Src and Pyk2 Mediate G-protein-coupled Receptor Activation of Epidermal Growth Factor Receptor (EGFR) but Are Not Required for Coupling to the Mitogen-activated Protein (MAP) Kinase Signaling Cascade*

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The epidermal growth factor receptor (EGFR) and the non-receptor protein tyrosine kinases Src and Pyk2 have been implicated in linking a variety of G-protein-coupled receptors (GPCR) to the mitogen-activated protein (MAP) kinase signaling cascade. In this report we apply a genetic strategy using cells isolated from Src-, Pyk2-, or EGFR-deficient mice to explore the roles played by these protein tyrosine kinases in GPCR-induced activation of EGFR, Pyk2, and MAP kinase. We show that Src kinases are critical for activation of Pyk2 in response to GPCR-stimulation and that Pyk2 and Src are essential for GPCR-induced tyrosine phosphorylation of EGFR. By contrast, Pyk2, Src, and EGFR are dispensable for GPCR-induced activation of MAP kinase. Moreover, GPCR-induced MAP kinase activation is normal in fibroblasts deficient in both Src and Pyk2 (Src−/−;Pyk2−/− cells) as well as in fibroblasts deficient in all three Src kinases expressed in these cells (Src−/−;Yes−/−;Fyn−/− cells). Finally, experiments are presented demonstrating that, upon stimulation of GPCR, activated Pyk2 forms a complex with Src, which in turn phosphorylates EGFR directly. These experiments reveal a role for Src kinases in Pyk2 activation and a role for Pyk2 and Src in tyrosine phosphorylation of EGFR following GPCR stimulation. In addition, EGFR, Src family kinases, and Pyk2 are not required for linking GPCRs with the MAP kinase signaling cascade.

The protein tyrosine kinases Src, Pyk2, and epidermal growth factor receptor (EGFR) have been implicated as intermediates in signaling networks that couple G-protein-coupled receptors (GPCRs) with the Ras/MAP kinase signaling cascade (1–4). Although the mechanisms underlying these pathways are not fully defined, at least EGFR activation by GPCRs was proposed to be mediated by intracellular (1, 4) and extracellular (5) processes. Many ligands acting via GPCRs are known to elicit a mitogenic response in a variety of cell types; MAP kinases appear to be a critical component of these growth-promoting pathways (6). It has been shown that GPCR-mediated activation of MAP kinases occurs via pertussis toxin-sensitive and -insensitive processes as well as by Ras-dependent and Ras-independent mechanisms (2, 4, 7, 8). Because many GPCRs couple to more than one G-protein subtype, their activation will initiate simultaneous stimulation of multiple effector systems. In fibroblasts, however, lysophosphatidic acid (LPA)-induced activation of the MAP kinase pathway is mediated solely by Gαi-regulated Ras activation (9, 10). Several protein tyrosine kinases have been implicated as intermediates between Gαi and Ras/MAP kinase activation, including Src, Pyk2, and EGFR (1–4, 11). It has been shown that EGFR, Pyk2, and Src family kinases are rapidly and transiently activated by various GPCRs. In PC12 cells, activated Pyk2 binds to Src, association-mediated through binding of the Src homology 2 domain of Src to pY402 of Pyk2 (11). Experiments employing a dominant interfering EGFR mutant or pretreatment of cells with inhibitors of the protein tyrosine kinase of EGFR suggested that EGFR plays a role in GPCR-induced mitogenic response (12–14). It has been proposed that Src kinases play a crucial role in activation of EGF receptors by GPCR stimulation (14, 15). Moreover, it has been demonstrated that inhibition of Src kinase activity impairs LPA and β2-adrenergic receptor-mediated activation of MAP kinase (16).

We have employed a genetic strategy to explore the role played by Src, Pyk2, and EGFR in GPCR-induced activation of EGFR and MAP kinase. Using cells isolated from Src- or Pyk2-deficient mice we show that Src kinases are critical for activation of Pyk2 and that Src and Pyk2 are essential for GPCR-induced activation of EGFR. However, these kinases are dispensable for GPCR-induced activation of MAP kinase in mouse embryonic fibroblasts. Moreover, GPCR-induced MAP kinase activation is normal in fibroblast deficient in both Src and Pyk2 as well as in fibroblasts deficient in all three Src kinases expressed in these cells (Src, Yes, and Fyn). Using fibroblasts isolated from EGFR-deficient mice, we showed that EGFR activation is dispensable for GPCR-induced activation of MAP kinase. The experiments described in this report reveal an intracellular mechanism for activation of Pyk2 and EGFR by Src kinases following GPCR stimulation and demonstrate

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‡ The abbreviations used are: EGFR, epidermal growth factor receptor; GPCR, G-protein-coupled receptor; MAP, mitogen-activated protein; LPA, lysophosphatidic acid; EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; RBD, Ras binding domain; MAPK, MAP kinase.
that EGFR, Src, and Pyk2 kinases are not required for linking GPCRs with the MAP kinase signaling cascade.

**EXPERIMENTAL PROCEDURES**

**Reagents, Antibodies and Plasmids—**LPA, carbachol, and bradykinin were purchased from Sigma. Recombinant human EGFR was from Intergen. EGFR kinase inhibitors SU1478/009 and SU1517/002 were provided by Sugen, Inc. Monoclonal anti-Pyk2 antibodies used for immunoprecipitation and anti-Ras antibodies were obtained from Transduction Laboratories. Polyclonal anti-Pyk2 antibodies that were used for immunoprecipitation were previously described (11). Monoclonal anti-phosphotyrosine antibodies (4G10) were from Upstate Biotechnology. Polyclonal anti-EGFR antibodies RK-2 and anti-C antibodies were used for immunoprecipitation and immunoblotting of EGFR (17). Polyclonal antibodies against phospho-MAP kinase were from New England Biolabs. Monoclonal antibodies against Src that were used for immunoprecipitation were from Calbiochem. The Pyk2 expression vector we used was previously described (18). The Src expression vector was obtained from J. Sap, New York University, NY. The EGFR expression vector was previously described (17). Kinase negative mutant of EGFR (EGFR-KA) was generated by substituting the codons of Lys-745 with an Ala residue using Stratagene site-directed mutagenesis kit (Stratagene).

**Cell Lines and Transfections—**Embryonic fibroblasts derived from wild type and knockout mice were generated by spontaneous immortalization. Mouse embryonic fibroblasts and human kidney (293) cells were grown in Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Subconfluent 293 cells were transfected using the calcium precipitation method (19) with 1 μg of DNA/well in a 6-well plate. Pyk2/−/−, Src/−/−, and Pyk2/−/−Src/−/−-deficient fibroblasts were isolated from Pyk2/−/−, Src/−/−, and Pyk2/−/−Src/−/− murine E12.5 embryos by standard procedures. EGFR/−/− mouse embryonic fibroblasts were obtained from Maria Sibilia and Erwin Wagner, Vienna, Austria. Src/−/− (LongEV) cells were purchased from ATCC (CRL-2459). Src/−/− Fyn/+/− cells were obtained from Sheila Thomas, Boston, MA. Src/−/− Fyn/+/− mouse embryonic fibroblasts expressing physiological levels of wild type Src and kinase negative mutant of Src were generated in Jonathan Cooper’s laboratory (Fred Hutchinson Cancer Research Center, Seattle, WA) by retroviral infection and selection of low expressing cultures.

**Immunoprecipitation and Immunoblotting—**Cells were washed in ice-cold phosphate-buffered saline and lysed in 50 mM Hepes, pH 7.2, 150 mM NaCl, 1 mM EDTA, 10% Glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 40 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin (lysis buffer). Cells extracts were preclarified by centrifugation and loaded on SDS-PAGE (total lysate) or incubated with antibodies that were cross-linked to protein A-Sepharose beads in lysis buffer and treated as described (17).

**Ras Activation Assay—**Cells were lysed in a buffer containing 20 mM Hepes, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 5 mM MgCl2, and 10% glycerol. The lysates were incubated with 20 μg of GST-Raf(RBD) provided by J. Bos, Utrecht, The Netherlands and were washed three times in lysis buffer. The amount of Ras pulled down was then assessed by immunoblotting with anti-Ras antibodies. In all pull-down experiments, the lysates were preclarified using GST. Analysis of Ras binding to GST alone was performed in each assay but was not detected, indicating that Ras binds to GST-RBD in RBD- and stimulus-dependent manners.

**RESULTS AND DISCUSSION**

Stimulation of mouse embryonic fibroblasts with LPA, bradykinin, or carbachol acting on their cognate GPCRs triggers tyrosine phosphorylation of EGFR (Fig. 1, A). The stimulation of EGFR tyrosine phosphorylation by these GPCR agonists is similar to the level of EGFR phosphorylation seen after stimulation with 2 ng/ml of EGF (Fig. 1, A). Higher concentrations of EGF caused higher levels of EGFR tyrosine phosphorylation, but the level of MAP kinase activation remained the same; it was similar to that induced by LPA (2.5 μM) (Fig. 1, B). It has been proposed that Src kinases play a pivotal role in EGFR activation in response to GPCR stimulation (2, 4, 15). Because we had previously demonstrated that Pyk2 and Src are activated by the same stimuli (11), we examined the effects of Src or Pyk2 deficiencies on LPA-induced activation of EGFR.

**Src and Pyk2 Are Essential for LPA-induced Activation of EGFR—**Src/−/− or Pyk2/−/− fibroblasts were stimulated with LPA, and lysates from unstimulated or stimulated cells were subjected to immunoprecipitation with anti-EGFR antibodies followed by immunoblotting with antibodies against phosphotyrosine (anti-pTyr). The experiment presented in Fig. 2 demonstrates that both the amplitude and kinetics of LPA-induced activation of EGFR were reduced in Src/−/− or Pyk2/−/− fibroblasts. We next examined the time course of LPA-induced activation of EGFR in fibroblasts derived from mice deficient in both Src and Pyk2. The experiment presented in Fig. 2, lower right panel, shows higher basal phosphorylation of EGFR in Src/−/−Pyk2/−/− cells, but LPA-induced stimulation of tyrosine phosphorylation of EGFR could not be detected in these fibroblasts. Similar results were obtained with Src/−/−Pyk2/−/− fibroblasts that were stimulated with bradykinin or carbachol (data not shown). In addition, similar results were obtained with several different primary cultures and with immortalized Src/−/−Pyk2/−/− fibroblasts (data not shown). These experiments demonstrate that Src and Pyk2 are essential for GPCR-induced activation of EGFR.

**Activation of EGFR Is Not Essential for GPCR-induced Stimulation of MAP Kinase—**If EGFR plays a critical role in the coupling of GPCRs to MAP kinase activation (12–14) and Src and Pyk2 are essential for tyrosine phosphorylation of EGFR,
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FIG. 2. Kinetics of LPA-induced tyrosine phosphorylation of EGFR are altered in Src−/− or Pyk2−/− fibroblasts and particularly in Src−/−Pyk2−/− fibroblasts. However, LPA-stimulated MAP kinase activation was readily detected in cells deficient in either Src (20) or Pyk2 and in cells deficient in both protein tyrosine kinases (Fig. 3A). In these experiments the cells were stimulated with LPA for 3 min and analyzed for MAP kinase activation using antibodies that recognize the phosphorylated (activated) form of MAP kinase. We next compared the kinetics of LPA-induced MAP kinase activation in Src−/−, Pyk2−/−, or Src−/−Pyk2−/− cells with the kinetics of LPA-induced MAP kinase activation in wild type fibroblasts. Again, no detectable differences in the kinetics of MAP kinase stimulation were observed (Fig. 3B). Interestingly, LPA-stimulated MAP kinase activation was also detected in Src−/−Yes−/−Fyn−/− fibroblasts, cells deficient in all Src family members that are expressed in these cells (21) (Fig. 3C, left panel). Moreover, LPA-induced activation of Ras and MAP kinase in Src−/−Yes−/−Fyn−/− fibroblasts was inhibited by pertussis toxin (Fig. 3C, right panel), indicating that MAP kinase activation in these cells is mediated by Gi, as it is in wild type cells (9, 10). These experiments demonstrate that LPA-induced MAP kinase activation is normal in fibroblasts deficient in all Src kinases and that Pyk2 and EGFR activation are not essential for MAP kinase response.

To address the role of EGFR in LPA-induced MAPK activation more directly, we compared LPA-stimulation of MAP kinase in wild type fibroblasts to fibroblasts derived from EGFR−/− mice. In this experiment, wild type or EGFR−/− fibroblasts were stimulated with LPA for different times. Lysates from unstimulated or stimulated cells were analyzed for MAP kinase activation with antibodies that recognize activated, phosphorylated MAP kinase. These experiments showed no difference in the kinetics or amplitude of LPA-induced MAP kinase activation of wild type and EGFR−/− fibroblasts at the indicated time points (Fig. 4A) and up to 90 min of LPA stimulation (data not shown). Surprisingly, pretreatment of wild type fibroblasts with two different inhibitors that block the tyrosine kinase activity of EGFR but not Src did not reduce EGF or LPA-induced MAP kinase activation (Fig. 4B). It is noteworthy that the tyrosine kinase inhibitors that were used in this experiment also block the tyrosine kinase activity of erbB2 and erbB4 (data not shown), demonstrating that erbB2 and erbB4 do not compensate for the loss of EGFR and that these receptors are not involved in this process. Taken together, these experiments demonstrate that activation of EGFR is not essential for GPCR-induced MAP kinase activation.

FIG. 3. LPA-stimulated MAP kinase activation is normal in fibroblasts deficient in all Src kinases and that Pyk2 and EGFR activation are not essential for MAP kinase activation.

We have previously shown that stimulation of PC12 cells with LPA leads to tyrosine phosphorylation of Pyk2, which in turn recruits Src via its Src homology 2 domain (11). The experiment presented in Fig. 5A shows that LPA stimulation leads to complex formation between endogenous Src and Pyk2 in fibroblasts. Moreover, in accordance with the data reported previously for different cell types (9, 14, 22), Src and EGFR form a complex in LPA-stimulated fibroblasts, as shown in Fig. 5A. However, we have detected association between EGFR and Src in lysates from unstimulated Pyk2−/− fibroblasts. It is possible that Src forms a complex with EGFR in unstimulated Pyk2−/− fibroblasts because of the higher basal activity of EGFR in Pyk2−/− cells. To further analyze the nature of the association between Src-Pyk2 complex and EGFR, 293 cells were transfected with expression vectors that direct the synthesis of EGFR and EGFR-KA (a kinase negative EGFR point mutant at Lys-745) together with expression vectors for Src or Pyk2. The experiment presented in Fig. 5B shows that, in transfected 293 cells, the kinase negative mutant of EGFR is tyrosine-phosphorylated by Src but not by Pyk2.
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**FIG. 3.** Activation of Src or Pyk2 is not essential for LPA-induced MAP kinase activation in fibroblasts. A, embryonic fibroblasts derived from wild type (WT), Src−/−, Pyk2−/−, or Pyk2−/−Src−/− mutant mice were starved for 48 h and either left untreated or stimulated with LPA (2.5 μM) for 3 min, lysed, subjected to SDS-PAGE, and immunoblotted with antibodies that recognize the activated form of MAP kinase (pMAPK). The filter was stripped and rebotted with anti-ERK1 antibodies as a loading control. All filters were developed simultaneously for quantitative comparison of the results. B, embryonic fibroblasts derived from Src−/−, Pyk2−/−, or Pyk2−/−Src−/− mutant mice were starved for 48 h and either left untreated or stimulated with EGF (2 nM) or LPA (2.5 μM) for the indicated times, lysed, subjected to SDS-PAGE, and immunoblotted with antibodies that recognize the activated form of MAP kinase (pMAPK). The filters were stripped and rebotted with anti-ERK1 antibodies. C, inhibitors of EGFR do not influence EGF- or LPA-induced MAP kinase activation in wild type fibroblasts. Mouse embryonic fibroblasts isolated from wild type mice were starved for 48 h and either left untreated or pre-treated with EGFR tyrosine kinase inhibitors SU1478009 or SU1517002 (1 μM) for 20 min, stimulated with EGF (2 nM) or LPA (2.5 μM) for 3 min, lysed, subjected to SDS-PAGE directly (Total lysate), and analyzed with antibodies that recognize the activated form of MAP kinase (pMAPK). lysates were also subjected to immunoprecipitation (IP) with anti-EGFR or anti-Src antibodies and analyzed with anti-phosphotyrosine antibodies (pY). The filters were stripped and rebotted with anti-ERK1, anti-EGFR, or anti-Src antibodies, respectively. Positions and sizes (kDa) of standard protein markers are indicated on the right.

**FIG. 4.** EGFR activation is not essential for LPA-induced MAP kinase activation in fibroblasts. A, time course of LPA-induced MAP kinase activation in wild type or EGFR−/− fibroblasts. Mouse embryonic fibroblasts derived from wild type (WT) or EGFR−/− mutant mice were starved for 48 h and either left untreated or stimulated with LPA (2.5 μM) for the indicated times, lysed, subjected to SDS-PAGE, and immunoblotted with antibodies that recognize the activated form of MAP kinase (pMAPK). The filters were stripped and rebotted with anti-ERK1 antibodies. B, inhibitors of EGFR do not influence EGF- or LPA-induced MAP kinase activation in wild type fibroblasts. Mouse embryonic fibroblasts isolated from wild type mice were starved for 48 h and either left untreated or pre-treated with EGFR tyrosine kinase inhibitors SU1478009 or SU1517002 (1 μM) for 20 min, stimulated with EGF (2 nM) or LPA (2.5 μM) for 3 min, lysed, subjected to SDS-PAGE directly (Total lysate), and analyzed with antibodies that recognize the activated form of MAP kinase (pMAPK). lysates were also subjected to immunoprecipitation (IP) with anti-EGFR or anti-Src antibodies and analyzed with anti-phosphotyrosine antibodies (pY). The filters were stripped and rebotted with anti-ERK1, anti-EGFR, or anti-Src antibodies, respectively. Positions and sizes (kDa) of standard protein markers are indicated on the right.

bodies followed by immunoblotting with anti-pTyr antibodies. The experiment presented in Fig. 6 shows that LPA failed to stimulate Pyk2 activation in Src−/−Yes−/−Fyn−/− fibroblasts, demonstrating that Src kinases are crucial for LPA-induced activation of Pyk2. Similar results were obtained with Src−/−Yes−/−Fyn−/− fibroblasts stimulated with bradykinin, carbachol, or angiotensin II (data not shown). However, LPA-induced activation of Pyk2 was normal in Src−/−fibroblasts, indicating that either Yes or Fyn are able to compensate for the loss of Src in these cells. Indeed, stimulation of Src−/−Fyn−/− fibroblasts with LPA resulted in normal activation of Pyk2, confirming that Yes is capable in mediating LPA-induced Pyk2 activation. Moreover, LPA-induced Pyk2 activation is completely restored in Src−/−Yes−/−Fyn−/− fibroblasts by stimulation was analyzed by immunoblotting of total lysates using antiphospho-MAPK antibodies. All filters were developed simultaneously for quantitative comparison of the results. Positions and sizes (kDa) of standard protein markers are indicated on the right.
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CONCLUSIONS

Previous studies performed in PC12 cells using dominant negative mutants of Pyk2 (11) and studies performed in Rat-1 fibroblasts using dominant interfering mutants of EGFR or Src (12, 23) demonstrated that EGFR, Src, and Pyk2 play a role in linking GPCR activation with the MAP kinase signaling pathway. Experiments performed in embryonic fibroblasts derived from EGFR−/−, Src−/−, Pyk2−/−, or Src−/−Pyk2−/− mice demonstrate that both Src and Pyk2 are essential for GPCR-induced tyrosine phosphorylation of EGFR. However, together with EGFR, they are dispensable for coupling to the MAP kinase signaling cascade. Moreover, GPCR-induced MAP kinase stimulation is normal in fibroblasts deficient in Src, Yes, and Fyn. Taken together, these studies show that different signaling networks may couple GPCRs with the Ras/MAP kinase signaling pathway in different cell types. In fibroblasts, different experimental strategies have produced conflicting results as to the role played by EGFR, Src, and Pyk2 in GPCR-induced MAP kinase activation. Experiments with dominant interfering EGFR and Src mutants could be misleading because vast overexpression of mutant proteins necessary for inhibition of MAP kinase may interfere with the action of other proteins involved in linking GPCR activation with the MAP kinase signaling cascade. Certain EGFR kinase inhibitors are certainly not sufficiently specific, and most likely block the action of protein tyrosine kinases other than EGFR. In addition, studies using a genetic approach are not always easy to interpret; deficiency in a protein tyrosine kinase may be compensated for by another member of the same family of enzymes. We have addressed this issue for Src kinases by using fibroblasts deficient in all Src kinases expressed in these cells. Our results clearly demonstrate that GPCR-induced activation of MAP kinase is normal even in Src−/−Yes−/−Fyn−/− fibroblasts. However, a single Src kinase is sufficient for mediating LPA-induced stimulation of Pyk2.

Taken together, these studies suggest that establishment of intracellular links between signaling proteins cannot be based upon the sole use of inhibitors or expression of dominant negative mutant proteins. Genetic tools should be used for establishing links between signaling pathways, for testing hypotheses, and confirming conclusions drawn from experiments based upon indirect approaches. Finally, an alternative and intriguing possibility is that the link between GPCR activation and the MAP kinase signaling cascade is mediated by several parallel signaling pathways. Inhibition of one of the pathways by a drug or mutation may lead to a bypass or rerouting of the information flow via alternative pathway(s) for coupling GPCR-activation with the MAP kinase signaling cascade.

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REFERENCES

1. Hackel, P. O., Zwick, E., Prenzel, N., Ullrich, A. (1999) Curr. Opin. Cell Biol. 11, 184–189
2. Carpenter, G. (1999) J. Cell Biol. 146, 697–702
3. Della Rocca, G. J., van Biesen, T., Daaka, Y., Luttrell, D. K., Luttrell L. M., Hawes, B. E., Lefkowitz, R. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1237–1241
4. Hawes, B. E., Luttrell, L. M., van Biesen, T., and Lefkowitz, R. J. (1996) J. Biol. Chem. 271, 12133–12136
5. van Corven, E. J., Hordijk, P. L., Medema, R. H., Bos, J. L., and Moolenaar, W. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1257–1261
6. Blumer, K. J., and Johnson, G. L. (1994) Trends Biochem. Sci. 19, 236–240
7. Hawes, B. E., Luttrell, L. M., van Biesen, T., and Lefkowitz, R. J. (1996) J. Biol. Chem. 271, 12133–12136
8. van Corven, E. J., Hardijk, P. L., Medema, R. H., Bos, J. L., and Moolenaar, W. H. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1257–1261
9. Hardijk, P. L., Verlaan, I., van Corven, E. J., and Moolenaar, W. H. (1994) J. Biol. Chem. 269, 645–651
10. Dikie, I., Tokiwa, G., Lev, S., Courteide, S. A., Schlessinger, J. (1996) Nature
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12. Daub, H., Weiss, F. U., Wallasch, C., and Ullrich, A. (1996) *Nature* **379**, 557–560
13. Della Rocca, G. J., Maudsley, S., Daaka, Y., Lefkowitz, R. J., and Luttrell, L. M. (1999) *J. Biol. Chem.* **274**, 13978–13984
14. Eguchi, S., Numaguchi, K., Iwasaki, H., Matsumoto, T., Yamakawa, T., Utsunomiya, H., Molley, E. D., Kawakatsu, H., Owada, K. M., Hirata, Y., Marumo, F., Inagami, T. (1998) *J. Biol. Chem.* **273**, 8890–8896
15. Luttrell, L. M., Ferguson, S. S., Daaka, Y., Miller, W. E., Maudsley, S., Della Rocca, G. J., Lin, F., Kawakatsu, H., Owada, K., Luttrell, D. K., Caron, M. G., and Lefkowitz, R. J. (1999) *Science* **283**, 655–661
16. Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (1997) *Nature* **390**, 88–91
17. Margolis, B., Li, N., Koch, A., Mohammadi, M., Hurwitz, D. R., Zilberstein, A., Ullrich, A., Pawson, T., and Schlessinger, J. (1990) *EMBO J.* **9**, 4375–4380
18. Lev, S., Moreno, H., Martinez, R., Canell, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B., and Schlessinger, J. (1995) *Nature* **376**, 737–745
19. Chen, C., and Okayama, H. (1987) *Mol. Cell. Biol.* **7**, 2745–2752
20. Kranenburg, O., Verlaan, I., Hordijk, P. L., and Mooldenaar, W. H. (1997) *EMBO J.* **16**, 3097–3105
21. Klinghoffer, R. A., Sachsenmaier, C., Cooper, J. A., and Soriano, P. (1999) *EMBO J.* **18**, 2459–2471
22. Thomas, S. M., and Brugge, J. S. (1997) *Annu. Rev. Cell Dev. Biol.* **13**, 513–609
23. Daub, H., Wallasch, C., Lankenau, A., Herrlich, A., and Ullrich, A. (1997) *EMBO J.* **16**, 7032–7044