Negative Feedback Regulation and Desensitization of Insulin- and Epidermal Growth Factor-stimulated p21\textsuperscript{ras} Activation*  

(Received for publication, July 10, 1995, and in revised form, August 31, 1995)  

W. John Langlois, Toshiyasu Sasaoka, Alan R. Saltiel, and Jerrold M. Olefsky  

From the Department of Medicine, Division of Endocrinology and Metabolism, University of California, San Diego, La Jolla, California 92093, the Veteran's Administration Medical Center, Medical Research Service, San Diego, California 92161, the ‡First Department of Medicine, Toyama Medical and Pharmaceutical University, Toyama 930-01, Japan, and the $Department of Signal Transduction, Parke-Davis Pharmaceutical Research Division, Warner Lambert Company, Ann Arbor, Michigan 48105  

Insulin and epidermal growth factor receptors transmit signals for cell proliferation and gene regulation through formation of active GTP-bound p21\textsuperscript{ras} mediated by the guanine nucleotide exchange factor Sos. Sos is constitutively bound to the adaptor protein Grb2 and growth factor stimulation induces association of the Grb2/Sos complex with Shc and movement of Sos to the plasma membrane location of p21\textsuperscript{ras}. Insulin or epidermal growth factor stimulation induces a rapid increase in p21\textsuperscript{ras} levels, but after several minutes levels decline toward basal despite ongoing hormone stimulation. Here we show that deactivation of p21\textsuperscript{ras} correlates closely with phosphorylation of Sos and dissociation of Sos from Grb2, and that inhibition of mitogen-activated protein (MAP) kinase kinase (also known as extracellular signal-related kinase (ERK) kinase, or MEK) blocks both events, resulting in prolonged p21\textsuperscript{ras} activation. These data suggest that a negative feedback loop exists whereby activation of the Raf/MEK/MAP kinase cascade by p21\textsuperscript{ras} causes Sos phosphorylation and, therefore, Sos/Grb2 dissociation, limiting the duration of p21\textsuperscript{ras} activation by growth factors. A serine/threonine kinase downstream of MEK (probably MAP kinase) mediates this desensitization feedback pathway.

The tyrosine kinase receptors for insulin, EGF, and other growth factors mediate cell proliferation and gene regulation, and it has been demonstrated that activation of p21\textsuperscript{ras} is essential for these effects (1, 2). p21\textsuperscript{ras} is one of a family of closely related membrane-bound guanine nucleotide-binding proteins, which bind GTP and catalyze its hydrolysis to GDP. The GTP-bound protein is active, while the GDP-bound form is not. The GTPase-activating protein GAP promotes inactivation of p21\textsuperscript{ras} by stimulating the hydrolysis of GTP (3), whereas the guanine nucleotide exchange factor (GEF) Sos (homologue of the Drosophila Son-of-sevenless protein) mediates activation by inducing the release of GDP (4–8). In the case of insulin and EGF, activation of p21\textsuperscript{ras} has been shown to be mediated by Sos rather than by GAP (8, 9).

In the basal state, Sos is a cytoplasmic protein constitutively bound to the adaptor protein Grb2. This interaction is mediated by the two Src homology 3 (SH3) domains of Grb2, which bind to proline-rich regions in the C terminus of Sos (6, 10–12). Insulin or EGF stimulation results in the translocation of Sos to the plasma membrane, where activation of p21\textsuperscript{ras} occurs (5). Growth factor stimulation also results in the tyrosine phosphorylation of the intermediary signaling molecule Shc on a tyrosine residue in a consensus binding motif for the Grb2 SH2 domain, resulting in the binding of the Grb2/Sos complex to Shc (6, 11). In the case of the EGF receptor the mechanism for targeting Sos to the plasma membrane is binding of the SH2 domain or phosphotyrosine binding domain of Shc to phosphotyrosine residues of the EGF receptor (5, 13–15); direct binding of the Grb2 SH2 domain to the EGF receptor most likely plays a lesser role (16). There is evidence that Shc plays a key role upstream of p21\textsuperscript{ras} in the mitogenic response to both insulin and EGF, suggesting that in fact the trimeric Shc/Grb2/Sos complex is important for activation of p21\textsuperscript{ras} (16–19).

The pattern of p21\textsuperscript{ras} activation after growth factor stimulation is one of a rapid increase in GTP binding, peaking in the first several minutes and then falling rather rapidly toward basal levels despite ongoing ligand stimulation (10, 20–24). The mechanism of this down-regulation is unknown. However, it has recently been demonstrated that Sos undergoes serine/threonine phosphorylation following growth factor stimulation (24–26), and dissociation of the Grb2/Sos complex appears to correlate temporally with Sos phosphorylation (24, 25). The purpose of this study was to elucidate the serine kinase pathway leading to Sos phosphorylation and to determine whether Grb2/Sos dissociation is causally related. In addition, we sought to learn whether this dissociation is a negative feedback mechanism resulting in the desensitization of p21\textsuperscript{ras} to prolonged activation by growth factor stimulation.

**EXPERIMENTAL PROCEDURES**

Materials—Rat1 cells expressing wild type human insulin receptors (HrRc) were maintained as described previously (27). Insulin was kindly provided by Lilly. Polyclonal antibodies used for immunoprecipitation were from sources as follows: anti-Sos1, Upstate Biotechnology (Lake Placid, NY); anti-Grb2, Santa Cruz Biotechnology (Santa Cruz, CA); anti-Shc, Transduction Laboratories (Lexington, KY). Monoclonal antibodies anti-Sos1, anti-Grb2 and anti-phosphotyrosine (PY20) used for immunoblotting were all from Transduction Laboratories. The rat anti-p21\textsuperscript{ras} antibody was from Santa Cruz Biotechnology, and the rabbit anti-rat antiseraum was from Cappel (Durham, NC). Carrier-free [32P]orthophosphate (40 mCi/ml) was from DuPont NEN. Polyethyleneimine cellulose TLC plates were from J. T. Baker (Phillipsburg, NJ). Electrophoresis reagents were from Bio-Rad. Enhanced chemiluminescence reagents were from Amersham Corp. All other reagents were purchased from Sigma.
Immunoprecipitation and Western Blotting Studies—HIRcB cells were serum-starved for 16 h, and then treated for 60 min with 20 μM PD098059 in 1% Me2SO, or 1% Me2SO alone. Cells were then stimulated with 17 nM insulin or 160 nM EGF for the indicated times. The cells were washed twice in ice-cold phosphate-buffered saline and collected in lysis buffer consisting of 25 mM HEPES, pH 7.4, 120 mM NaCl, 20 mM MgCl2, 0.5% Nonidet P-40, 10 μg/ml aprotinin, 1% phenylmethylsulfonyl fluoride, 800 KIU/ml aprotinin, 8 mM EDTA, 0.5 mg/ml bacitracin, 2 mM dichloroacetate, 15 mM benzoamide, 160 mM NaF, 2 mM Na3VO4, 10 mM Na3P2O7, 10 mM H2O. Lysates were clarified by centrifugation, and the supernatants were divided for immunoprecipitation with polyclonal antibodies to Sos, Grb2, or Shc. Immunoprecipitations were performed for 4 h at 4°C with the addition of protein A-agarose for the final hour, and the pellets were washed three times and boiled in SDS-PAGE sample buffer. Samples were resolved on 5–15% SDS-PAGE gels and electroblotted onto nitrocellulose. After blocking in 50 mM Tris-HCl, pH 7.5, NaCl 150 mM, 0.1% Tween 20, and 2.5% bovine serum albumin, membranes were incubated with the indicated primary antibody, washed, incubated with 1/1000 dilution of Amersham sheep anti-mouse Fc horseradish peroxidase-linked antibody, washed, and developed by ECL (Amersham).

Measurement of GTP- and GDP-bound p21ras—Confluent 60-mm dishes of HIRcB cells were serum-starved for 24 h and labeled with 0.5 mCi of [32P]orthophosphate/dish for 4 h. During the final hour of labeling, 20 μM PD098059 with 1% Me2SO, or 1% Me2SO alone, was added. After labeling, cells were stimulated with 17 nM insulin for the indicated times, and quantitation of p21rasbound GTP and GDP was performed essentially as described (20). Medium was aspirated, and cells were washed twice in ice-cold Tris-buffered saline and lysed in 1 ml of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM MgCl2, 0.5% Nonidet P-40, 10 μg/ml aprotinin, 1% phenylmethylsulfonyl fluoride, and 1 mM Na3VO4. The lysates were cleared by centrifugation, excess free nucleotides were removed by incubation with activated charcoal, and samples were frozen in a dry ice/methanol bath. After thawing at 37°C for 3 min, anti-Ras monoclonal rat antibody Y13-259 and protein A-agarose precoupled to rabbit anti-rat IgG were added for 1 h at 4°C. Immune complexes were washed twice with lysis buffer and twice with lysis buffer lacking Nonidet P-40, and nucleotides were eluted in 20 μl Tris-HCl, pH 7.5, 20 mM EDTA, 2% SDS, 0.5 mM GTP, and 0.5 mM GDP by heating at 65°C for 5 min. Thin layer chromatography was performed on polyethyleneimine cellulose in 0.75 mM KH2PO4, pH 3.4. Results were quantitated directly using a PhosphorImager.

RESULTS AND DISCUSSION

Growth-factor-induced serine/threonine phosphorylation of Sos is mediated by kinases downstream of Ras in the Raf1/MEK/MAP kinase cascade activated by p21ras (28). To examine the role of Sos phosphorylation in insulin signaling, an inhibitor of MEK activity, PD098059, was used. This compound is relatively specific for MEK with no inhibitory activity against a number of other serine/threonine and tyrosine kinases (29). Insulin stimulation of HIRcB cells resulted in a significant retardation of mobility of Sos on SDS-PAGE, reflecting Sos phosphorylation, and mobility shift compared to total cellular Sos, suggesting that the Sos which remained bound to Grb2 represented a subpopulation of Sos that was less heavily phosphorylated. MAP kinase has been shown to phosphorylate Sos in vitro (30), and there are several MAP kinase consensus phosphorylation sequences in the C terminus of Sos (31, 32), suggesting that phosphorylation of Sos by MAP kinase may disrupt binding of Sos to the SH3 domains of Grb2.

Insulin stimulation leads to binding of the Grb2 SH2 domain to tyrosine-phosphorylated Shc, which plays a key role in insulin-stimulated mitogenesis, presumably by targeting Sos to the plasma membrane where it activates p21ras (19, 33, 34). Thus, the importance of Grb2/Sos dissociation may be that it decreases linkage of Sos to Shc. Consistent with this notion, Shc/Sos coprecipitation studies showed only a transient association, seen at 1 and 5 min and gone by 10 min (Fig. 1C, lanes 1–6), while in cells treated with PD098059, the Sos/Shc interaction persisted for 120 min (Fig. 1C, lanes 7–12). Shc/Grb2 coimmunoprecipitation analysis revealed minimal or no basal coprecipitation; insulin treatment induced significant Shc/Grb2 association by 1 min, which was maximal by 5 min and unchanged thereafter, and was unaffected by the MEK inhibitor (Fig. 1D). As expected, Shc/Grb2 association correlated closely with the course of Shc tyrosine phosphorylation (Fig. 1E), which was also unaffected by the inhibitor. Thus, initial formation of the Shc/Grb2/Sos complex is caused by binding of preformed Grb2/Sos complexes to tyrosine-phosphorylated Shc. However, disassembly of the Shc/Grb2/Sos trimer is caused by rapid phosphorylation of Sos and dissociation of Sos from Grb2, leaving the Shc/Grb2 complex intact, while Sos becomes free (and presumably unable to activate p21ras). As Shc remains bound to Grb2, it is unavailable to bind to any residual free Grb2/Sos complexes, which might otherwise contribute to p21ras activation. MEK inhibition blocks Sos phos-
The association of Grb2 with Shc, illustrated in Fig. 3, is transient, despite ongoing growth factor stimulation (10, 20–24). p21ras activation by insulin in HIRcB cells was transient, with peak p21ras-GTP levels by 7 min and a subsequent rapid decline (Fig. 2). This correlates closely with the time course of Shc/Grb2/Sos association (Fig. 1C), which peaked at 5 min and fell by 10 min. In contrast, MEK inhibition resulted in prolonged stimulation of p21ras-GTP, with peak levels not seen until 15 min, and only a gradual decline thereafter. Taken together, the data strongly suggest that dissociation of SOS from Grb2 plays a key role in limiting the duration of activation of p21ras in response to ongoing insulin stimulation. This would be analogous to a negative feedback loop described in Saccharomyces cerevisiae, in which glucose induced elevated levels of cAMP via a Ras adenyl cyclase pathway activated by the GEF Cdc25. The resultant rapid activation of cAMP-dependent protein kinase then caused phosphorylation of Cdc25 and caused it to dissociate from Ras, resulting in Ras deactivation (35).

Rat1 fibroblasts express \(-10^5\) EGF receptors/cell, and a similar analysis of the association patterns of Shc, Grb2, and Sos upon EGF stimulation was performed. EGF also caused Sos phosphorylation (Fig. 3A), and this was correlated with dissociation from Grb2 and from Shc (Fig. 3B and C). The MEK inhibitor PD098059 prevented Sos phosphorylation, as well as dissociation of Sos from Grb2 and Shc (Fig. 3, A–C). Interestingly, the association of Grb2 with Shc, illustrated in Fig. 3D, occurred rapidly but was not as sustained as it was with insulin stimulation (Fig. 1D). This may reflect down-regulation of the low number of endogenous EGFRs, and could explain why at 60 and 120 min there was only a minimal Sos mobility shift (Fig. 3A, lanes 5 and 6), and significant Grb2/Sos reassociation (Fig. 3B, lanes 5 and 6). Furthermore, it explains why, in the presence of PD098059, Sos coprecipitation with Shc declines by 30 min (i.e., although Sos and Grb2 remain in a complex, much of Grb2 has dissociated from Shc). Interestingly, while some Grb2/Sos reassociation was seen at 40 and 120 min, there was no concomitant coprecipitation of Sos with Shc (Fig. 3C, lanes 5 and 6), suggesting that only Grb2 already dissociated from Shc reassociated with Sos. Thus, the phenomenon of Sos phosphorylation, with Grb2/Sos complex dissociation reducing the duration of activation of the active Shc/Grb2/Sos complex, is also observed with signaling through the EGFR receptor.

Prolonged p21ras activation, either by oncogenic forms of p21ras (36) or by activation of endogenous p21ras by the introduction of upstream activators such as membrane-targeted Sos (34), is a transforming event that plays a significant role in many malignancies (37). Thus, tight control of p21ras activation is important in growth regulation. Growth factors typically cause a transient rise in p21ras-GTP formation and the subsequent fall back to baseline values serves to attenuate the hormonal signal, and may make cells refractory to subsequent growth factor stimulation. The current studies indicate that the mechanism underlying this attenuation process involves hyperphosphorylation of Sos on serine/threonine residues with subsequent dissociation of Grb2/Sos complexes; p21ras activates the MAP kinase pathway, and this feedback signal is generated from MEK or a serine/threonine kinase downstream of MEK.

Acknowledgment—We thank L. Collins for assistance with the p21ras-GTP assay.

REFERENCES
1. Burgering, B. M. T., Medema, R. H., Maassen, J. A., van de Wetering, M. L., van der Eb, A. J., McCormick, F., and Bos, J. L. (1993) EMBO J. 5, 1103–1109
2. J. hun, B. H., Meinloth, J. L., Letner, J. W., Draznin, B., and Olefsky, J. M. (1994) J. Biol. Chem. 269, 5699–5704
3. Trahey, M., and McCormick, F. (1997) Science 278, 542–545
4. Simon, M. A., Bowtell, D. D. L., Dodson, G. S., Laverty, T. R., and Rubin, G. M. (1991) Cell 67, 701–716
5. Buday, L., and Downward, J. (1993) Cell 73, 611–620
6. Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M., and Weinberg, R. A. (1993) Nature 363, 45–51
7. Baltensperger, K., Koama, L. M., Cherniak, A. D., Klarlund, J. K., Chawla, A., Banejee, U., and Czech, M. P. (1993) Science 260, 1950–1952
8. Medema, R. H., de Vries-Smit, A. M. M., van der Zon, G. C. M., Maassen, J. A., and Bos, J. L. (1993) Mol. Cell. Biol. 13, 155–162
9. Draznin, B., Chang, L., Letner, J. W., Takata, Y., and Olefsky, J. M. (1993) J. Biol. Chem. 268, 19998–20001
10. Chardin, P., Camonis, J. H., Gale, N. W., Van Aelst, L., Schlessinger, J., Wagler, M. H., and Bar-Sagi, D. (1993) Science 260, 1338–1343
11. Rozakis-Adcock, M., Fennelly, R., Wade, J., and Bown, T., and Bowtell, D. (1993) Nature 363, 45–51
12. Liu, N., Batzer, A., Daly, R., Jahnk, V. Skudnik, E., Chardin, P., Bar-Sagi, D., Margolis, B., and Schlessinger, J. (1993) Nature 363, 85–88
13. Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallio, F., Forini, G., Nicoletti, L., and Melchiorri, P. (1992) Cell 70, 93–104
14. Kavanaugh, W. M., and Williams, L. T. (1994) Science 266, 1626–1628
15. Blakie, P., Immanuel, D., Wu, J., Li, N., Jahnk, V., and Margolis, B. (1994) J. Biol. Chem. 269, 19998–20001
Negative Feedback Regulation of Ras Activation

25323

J. Biol. Chem. 269, 32031–32034
16. Sasaoka, T., Langlois, W. J., Leitner, J. W., Draznin, B., and Olefsky, J. M. (1994) J. Biol. Chem. 269, 32031–32034
17. Sasaoka, T., Draznin, B., Leitner, J. W., Langlois, W. J., and Olefsky, J. M. (1994) J. Biol. Chem. 269, 10734–10738
18. Sasaoka, T., Rose, D. W., Jhun, B. H., Saltiel, A. R., Draznin, B., and Olefsky, J. M. (1994) J. Biol. Chem. 269, 13689–13694
19. Pronk, G. J., de Vries-Smits, A. M. M., Buday, L., Downward, J., Maassen, J. A., Medema, R. H., and Bos, J. L. (1994) Mol. Cell. Biol. 14, 1575–1581
20. Gibbs, J. B., Marshall, M. S., Schnick, E. M., Dixon, R. A. F., and Vogel, U. S. (1995) J. Biol. Chem. 265, 20437–20442
21. Nakafuku, M., Satoh, T., and Kaziro, Y. (1992) J. Biol. Chem. 267, 19448–19454
22. Buday, L., and Downward, J. (1993) Mol. Cell. Biol. 13, 1903–1910
23. Hashimoto, Y., Matsuoka, K., Takenawa, T., Muroya, K., Hattori, S., and Nakamura, S. (1994) Oncogene 9, 869–875
24. Cherniack, A. D., Klarlund, J. K., Conway, B. R., and Czech, M. P. (1995) J. Biol. Chem. 270, 1485–1488
25. Waters, S. B., Yamauchi, K., and Pessin, J. E. (1995) Mol. Cell. Biol. 15, 2791–2799
26. de Vries-Smits, A. M. M., Pronk, G. J., Medema, J. P., Burgering, B. M. T., and Bos, J. L. (1995) Oncogene 10, 939–925
27. McClain, D. A., Maegawa, H., Lee, J., Dull, T. J., Ullrich, A., and Olefsky, J. M. (1987) J. Biol. Chem. 262, 14663–14671
28. Burgering, B. M. T., Pronk, G. J., van Weeren, P. C., Chardin, P., and Bos, J. L. (1993) EMBO J. 12, 4211–4220
29. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) Proc. Natl. Acad. Sci. U. S. A., in press
30. Cherniack, A. D., Klarlund, J. K., and Czech, M. P. (1994) J. Biol. Chem. 269, 4717–4720
31. Gonzalez, F. A., Raden, D. L., and Davis, R. J. (1991) J. Biol. Chem. 266, 22159–22163
32. Clark-Lewis, I., Sanghera, J. S., and Pelech, S. L. (1991) J. Biol. Chem. 266, 15180–15184
33. Sasaoka, T., Draznin, B., Leitner, J. W., Langlois, W. J., and Olefsky, J. M. (1994) J. Biol. Chem. 269, 10734–10738
34. Aronhein, A., Engelberg, D., Li, N., al-Alawi, N., Schlessinger, J., and Karin, M. (1994) Cell 78, 949–961
35. Gross, E., Goldberg, D., and Levitzki, A. (1992) Nature 356, 762–765
36. Stacey, D. M., and Kung, H. F. (1984) Nature 310, 508–511
37. Bos, J. L. (1989) Cancer Res. 49, 4662–4669