment of Grb2-mSos complexes to the plasma membrane requires tyrosine phosphorylation of Grb2-binding sites on membrane-associated scaffolds. Several proteins that contain potential Grb2-binding sites undergo GPCR-mediated tyrosine phosphorylation, including Shc, FAK family kinases, and some RTKs. Tyrosine phosphorylation of the Shc adapter protein following stimulation of thyrotropin-releasing hormone (20), endothelin I (42), bradykinin (38), LPA (10, 17), α2-adrenergic (10, 17), and formyl-methionyl peptide chemoattractant receptors (43) has been reported. Depending upon receptor and cell type, GPCR-induced Shc phosphorylation may be mediated via either pertussis toxin-sensitive or -insensitive G proteins. LPA-stimulated Shc phosphorylation in COS-7 cells results in its rapid association with Grb2 and Ras-GEF activity (17). Pertussis toxin-sensitive phosphorylation of Shc by formyl-methionyl peptide correlates with formation of a protein complex containing Shc and the Src family kinase, Lyn (43). Complex formation between Shc and c-Src accompanies α2A adrenergic and LPA receptor-stimulated Shc phosphorylation in COS-7 cells (10, 16). Overexpression of Csk, which inactivates Src family kinases, inhibits LPA-stimulated Shc phosphorylation and Erk 1/2 activation in COS-7 cells (10, 16), suggesting a role for Src kinases in the calcium-independent, Gi-mediated pathway.

Recruitment of FAK into focal adhesion complexes following integrin-engagement results in FAK autophosphorylation, association with c-Src, and binding of the Grb2-SH2 domain to a Src phosphorylation site on FAK (44, 45). FAK family kinases also provide Grb2-binding sites following GPCR stimulation. In PC-12 cells, calcium-dependent activation of Pyk2 results in Src kinase recruitment, Src-mediated Pyk2 phosphorylation, and binding of Grb2 (18). Overexpression of Csk inhibits both bradykinin- and LPA-stimulated Shc phosphorylation and Erk 1/2 activation in PC-12 cells (18), also implicating Src kinases in the calcium-dependent Pyk2 pathway.

Some GPCRs stimulate rapid tyrosine phosphorylation of RTKs, including the EGF (13, 16), PDGF (14), and IGF-1 (15) receptors, via a poorly understood “transactivation” mechanism (15). Like activated FAK, the RTKs for EGF and PDGF bind Src kinases, and Src kinase activity is thought to be important for RTK-mediated mitogenic signal transduction (46). Since these receptors possess intrinsic docking sites for Grb2, Shc, and other signaling molecules, transactivated RTKs also represent potential scaffolds for GPCR-mediated assembly of a Ras activation complex. Indeed, communoprecipitation of EGF receptor, c-Src, Shc, and Grb2 has been observed following LPA stimulation of COS-7 cells (16).

As we demonstrate, GPCR-induced FAK phosphorylation is not required for rapid GPCR-induced Erk 1/2 activation in fibroblasts, which lack detectable expression of Pyk2 (38). Re-
Recruitment and activation of Src kinases in focal adhesions may, however, explain apparent discrepancies between the pertussis toxin insensitivity of Src kinase activation and pertussis toxin sensitivity of Erk 1/2 activation in some systems (24). LPA, α-thrombin, and bombesin receptors mediate equivalent, pertussis toxin-insensitive, phosphorylation of FAK in Rat 1 fibroblasts. Since activation of FAK should result in recruitment of Src to focal adhesion complexes (44, 45), pertussis toxin-insensitive Src activation would thus be expected in these cells. As noted, these data do not exclude a role for Src kinases recruited...
to other loci, such as transactivated RTKs (13–16) or Shc-containing protein complexes (10, 43), in mediating pertussis toxin-sensitive Erk activation.

The finding that Ras-dependent activation of Erk 1/2 by both RTKs and GPCRs is blocked by inhibitors of receptor endocytosis adds a new dimension of complexity to our understanding of mitogenic signal transduction. The dissociation of FAK-Grb2 complex formation from receptor-mediated activation of Erk 1/2 indicates that recruitment of Ras-GEF to the plasma membrane is not sufficient to mediate rapid Erk activation. An additional step involving clathrin-coated vesicle-mediated endocytosis (4, 29), and thus sensitive to inhibitors of receptor sequestration, is apparently required.

The precise role of clathrin-coated vesicle-mediated endocytosis in the Ras-dependent activation of Erk kinases is unclear. Perhaps the clathrin-coated pit represents a specialized microdomain within the plasma membrane where critical components of the mitogenic signaling complex are assembled. Such a role has been proposed for caveolae (47–49). It may also be significant that recruitment of the Raf kinase to activated GTP-bound Ras is thought to be the last membrane-delimited event in the Erk activation cascade (50, 51). The subsequent steps, Raf-dependent phosphorylation of MEK kinases, and

**FIG. 7.** Effects of concanavalin A treatment on GPCR- and RTK-stimulated FAK and Shc phosphotyrosine content and on Erk 1/2 phosphorylation in Rat 1a fibroblasts. A, quantitation of GPCR- and EGF-stimulated FAK tyrosine phosphorylation. Serum-starved cells were preincubated for 20 min in the presence or absence of concanavalin A (Con A, 250 μg/ml) and stimulated for 1 min with LPA, SFLLRN, bombesin, EGF, PMA, or vehicle (NS). Stimulated monolayers were lysed in RIPA buffer, and FAK was immunoprecipitated and immunoblotted for phosphotyrosine as described. B, quantitation of GPCR- and EGF-stimulated Shc tyrosine phosphorylation. Concanavalin A-treated and -untreated cells were stimulated for 3 min with LPA, SFLLRN, bombesin, EGF, PMA, or vehicle. Stimulated monolayers were lysed in RIPA buffer, and Shc was immunoprecipitated and immunoblotted for phosphotyrosine as described. C, quantitation of GPCR- and EGF-stimulated Erk 1/2 phosphorylation. Concanavalin A-treated and -untreated cells were stimulated for 5 min with LPA, SFLLRN, bombesin, EGF, PMA, or vehicle. Stimulated monolayers were lysed in Laemmli sample buffer, and Erk 1/2 phosphorylation was determined as described. In each panel, data were normalized as -fold increase in phosphorylation, assigning a value of 1 to that obtained for nonstimulated cells that were not treated with concanavalin A. Data shown represent the means ± S.E. for three separate experiments.

**FIG. 8.** Effects of hypertonic medium, intracellular potassium depletion, and monodansylcadaverine treatment on GPCR- and RTK-stimulated Erk 1/2 phosphorylation in Rat 1a fibroblasts. A, serum-starved cells were preincubated for 15 min in the presence or absence of monodansylcadaverine (MDC, 1 μM), or for 30 min in serum-free medium containing hypertonic sucrose (450 mM), prior to stimulation for 5 min with LPA, SFLLRN, bombesin, EGF, PMA, or vehicle (NS). Stimulated monolayers were lysed with Laemmli sample buffer, and Erk 1/2 phosphorylation was determined as described. B, serum-starved cells were depleted of intracellular potassium as described, prior to stimulation for 5 min with LPA, SFLLRN, bombesin, EGF, PMA, or vehicle. Stimulated monolayers were lysed with Laemmli sample buffer, and Erk 1/2 phosphorylation was determined as described. In each panel, data were normalized as -fold increase in phosphorylation, assigning a value of 1 to that obtained for untreated, nonstimulated cells. Data shown represent the means ± S.E. for three separate experiments.
MEK-dependent phosphorylation and activation of Erk kinases occur in the cytoplasm. Thus, the endocytic machinery of the cell may play an as yet undefined role in linking signals generated at the plasma membrane with the cytoplasmic kinases that ultimately convey regulatory information to the nucleus.

Acknowledgments—We thank D. Addison and M. Holben for excellent secretarial assistance.

REFERENCES

1. Pawson, T. (1992) Curr. Opin. Struct. Biol. 2, 432–437
2. van der Geer, P., and Pawson, T. (1995) Trends Biochem. Sci. 20, 277–280
3. Medema, R. H., and Bos, J. L. (1993) Crit. Rev. Oncogenesis 4, 615–661
4. Vieira, A. V., Lamaze, C., and Schmid, S. L. (1996) Science 274, 2086–2089
5. Clark, E. A., and Brugge, J. S. (1995) J. Biol. Chem. 270, 112563–12568
6. Luttrell, L. M., Daaka, Y., Luttrell, D. K., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 27372–27377
7. Simonson, M. S., Wang, Y., and Herman, W. H. (1996) J. Biol. Chem. 271, 77–82
8. Schieffer, B., Paxton, W. G., Chai, Q., Marrero, M. B., and Bernstein, K. E. (1996) J. Biol. Chem. 271, 10329–10333
9. Ishida, M., Marrero, M. B., Schieffer, B., Ishida, T., Bernstein, K. E., and Berk, E. C. (1995) Circ. Res. 77, 1053–1059
10. Luttrell, L. M., Hawes, B. E., van Biesen, T., Luttrell, D. K., Lansing, T. J., and Lefkowitz, R. J. (1996) J. Biol. Chem. 271, 19443–19450
11. Rodriguez-Fernandez, J. L., and Rozengurt, E. (1996) J. Biol. Chem. 271, 27965–27971
12. Nordjik, P. L., Verlaan, I., van Corven, E. J., and Moolenaar, W. H. (1994) J. Biol. Chem. 269, 645–651
13. Dauh, H., Weiss, F. U., Wallasch, C., and Ulrich, A. (1996) Nature 379, 557–560
14. Linseman, D. A., Benjamin, C. W., and Jones, D. A. (1995) J. Biol. Chem. 270, 12563–12568
15. Rao, G. N., Delafontaine, P., and Runge, M. S. (1995) J. Biol. Chem. 270, 27871–27875
16. Luttrell, L. M., Della Rocca, G. J., van Biesen, T., Luttrell, D. K., and Lefkowitz, R. J. (1992) J. Biol. Chem. 267, 4837–4844
17. van Biesen, T., Hawes, B. E., Luttrell, D. K., Krueger, K. M., Touhara, K., Pursi, E., Sakaue, M., Luttrell, L. M., and Lefkowitz, R. J. (1995) Nature 379, 557–560
18. Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A., and Schlessinger, J. (1996) Nature 383, 547–550
19. Luttrell, L. M., Della Rocca, G. J., van Biesen, T., Luttrell, D. K., and Lefkowitz, R. J. (1992) J. Biol. Chem. 267, 4837–4844
20. Ohmichi, M., Sawada, T., Kanda, Y., Koike, K., Hirota, K., Miyake, A., and Saltiel, A. R. (1994) J. Biol. Chem. 269, 3783–3788
21. Luttrell, D. K., Lee, A., Lansing, T. J., Crosby, R. M., Jung, K. D., Willard, D., Luttrell, D. K., Rodriguez, M., Berman, J., and Gilmer, T. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 83–87
22. Widmann, C., Dolci, W., and Thoren, B. (1995) Biochem. J. 310, 203–214
23. Barak, L. S., Tiberi, M., Freedman, N. J., Kwarra, M. M., Lefkowitz, R. J., and Couraud, P. O. (1994) J. Biol. Chem. 269, 25071–25077
24. Moolenaar, W. H., Kranenburg, O., Postma, F. R., and Zondag, G. C. M. (1997) Curr. Opin. Cell. Biol. 9, 168–173
25. Rozengurt, E. (1995) Cancer Surv. 24, 81–96
26. Koch, W., Hawes, B. E., Allen, L. F., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 16485–16498
27. Faull, D., McKinna, J. M., and Cohen, S. (1997) J. Biol. Chem. 272, 382–395
28. Riccio, A., Pirechala, B. A., Ciarello, C. L., and Gimy, D. D. (1997) Science 277, 1097–1100
29. Wang, H. Y., Berrios, M., and Malbon, C. C. (1989) Biochem. J. 263, 533–538
30. Pippig, S., Andexinger, S., and Lohe, M. J. (1995) Mol. Pharmacol. 47, 666–676
31. Schlessinger, J., and Ulrich, A. (1992) Neuron 9, 383–391
32. Va, S. S., Lefkowitz, R. J., and Hausdorff, W. P. (1993) J. Biol. Chem. 268, 337–341
33. Hansen, S. H., Sandvig, K., and van Duers, B. (1993) J. Cell. Biol. 121, 61–72
34. Phosphoryl, Y., and Rosenthal, K. S. (1991) FEBS Lett. 281, 188–190
35. Eguchi, S., Matsumoto, T., Motley, E. D., Utsunomiya, H., and Inagami, T. (1996) J. Biol. Chem. 271, 14169–14175
36. Watanabe, T., Waga, I., Honda, Z., Kurokawa, K., and Shimizu, T. (1995) J. Biol. Chem. 270, 8984–8990
37. Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B., and Schlessinger, J. (1995) Nature 376, 737–745
38. Della Rocca, G. J., van Biesen, T., Daaka, Y., Luttrell, D. K., Luttrell, L. M., and Lefkowitz, R. J. (1997) J. Biol. Chem. 272, 19125–19132
39. van Corven, E. J., Groenik, A., Jalink, K., Eicholtz, T., Moolenaar, W. H. (1989) Cell 59, 45–54
40. Alblas, J., van Corven, E. J., Nordjik, P. L., Milligan, G., and Moolenaar, W. H. (1993) J. Biol. Chem. 268, 22235–22238
41. Cazaubon, S. M., Ramos-Morales, F., Fischer, S., Schweighoffer, F., Strosberg, A. D., and Couraud, P. O. (1994) J. Biol. Chem. 269, 24805–24809
42. Ptasznik, A., Traynor-Kaplan, A., and Bokoch, G. M. (1995) J. Biol. Chem. 270, 24805–24809
43. Slaper, D. F., and Hecker, T. (1996) Mol. Cell. Biol. 16, 5623–5633
44. Slaper, D. F., Broome, M. A., and Hunter, T. (1996) Mol. Cell. Biol. 17, 1702–1713
45. Eppel, T., Alonso, G., Roche, S., and Courteigne, S. A. (1996) J. Biol. Chem. 271, 16807–16812
46. Li, S., Okamoto, K., Chung, S. R., and Sapp, R. (1996) J. Biol. Chem. 271, 16807–16812
47. Liu, P., Ying, Y., and Anderson, R. G. W. (1996) J. Biol. Chem. 271, 10293–10303
48. Mineo, C., James, G. L., Smart, E. J., and Anderson, R. G. W. (1996) J. Biol. Chem. 271, 10979–11003
49. Katz, M. E., and McCormick, F. (1997) Curr. Opin. Genet. Dev. 7, 75–79
50. Morrison, D. K., and Cutler, R. E. (1997) Curr. Opin. Cell Biol. 9, 174–179