Desensitization of p21\(^{ras}\) after stimulation of cells by growth factors and phorbol 12-myristate 13-acetate (PMA) correlates with hyperphosphorylation of the guanine nucleotide exchange factor Son-of-sevenless (Sos) and its dissociation from the adaptor protein Grb2 (Cherniack, A.; Klarlund, J. K.; Conway, B. R.; and Czech, M. P. (1995) J. Biol. Chem. 270, 1485–1488). To test the role of the Raf/mitogen-activated protein (MAP) kinase pathway, we utilized cells expressing a chimera composed of the catalytic domain of p74Raf-1 and the hormone binding domain of the estradiol receptor (ΔRaf-1:ER). Estradiol markedly stimulated ΔRaf-1:ER and the downstream MEK and MAP kinases in these cells as well as Sos phosphorylation. However, the dissociation of Grb2 from Sos observed in response to PMA was not apparent upon ΔRaf-1:ER activation. Furthermore, stimulation of ΔRaf-1:ER did not impair GTP loading of p21\(^{ras}\) in response to platelet-derived growth factor or epidermal growth factor. We conclude that activation of the Raf/MAP kinase pathway alone in these cells is insufficient to cause disassembly of Sos from Grb2 or to interrupt the ability of Sos to catalyze activation of p21\(^{ras}\).

The proto-oncogene product p21\(^{ras}\) serves as a pivotal intermediate in the transmission of signals from numerous growth factor receptors and the insulin receptor to downstream effectors (1–8). p21\(^{ras}\) exists in an inactive GDP-bound state but is converted to the active GTP-bound state through the activities of guanine nucleotide exchange factors, such as Son-of-sevenless (Sos\(^2\)). Proline-rich regions in the COOH terminus of Sos bind src homology 3 (SH3) groups present in adaptor proteins such as Grb2, which also contains an src homology 2 (SH2) group. Upon stimulation of cells with growth factors, the Sos/Grb2 complex is recruited to the plasma membrane via the binding of the SH2 group of Grb2 to tyrosine-phosphorylated sites on activated receptors, to phospholipid-tyrosine 317 on SHC, or possibly to other tyrosine phosphates on membrane-associated proteins (9–16).

GTP-loaded p21\(^{ras}\) binds to the amino-terminal part of the protein kinase Raf, which is thereby translocated to the plasma membrane where it undergoes an activation process (17–21). These events initiate the activation of a cascade of protein kinases. Mitogen-activated protein kinase kinase (MEK) is apparently directly phosphorylated by Raf, resulting in its activation. MEK in turn stimulates mitogen-activated protein kinase (MAPK) by causing its phosphorylation on a tyrosine and a threonine residue. Whereas the substrate specificities of Raf and MEK are extremely limited, MAPK can phosphorylate a broad spectrum of proteins and has been shown to regulate numerous cellular enzymes such as phospholipase A\(_2\), nuclear transcription factors, and other protein kinases (22).

An important aspect of the function of p21\(^{ras}\) and other intracellular signaling molecules is that their activation is reversible. The activity of p21\(^{ras}\) is typically found to peak 2–5 min after growth factor stimulation, and it declines to near basal levels after 20 min. The deactivation mechanism, however, is not well understood. Grb2 in 3T3-L1 adipocytes and CHO-IR cells partially dissociates from Sos after stimulation with insulin or platelet-derived growth factor, which presumably results in disruption of p21\(^{ras}\)-activating complexes (23–25). After stimulation and desensitization of 3T3-L1 adipocytes with epidermal growth factor (EGF), no such dissociation is seen, but p21\(^{ras}\) deactivation in this case may be explained by the rapid down-regulation of EGF receptors observed in this system (24).

As has been noted by several groups, Sos is highly phosphorylated after growth factor stimulation (12, 23, 26–28). The time course of this phosphorylation suggests a potential role in the deactivation rather than the activation phase of p21\(^{ras}\) regulation. A negative feedback role of MAPK in deactivating p21\(^{ras}\) is attractive for several reasons. The existence of negative feedback by a downstream kinase is likely in Saccharomyces cerevisiae since the p21\(^{ras}\) exchange factor CDC25 is phosphorylated by protein kinase A, and the exchange factor is concomitantly released from the membrane (29). A large number of consensus sites for MAPK phosphorylation are found in the COOH-terminal region of Sos protein, and we have previously reported that at least two sites are phosphorylated in vitro and in intact cells heterologously expressing Drosophila Sos (30). Inhibition of the Raf/MAPK pathway at the level of MEK either by a cell-permeable inhibitor or by a dominant negative mutant of MEK blocked Grb2/Sos dissociation in response to insulin and the deactivation of p21\(^{ras}\) (28, 31). Taken together, these observations strongly imply that MAPK or other protein kinases downstream of MEK are necessary for both Grb2/Sos dissociation and for the deactivation of p21\(^{ras}\) seen after the initial activation by insulin. The aim of the work presented here was to assess whether the protein kinases in the Raf/MAPK pathway or other downstream kinases are indeed sufficient to desensitize p21\(^{ras}\) to growth factor stimulation or whether other events are also necessary. We report here that activation of the Raf/MAPK pathway, independent of growth factor stimulation, does not cause dissociation of Grb2.
Role of Raf/MAP Kinase Pathway in p21ras Desensitization

EXPERIMENTAL PROCEDURES

Materials—The anti-phosphotyrosine antibody PY20 and monoclonal anti-Grb2 antibody was from Transduction Laboratories. For immunoprecipitation of Sos, an antibody raised against a peptide corresponding to amino acids 100–120 was used (23). For immunoblotting of Sos, an antibody to a COOH-terminal part of the Sos-1 was kindly provided by Dr. H. Meisner (32). PMA was from LC laboratories. Estradiol was from Sigma. Platelet-derived growth factor-BB (PDGF-BB) and EGF were from Upstate Biotechnology.

Cell Culture—3T3Raf-1:ER cells (33) were grown in 10-cm plates (Corning) in Dulbecco’s modified Eagle’s medium (DMEM), with 10% fetal calf serum (Upstate Biotechnology), 50 μg/ml streptomycin sulfate, 50 μg/ml penicillin, and 50 μM ganciclovir.

Cell Lysis, Immunoprecipitation, and Immunoblotting—3T3Raf-1:ER cells were serum starved and labeled with 32P, overnight and stimulated with 10−7 M EGF or 10−8 M PDGF for the indicated times. p21ras was immunoprecipitated, and the bound guanosine nucleotide was analyzed on polyethylamine thin-layer plates as described under “Experimental Procedures.” A, autoradiography of plates. B, calculated ratio of [32P]GTP/[32P]GDP + [32P]GTP as a function of time. The values are means of duplicate measurements, and the error bars represent the standard deviations.

RESULTS AND DISCUSSION

A chimeric protein containing the catalytic domain of oncogenic Raf-1 fused to the hormone binding region of the estradiol receptor (ΔRaf:1:ER) exhibits protein kinase activity that can be rapidly activated by addition of estradiol, resulting in activation of endogenous MEK and MAPK (33, 36). Cells stably expressing ΔRaf:1:ER therefore allow examination of the effects of activation of Raf-1/MAPK kinase cascade independently of other signaling pathways. We first examined the time course of p21ras activation in response to EGF and PDGF in C7 3T3 cells expressing ΔRaf:1:ER (3T3Raf:1:ER cells) to verify that deactivation does occur in these cells after initial activation by the growth factors. As is seen in Fig. 1, both hormones rapidly induce a 3–4-fold increase in GTP loading of p21ras, and this is followed by a deactivation phase resulting in the return of GTP-p21ras concentrations to basal levels after 20–30 min. As seen in other systems, the deactivation of p21ras occurs in parallel with hyperphosphorylation of mSos as evidenced by its decreased mobility on SDS-PAGE (data not shown).
Sos contains numerous consensus sites for MAPK phosphorylation in its COOH-terminal portion and can be phosphorylated by MAP kinase both in vitro and in intact cells (30). We thus tested whether MEK inhibition abolishes Sos hyperphosphorylation and Grb2 dissociation from Sos, as reported recently for Chinese hamster ovary cells or rat-1 cells overexpressing the insulin receptor (28, 31). 3T3Δraf-1:ER cells were treated with or without the potent MEK inhibitor, PD98059 (37), and incubated in the presence or absence of PMA to initiate the phosphorylation and disassembly of Sos from Grb2. Fig. 2 shows that these two previously reported effects of PMA in 3T3-L1 adipocytes are also observed in 3T3Δraf-1:ER cells. Importantly, the MEK inhibitor blocked both the retarded electrophoretic mobility of Sos (panel A) as well as the 50% loss of Grb2 from immunoprecipitated Sos (panels B and C) caused by PMA. These data confirm that MEK and presumably MAPK are required for these processes to proceed normally.

To determine whether selective activation of the Raf/MAPK pathway downstream of receptor or protein kinase C elements causes phosphorylation of Sos and its dissociation from Grb2, 3T3Δraf-1:ER cells were treated with or without estradiol or PMA. Estradiol treatment of 3T3Δraf-1:ER cells caused a marked electrophoretic mobility shift of the Sos protein, which was even more extensive than that caused by PMA (Fig. 3A). It is noteworthy that all of the Sos from the untreated cells appears to be converted to the slower migrating species, indicating that virtually all Sos molecules are phosphorylated in response to Δraf-1:ER activation. This system therefore allows rigorous testing of the hypothesis that phosphorylation of Sos by protein kinases in the Raf/MAPK pathway constitutes a feedback mechanism that results in Grb2/Sos dissociation and desensitization of p21ras to growth factor stimulation. Surprisingly, the apparent hyperphosphorylation of Sos by Δraf-1:ER activation was not accompanied by any detectable diminution of Grb2 from anti-Sos immune complexes (Fig. 3, B and C). Under these same experimental conditions, PMA treatment of 3T3Δraf-1:ER cells elicited release of over half of the Grb2 band to cellular Sos protein. These data lead to the unexpected conclusion that selective stimulation of MAPK via Δraf-1:ER activation can cause Sos hyperphosphorylation but is insufficient to signal Grb2/Sos disassembly in 3T3Δraf-1:ER cells.

Although Sos disassembly from Grb2 does not occur in response to MAP kinase activation (Fig. 3), it is possible that GTP:p21ras levels are regulated by MAP kinase through other mechanisms. We tested this hypothesis by treating 32p-labeled 3T3Δraf-1:ER cells with or without estradiol for 30 min prior to a 5-min exposure to either EGF or PDGF and then analyzing the labeled guanosine nucleotide content of p21ras. Fig. 4 demonstrates the failure of estradiol to modulate the extent of p21ras activation by EGF or PDGF under these experimental conditions. Parallel plates of cells treated with estradiol for 30 min were shown to exhibit marked hyperphosphorylation of endogenous Sos, verifying the effectiveness of estradiol to stimulate the Raf/MAPK pathway (data not shown, see Figs. 2 and 3). Thus, activation of MAPK via the Δraf-1:ER is not sufficient to cause desensitization of p21ras under these conditions.

In this same series of experiments (Fig. 3), the cells were also incubated with or without okadaic acid, a potent phosphatase
Role of Raf/ MAP Kinase Pathway in p21\textsuperscript{ras} Desensitization

Inhibitor known to induce or potentiate the activation of several cellular protein kinases including MAPK (38, 39). When added alone to 3T3Rafl:ER cells, this agent only slightly decreased the electrophoretic mobility of Sos (Fig. 3A) and did not detectably dissociate Grb2 from Sos (Fig. 3, B and C). However, incubation of okadaic acid with estradiol-treated 3T3Rafl:ER cells potentiated Sos hyperphosphorylation and caused a marked, 60% decrease in Grb2 associated with Sos protein. Thus, the combined signaling pathways elicited by ΔRafl:ER and okadaic acid are able to cause disassembly of complexes containing Sos and Grb2, whereas each of these pathways acting alone is insufficient to mediate this response.

Previous results showed that prolonged activation of ΔRafl:ER in 3T3 cells induces a partial block in activation of endogenous Raf/MAPK pathway by PDGF, which does not appear to be due to impaired function of the PDGF receptor (36). However, estradiol-treated 3T3Rafl:ER cells induce a partial block in activation of p21\textsuperscript{ras} in response to insulin and PMA (37). This conclusion is reinforced by the observation that EGF does not cause Grb2/Sos dissociation in these cells (24, 40–43). On the other hand, MAPK seems necessary for dissociation of Sos from Grb2 (29, 30). One reasonable hypothesis is that insulin and PMA activate another protein kinase or kinases that are necessary for Grb2/Sos dissociation but that this kinase is not activated by other growth factors such as EGF. Thus, activation of both MAP kinase and another unidentified protein kinase might be required to phosphorylate the putative multiple sites on Sos necessary for Grb2 dissociation. This dissociation presumably contributes to the desensitization of p21\textsuperscript{ras} (for detailed discussion, see Ref. 24). The deactivation that occurs after initial p21\textsuperscript{ras} activation in response to EGF could be caused by a different mechanism: down-regulation of EGF receptors (24, 44). Clearly, a more precise definition of the protein kinases that phosphorylate Sos will be of importance in understanding the molecular basis of Grb2/Sos dissociation in response to insulin and PMA.

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