Grb2 Interaction with MEK-Kinase 1 Is Involved in Regulation of Jun-Kinase Activities in Response to Epidermal Growth Factor*

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Epidermal growth factor (EGF) receptor was shown to be involved in the activation pathway of the stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) cascade not only by EGF, but also by UV radiation or osmotic stress. This paper describes a specific interaction between the COOH-terminal SH3 domain of Grb2 and the NH2-terminal regulatory domain of MEKK1 in ER22 cells overexpressing the EGF receptor. This interaction results in the formation of a constitutive complex between Grb2 and MEKK1 in both proliferating and resting cells. EGF stimulation causes this complex to be rapidly and transiently recruited by Shc proteins. The subsequent release of the Grb2-MEKK1 complex from Shc proteins correlates with JNK activation. Transfection of the NH2-terminal regulatory domain of MEKK1 specifically inhibits EGF-dependent JNK activation indicating that Grb2 is involved in MEKK1 activation. Thus, adaptor proteins have a new role in the regulation of the SAPK/JNK cascadeca after EGF stimulation.

Several mammalian mitogen-activated protein (MAP)1 kinases have been identified, including extracellular signal-regulated protein kinases (ERKs) (1, 2), stress-activated protein kinases (SAPKs), also known as c-Jun NH2-terminal protein kinases (JNKS) (3, 4), and p38/Hog1 MAP kinases (5, 6). The activation of MAP kinases appears to be a key event in many cell responses to external stimuli. The JNK cascade is activated in response to a variety of cell stresses, including UV irradiation, g-rays, pro-inflammatory cytokines, ceramides, vasoactive peptides, protein synthesis inhibitors, heat shock, osmotic shock, and also in response to mitogens such as epidermal growth factor (EGF) and nerve growth factor. The JNK cascade leads to the activation by phosphorylation of a series of transcription factors including c-Jun, Elk-1, and ATF-2 (7). The stimulation of the JNKS (JNK1, JNK2, and JNK3) is associated with proliferation and differentiation but also with the arrest of cell growth and apoptosis. It was recently reported that stimulation of the JNKS mediates Ras transformation (8).

The involvement of small GTP-binding proteins, such as Ras proteins and the members of the Rho subfamily, in the activation of the JNK cascade is well documented in cell responses to stress or growth factors. EGF activates the JNK pathway (9), and phosphatidylinositol 3-kinase was recently reported to be a link between JNK signaling mediated by the Rho family and the Ras pathway upon EGF stimulation (10). Exposure to UV radiation or to osmotic shock was also found to induce clustering and internalization of EGF receptors and their activation by tyrosine autophosphorylation, with subsequent activation of the JNK cascade through Ras activation (11).

The JNKS are activated by dual phosphorylation on tyrosine and threonine residues by the JNK kinases (MKK4/SEK1), which are, in turn, activated by upstream kinases referred to as MEK kinases (MEKKs) (9, 12). MEKKs are an expanding family of kinases (13). Mammalian MEKK1 cDNA encodes a 78-kDa protein, but several forms of MEKK1 have been found in various cell lines (50, 78, or 98 kDa) (14). Thereafter, a rat MEKK1 full-length cDNA was cloned, encoding a 195-kDa protein (15). The 98-kDa protein corresponds to the 625 COOH-terminal amino acids of the full-length MEKK1 and might be produced by specific cleavage (16).

Few data describe the mechanism of transient activation of MEKK1 in response to growth factors. We have therefore studied the activation of MEKK1 in response to EGF in Chinese hamster lung fibroblasts (ER22 cells), in which the main immunoreactive form of MEKK1 is a 98-kDa protein. We found a constitutive Grb2-MEKK1 complex in the ER22 cells. In response to EGF stimulation, this complex is recruited by Shc proteins. The JNK activation occurs when the complex is released from Shc proteins.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfections—ER22 cells, derived from CCL39 cells by stable expression of the EGF receptor (17), were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS). Transient transfections were performed using LipofectAMINE, and cells were harvested 48 h later.

cDNA Constructs and Reconstitvent Proteins—The mutant Grb2 YTV cDNA was obtained from the Grb2 cdNA by PCR mutagenesis with the following primers: sense (5’-ATGGAAGCCATCGCCAAAGTCGACT-3’) and antisense (5’-AGATCTCATTAGTGGTCAGCT-3’). The Grb2SH3C cDNA was obtained from the Grb2 cDNA by deletion of the region encoding the COOH-terminal domain from amino acid Q157. HA-tagged MEKK1COOH(301–672) and VSV-tagged MEKK1,1–672 were a generous gift from Dr. J. Pouyssegur (UMR134, CNRS Nice, France). MEKK1,1–410 was constructed from the MEKK1,1–672 cDNA by PCR and subsequent sequencing. The Myc-tagged Grb2, Grb2, and Grb3-3 expression vectors have been described (18). GST-Crk and GST-
Nck were a gift from Dr. S. Fischer (INSERM U363, Paris, France). GST-cJun(1–223) was kindly provided by Dr. B. Wasylyk (IGBMC, Strasbourg, France). GST fusion proteins were produced using the pGEX-2T vector (Pharmacia). The recombinant His-MEKK1(1–301) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Transfection Analysis—ER22 cells were transfected with the reporter plasmid Gal4-CAT, the activator plasmid encoding Gal4-cJun(1–223), and pSV2 plasmid without a cDNA insert or with MEKK1(1–410) or MEKK1(301–672) cDNAs. Cells were incubated for 24 h after transfection, deprived of FCS for an additional 24 h, and treated with or without 100 ng/ml EGF. CAT activity was measured using an InstantImager (Packard Instrument Corp.).

Protein Kinase Assays—INK activities were assayed using an in vitro c-Jun kinase assay (20). Cell lysates were prepared as described previously (21) and incubated with GST-cJun(1–223), bound to GSH-c-Jun kinase assay (20). Cell lysates were subjected to SDS-PAGE and Western blot analysis. Bound proteins were dissolved in Laemmli sample buffer and subjected to SDS-PAGE and Western blot analysis were performed as described previously (19).

Antibody binding was revealed by enhanced chemiluminescence (ECL) (Amersham) or by the alkaline phosphatase 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma). Proteins were detected by far-Western blot analysis with anti-GST antibodies (0.25 μg/ml) and exposed with the alkaline phosphatase 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium system (Sigma).

GST Fusion Protein Purification and Affinity Chromatography—GST fusion proteins (Grb2, Grb2YTV, Grb2SH3C, Grb3-3, Nck, Crk) were adsorbed onto glutathione-agarose beads equilibrated in buffer A containing 0.2% Triton X-100 and incubated with the cell lysates for 3 h at 4 °C. Bound proteins were dissolved in Laemmli sample buffer and subjected to SDS-PAGE and Western blot analysis.

Protein Kinase Assay—INK activities were assayed using an in vitro c-Jun kinase assay (20). Cell lysates were prepared as described previously (21) and incubated with GST-cJun(1–223), bound to GSH-agarose. Proteins were incubated (20 min at 30 °C) in 20 mM HEPES, pH 7.4, 5 mM MgCl2, 2 mM EGTA, 2 mM dithiothreitol, 40 μM ATP, and 10 μCi/ml [γ-32P]ATP (3 Ci/mmol, Amersham). 32P incorporation in GST-cJun(1–223) was monitored by 10% SDS-PAGE and autoradiography.

RESULTS AND DISCUSSION

Grb2 Binds to MEKK1 in Vitro and in Vivo—The MEKK1(1–672) sequence (14) contains several proline-rich stretches in the NH2-terminal region that could interact with protein SH3 domains. We examined the interaction of MEKK1 with various GST-adaptor proteins (containing mainly SH2 and SH3 domains). Lysates from FCS-cultured ER22 cells were subjected to affinity chromatography on glutathione-agarose coupled to GST-Grb2, GST-Nck, or GST-Crk fusion proteins (Fig. 1A). Immobilized GST-Grb2 specifically retained the 98-kDa MEKK1, as revealed by Western blot analysis using antibodies directed against the COOH-terminal sequence of MEKK1. This molecular weight has already been reported for the MEKK1 in NIH or Swiss 3T3 fibroblasts (22). In contrast, the anti-MEKK1 antibodies detected no immunoreactive protein in eluates from GST-Nck and GST-Crk affinity chromatography columns. We also found a 78-kDa protein associated with Grb2 in primary cultures of rat astrocytes (results not shown). This corresponds to the molecular weight of MEKK1 in PC12 cells (14).

No immunoreactive protein was detected with antibodies against other MAP kinase kinase kinases, such as c-Raf1 or B-Raf, or with antibodies against the MAP kinase kinase (MEK1), which contains many proline-rich stretches (results not shown). In contrast, the Ras exchange factor, Sos1, was found associated with Grb2 and Crk (results not shown). These data suggest that MEKK1 interacts specifically with Grb2 in vitro.

We then examined the Grb2-MEKK1 interaction in vivo (Fig. 1B). Extracts of proliferating ER22 cells were immunoprecipitated with anti-Grb2 antibodies and analyzed by Western blotting with anti-MEKK1 antibodies (lane 1). Two major polyepitides were detected, a 98-kDa protein corresponding to the molecular mass of the wild type MEKK1 and a 195-kDa protein having the same size as the large form of MEKK1 (15). The cell extracts were immunoprecipitated with anti-MEKK1 antibodies, and the 25-kDa Grb2 protein was detected using anti-Grb2 antibodies (lane 3). These data indicate that endogenous Grb2 and MEKK1 associate in vivo.

To confirm this interaction, ER22 cells were transiently transfected with plasmids carrying VSV-tagged MEKK1 (lane 2). Grb2 was associated with VSV-tagged MEKK1 after immunoprecipitation of Grb2 followed by Western blot analysis with anti-VSV tag antibodies. ER22 cells were also transfected with plasmids carrying Myc-tagged Grb2. Anti-MEKK1 immunoprecipitates of cell extracts, blotted with anti-Grb2 antibodies, detected two proteins, one corresponding to endogenous Grb2 and the other to Myc-Grb2 (lane 4), the latter was also detected by anti-Myc antibodies (results not shown). This confirms that MEKK1 interacts with Grb2 in ER22 cells.

Mapping of the Interaction Domains on Grb2 and on MEKK1—The functional domains of Grb2 that interact with MEKK1 were identified by affinity chromatography using mutants or variants of Grb2 (Fig. 2A). Grb3-3 is a natural splicing isoform of Grb2 with a crippled SH2 domain that cannot interact with the phosphorylated EGF receptor (18). It interacted with MEKK1 as well as did unspliced Grb2, indicating that the
SH2 domain of Grb2 is not implicated in the binding with MEKK1. Grb2Y7V is an NH2-terminal SH3 mutant that has lost the ability to bind dynamin.2 It still bound to MEKK1, although less strongly than Grb2, indicating that MEKK1 does not bind to the same site as dynamin. Grb2ΔSH3C is deleted from its COOH-terminal SH3 domain and did not interact any more with MEKK1 (Fig. 2A), although it did interact with Sos1 (result not shown). The Grb2 G203R mutant did not bind to Sos1 under our experimental conditions2 and is known to block DNA synthesis induced by activation of the Ras pathway (23).

SH3 domain of Grb2 associates with the proline-rich NH2-terminal domain of MEKK1. A, lysates (1 mg of protein) from ER22 cells were incubated with glutathione-agarose beads coupled to GST fusion proteins (60 μg): GST-Grb2 (lane 1), GST-Grb2Δ3 (lane 2), GST-Grb2Y7V (lane 3), GST-Grb2ΔSH3C (lane 4). Bound proteins were resolved by 8.5% SDS-PAGE and analyzed by immunoblotting using anti-MEKK1 antibodies. B, ER22 cells were transfected (lanes 2 and 4) or not (lanes 1 and 3) with 5 μg of either VSV-MEKK1 (lane 2) or HA-MEKK1ΔCOOH (lane 4) expression plasmids. Cells were harvested 48 h later, and protein extracts were immunoprecipitated with anti-VSV or anti-HA (12CA5, Berkeley Antibody Co., Richmond, CA) antibodies. Immunoprecipitated proteins were analyzed by immunoblotting using monoclonal anti-Grb2 antibodies. C, recombinant His-MEKK1 (1–301) was fractionated by SDS-PAGE, transferred onto an Immobilon-P membrane, and probed with either recombinant GST (lane 1) or GST-Grb2 (lane 2) for 12 h at 4 °C. After washes in phosphate-buffered saline, 0.05% Tween 20, bound proteins were detected after incubation with (0.25 μg/ml) anti-glutathione S-transferase monoclonal antibodies (HybriSolv Pasteur Institute).

We identified the domain of MEKK1 that interacted with Grb2 by transfecting ER22 cells with plasmids encoding VSV-tagged MEKK1K1–672 or HA-tagged MEKK1K1(301–672) (Fig. 2B). Tagged MEKK1s were immunoprecipitated using anti-tag antibodies and blotted with anti-Grb2 antibodies. We found that Grb2 interacted in vivo with MEKK1K1(1–672) but not with its COOH-terminal catalytic domain.

The identity of the MEKK1 interaction domain was confirmed by far-Western blot analysis. Recombinant polyhistidine-tagged MEKK1K1(1–301) was probed with GST or GST-Grb2 (Fig. 2C). As expected, GST-Grb2 bound to a single polypeptide (Fig. 2D), whereas GST alone showed only background activity.

Relationship between the time course of the Shc-Grb2-MEKK1 ternary complex and JNK activity in response to EGF. ER22 cells were grown to confluence in Dulbecco's modified Eagle's medium/F-12 containing 10% FCS and then in medium without serum. Cells were treated for the indicated time with 100 ng/ml EGF. A, the protein extracts were immunoprecipitated with anti-MEKK1 antibodies and the immunoprecipitated proteins were analyzed by Western blot with monoclonal anti-Grb2 antibodies. B and C, Shc proteins were immunoprecipitated with polyclonal anti-Shc antibodies (Transduction Laboratories Inc.). Association between Shc proteins and Grb2 or MEKK1 was monitored by Western blot analysis of the immunoprecipitated proteins using monoclonal anti-Grb2 antibodies (B) or anti-MEKK1 antibodies (C). D, cell extracts were prepared and analyzed for JNK activity in vitro using GST-Jun(1–223) adsorbed onto glutathione-agarose beads as a substrate. Phosphorylation products were analyzed by 10% SDS-PAGE and autoradiography.

Fig. 2. The SH3 COOH-terminal domain of Grb2 associates with the proline-rich NH2-terminal domain of MEKK1. A, lysates (1 mg of protein) from ER22 cells were incubated with glutathione-agarose beads coupled to GST fusion proteins (60 μg): GST-Grb2 (lane 1), GST-Grb2Δ3 (lane 2), GST-Grb2Y7V (lane 3), GST-Grb2ΔSH3C (lane 4). Bound proteins were resolved by 8.5% SDS-PAGE and analyzed by immunoblotting using anti-MEKK1 antibodies. B, ER22 cells were transfected (lanes 2 and 4) or not (lanes 1 and 3) with 5 μg of either VSV-MEKK1 (lane 2) or HA-MEKK1ΔCOOH (lane 4) expression plasmids. Cells were harvested 48 h later, and protein extracts were immunoprecipitated with anti-VSV or anti-HA (12CA5, Berkeley Antibody Co., Richmond, CA) antibodies. Immunoprecipitated proteins were analyzed by immunoblotting using monoclonal anti-Grb2 antibodies. C, recombinant His-MEKK1 (1–301) was fractionated by SDS-PAGE, transferred onto an Immobilon-P membrane, and probed with either recombinant GST (lane 1) or GST-Grb2 (lane 2) for 12 h at 4 °C. After washes in phosphate-buffered saline, 0.05% Tween 20, bound proteins were detected after incubation with (0.25 μg/ml) anti-glutathione S-transferase monoclonal antibodies (HybriSolv Pasteur Institute).

Fig. 3. Relationship between the time course of the Shc-Grb2-MEKK1 ternary complex and JNK activity in response to EGF. ER22 cells were grown to confluence in Dulbecco’s modified Eagle’s medium/F-12 containing 10% FCS and then in medium without serum. Cells were treated for the indicated time with 100 ng/ml EGF. A, the protein extracts were immunoprecipitated with anti-MEKK1 antibodies and the immunoprecipitated proteins were analyzed by Western blot with monoclonal anti-Grb2 antibodies. B and C, Shc proteins were immunoprecipitated with polyclonal anti-Shc antibodies (Transduction Laboratories Inc.). Association between Shc proteins and Grb2 or MEKK1 was monitored by Western blot analysis of the immunoprecipitated proteins using monoclonal anti-Grb2 antibodies (B) or anti-MEKK1 antibodies (C). D, cell extracts were prepared and analyzed for JNK activity in vitro using GST-Jun(1–223) adsorbed onto glutathione-agarose beads as a substrate. Phosphorylation products were analyzed by 10% SDS-PAGE and autoradiography.

Fig. 4. Effect of MEKK1(1–410) transfection on EGF-induced Jun-kinase activity. ER22 cells were cotransfected with the Gal4-CAT and the Gal4-Jun(1–223) plasmids and pSV2 plasmid without a cDNA insert or with MEKK1(1–410) or MEKK1(301–672) cDNAs. Cells were incubated for 24 h after transfection, deprived of FCS for an additional 24 h, and treated with 100 ng/ml EGF. CAT activity was measured using an InstantImager (Packard Instrument Corp.). Values were normalized to equivalent amounts of proteins.

Fig. 5. Relationship between the time course of the Shc-Grb2-MEKK1 ternary complex and JNK activity in response to EGF. ER22 cells were grown to confluence in Dulbecco’s modified Eagle’s medium/F-12 containing 10% FCS and then in medium without serum. Cells were treated for the indicated time with 100 ng/ml EGF. A, the protein extracts were immunoprecipitated with anti-MEKK1 antibodies and the immunoprecipitated proteins were analyzed by Western blot with monoclonal anti-Grb2 antibodies. B and C, Shc proteins were immunoprecipitated with polyclonal anti-Shc antibodies (Transduction Laboratories Inc.). Association between Shc proteins and Grb2 or MEKK1 was monitored by Western blot analysis of the immunoprecipitated proteins using monoclonal anti-Grb2 antibodies (B) or anti-MEKK1 antibodies (C). D, cell extracts were prepared and analyzed for JNK activity in vitro using GST-Jun(1–223) adsorbed onto glutathione-agarose beads as a substrate. Phosphorylation products were analyzed by 10% SDS-PAGE and autoradiography.

M. Pomerance, M.-C. Mulate, F. Parker, C. Venot, J.-P. Blondeau, B. Tocqué, and F. Schweighoffer, unpublished results.
Therefore, we conclude that the Grb2-MEKK1 association is not modulated by EGF or serum and that the complex is constitutive, as is the Grb2-Sos complex (25–28).

**EGF Receptor Tyrosine Phosphorylation Promotes Grb2-MEKK1-She Complex Formation**—She proteins were immunoprecipitated from ER22 cells and were found to be associated with Grb2 upon EGF stimulation (Fig. 3B), as described previously (29, 30). This association was transient, reaching a peak after 5–10 min of EGF treatment. Concomitantly, MEKK1 was detected in the Shc immunoprecipitates (Fig. 3C). This association was confirmed using ER22 cells transfected with plasmids carrying VSV-tagged MEKK1. Immunoprecipitation with anti-SH antibodies followed by Western blot analysis with anti-Shc antibodies revealed p66, p52, and p46 Shc proteins (results not shown). The JNK activities in ER22 cells were stimulated by EGF and reached a peak 20 min after adding EGF (Fig. 3D), following the release of MEKK1 from Shc proteins.

Dissociation of the Grb2-MEKK1 complex from Shc, 10 min after EGF stimulation, may allow this complex to be recycled to the cytoplasm, in a way similar to the Grb2-Sos complex. Our results suggest that the activation of JNK by EGF requires a transient association of a Grb2-MEKK1 complex with Shc proteins and perhaps with the EGF receptor. This may result in a transient translocation of MEKK1 to the plasma membrane. Other protein kinases involved in JNK activation such as phosphatidylinositol 3-kinase, PAKs, and HKP1 require recruitment at the membrane for their activation (31–33). MEKK1 could interact with GTP-loaded small GTPases at the membrane. Indeed, JNK activation has been found to depend on Ras (34). Other protein kinases involved in JNK activation such as p38 kinase like kinase (GLK), which shares homology with the MEKK1 and suggest that Grb2 is involved in several signaling pathways. The recruitment of MEKK1 to the cell membrane may promote its activation by locating it close to a suitable kinase. This mechanism is an integral part of the signal generated by the EGF receptor to activate JNKs.

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**REFERENCES**

1. Boulton, T. G., Yancopoulos, G. D., Gregory, J. S., Slaughter, C., Moomaw, C., Hsu, J., and Cobb, M. H. (1996) Science 274, 64–67
2. Hunter, T. (1995) Cell 80, 225–236
3. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994) Nature 369, 156–160
4. Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) Cell 76, 1025–1037
5. Han, J., Lee, J. D., Bibbs, L., and Ulevitch, R. J. (1984) Science 265, 808–811
6. Kumar, S., McDoungell, P. C., Gum, R. J., Hand, A. T., Lee, J. C., and Young, P. R. (1997) Biochem. Biophys. Res. Commun. 235, 533–538
7. Minden, A., and Karin, M. (1997) Biochem. Biophys Acta 1333, 85–104
8. Rodrigues, G. A., Park, M., and Schlessinger, J. (1997) EMBO J. 16, 2844–2845
9. Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R. J., Johnson, G. L., and Karin, M. (1994) Science 266, 1719–1723
10. Logan, S. K., Falasca, M., Hu, P., and Schlessinger, J. (1997) Mol. Cell. Biol. 17, 5784–5790
11. Rossette, C., and Karin, M. (1996) Science 274, 1194–1197
12. Yan, M., Dai, T., Desk, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R., and Templeton, D. J. (1994) Nature 372, 788–800
13. Fanger, G. R., Johnson, N. L., and Johnson, G. L. (1997) EMBO J. 16, 4961–4972
14. Lange-Carter, C., Pleiman, C. M., Gardener, A. M., Blumer, K. J., and Cobb, M. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5291–5295
15. Cardone, M. H., Salvesen, G. S., Widmann, C., Johnson, G., and Frisch, M. (1997) Cell 90, 315–323
16. Sardet, C., Fabunhonx, P., and Pouysségur, J. (1991) J. Biol. Chem. 266, 19166–19171
17. Fathi, I., Schweighoff, F., Rey, I., Multon, M. C., Boiziu, J., Duchesne, M., and Toqué, B. (1994) Science 264, 971–974
18. Parker, F., Maurier, F., Delumeau, I., Duchesse, D., Debusche, L., Dugué, A., Schweighoff, F., and Toqué, B. (1996) Mol. Cell. Biol. 16, 2561–2569
19. Westwick, J. K., and Brenner, D. A. (1995) Methods Enzymol. 255, 342–359
20. Pomerance, M., Thang, M. N., Toqué, B., and Pierre, M. (1996) Mol. Cell. Biol. 16, 3179–3186
21. Lange-Carter, C. A., and Johnson, G. L. (1994) Science 265, 1458–1461
22. Levine, E. J., Daly, R. J., Bater, A. G., Li, W., Margolis, B., Lammens, R., Ulrich, A., Skolnik, E. Y., Bar-Sagi, D., and Schlessinger, J. (1992) Cell 70, 431–442
23. Cohen, G. B., Ben, R., and Baltimore, D. (1995) Cell 80, 237–248
24. Chardin, P., Camonis, J. H., Gale, N. W., van Aelst, L., Schlessinger, J., Wigler, M. H., and Bar-Sagi, D. (1993) Science 260, 1338–1343
25. Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M., and Weinberg, R. A. (1993) Nature 363, 45–51
26. Li, N., Bater, A. D., Ral, Yajnik, V., Skolnik, E. Y., Chardin, P., Bar-Sagi, D., Margolis, B., and Schlessinger, J. (1993) Nature 363, 85–88
27. Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T., and Bowtell, D. (1993) Nature 363, 83–92
28. Rozakis-Adcock, M., Miglade, J., Mabamulu, G., Pelici, G., Daly, R., Li, W., Bater, A. Thomas, S., Brugge, J., and Pelici, P. G. (1992) Nature 360, 699–692
29. Holt, K. H., Waters, S. B., Okada, S., Yamauchi, K., Decker, S. J., Saltiel, A. R., Motto, D. G., Korzetzy, G. A., and Pessin, J. E. (1996) J. Biol. Chem. 271, 8303–8306
30. Vanhaesebroeck, B., Leevers, S. J., Panayotou, G., and Waterfield, M. D. (1997) Trends Biochem. Sci. 22, 267–272
31. Manser, E., Leung, T., Salhuddin, H., Zhao, Z., and Lim, L. (1994) Nature 367, 40–46
32. Anafi, M., Kiefier, F., Gish, G. D., Mabamulu, G., Iscoe, N. N., and Pawson, T. (1997) J. Biol. Chem. 272, 27804–27811
33. Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gotkind, S. (1995) Cell 81, 1137–1146
34. Russell, M., Lange-Carter, C. A., and Johnson, G. L. (1995) J. Biol. Chem. 270, 11757–11760
35. Fanger, G. R., Widmann, C., Porter, A. C., Sather, S., Johnson, G. L., and Vaillancourt, R. R. (1998) J. Biol. Chem. 273, 3476–3483
36. Diener, K., Wang, X. S., Chen, C., Meyer, C. F., Keesler, G., Zoukowitzki, M., Tan, T., and Yao, Z. (1997) Proc Natl. Acad. Sci. U. S. A. 94, 9687–9692