Pleiotropic Coupling of G Protein-coupled Receptors to the Mitogen-activated Protein Kinase Cascade

ROLE OF FOCAL ADHESIONS AND RECEPTOR TYROSINE KINASES*

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Gregory J. Della Rocca‡, Stuart Maudsley, Yehia Daaka, Robert J. Lefkowitz§, and Louis M. Luttrell

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

G protein-coupled receptors (GPCRs) initiate Ras-dependent activation of the Erk 1/2 mitogen-activated protein kinase cascade by stimulating recruitment of Ras guanine nucleotide exchange factors to the plasma membrane. Both integrin-based focal adhesion complexes and receptor tyrosine kinases have been proposed as scaffolds upon which the GPCR-induced Ras activation complex may assemble. Using specific inhibitors of focal adhesion complex assembly and receptor tyrosine kinase activation, we have determined the relative contribution of each to activation of the Erk 1/2 cascade following stimulation of endogenous GPCRs in three different cell types. The tetrapeptide RGDS, which inhibits integrin dimerization, and cytochalasin D, which depolymerizes the actin cytoskeleton, disrupt the assembly of focal adhesions. In PC12 rat pheochromocytoma cells, both agents block lysophosphatidic acid (LPA)- and bradykinin-stimulated Erk 1/2 phosphorylation, suggesting that intact focal adhesion complexes are required for GPCR-induced mitogen-activated protein kinase activation in these cells. In Rat 1 fibroblasts, Erk 1/2 activation via LPA and thrombin receptors is completely insensitive to both agents. Conversely, the epidermal growth factor receptor-senpecific tyrphostin AG1478 inhibits GPCR-mediated Erk 1/2 activation in Rat 1 cells but has no effect in PC12 cells. In HEK-293 human embryonic kidney cells, LPA and thrombin receptor-mediated Erk 1/2 activation is partially sensitive to both the RGDS peptide and tyrphostin AG1478, suggesting that both focal adhesion and receptor tyrosine kinase scaffolds are employed in these cells. The dependence of GPCR-mediated Erk 1/2 activation on intact focal adhesions correlates with expression of the calcium-regulated focal adhesion kinase, Pyk2. In all three cell types, GPCR-stimulated Erk 1/2 activation is significantly inhibited by the Src kinase inhibitors, herbimycin A and 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo-n-3,4-pyrimidine (PP1), suggesting that Src family nonreceptor tyrosine kinases represent a point of convergence for signals originating from either scaffold.

Many GPCRs1 initiate Ras-dependent activation of the Erk 1/2 MAP kinase cascade by inducing the tyrosine phosphorylation of proteins that serve as scaffolds for the plasma membrane recruitment of Ras guanine nucleotide exchange factors. Receptor stimulation results in a rapid increase in the tyrosine phosphorylation of docking proteins, such as She (1, 2) and Gab1 (3), followed by the Grb2-mediated recruitment of the Ras guanine nucleotide exchange factor, mSos, to the plasma membrane (4). These tyrosine phosphorylation events are sensitive to the inhibition of Src family nonreceptor tyrosine kinases in many cell types (2, 3, 5).

Although the requirement for tyrosine kinases in GPCR-mediated Erk 1/2 activation has been well documented, the proximal signaling events whereby these receptors initiate tyrosine phosphorylation remain poorly understood. Recent data have implicated FAK family kinases and RTKs, both of which regulate the activity of Src kinases, as proximal mediators of GPCR-induced tyrosine phosphorylation. FAKs are nonreceptor tyrosine kinases that compose part of the focal adhesion complex. These complexes assemble on αβ integrin heterodimers following integrin engagement of extracellular matrix proteins. Following recruitment, FAKs autophosphorylate and provide docking sites for several signaling proteins, including c-Src and Grb2 (6). In many cell types, stimulation of Gαi- or Gαq-coupled receptors causes FAK activation (7–9). This activation is cell adhesion-dependent, because disruption of focal adhesions prevents the response (10). In neuronal cells, stimulation of either LPA or bradykinin receptors activates the calcium-regulated FAK family kinase, Pyk2 (11), and overexpression of Pyk2 mutants that are either catalytically inactive or unable to bind to c-Src prevents GPCR-induced Erk 1/2 activation (5, 11). In other systems, however, GPCR-mediated Erk 1/2 activation is apparently dissociable from FAK phosphorylation (7–9).

Classical RTKs, such as the receptor for epidermal growth factor (EGF), are single transmembrane domain proteins that dimerize and transphosphorylate upon ligand binding. Tyrosine phosphorylation of RTKs promotes their association with SH2 or PTB domain-containing signaling proteins, which assemble on the receptor to form a Ras activation complex (12). “Transactivation” of RTKs following GPCR stimulation has been implicated in GPCR-mediated activation of Erk 1/2 (13–15). In Rat 1 fibroblasts and COS-7 cells, inhibition of EGF receptor function inhibits LPA-, endothelin-1-, and thrombin

1 The abbreviations used are: GPCR, G protein-coupled receptor; MAP, mitogen-activated protein; FAK, focal adhesion kinase; RTK, receptor tyrosine kinase; EGF, epidermal growth factor; LPA, lysophosphatidic acid; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo-n-3,4-pyrimidine; PAGE, polyacrylamide gel electrophoresis.
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receptor-mediated tyrosine phosphorylation of Shc and Gab1 and activation of Erk 1/2 (3, 15). In this model, the transacti-

lated RTK forms the structural core of a GPCR-induced mi-
togenic signaling complex, as receptor phosphorylation creates
docking sites for the components of the Ras activation complex.

We compared the role of focal adhesions and EGFR receptors in
mediating Erk 1/2 activation via endogenously expressed LPA, thrombin, and bradykinin receptors in three different cell
types: PC12 rat pheochromocytoma cells, Rat 1 fibroblasts, and
HEK-293 embryonic kidney cells. Surprisingly, we found that the
preferred scaffold was independent of the specific G/Go-
coupled GPCR being stimulated. Rather, the utilization of scaff-
olds varied between cell types, with PC-12 cells and Rat 1
fibroblasts apparently representing opposite ends of a contin-
uum. In PC-12 cells GPCR-mediated Erk 1/2 activation was
almost exclusively focal adhesion-dependent, whereas in Rat 1
fibroblasts it was almost exclusively RTK-dependent. In HEK-
293 cells, both scaffolds contributed to the GPCR signal. Utili-
zation of the focal adhesion scaffold correlated with signaling via
pertussis toxin-insensitive G proteins and with cellular
expression of the calcium-regulated FAK family kinase, Pyk2.
In each case, GPCR-stimulated Erk 1/2 activation was sensi-
tive to Src kinase inhibitors, suggesting that a critical role of
both scaffolds is to support the GPCR-induced activation of Src
family nonreceptor tyrosine kinases.

EXPERIMENTAL PROCEDURES

Materials—Cytochalasin D, EGF, herbinymcin A, tyrophostin AG1478,
and 4-amino-5-(4-methylphenyl)-7-t-tetrahydropyrazolo-b-d-4-pyrimidine
(PPI) were from Calbiochem. Bordetella pertussis toxin was from List
Biologica. LPA and bradykinin were from Sigma. The tetrapeptides
H3N-arginine-glycine-aspartate-serine-COO (RGDS) and H3N-argi-
ine-glycine-glutamate-serine-COO (RGES) and the thrombin agonist
hexapeptide H3N-arginine-phenylalanine-leucine-arginine-as-
paragine-CONH2 (SFLLRN) were synthesized at the Howard Hughes
Medical Institute peptide facility (Duke University Medical Center).
Cell Culture—Rat pheochromocytoma PC12 cells, Rat-1 fibroblasts,
and HEK-293 cells were from the American Type Culture Collec-
tion. PC12 cells were maintained in RPMI 1640 media with 1-glutamine
(Life Technologies, Inc.) supplemented with 10% horse serum (Life
Technologies, Inc.) and 5% fetal bovine serum (Life Technologies, Inc.) at
37 °C in a humidified, 5% CO2 atmosphere. Rat-1 fibroblasts were
maintained in Dulbecco’s modified Eagle’s medium (Life Technologies,
Inc.) supplemented with 10% fetal bovine serum and 50 μg/ml genta-
imic (Life Technologies, Inc.). HEK-293 cells were maintained in
minimum essential medium with Earle’s salts (Life Technologies, Inc.)
supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin
(Life Technologies, Inc.).

Immunoprecipitation and Immunoblotting—For the determina-
tion of phospho-specific MAP kinase IgG (New England Biolabs) with alkaline
phosphatase-conjugated goat anti-rabbit IgG (Amersham Pharmacia
Biotech) as secondary antibody. Quantitation of Erk 1/2 phosphoryla-
tion was performed after exposure of nitrocellulose membranes to
Vistra ECF reagent (Amersham Pharmacia Biotech) and scanning on a
Storm PhosphorImager (Molecular Dynamics). After quantitation of
Erk 1/2 phosphorylation, nitrocellulose membranes were stripped of
immunoglobulin and reprobed using rabbit polyclonal anti-Erk2 IgG
(Santa Cruz Biotechnology) to confirm equal loading of Erk2 protein.

RESULTS

Erk 1/2 Phosphorylation following Stimulation of Endog-
neous GPCRs in PC12, Rat 1, and HEK-293 Cells—To select
endogenous GPCRs capable of activating the Erk 1/2 cascade in
a variety of cell types, we assayed Erk 1/2 phosphorylation fol-
lowing stimulation of PC12, Rat 1, and HEK-293 cells with agonists
for LPA, thrombin, or bradykinin receptors. Each of these
receptors has been shown to mediate both pertussis tox-
in-sensitive and -insensitive signals resulting from dual cou-
pling to Gαi-family and Gq/11-family heterotrimeric G proteins
(16–18). Erk 1/2 activation via endogenous EGF receptors was
also determined as a control for cellular responsiveness and
inhibitor specificity.

Fig. 1 compares GPCR-induced Erk 1/2 phosphorylation in
each of the three cell lines. In PC12 cells, both LPA- and
bradykinin-stimulated Erk 1/2 phosphorylation was pertussis
toxin-insensitive (Fig. 1A). In these cells, the thrombin agonist
peptide, SFLLRN, provoked a less than 2-fold stimulation of
Erk 1/2 phosphorylation (data not shown). In contrast, LPA-
and SFLLRN-stimulated Erk 1/2 phosphorylation in Rat 1
fibroblasts was completely pertussin toxin-sensitive (Fig. 1B).
In HEK-293 cells, pertussis toxin only partially blocked the
LPA and thrombin receptor responses (Fig. 1C). Here, LPA-
stimulated Erk 1/2 phosphorylation was predominantly pertus-
sis toxin-sensitive, whereas the response to SFLLRN was pre-
dominantly pertussin toxin-insensitive. This differential
sensitivity to pertussis toxin indicates that dual Gαi/Gq-coupled
GPCRs activate the Erk 1/2 cascade via distinct G protein pools
in different cell types. As expected, EGF-stimulated Erk 1/2
phosphorylation was insensitive to pertussis toxin in all three
cell lines.

As shown in Fig. 2, the Src-selective tyrosine kinase inhibi-
tors herbinymcin A (left panels) and PPI (right panels) signifi-
cantly inhibited LPA-, bradykinin- and SFLLRN-stimulated
Erk 1/2 phosphorylation in PC12, Rat 1, and HEK-293 cells.
Although neither inhibitor affected EGF receptor autophos-
phorylation at the concentrations employed (data not shown), both
herbinymcin A and PPI also impaired EGF-stimulated Erk 1/2
phosphorylation. These data suggest that Src kinase activation
contributes to both the GPCR- and RTK-mediated Erk 1/2
cascades.

Because Src family kinases associate with both integrin-
based focal adhesion complexes (19) and receptor tyrosine ki-
nases (12), it is likely that signals originating from either locus
would be sensitive to Src inhibitors. If Gαi/Gq-coupled receptors
in different cell types selectively employ focal adhesions or
RTKs as signaling platforms, then the differential use of these
tscaffolds might account for some of the observed heterogeneity
in GPCR-mediated Erk 1/2 activation. To test this hypothesis, we determined the relative dependence of GPCR signals on the presence of functional focal adhesions and receptor tyrosine kinases in PC12, Rat 1, and HEK-293 cells.

**Focal Adhesion Complexes as Scaffolds for GPCR-stimulated Erk 1/2 Activation—**Two FAK family kinases, p125FAK and Pyk2 (also known as CADTK, CAKβ0, RAFTK, and FAK2) have been shown to autophosphorylate in response to GPCR stimulation (7–9, 11). As shown in Fig. 3A, PC12, HEK-293, and Rat 1 cells exhibit distinct patterns of p125FAK and Pyk2 expression as detected by protein immunoblotting. Although all three lines express abundant p125FAK, only the neuronal PC12 cells express abundant Pyk2. In contrast, HEK-293 cells contain little, and Rat 1 cells no, detectable Pyk2 immunoreactivity. In quiescent PC12 cells, LPA, bradykinin, and EGF stimulation increased tyrosine phosphorylation of both Pyk2 and p125FAK, indicating that both kinases, when present, are activated following GPCR stimulation (Fig. 3B).

Intact focal adhesions are required for the activation of FAK family kinases and formation of FAKc-Src complexes (20–22). Proper assembly of focal adhesions requires both cytoskeletal rearrangement (23) and integrin-mediated attachment to the extracellular matrix (24). Peptides containing the motif RGD, which mimic the integrin ligand found in extracellular matrix proteins such as fibronectin, have been shown to block integrin heterodimerization (25, 26) and thereby disrupt the formation of focal adhesions. Similar effects are produced by depolymerization of actin stress fibers following exposure to cytochalasin D. In HEK 293 cells, blocking integrin dimerization using the synthetic oligopeptide GRGDS inhibits m1 and m3 muscarinic receptor-stimulated tyrosine phosphorylation of p125FAK and paxillin (10).

To determine the extent to which intact focal adhesions might be required for GPCR-stimulated Erk 1/2 activation, we determined the effects of RGD peptides and cytochalasin D on Erk 1/2 phosphorylation in PC12, Rat 1, and HEK-293 cells. In each experiment, agonist-stimulated tyrosine phosphorylation of p125FAK was measured as a marker for the integrity of focal adhesion complexes. As shown in Fig. 4, stimulation of LPA, thrombin, bradykinin, or EGF receptors rapidly induced the tyrosine phosphorylation of p125FAK, indicating that each of these receptors promoted focal adhesion complex assembly.

Preincubation of cells with the RGDS peptide, but not the control RGES peptide, inhibited agonist-stimulated p125FAK phosphorylation in each of the three cell lines (Fig. 4, A–C, left panels). In PC12 cells, LPA- and bradykinin-stimulated Erk 1/2 phosphorylation, like p125FAK phosphorylation, was markedly inhibited by the RGDS peptide (Fig. 4A, right panel). In contrast, LPA and SFLLRN-stimulated Erk 1/2 phosphorylation in Rat 1 fibroblasts was completely insensitive to the RGDS peptide despite the significant inhibition of agonist-induced p125FAK phosphorylation (Fig. 4B, right panel). In HEK-293 cells, LPA and SFLLRN-stimulated Erk 1/2 phosphorylation was partially inhibited (Fig. 4C, right panel). EGF receptor-mediated Erk 1/2 activation was insensitive to the RGDS peptide in all three cell lines, indicating that intact focal adhesions are not required for acute stimulation of Erk 1/2 by RTKs.

Similar results were obtained using cytochalasin D to inhibit focal adhesion assembly. As shown in Fig. 5, cytochalasin D treatment markedly reduced GPCR- and EGF receptor-stimulated p125FAK phosphorylation in PC12, Rat-1, and HEK-293 cells (Fig. 5, A–C, left panels). As with the RGDS peptide, cytochalasin D completely blocked LPA- and SFLLRN-stimulated Erk 1/2 phosphorylation in PC12 cells (Fig. 5A, right panel). In Rat 1 fibroblasts, LPA- and SFLLRN-stimulated Erk 1/2 phosphorylation was cytochalasin D-insensitive (Fig. 5B, right panel), whereas a partial inhibition of LPA- and SFLLRN-stimulated Erk 1/2 phosphorylation was observed in HEK-293 cells (Fig. 5C, right panel).

**RTKs as Scaffolds for GPCR-stimulated Erk 1/2 Activation—**Like FAKs, RTKs including the EGF (3, 15, 27), platelet-derived growth factor (13), and insulin-like growth factor-1 (14) receptors can be activated in response to GPCR stimulation. In Rat 1 and COS-7 cells, inhibition of EGF receptor transactivation blocks GPCR-mediated MAP kinase activation (3, 15). Because both activated RTKs and focal adhesions represent potential docking sites for proteins involved in the regulation of mitogenesis, either or both might function as a scaffold for GPCR-mediated activation of Erk 1/2.

As shown in Fig. 6, the EGF receptor-specific tyrphostin AG1478 has markedly different effects on GPCR-stimulated Erk 1/2 phosphorylation in PC12, Rat 1, and HEK-293 cells. As expected, exposure to tyrphostin AG1478 had no effect on LPA, bradykinin- or SFLLRN-stimulated FAK phosphorylation, where the basal amount of Erk 1/2 phosphorylation in untreated cells is assigned a value of 1.0. Data shown represent the mean ± S.E. values of duplicate determinations from each of three separate experiments. *, less than control; p < 0.05, paired t test. IB, immunoblot.
Scaffolds for GPCR-mediated MAP Kinase Activation

Our data indicate that both focal adhesions and RTKs can function independently to support activation of the Erk 1/2 MAP kinase cascade following activation of endogenous G₁/Gᵢ-coupled receptors. Fig. 7 schematically depicts a model consistent with these data. In PC12 cells, G₁/Gᵢ-coupled receptors such as those for LPA and bradykinin mediate Erk 1/2 activation predominantly via pertussis toxin-insensitive G proteins and a focal adhesion-based scaffold. The lack of pertussis toxin sensitivity is consistent with the recent report that the G₁-coupled α₂A adrenergic receptor does not mediate Erk 1/2 activation in stably transfected PC12 cells (28). Despite the presence of functional receptors, EGF receptor transactivation does not contribute detectably to GPCR-stimulated Erk 1/2 activation in these cells.

Rat 1 fibroblasts apparently represent the opposite end of a continuum. In these cells, LPA and thrombin receptors mediate Erk 1/2 activation largely via pertussis toxin-sensitive G proteins and transactivation of the EGF receptor. Unlike PC12 cells, GPCR-stimulated Erk 1/2 activation in these cells is unaffected by the disruption of focal adhesion complexes. HEK-293 cells apparently employ both scaffolds, as these cells exhibit LPA- and thrombin-stimulated Erk 1/2 activation that is partially pertussis toxin-sensitive and partially sensitive to inhibitors of focal adhesion complex assembly and of EGF receptor transactivation. Signals arising from either scaffold apparently converge on Src family nonreceptor tyrosine kinases, as Src inhibitors impair Erk 1/2 activation in each cell type.

G₁/Gᵢ-coupled receptors are known to activate the Erk pathway via both tyrosine kinase-dependent and -independent pathways (29). Our data indicate that about a third of the Erk 1/2 phosphorylation mediated by LPA receptors in Rat 1 fibroblasts and by LPA and thrombin receptors in HEK-293 cells is insensitive to both Src- and EGF receptor-selective kinase inhibitors. This residual, tyrosine kinase-independent signal may reflect protein kinase C-mediated Erk 1/2 activation, which we have shown is Ras-independent and herbimycin A-insensitive in HEK-293 cells (29).

Although it is clear that GPCR-mediated Erk 1/2 activation arising from focal adhesion- and RTK-based scaffolds are dissociable, the factors that determine scaffold preference are poorly understood. Our data suggest that cell type-specific expression of calcium-regulated FAK kinases such as Pyk2 in neuronal (11) or hematopoietic cells (30) may dictate whether...
GPCRs employ the focal adhesion complex as a signaling scaffold. Pyk2 and p125 FAK share approximately 60% sequence identity within their catalytic domain and 40% within their N- and C-terminal domains (11) but appear to differ significantly in their regulation by extracellular stimuli. Unlike p125 FAK, activation of the Pyk2 homologue CADTK apparently occurs by a two-stage process dependent upon both cellular adhesion and a costimulatory calcium- or protein kinase C-dependent signal (11, 22). Similarly, phosphorylation of both endogenous Pyk2 and p125 FAK occurs following cell adhesion in rat aortic smooth muscle cells, but Pyk2 phosphorylation is further increased by costimulation with calcium ionophore or angiotensin II (31). This differential regulation of Pyk2 and p125 FAK activity suggests a basis for their distinct roles in the regulation of MAP kinase pathways. Consistent with this, GPCR-induced p125 FAK phosphorylation is dissociated from Erk 1/2 activation in Rat 1 cells (8, 9), which do not detectably express Pyk2.

Conversely, overexpression of Pyk2 in 293T cells is sufficient to confer robust LPA-stimulated Erk 1/2 activation that is calcium- and Src kinase-dependent (5).

Several distinct RTKs, including those for platelet-derived growth factor, EGF, and insulin-like growth factor-1, can undergo transactivation (13–15). In a given cell type, GPCR-stimulated Erk 1/2 activation may involve transactivation of multiple RTKs. For example, in Chinese hamster ovary cells, which lack endogenous EGF receptors, LPA stimulation results in Erk 1/2 activation that is dependent upon transactivation of platelet-derived growth factor receptors. However, when EGF receptors are expressed in these cells, signaling proceeds in an EGF receptor-dependent manner (32). Although such data suggest that “generic” mechanisms for the pleiotropic transactivation of RTKs may exist, the molecular mechanisms behind RTK transactivation are poorly understood. In COS-7 cells, EGF receptor transactivation is pertussis toxin-sensitive and inhibited by sequestration of free G protein Gβγ subunits (27, 32).
Conversely, protein kinase C-dependent EGF receptor transactivation has been described in HEK-293 cells stably overexpressing m1 muscarinic acetylcholine receptors (33).

Considerable evidence supports the role of Src family kinases in GPCR stimulation of Erk 1/2 activation. Activation of Src kinases by α-thrombin (34), LPA (2), angiotensin II (35), N-formylmethionyl peptide chemoattractant (1), α2A adrenergic (2, 34), and m1 muscarinic (34) receptors has been reported. Recruitment of c-Src to Pyk2 is required for its action, because a point mutant of Pyk2 that cannot complex with c-Src behaves as a dominant negative inhibitor of GPCR-stimulated Erk 1/2 phosphorylation (5). Similarly, inhibition of Src kinase activity using either dominant inhibitory c-Src mutants (2) or pharmacologic agents (3) dramatically reduces LPA receptor-mediated tyrosine phosphorylation of Shc and Gab1 and Erk 1/2 activation in COS-7 cells. Although these data clearly support a role for Src kinases “downstream” of both FAK family kinases and activated RTKs, additional evidence suggests that Src kinase activity may also play an “upstream” role in GPCR-induced RTK transactivation. Overexpression of Src inhibitor kinase Csk impairs LPA and α2A adrenergic receptor-mediated EGF receptor phosphorylation in COS-7 cells (27). In addition, angiotensin II stimulation has recently been shown to induce association of activated c-Src with the EGF receptor independent of EGF receptor catalytic activity, suggesting that c-Src activation may precede EGF receptor transactivation (36).

Previous work has revealed significant heterogeneity in the mechanisms whereby GPCRs mediate activation of the Erk 1/2 MAP kinase pathway. In most systems studied, GPCR-stimulated Erk 1/2 activation involves the assembly of a Ras activation complex on the plasma membrane, which is dependent upon regulated tyrosine phosphorylation of adapter proteins such as Shc and Gab1 and recruitment of Grb2-mSos. Our data strongly suggest that both focal adhesions and RTKs can function as independently regulated scaffolds for the assembly of this complex and indicate that the preferred scaffold is determined primarily by the cellular context in which the receptor is expressed. Considerable care is therefore warranted in using ectopic expression systems to characterize the signal transduction pathways employed by GPCRs which exhibit tissue-specific expression in vivo. Further examination of the functional significance of these different scaffolds will ultimately enhance our understanding of the diversity of proliferative and differentiative signals originating from GPCRs.
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