Divergent Mechanisms for Homologous Desensitization of p21\textsuperscript{ras} by Insulin and Growth Factors*

(Received for publication, July 14, 1995, and in revised form, August 2, 1995)

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Previous work suggested that desensitization of p21\textsuperscript{ras} in response to growth factors such as epidermal growth factor (EGF) results from receptor down-regulation. Here we show that p21\textsuperscript{ras} is desensitized by insulin in 3T3-L1 adipocytes in the continued presence of activated insulin receptors, while loss of epidermal growth factor and platelet-derived growth factor (PDGF) receptors in response to their ligands correlates with p21\textsuperscript{ras} desensitization. Furthermore, elevated amounts of Grb2/Sc3 complexes persisted throughout p21\textsuperscript{ras} desensitization by insulin. However, immunoblotting of anti-Son-of-sevenless (Sos) 1 and 2 immunoprecipitates with anti-Grb2 antisera revealed that p21\textsuperscript{ras} desensitization in response to insulin and PDGF, but not EGF, is associated with a marked decrease in cellular complexes containing Sos and Grb2 proteins. Nonetheless, the desensitization of p21\textsuperscript{ras} in response to these stimuli was homologous, in that each peptide could reassociate [32P]GTP loading of p21\textsuperscript{ras} after desensitization by any of the others. Taken together, these data indicate that insulin, EGF, and PDGF all cause disassembly of Sos proteins from signaling complexes during p21\textsuperscript{ras} desensitization, but at least two mechanisms are involved. Insulin elicits dissociation of Sos from Grb2 SH3 domains, whereas EGF signaling is reversed by receptor down-regulation and Shc dephosphorylation, releasing Grb2 SH2 domains. PDGF action triggers both mechanisms of Grb2 disassembly, which probably operate in concert with GAP to attenuate p21\textsuperscript{ras} signaling.

Peptide growth factors are key extracellular regulators that modulate pathways of intermediary metabolism, protein synthesis, and mRNA transcription. Growth factors also mediate critical steps in cell cycle control and DNA synthesis. This remarkable multitude and diversity of biological effects raise important questions about the molecular signaling mechanisms involved in the actions of these peptides. Recent work has revealed that several signaling pathways are simultaneously stimulated by growth factors, including the phosphoinositide cycle (1–3), p21\textsuperscript{ras} (4–10), and the phosphatidylinositol 3-kinase reaction (11–14). These pathways initiate downstream events that must be highly coordinated, controlled, and ultimately extinguished to elicit appropriate types and duration of biological effects. Thus, understanding mechanisms that restrain these signaling circuits is an important aspect of the knowledge base required to fully describe them in molecular terms.

Cellular stimulation by peptide growth factors through small GTP binding proteins exemplifies the highly regulated nature of intermediary steps in signaling pathways. The p21\textsuperscript{ras} proteins cycle between the inactive, GDP-bound form and a GTP-bound, biologically active state through the actions of guanosine nucleotide exchange factors that catalyze release of GDP from p21\textsuperscript{ras}, allowing GTP to bind, and GTPase activating proteins which enhance p21\textsuperscript{ras}-bound GTP hydrolysis to GDP (4–10). Growth factors and insulin are thought to activate p21\textsuperscript{ras} by recruitment of guanosine nucleotide exchange factors such as the Son-of-sevenless (Sos)\textsuperscript{1,2} to tyrosine-phosphorylated Shc proteins through the adaptor Grb2 (15–19). Grb2 binds to tyrosine phosphate on Shc (Tyr-317) through its Src homology SH2 domain and binds proline-rich regions on Sos proteins through its SH3 domains (17, 20). In the case of insulin but not EGF or PDGF, another tyrosine-phosphorylated protein, IRS-1, also binds Grb2 and may be involved in p21\textsuperscript{ras} activation (21, 22). Rapid increases in GTP loading of p21\textsuperscript{ras} proteins in response to growth factors is followed by a deactivation phase whereby GTP-p21\textsuperscript{ras} concentrations return to near basal levels (23–27). Activated, GTP-bound p21\textsuperscript{ras} associates with the N-terminal, regulatory domain of Raf protein kinases, leading to events that elevate Raf kinase activity (28–30). The Raf kinases in turn phosphorylate and activate MEK protein kinases, which further activate a cascade of protein kinases, including the MAP kinases (6, 28, 31–34). Importantly, MAP kinase activation by growth factors through this mechanism is also transient and returns to near basal levels with about the same time course as p21\textsuperscript{ras} deactivation (35–38). Thus, important feedback mechanisms operate to restrain this signaling pathway and control the extent of cellular modulation.

Previous work indicated that desensitization of p21\textsuperscript{ras} caused by EGF is associated with rapid disappearance of EGF receptors from the cell surface (39), suggesting a simple mechanism for the desensitization. Consistent with the concept that EGF-mediated down-regulation of its specific receptors causes the loss of p21\textsuperscript{ras} responsiveness to EGF, insulin was found to reactivate p21\textsuperscript{ras} after desensitization by EGF (39). However, interpretation of those studies is difficult because insulin itself did not elicit p21\textsuperscript{ras} desensitization under the conditions of the experiments. This was perhaps due to the use of a unique cell line heterologously expressing very high levels of human insulin receptors because we have recently reported marked p21\textsuperscript{ras} desensitization in response to insulin in 3T3-L1 adipocytes (40). We and others have also found that insulin caused partial dissociation of Grb2:Sos complexes, suggesting an alternative mechanism of deactivation (40, 41). Furthermore, no detailed studies have appeared which evaluate the basis for PDGF-induced p21\textsuperscript{ras} desensitization. Thus, the aim of the present investigation was to characterize the molecular nature of

1 The abbreviations used are: Sos, son-of-sevenless; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol 12-myristate 13-acetate.
p21\textsuperscript{ras} activation and deactivation in a well established model system, the 3T3-L1 adipocyte, that responds to insulin, EGF, and PDGF without overexpressed receptors. We demonstrate here that insulin, EGF, or PDGF treatment of 3T3-L1 adipocytes causes a rapid desensitization of p21\textsuperscript{ras} following the initial activation phase, but that reactivation can be achieved by either of the two other peptides. Importantly, we show that insulin-mediated p21\textsuperscript{ras} desensitization occurs without loss of activated cell surface insulin receptors or Shc/Grb2 complexes, in contrast to EGF-mediated p21\textsuperscript{ras} desensitization. Furthermore, both insulin and PDGF cause disassembly of Grb2:Sos complexes in these cells, while EGF does not. These data demonstrate that receptor down-regulation cannot explain the homologous p21\textsuperscript{ras} desensitization caused by insulin and that regulation of Sos function through its dissociation from Grb2 by both insulin and PDGF may play an important role in this process.

MATERIALS AND METHODS

Cell Culture—3T3-L1 mouse fibroblasts were grown to confluence in Dulbecco's modified Eagle's medium with 10% calf serum, 50 units/ml penicillin, and 50 \mu g/ml streptomycin sulfate and were differentiated to adipocytes as described previously (42). Adipocytes were used 9–14 days after the start of differentiation. Prior to stimulation with growth factors, cells were serum starved for 16–24 h in Dulbecco's modified Eagle's medium with 0.5% bovine serum albumin.

Antibodies—Anti-mSos antibody was raised against a peptide corresponding to amino acids 100–120 which are identical in mSos1 and mSos2 (15). Antibodies to mSos1 and 4G10 anti-phosphotyrosine antibody were from Upstate Biotechnology Incorporated. Monodonal antibody to Grb2 and anti-phosphotyrosine (PY20) antibody were from Transduction Laboratories. Monodonal anti-p21\textsuperscript{ras} antibody was obtained from supernatants of the hybridoma cell line Y13–259 (American Type Culture Collection).

Plasma Membrane Preparation—Plasma membranes were prepared from 3T3-L1 adipocytes in 15-cm plates as described previously (43).

Ras Activation Assay—3T3-L1 adipocytes in 10-cm plates were incubated with 3.5 \mu l of phosphate-free Dulbecco's modified Eagle's medium with 25 \mu M HEPES, pH 7.4, 2 \mu M sodium pyruvate, and 1 miU of carrier-free \([\text{32P}]\text{orthophosphate}\) at 37°C. After 16 h, the cells were transferred to 22°C for 30 min and stimulated for the indicated times with growth factors. The cells were then rapidly washed and lysed by addition of 800 \mu l of lysis buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM MgCl\(_2\), 1\% Triton X-100, 1\% dithiothreitol, 1\% phenylmethylsulfonyl fluoride, 1 mM benzamidine, and leupeptin at 0.5 \mu g/ml each with 10% tissue culture supernatant from the hybridoma cell line Y13–259). After clarification by centrifugation, the lysates were incubated for 1 h with 5 \mu l of Sepharose 4B coupled to goat anti-rat antibodies (Organon Teknika-Cappel). The beads were collected by centrifugation and washed extensively in 50 \mu l of PBS supplemented with 0.1% Triton X-100, 2 mM sodium orthophosphate, 50 mM NaF, 1 mM sodium pyruvate, and 1 mM benzamidine. The lysates were spun at 15,000 \times g for 10 min at 4°C. The supernatants were removed and assayed for total protein content using the Bradford (45) method. After normalization of protein, the lysates were then preclayed by the addition of 10 \mu l of protein A-Sepharose (Pharmacia Biotech Inc.) and incubated on an end-over-end mixer at 4°C for 1 h. Samples were then centrifuged at 15,000 \times g for 2 min at 4°C, and supernatants were incubated on an end-over-end mixer with 10 \mu l of antibody and 25 \mu l of protein A-Sepharose for 16 h. The Sepharose was pelleted by centrifugation at 15,000 \times g for 2 min at 4°C. Pellets were washed five times with cold wash buffer (PBS with 0.1% Triton X-100, 2\% p-nitrophenyl phosphate, 50 mM NaF, 1 mM Na\textsubscript{2}VO\textsubscript{4}), and the protein was dissolved in SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE and transferred to nitrocellulose filters. Filters were probed with the indicated antibodies and bound antibody was visualized using Renaissance (DuPont NEN) according to the manufacturer's specifications. For visualization of the tyrosine phosphorylated insulin receptor, the anti-phosphotyrosine antibody 4G10 was used. For visualization of the EGF receptor, the anti-phosphotyrosine antibody PY20 was used, since the 4G10 antibody was found to react poorly with the tyrosine-phosphorylated EGF receptor.

RESULTS

Most studies on p21\textsuperscript{ras} regulation by insulin have been performed on cells overexpressing insulin receptors rather than primary fat or muscle cells. Cultured 3T3-L1 adipocytes were chosen for the present studies because they have been extensively used as a model system for insulin sensitive tissues and are highly responsive to the hormone. In order to characterize the dynamics of GTP loading of p21\textsuperscript{ras} in response to insulin, EGF, and PDGF in 3T3-L1 adipocytes, the GTP and GDP contents of p21\textsuperscript{ras} were determined at various times after addition of insulin, EGF (Fig. 1) or PDGF (Fig. 2) to \(^{32P}\)-labeled cells. These measurements were conducted at room temperature because greater responses to growth factors were observed compared to 37°C. Nucleotides bound to p21\textsuperscript{ras} from \(^{32P}\)orthophosphate-labeled cells were analyzed by thin layer chromatography. GTP accounted for about 10% of total labeled GTP plus GDP in p21\textsuperscript{ras} immunoprecipitates from control cells (Figs. 1 and 2).

Treatment of the cultured adipocytes with insulin, EGF, or PDGF caused a 2–3-fold increase in labeled GTP recovered from p21\textsuperscript{ras} immunoprecipitates within 5 min. A gradual decay in this effect was observed in the continued presence of these growth factors, resulting in the return of GTP-p21\textsuperscript{ras} concentrations to near basal levels by 120–180 min (Figs. 1 and 2). Interestingly, maximal \(^{32P}\)GTP loading of p21\textsuperscript{ras} in response...
to stimulation of 3T3-L1 adipocytes at 37 °C was also observed by 5 min, but the deactivation phase was much more rapid at the higher temperature (not illustrated). These data demonstrate that insulin, EGF, and PDGF cause similar stimulatory effects on steady-state GTP binding to endogenous p21\textsuperscript{ras} in these cells, followed by a decay to GTP-p21\textsuperscript{ras} levels approaching those observed in the basal state within 2–3 h at room temperature and 20–30 min at 37 °C.

It has been proposed (39) that receptor down-regulation accounts for p21\textsuperscript{ras} desensitization in response to EGF, based on the observed rapid loss of cell surface receptors that paralleled the deactivation of p21\textsuperscript{ras}. However, certain receptor tyrosine kinases known to desensitize p21\textsuperscript{ras}, such as the insulin receptor, recycle to the plasma membrane in response to ligand-mediated endocytosis, ensuring a high steady-state cell surface receptor content (46). Experiments were designed to determine whether tyrosine-phosphorylated receptors in the plasma membrane of 3T3-L1 adipocytes remain at high levels during prolonged insulin, EGF, or PDGF treatment. As shown in Fig. 3, insulin receptor β subunit, EGF, and PDGF receptors in plasma membranes were readily visualized by immunoblotting with anti-tyrosine phosphate antibody 5 min after incubation of cultured adipocytes with 100 nM insulin, 100 nM EGF, or 10 nM PDGF, respectively. Little or no receptor tyrosine phosphorylation could be detected in the absence of these peptides. The level of tyrosine-phosphorylated insulin receptor in the plasma membrane fraction of 3T3-L1 adipocytes remained elevated throughout the 2-h incubation period during which p21\textsuperscript{ras} desensitization was observed (Fig. 3). In contrast, EGF or PDGF receptor tyrosine phosphorylation in response to EGF, and PDGF, respectively, markedly decreased during the 3-h p21\textsuperscript{ras} desensitization phase elicited by EGF or PDGF treatment (Fig. 3). These data indicate that EGF and PDGF receptor down-regulation or dephosphorylation correlates with p21\textsuperscript{ras} desensitization, whereas insulin receptors in the plasma membranes of cultured adipocytes remain tyrosine phosphorylated and active throughout the course of p21\textsuperscript{ras} desensitization.

In order to extend the results in Fig. 3, we reasoned that tyrosine phosphorylation of Shc proteins should correlate with the presence of activated receptors in the plasma membrane. Thus, for example, Shc tyrosine phosphorylation should be transient and decrease with a time course similar to the loss of activated EGF receptors from plasma membranes. This should be accompanied by dissociation of Grb2 from Shc. This was tested by immunoprecipitation of Shc proteins from lysates of 3T3-L1 adipocytes treated with insulin or EGF for various times. As shown in Fig. 4, immunoblotting such Shc immunoprecipitates after SDS-PAGE with anti-Grb2 antiserum revealed both insulin and EGF rapidly increased the amount of Grb2 associated with Shc. In the case of insulin stimulation, the amount of Grb2 in Shc complexes remain elevated for 2 h. In contrast, complexes containing Shc and Grb2 formed in response to EGF receptor activation are mostly dissociated during the time course of p21\textsuperscript{ras} desensitization. The time course of this disassembly is consistent with the hypothesis that it constitutes, at least in part, an underlying mechanism of p21\textsuperscript{ras} desensitization in response to EGF.

The results indicating that Grb2 proteins remain associated with Shc during insulin-mediated p21\textsuperscript{ras} desensitization prompted us to examine the interaction of Grb2 and Sos proteins under these conditions. It was recently shown that insulin causes disassembly of Sos from Grb2 during p21\textsuperscript{ras} desensitization (40, 41). Cultured 3T3-L1 adipocytes were incubated at 37 °C with or without insulin, EGF, or PDGF for 20 min to cause p21\textsuperscript{ras} desensitization. Lysates were immunoprecipitated with rabbit anti-mSos antibodies raised against a peptide corresponding to an N-terminal region of murine mSos1 that is identical to mSos2. Immunoblot analysis of such immunoprecipitates with anti-mSos1 antibodies specific to that isoform revealed equivalent amounts of mSos1 present in the lysates under all experimental conditions (Fig. 5). Treatment of cells with insulin, EGF, or PDGF caused a shift in electrophoretic migration of mSos1 proteins, reflecting hyperphosphorylation on serine and threonine residues (23, 47). Immunoblotting of the anti-mSos precipitates with anti-Grb2 antibodies revealed Grb2 associated with Sos proteins (Fig. 5B). Importantly, insulin- and PDGF-mediated p21\textsuperscript{ras} desensitization was associated with a marked decrease in Grb2 content in these Sos immunoprecipitates. PMA, which also causes Sos hyperphosphorylation, mimicked the ability of insulin or PDGF to dissociate Grb2 from Sos (Fig. 5). In contrast, EGF action failed to cause a detectable reduction in complexes containing Sos and Grb2 proteins. Densitometry of the autoradiographs from several such experiments demonstrated a mean inhibition by insulin, PDGF, and PMA of about 60% in the amount of Grb2...
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**FIG. 4.** Association of Grb2 with Shc proteins in cultured adipocytes. 3T3-L1 adipocytes were stimulated with either 10 \textsuperscript{-7} M insulin or 10 \textsuperscript{-7} M EGF for the indicated times. The cells were then lysed, and 500 µg of total cell protein was immunoprecipitated with 2 µg of Shc antibody. A quarter of the immunoprecipitates was transferred to nitrocellulose, and blotted with antibodies to Grb2. A, autoradiography of the blots. NI is precipitation of extracts from the 30 min time point with a non-immune serum. NE is mock precipitation using the anti-Shc antibody, but no extract. B, quantification of Grb2 in the immunoprecipitates by densitometry. The data were normalized to the initial stimulated level of Grb2 (10 min) and were compiled from two experiments.

The fact that p21\textsuperscript{ras} desensitization due to insulin and PDGF action appears associated with the disassembly of cellular Grb2-Sos complexes suggested a desensitization mechanism that may be general rather than selective. On the other hand, EGF-mediated deactivation might be expected to block the action of EGF specifically, based on the rapid down-regulation of its specific receptors. These hypotheses were tested by prolonged stimulation of \textsuperscript{32}P-labeled 3T3-L1 adipocytes with either EGF, PDGF, or insulin to cause activation and deactivation of p21\textsuperscript{ras} proteins, followed by a further addition of either ras after its desensitization to growth factors in other cell types (23–27), we observe a marked decrease of this effect. Consistent with this hypothesis, the potent activation of protein kinases (28, 31–34, 48) and the strong biological enhancement of cell proliferation (4–10). The potent activation of protein kinases (28, 31–34, 48) and the strong biological enhancement of cell proliferation (4–10).

**DISCUSSION**

Stimulated GTP loading of p21\textsuperscript{ras} proteins by growth factors is now well established as a key signaling element in their enhancement of cell proliferation (4–10). The potent activation of protein kinases (28, 31–34, 48) and the strong biological enhancement of cell proliferation (4–10). The potent activation of protein kinases (28, 31–34, 48) and the strong biological enhancement of cell proliferation (4–10).
pocytes (Figs. 1 and 2). EGF and PDGF were more effective in stimulating levels of \( p21^{ras} \)GTP (3-fold) than was insulin (2-fold) at 22°C in these cells, and the deactivation phase was somewhat slower during treatment with EGF or PDGF (3 h) versus insulin (2 h). However, in all cases, the cellular concentrations of \( p21^{ras} \)GTP fell to levels approaching those measured under basal conditions (Figs. 1 and 2), indicating the operation of a strong desensitization process. This was confirmed by the observation that readdition of the growth factor that had initiated the \( p21^{ras} \) activation and deactivation phases resulted in no further stimulation (Figs. 6 and 7).

Experiments with 3T3-L1 adipocytes performed at 37°C showed more rapid desensitization phases for both insulin and EGF treatment, which were complete within 30 min of initial incubation with peptide (not illustrated). Desensitization at 37°C led to levels of \( p21^{ras} \)GTP that were not significantly different than those measured in untreated adipocytes. Furthermore, this rapid time course of \( p21^{ras} \) activation and deactivation observed at 37°C paralleled that of the activation and deactivation phases for MAP kinase activity in response to insulin or EGF in 3T3-L1 cells (49 and data not shown). Taken together, these data demonstrate that effective cellular mechanisms operate in this cultured adipocyte model system to limit the duration of maximal \( p21^{ras} \) signaling in response to insulin and growth factors.

A recent report suggested that little deactivation and desensitization of \( p21^{ras} \) occurs in response to insulin treatment of transfected cultured cells overexpressing insulin receptors (39). In contrast, our present results show unequivocal \( p21^{ras} \) desensitization during prolonged incubation of 3T3-L1 adipocytes with insulin (Figs. 1 and 6). Furthermore, more recent work in our laboratory has demonstrated insulin-mediated \( p21^{ras} \) desensitization in primary rat adipocytes as well (not illustrated).

The failure to observe \( p21^{ras} \) desensitization in response to insulin in the previous study (39) probably reflects the different cell types used. Cells expressing high levels of insulin receptors were employed in those studies. Experiments in our laboratory show that Chinese hamster ovary cells expressing human in-
Insulin receptors at high levels also exhibit both activation and deactivation phases with respect to GTP loading of p21^{ras} upon insulin treatment (not illustrated). Thus it is unlikely that heterologous expression of insulin receptors in cultured cells eliminates the desensitization phenomenon, although perhaps extraordinarily high levels of insulin receptors used in the previous study (39) may account for the difference in results.

It has been suggested that p21^{ras} desensitization occurs through specific down-regulation of growth factor receptors based on studies with EGF (39). In those experiments, EGF-mediated p21^{ras} desensitization was accompanied by rapid disappearance of cell surface EGF receptors, as detected by binding of cells to labeled EGF peptide. The present work demonstrates that stimulated GTP loading of p21^{ras} can be reversed and p21^{ras} desensitized even in the continued presence of activated receptors (Figs. 1 and 3).

Our experimental approach took advantage of the fact that relatively pure preparations of plasma membranes can be prepared from adipocytes (40, 50). Tyrosine-phosphorylated EGF, PDGF, or insulin receptors are readily identified in these membranes very rapidly after treatment of intact cells with growth factors by SDS-PAGE and immunoblotting with anti-tyrosine phosphate antibody (Fig. 3). Three hours after incubation of cultured adipocytes with EGF or PDGF, tyrosine-phosphorylated EGF or PDGF receptors, respectively, were greatly diminished, while tyrosine-phosphorylated insulin receptors remained in these plasma membranes at high levels. These data are consistent with extensive literature documenting the rapid cellular internalization and degradation of EGF and PDGF receptors in response to their respective ligands (51–59), and the rapid recycling of insulin receptors back to the cell surface during insulin treatment (46, 60, 61). The present data, in combination with results of others, show that the decay of EGF or PDGF action on p21^{ras} under the conditions of our experiments could result from loss of their functional cell surface receptors, but this mechanism cannot explain p21^{ras} deactivation in response to insulin.

A major pathway of p21^{ras} activation appears to be phosphorylation of Shc proteins at tyrosine 317, which in turn binds the SH2 domain of Grb2 (20, 47, 62–65). Shc proteins, which contain an SH2 domain and a domain with sequence similarity to collagen, are the products of a single gene that apparently gives rise to multiply spliced mRNA transcripts (63). Upon its activation and autophosphorylation in response to EGF, the EGF receptor binds the SH2 domain of Shc directly through its phosphorylated tyrosines 1173 and 992. Activated EGF receptors also bind Grb2 directly through phosphorylated tyrosines 1068 and 1086. Activated insulin and PDGF receptors do not appear to directly bind Shc or Grb2, but initiate tyrosine phosphorylation of Shc through an unknown mechanism (64, 65). This same alternate mechanism may be shared by EGF receptors because the latter can cause Shc tyrosine phosphorylation even when the tyrosines on the receptor are removed (66, 67). Thus, EGF receptors can apparently act to mobilize Sos proteins through direct binding of Grb2 or Shc, and by an independent pathway leading to Shc phosphorylation. These considerations may explain the observation that GTP loading of p21^{ras} is enhanced more markedly by EGF than by insulin (Fig. 1). Similarly, the time course of EGF-mediated p21^{ras} desensitization is longer than that for insulin (Fig. 1). Consistent with this concept, the present studies show Shc association with Grb2 is more pronounced in response to EGF than insulin at the earliest time point measured in 3T3-L1 adipocytes (Fig. 4).

The time course of Shc/Grb2 association in response to EGF and insulin (Fig. 4) further confirm the postulate that EGF receptors rapidly down-regulate while insulin receptors remain active. The extensive decline in Grb2 associated with Shc after EGF stimulation apparently reflects a decrease in Shc tyrosine phosphorylation state. Insulin action, on the other hand, is not associated with a decrease in cellular Grb2/Shc complexes following the initial stimulation of complex formation observed at 10 min. Tyrosine phosphatase activity is presumably responsible for Shc dephosphorylation during loss of active EGF receptors, although the tyrosine phosphatase involved is not known. In any case, these results provide an independent confirmation that a decay in relevant tyrosine phosphorylation (EGF receptors and Shc) correlates with p21^{ras} desensitization caused by EGF, but not insulin. Thus, as shown in Fig. 9, the activation complex containing EGF receptor, Shc, and Grb2-Sos proteins is hypothesized to disassemble during desensitization, yielding dephosphorylated (and degraded) EGF receptor, dephosphorylated Shc, and released Grb2-Sos complexes. According to this model (Fig. 9, bottom), all of these components are removed from the plasma membrane, while p21^{ras} remains. PDGF receptor down-regulation also occurs in response to PDGF, and thus PDGF-mediated desensitization would be expected to be similar to the EGF system in this respect. In contrast, our data indicate that Sos proteins desensitize p21^{ras} in response to insulin while Shc/Grb2 complexes remain present (Fig. 9, top). PDGF is also able to cause disassembly of Sos/Grb2 complexes (Fig. 5), suggesting that disengagement of both SH2 and SH3 domains of Grb2 from Shc and Sos, respectively, occurs in response to PDGF.

The disassembly of Sos from Grb2 complexes during insulin- and PDGF-mediated p21^{ras} desensitization may reflect an important feedback mechanism elicited by a protein kinase or kinases. Sos is known to be hyperphosphorylated in response to insulin (68) or EGF (69), and PDGF also causes this effect as indicated in Fig. 5A by the shift in electrophoretic mobility of Sos on SDS-PAGE. We previously reported that at least two of the sites phosphorylated in heterologously expressed Drosophila Sos protein in intact cells matched sites phosphorylated in vitro by MAP kinase (70). Phosphorylation of the yeast (Saccharomyces cerevisiae) CDC25 Ras exchange factor has also
been reported to correlate with release of this protein from the plasma membrane and Ras deactivation (71). Recently, it was proposed that MAP kinase phosphorylation of Sos results in its dissociation from Grb2. However, a key finding presented in this study is the failure of EGF to cause Sos dissociation from Grb2 (Fig. 5), in spite of its ability to cause Sos hyperphosphorylation. Both insulin and EGF cause similar stimulations of MAP kinases in 3T3-L1 adipocytes (49 and data not shown). These considerations indicate that phosphorylation of Sos by MAP kinases is not sufficient to cause disassembly of Grb2-Sos complexes. Perhaps a unique protein kinase or kinases are stimulated by insulin and PDGF and cause Sos phosphorylation on a unique site that regulates Grb2 binding. This hypothesis will require further testing. Protein kinase C or protein kinases activated in response to protein kinase C stimulation by PMA appear to catalyze this response as well (Fig. 5).

The fact that only about 60% of the cellular Grb2-Sos complexes are dissociated during p21
\(^{ras}\) by PMA appear to catalyze this response as well (Fig. 5). Thus, the magnitude of the decrease in Sos-Grb2 complexes in response to prolonged insulin treatment approximately coincides with the extent of decrease in GTP-p21
\(^{ras}\) levels during deactivation and desensitization. The amount of Shc-Grb2 complexes present at the plasma membrane probably reflects an equilibrium involving the concentration of tyrosine-phosphorylated Shc and concentration of available Sos-Grb2 complexes. In the case of insulin action, activated receptors and tyrosine-phosphorylated Shc, reflected by Shc phosphorylation. Both insulin and EGF cause similar stimulations of MAP kinases in 3T3-L1 adipocytes (49 and data not shown).

Grb2/Sos complexes are dissociated during p21
\(^{ras}\) desensitization. The amount of Shc,
\(z\)

Phosphorylated Shc than are required for the 3-fold activation of p21
\(^{ras}\). This may be the case because EGF treatment for 10 min is observed to cause much greater recruitment of Grb2 to Shc than does insulin (Fig. 4). Thus, EGF may be able to cause recruitment of sufficient Shc-Grb2-Sos complexes even after depletion of about half of the cellular Grb2-Sos complexes due to insulin action. It is also possible that specific cellular pools of Sos-Grb2 are subject to insulin-mediated dissociation, leaving other pools available for reactivation of p21
\(^{ras}\) by EGF or other external stimuli. Alternatively, EGF and PDGF may recruit a different exchange factor or factors such as the newly reported C3G protein (72). Further experiments are required to definitively link the Grb2/Sos disassembly observed here to the p21
\(^{ras}\) desensitization mechanism. However, the data presented here and elsewhere (40) by our laboratory strongly implicate a link between these two processes. It is also likely that additional mechanisms, presumably involving GAP function, act in concert with receptor down-regulation and Sos/Grb2 dissociation to attenuate p21
\(^{ras}\) signaling.
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