Identification of the Mechanisms Regulating the Differential Activation of the MAPK Cascade by Epidermal Growth Factor and Nerve Growth Factor in PC12 Cells*

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In PC12 cells, epidermal growth factor (EGF) transiently stimulates the mitogen-activated protein (MAP) kinases, ERK1 and ERK2, and provokes cellular proliferation. In contrast, nerve growth factor (NGF) stimulation leads to the sustained activation of the MAPKs and subsequently to neuronal differentiation. It has been shown that both the magnitude and longevity of MAPK activation governs the nature of the cellular response. The activations of MAPKs are dependent upon two distinct small G-proteins, Ras and Rap1, that link the growth factor receptors to the MAPK cascade by activating c-Raf and B-Raf, respectively. We found that Ras was transiently stimulated upon both EGF and NGF treatment of PC12 cells. However, EGF transiently activated Rap1, whereas NGF stimulated prolonged Rap1 activation. The activation of the ERKs was due almost exclusively (>90%) to the action of B-Raf. The transient activation of the MAPKs by EGF was a consequence of the formation of a short lived complex assembling on the EGF receptor itself, composed of Crk, C3G, Rap1, and B-Raf. In contrast, NGF stimulation of the cells resulted in the phosphorylation of FRS2. FRS2 scaffolded the assembly of a stable complex of Crk, C3G, Rap1, and B-Raf resulting in the prolonged activation of the MAPKs. Together, these data provide a signaling link between growth factor receptors and MAPK activation and a mechanistic explanation of the differential MAPK kinetics exhibited by these growth factors.

The MAPK cascade is one of the principal intracellular signaling pathways linking activation of cell surface receptors to cytoplasmic and nuclear effectors. The MAP kinases (MAPKs), ERK1 and ERK2, have been shown to be essential for cellular proliferation as well as for acquisition and mainte-

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‡ The abbreviations used are: MAP, mitogen-activated protein; MAPK, MAP kinase; NGF, nerve growth factor; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; GEF, guanine nucleotide exchange factors; DMEM, Dulbecco’s minimal essential medium; FBS, fetal bovine serum; RB, Ras binding domain; GST, glutathione-S-transferase; PAGE, polyacrylamide gel electrophoresis; MEK, MAPK/ERK kinase; FGF, fibroblast growth factor.

nance of a differentiated phenotype. One of the best studied models employed to examine how the MAPKs act to regulate cellular phenotypes is the rat pheochromocytoma cell line, PC12 cells. These cells respond to epidermal growth factor (EGF) treatment by an increase in mitotic rates (2). In contrast, nerve growth factor (NGF) stimulation of the PC12 cells results in their differentiation into a sympathetic neuron-like phenotype (3). There is compelling evidence that the longevity of MAPK activation governs whether these cells are stimulated either to proliferate or to withdraw from the cell cycle and differentiate into a neuronal phenotype. EGF and other mitogens provoke the evanescent activation of the MAPKs, whereas NGF treatment results in the sustained activation of this signaling pathway (4–6). The question of how different growth factors elicit distinct biological outcomes through common signal transduction elements has provoked considerable interest resulting in a complex and controversial literature.

The MAPK cascade transduces signals from receptor tyrosine kinases to two members of the Ras family of small G-proteins, Ras and Rap1, which then stimulates the sequential activation of Raf serine/threonine kinases (c-Raf and B-Raf), MEK, and the MAPKs (ERK1 and ERK2) (7, 8). Recent studies of NGF-stimulated PC12 cells suggest that Ras functions principally to activate c-Raf, whereas Rap1 stimulates B-Raf activity (9). It is now widely appreciated that the activation of the small G-proteins, Ras and Rap1, is a pivotal step governing the kinetics of MAPK activation. The activation of the Ras family of small G-proteins is mediated through a complex of adaptor proteins and guanine nucleotide exchange factors (GEF). Ras is regulated through its association with an adaptor complex containing Grb2 and the GEF, SOS (10). SOS is a GEF of Ras whose activity is positively regulated by its recruitment to a membrane-associated signaling complex and negatively regulated in part by an auto-regulatory loop through the MAPK pathway resulting in its serine/threonine phosphorylation (11, 12). Similarly, Rap1 activation is accomplished by assembly of a complex including the adaptor molecule Crk and the GEF, C3G (13, 14). The mechanisms regulating activation of C3G are presently unclear. It is thought that C3G is activated by binding to the Crk protein complex. It also has been suggested that the tyrosine phosphorylation of C3G is important for its activation (15).

The EGF receptor and the NGF receptor, TrkA, both recruit a variety of signaling molecules to their receptor complexes upon growth factor stimulation. Some of these effectors are shared by the two receptor tyrosine kinases including phosphatidylinositol 3-kinase, phospholipase Cγ, and the adaptor proteins Shc and Grb2, whereas others are specific to the individual receptors (8). TrkA associates with additional sig-
naling molecules, most prominently FRS2 (16). FRS2 is a lipid-anchored docking protein that is highly tyrosyl-phosphorylated upon neurotrophin or FGF stimulation (17). FRS2 has been shown to associate directly with TrkA and FGF receptors through its phosphotyrosine binding domain and participates in MAPK activation (16). FRS2 has four Grb2- and two SHP-2 (a tyrosine phosphatase)-binding epitopes and recruits both Grb2 and SHP-2 upon stimulation, forming a protein complex in response to receptor stimulation. The formation of these FRS2-associated protein complexes has been postulated to play an important role in the sustained MAPK activation elicited by NGF and FGF and thus PC12 cell differentiation (17, 18).

The mechanisms of EGF receptor signaling have been well described. A unique feature of EGF signaling is that upon ligand binding the EGF receptor becomes associated with c-Cbl (19). c-Cbl acts both as an adaptor protein whose phosphorylation leads to formation of activation-dependent complexes with Crk and phosphatidylinositol 3-kinase, but also possesses a ubiquitin ligase activity (19). Following EGF binding, c-Cbl becomes associated with the receptor and ubiquitinates it, thus triggering its proteasomal degradation resulting in the down-regulation of EGF signaling (20–22).

The mechanisms governing MAPK activation in this model system have been the subject of substantial controversy. Stork and colleagues (9) have advanced a model in which both NGF and EGF activate Ras and c-Raf resulting in the transient activation of the MAPKs. They have argued that NGF elicits the sustained stimulation of the MAPKs through stimulation of Rap1 and B-Raf, whereas EGF is reported not to activate Rap1 (9). This model has been challenged by Bos and colleagues (23, 24) who failed to detect Rap1 activation upon NGF treatment of PC12 cells, suggesting that persistent ERK activation is mediated principally through Ras. Much of the controversy over the mechanisms regulating MAPK activation has arisen over the interpretation of experiments in which elements of the MAPK cascade have been overexpressed.

The aim of the present study was to investigate the molecular mechanisms subserving the differential regulation of the MAPKs by NGF and EGF through examination of the interactions and activity of the endogenous signaling molecules in a well characterized line of PC12 cells. We have arrived at different conclusions regarding the regulatory mechanisms governing the activation of the MAPK pathway. We report that the activation of the MAPKs in response to both NGF and EGF is due almost exclusively to the action of B-Raf, with c-Raf contributing less than 10% of the signal flux through this pathway. We found that both NGF and EGF activated Rap1 through formation of a receptor-linked signaling complex composed of Crk and C3G. The stability of this complex was the critical factor governing the longevity of MAPK activation. The Crk-C3G complex was formed through direct interactions with the EGF receptor and dissociated rapidly following ligand binding, concomitant with the ubiquitination of the receptor and its targeting for degradation. In contrast, NGF catalyzed the assembly of a long lived complex of Crk and C3G with the docking protein FRS2. FRS2 becomes associated with, and is tyrosine-phosphorylated by, the NGF receptor TrkA following NGF treatment and acts to scaffold this complex leading to MAPK activation.

**Experimental Procedures**

**Antibodies**—Anti-Ras was from Calbiochem (La Jolla, CA). Anti-TrkA, anti-EGF receptor, anti-SOS, anti-C3G, anti-c-Cbl, anti-Raf1, anti-FRS2, and anti-ERK2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-c-Crk, anti-p130Cas, and anti-Sin/Effl were from Transduction Laboratories (Lexington, KY). Anti-phosphotyrosine, 4G10 was from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-B-Raf and anti-c-Raf antibodies were described previously (25). The anti-ubiquitin antibody was from Stressgen Biotechnologies (Com-}

**Cell Lines**—PC12 cells were cultured at 10% CO2 in Dulbecco's minimal essential medium (DMEM) supplemented with 10% horse serum and 5% fetal bovine serum (FBS). PC12 cells stably expressing wild type FRS2, FRS2–4F mutant, FRS2–1F mutant, or pLXSN were gifts from Dr. J. Schlessinger (New York University Medical Center) and were grown at 5% CO2 in DMEM containing 10% donor horse serum and 10% FBS.

**Growth Factor Stimulation and Cell Lysate Preparation**—PC12 cells were cultured overnight in DMEM containing 0.5% FBS and then stimulated for the indicated time at 37 °C with 100 ng/ml EGF or 100 ng/ml NGF (Austral Biological, San Ramon, CA) in DMEM supplemented with 5 mM HEPES, pH 7.4, and 0.1% bovine serum albumin. After stimulation, cells were washed once with cold phosphate-buffered saline, solubilized with lysis buffer as described below, and pulse-sonicated for 5 s twice. Lysates were clarified by centrifugation at 13,000 g for 10 min at 4 °C. Protein concentration was determined by the method of Bradford (26), and equal amounts of protein were loaded in each experiment.

**Generation of GST-B-Raf and GST-c-Raf Ras Binding Domain Fusion Proteins**—Glutathione S-transferase (GST)-B-Raf RBD (BRBD) and GST-c-Raf RBD (CRBD) fusion proteins, containing the Ras binding domain (RBD) of the B-Raf (amino acids 1–272) and c-Raf (amino acids 1–149), respectively, were constructed by polymerase chain reaction and cloned into the bacterial expression vector, pGEX-KG. BRBD and CRBD fusion proteins were purified and immobilized on glutathione-Sepharose 4B (Amersham Pharmacia Biotech). Fusion protein-bound beads were stored at −80 °C in phosphate-buffered saline containing 30% ethylene glycol.

**Ras Binding Domain Affinity Binding Assay**—PC12 cells were lysed in RBD assay buffer containing 50 mM Tris, pH 7.5, 10 mM MgCl2, 1% Triton, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and leupeptin. The lysates (3 mg of protein) were incubated with 30 μg of BRBD or CRBD at 4 °C for 45 min. The affinity precipitates were washed 3 times with lysis buffer and eluted with Laemmli sample buffer (10% glycerol, 150 mM β-mercaptoethanol, 3% SDS, 150 mM Tris-HCl, pH 6.8). Proteins were resolved on 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes.

**Immunoprecipitation and Immunoblotting Analysis**—Immunoprecipitation was performed by lysing the cells in immunoprecipitation (IP) buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM MgCl2, 1 mM EGTA, 1 mM sodium orthovanadate, 20 mM β-mercaptoethanol, 1 mM sodium fluoride, 1 μM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin and leupeptin). The lysates (4 mg of protein) were incubated with 1–1.5 μg of the indicated antibodies for 1 h at 4 °C; protein A-agarose (40 μl, Santa Cruz Biotechnology, Santa Cruz, CA) was added, and the tubes were rocked at 4 °C for an additional 1.5 h. The FRS2 protein complex was immunoprecipitated with anti-FRS2 (2 μg) antibodies conjugated to the protein A-agarose (40 μl) and then incubated with a covalent linker to immobilize the FRS2–conjugated beads were then incubated with cell lysates for 2.5 h. The immunoprecipitates were washed three times with lysis buffer and resuspended in Laemmli sample buffer. Proteins were resolved using SDS-polyacrylamide gels (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes. The membranes were blocked in TBST (10 mM Tris, pH 7.5, 137 mM NaCl, 0.1% Tween 20) containing 3% bovine serum albumin. The blots were first probed with indicated primary antibodies in 3% bovine serum albumin/TBST and then with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech) in 5% dried milk/TBST. The bound proteins were visualized by enhanced chemiluminescence (Pierce).

**Raf Kinase Assays**—Raf kinase activities were purified by MonoQ and gel filtration chromatography and then measured in a coupled kinase assay as described previously (27). Briefly, PC12 cell lysates were resolved by applying clarified cell lysates to a Mono Q HR 5/5 column (Amersham Pharmacia Biotech), and the ability of the resulting fractions to activate MEK was measured. The MEK kinase-containing fractions were collected and applied to a Superose 12 HR 10/30 column (Amersham Pharmacia Biotech) to separate B-Raf and c-Raf proteins, and the kinase activities of the resulting fractions were measured. For immunoblot analysis, cell lysates were prepared by lysis in kinase lysis buffer containing 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1 mM sodium orthovanadate, 50 μM β-glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors, 1 μg/ml aprotinin and leupeptin. Raf proteins were isolated by immunoprecipitation using anti-B-Raf or c-Raf antibodies. Immunoprecipitates were washed twice with
kinase lysis buffer and twice with assay dilution buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 75 mM NaCl, and 5 mM EGTA). Kinase buffer (25 μl; 0.5 μg of MEK, 1.1 μg of ERK2, 160 μM ATP in assay dilution buffer) was added to initiate the kinase reaction for 20 min at room temperature. A 10-μl aliquot of the reaction mixture was diluted with 40 μl of assay dilution buffer supplemented with 1 mM sodium orthovanadate and 1 mM dithiothreitol. An aliquot of the diluted mixture (10 μl) was then incubated for 20 min at room temperature with 40 μl of kinase buffer containing (γ⁻³²P)ATP, 2 μg of myelin basic protein, 2 mM MgCl₂, 62.5 μM ATP, 22 dpm/nmol (γ⁻³²P)ATP, 1 mM sodium orthovanadate, and 1 mM dithiothreitol in assay dilution buffer. An aliquot of the final reaction mixture (40 μl) was spotted onto Whatman P81 filter papers. The papers were washed 3 times with 75 mM phosphoric acid and dried, and incorporated radioactivity was measured by scintillation counting.

RESULTS

Differences in the Activation Kinetics of B-Raf and c-Raf by NGF and EGF Stimulation—We have examined the activation kinetics of B-Raf and c-Raf in response to NGF and EGF stimulation. In PC12 cells, both EGF and NGF stimulated transient c-Raf activation with similar kinetics. The maximum c-Raf activities were detected after 2–5 min of stimulation and then declined to basal levels after 30 min (Fig. 1A). However, the pattern of B-Raf activation was quite different in response to NGF or EGF stimulation (Fig. 1B). EGF-stimulated B-Raf activity was transient, decreasing to basal level within 1 h of stimulation. In contrast, NGF stimulation produced the sustained activation of B-Raf, which was maximally activated within 5 min and was maintained for longer than 1 h. Both EGF and NGF stimulated a 3–5-fold increase in the kinase activities of B-Raf and c-Raf. These findings are consistent with the other report in the literature (28).

Growth Factor Stimulation of the MAP Kinases Is Due Principally to B-Raf—The degree of activation of both B-Raf and c-Raf by NGF was similar; however, this widely employed type of analysis does not reveal the dramatic differences in MEK kinase activity exhibited by the two Raf isoforms. B-Raf exhibits a much higher intrinsic kinase activity toward MEK. We quantified the proportional contribution of B-Raf and c-Raf to total MEK kinase activity elicited by NGF treatment of PC12 cells (Fig. 2). B-Raf and c-Raf proteins were isolated by MonoQ gel filtration chromatography from NGF-stimulated PC12 cells. Quantitative analysis of NGF-stimulated Raf activities revealed that after 5 min of NGF treatment, ~90% of MEK kinase activity was due to the activity of B-Raf, whereas less than 10% was found to be associated with c-Raf. A similar analysis of B-Raf and c-Raf activity measured in immune kinase assays yielded essentially identical results. We conclude that greater than 90% of MAPK activation in response to NGF is mediated by B-Raf. c-Raf plays a minor role in MAPK induction, contributing less than 10% of total MEK kinase activity at its maximum level of activation. Identical results were obtained from EGF-stimulated cells (data not shown). These data demonstrate that B-Raf is the principal MEK kinase stimulated by these growth factors.

Kinetic Analysis of Ras and Rap1 Activation—To determine if differential regulations of B-Raf and c-Raf were mediated by selective interactions with their small G-protein regulators, Ras and Rap1, we have investigated the dynamic interactions between Ras, Rap1, and Raf isoforms. We have used the RBDs of c-Raf and B-Raf to capture the activated, GTP-bound, forms of Ras and Rap1. Since these RBDs only bind to active form of Ras and Rap1, the activation kinetics of the individual small G-proteins can be monitored. Thus, GST fusion proteins of Ras binding domains (RBDs) of the B-Raf and c-Raf were used to bind the active forms of these Ras family members following stimulation by NGF and EGF at different times (29, 30). The RBDs of B-Raf and c-Raf were able to selectively capture the activated forms of both Ras and Rap1 (Fig. 3). In EGF-stimulated cells, the activation kinetics of both Ras and Rap1 were transient with maximum activation detected at 2 min (Fig. 3A). The amount of activated Ras and Rap1 declined rapidly to base-line levels after 30 min of EGF stimulation. This transient Ras and Rap1 activation correlated well with the transient MAPK activation as detected by ERK phosphorylation (Fig. 3C). In contrast, NGF stimulated a transient
activation of Ras but sustained Rap1 activation (Fig. 3B). Substantial levels of the active forms of Rap1 could still be detected after an hour of NGF stimulation. In addition, Ras activation was more rapid, starting at 2 min, whereas Rap1 activation was slightly delayed until 5 min. Furthermore, by comparing Ras and Rap1 activation kinetics to MAPK activation, we found that in EGF-stimulated cells transient MAPK activation is supported by both transient Ras and Rap1 activities. Importantly, in NGF-treated cells, the early phase of MAPK activity is correlated well with Ras activity, whereas that of the later phase is correlated with Rap1 activity, consistent with findings of York et al. (9).

**Growth Factor Regulation of Guanine Nucleotide Exchange Factors**—The differential activation of the GEFs by NGF and EGF plays a pivotal role in governing the longevity of the signals driven by Ras and Rap1. The inactivation of Ras occurs concurrently with the dissociation of the Grb2-SOS protein complex following the phosphorylation of SOS by ERKs and pp90<sup>rsk2</sup>, providing feedback inhibition of this pathway (11, 12). The phosphorylation of SOS after growth factor stimulation can be detected by its reduced electrophoretic mobility on SDS-PAGE. The mobility shift pattern of SOS revealed that the activation and inactivation of SOS were identical in the NGF- and EGF-treated cells (Fig. 4A). SOS exhibited its maximum mobility shift after 5 min of growth factor treatment. These data are consistent with the transient activation of Ras in response to both stimuli.

The inactivation of Rap1 is a consequence of the dissociation of the Crk-C3G complex (31, 32). We analyzed the interaction of C3G and Crk by immunoprecipitation of Crk (Fig. 4B). There is constitutive association of C3G with Crk in unstimulated cells. However, upon stimulation of the cells with EGF, this complex dissociates after 5 min. In contrast, upon NGF treatment, the levels of the Crk-C3G complex were enhanced and then remained stably associated for longer than 1 h, suggesting that the stabilized Crk-C3G complex is responsible for the sustained Rap1 activation and persistent activation of B-Raf observed upon NGF stimulation. The data shown in Fig. 4B were obtained from the same experiment, but different exposures have been provided to show clearly the changes in the Crk-C3G complexes.

**NGF and EGF Stimulate the Formation of Molecularly Distinct Complexes with Crk**—By having observed that EGF and NGF differentially regulated the amount and stability of the Crk-C3G protein complex, we proceeded to investigate the possible mechanisms governing Crk function. PC12 cells express three isoforms of Crk proteins, with c-CrkII and CrkL predominating (9). Crk possesses an SH2 and two SH3 domains and thus can participate in the linkage of a complex arrays of proteins to the receptors (33). Crk undergoes tyrosine phosphorylation after growth factor stimulation (34, 35). Examination of Crk tyrosyl phosphorylation following EGF stimulation revealed only a brief period of phosphorylation, whereas the time course of NGF-stimulated tyrosyl phosphorylation was significantly more protracted (Fig. 5A).

Importantly, we found that different tyrosyl-phosphorylated proteins associated with Crk in response to EGF or NGF, detected using the anti-phosphotyrosine antibody, 4G10 (Fig. 5B). EGF stimulation of the cells results in the transient dissociation of the 130-kDa (pp130) and 97-kDa proteins (pp97), concurrent with the association of the complex with the 180-kDa (pp180) and 120-kDa (pp120) proteins. The cohort of Crk-associated proteins in NGF-stimulated cells is less complex. NGF treatment resulted in an increase in the amount of pp130 and pp97 associated with Crk. We have determined that pp130 in the EGF- and NGF-stimulated Crk protein complexes is p130<sup>Cas</sup> (Fig. 5C). The pp97 protein has been identified as Sin/Eff (Fig. 5D). The pp180 and pp120 from EGF-stimulated cells were identified as the EGF receptor and c-Cbl, respectively (Fig. 6A and 5C). Identical immunoprecipitation results were obtained using antibodies either to c-Crk or CrkL (data not shown). We verified that the association of Sin and p130<sup>Cas</sup> with Crk was differentially regulated by EGF and NGF (Fig. 5D). Examination of the Crk immunoprecipitates using an anti-Sin or anti-p130<sup>Cas</sup> antibody demonstrated that EGF treatment resulted in displacement of Sin and p130<sup>Cas</sup> from Crk, whereas in NGF-stimulated cells there was a dramatic increase in the association of Sin and p130<sup>Cas</sup> with Crk. Interestingly, the increased association of p130<sup>Cas</sup> and Sin with Crk could only be detected in adherent cells treated with NGF. When the cells were stimulated with NGF in suspension, a dissociation of the Crk-p130<sup>Cas</sup> and Crk-Sin protein complexes was observed (data not shown), suggesting that these complexes are regulated by cytoskeletal organization.

**The EGF Receptor but Not TrkA Is Present in the Crk Protein**
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**FIG. 5.** NGF and EGF stimulate the formation of molecularly distinct complexes with Crk. Crk protein complexes were immunoprecipitated (IP) from EGF- or NGF-stimulated PC12 cells treated for the indicated periods. A and B, Crk-associated proteins were analyzed by probing Western blots with the anti-phosphotyrosine (4G10) antibody. A and B were obtained from the same blot. However, A represents a longer exposure, allowing visualization of phospho-Crk. The two Western blots shown in A were obtained from films with the same exposure periods. The blots were then reprobed with an anti-Crk antibody to verify protein loading. C and D, Crk immunoprecipitates were analyzed by Western analysis and probed with antibodies to p130Cas, Sin/Fes, and Cbl. The blots were then reprobed with anti-Crk antibody to verify protein loading. Data shown in this figure are representative of three experiments.

**FIG. 6.** EGF receptor, but not TrkA, is present in a Crk protein complex. Crk protein complexes were immunoprecipitated from a time course of EGF- (A) or NGF- (B)-stimulated PC12 cells using anti-Crk antibody. The immunoprecipitates (IP) were analyzed by probing Western blots with anti-EGF receptor (A) or anti-TrkA antibodies (B). The blots were then reprobed with anti-Crk antibody to verify protein loading. Representative data from three independent experiments are shown.

Complex—We found that Crk is stably associated with the EGF receptor in the unstimulated cells. Upon EGF stimulation, the EGF receptor becomes highly tyrosine-phosphorylated within 2 min (Fig. 7A). However, the level of EGF receptor in the Crk immunoprecipitates fell rapidly (Fig. 6A). This diminution in the amount of EGF receptor associated with Crk is also readily observed in the phosphotyrosine immunoblots of Crk immunoprecipitates (Fig. 5B). In contrast, even though TrkA is highly tyrosine-phosphorylated after NGF stimulation, we failed to detect a tyrosyl-phosphorylated protein corresponding to TrkA in the Crk complex (Fig. 5B and 6B), nor could we detect any TrkA protein in the Crk immunoprecipitates (Fig. 6A). These data demonstrate that Crk and TrkA do not form stable complexes.

**Decreased EGF Receptor-Crk Interaction after EGF Stimulation Is Due to Ubiquitination and Degradation of the EGF Receptor—**Upon ligand binding, the EGF receptor undergoes tyrosyl phosphorylation and recruits c-Cbl to the receptor complex (36, 37). c-Cbl possesses a ubiquitin ligase activity that extensively modifies the receptor resulting in its removal from the cell surface and targeting it to the proteasome for degradation (20, 22). We found both the EGF receptor and c-Cbl in Crk immune complexes after EGF stimulation, suggesting that the complex can be down-regulated upon targeting of the EGF receptor to the ubiquitin-proteasome degradation pathway (Fig. 5B). Therefore, in order to determine why the levels of the EGF receptor-Crk complex decreased after EGF treatment, we examined the ubiquitination levels of the EGF receptor. Interestingly, we found that the EGF receptor was rapidly and robustly ubiquitinated within 2 min of EGF stimulation (Fig. 7A). The receptor is actively de-ubiquitinated over the next few minutes, reflecting the acute regulation of this modification that is linked to endocytosis and degradation of the EGF receptor. After 1 h of EGF treatment, no ubiquitination could be detected concomitant with decreased EGF receptor protein levels in these cells. Therefore, the ubiquitination of the EGF receptor provides a mechanism for down-regulating the Crk signaling complex and thus explains the transient nature of Rap1 activation following EGF stimulation. In contrast, no ubiquitination of TrkA was detected, and we were unable to detect any changes in the protein levels of TrkA in response to NGF treatment (Fig. 7B).

Crk Is Recruited to a FRS2 Protein Complex after NGF Treatment—A number of signaling proteins have been shown to interact with Crk in different cell types and in response to
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**FIG. 8.** Crk is recruited to FRS2 protein complex after NGF treatment. Serum-starved PC12 cells were stimulated with 100 ng/ml NGF for the indicated periods. A, The Crk protein complex was immunoprecipitated (IP) using anti-Crk antibody. The immunoprecipitates were analyzed by probing a Western blot with anti-FRS2 antibodies. The blot was then reprobed with anti-Crk antibody to verify protein loading. B, FRS2-associated Crk protein was examined by immunoprecipitating FRS2 from the cell lysates using anti-FRS2 antibodies. The immunoprecipitates were then examined by Western blotting analysis using anti-phosphotyrosine (4G10) or anti-Crk antibodies. The blot was reprobed with anti-FRS2 antibodies to verify protein loading. The experiments were repeated twice with similar results.

**FIG. 9.** Crk interacts with the SHP-2-binding site on FRS2. Tyr-436. Cells were starved overnight and stimulated with 100 ng/ml NGF for the indicated periods. Cell lysates generated from PC12 cells expressing pLXSN vector alone or overexpressing wild type FRS2 or mutant FRS2 (4F-FRS2 or 1F-FRS2) were subjected to immunoprecipitation using anti-Crk antibodies. The immunoprecipitates were analyzed by Western blot analysis using anti-FRS2 antibodies. The blots were reprobed with anti-Crk antibody to confirm protein loading. Data shown are representative of three experiments.

**DISCUSSION**

The recognition that the differential regulation of MAPK activation governs cellular proliferation and differentiation has served to focus attention on the signaling pathways linking growth factor receptors to the MAPKs themselves. The present study documents the central importance of Rap1 and B-Raf in MAPK activation in PC12 cells. Significantly, a conclusion of this study is that signaling through the c-Raf pathway is a very minor contributor to growth factor-stimulated MAPK activation in this cell line. The involvement of Ras in the regulation of sustained MAPK activation in PC12 cells is controversial. Many of the previous studies were performed by overexpressing oncopgenic v-Ras (RasVal-12) or the dominant negative RasAro-17 mutant. Introducing v-Ras into PC12 cells led to prolonged Ras and MAPK activation, whereas RasAro-17 inhibited these effects (4, 5, 42, 45). It is now evident that the interpretation of these studies has been confounded by the interference of high levels of Ras with other small G-protein signaling. Recent studies demonstrate that overexpressing one small G-protein will interfere with the others binding to their shared effectors. Specifically, Okada and co-workers (32) have shown that by overexpressing Rap1 in Chinese hamster ovary cells, the direct association of Ras and Raf-1 induced by insulin is decreased. Thus, studies in which elements of the cascade
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have been expressed at supraphysiological levels have been misleading as to the importance of Ras in stimulating MAPK activation.

We have investigated the activation kinetics of Ras and Rap1 in response to NGF and EGF stimulation. In PC12 cells, by using Raf RBDs as affinity probes, we observed that NGF stimulated transient Ras, but sustained Rap1 activation, whereas EGF induced both Ras and Rap1 activity transiently (Fig. 3). The observation that Ras is transiently activated by both growth factors has been observed previously by other investigators (9, 44). There is, however, considerable disagreement over whether and how Rap1 is regulated. We and York et al. (9) have found a robust activation of Rap1 by NGF. This finding conflicts with data reported by Zwartrkuis and colleagues (24) who failed to detect Rap1 stimulation following NGF treatment. Conversely, Zwartrkuis et al. (24) found EGF treatment of PC12 cells resulted in Rap1 activation. This finding is similar to our observations (Fig. 3A). Stork and colleagues (45) have argued that EGF does not provoke Rap1 activation. There is no clear explanation for the different experimental outcomes.

In much of the literature investigating MAPK regulation, there has been the implicit assumption that B-Raf and c-Raf contribute equally to the magnitude of MAPK activation in NGF signaling in PC12 cells. Most of the previous studies report only the relative kinase activity of the enzymes instead of the absolute levels of kinase activity. When the total kinase activity of B-Raf and c-Raf derived from equal amount of cell lysates was measured by two different methods, B-Raf was found to contribute >90% of MEK kinase activity, whereas c-Raf activity accounted for <10% of the total activity (Fig. 2) (46). These data demonstrate that B-Raf is the principal MEK kinase stimulated by NGF, and c-Raf plays a very minor role (Fig. 2). Consistent with these findings, Moodie et al. (47) reported that immunodepletion of B-Raf from rat brain extract reduced MEK kinase activity by 96%. Papin et al. (48) have shown that B-Raf is a much more efficient MEK kinase than c-Raf, exhibiting ~10-fold higher specific activities toward MEK, as a consequence of the more avid binding of B-Raf to MEK than c-Raf. Therefore, we suggest that the involvement of c-Raf in NGF- and EGF-dependent MAPK activation in PC12 cells is quantitatively insignificant, and we argue that it is the magnitude and longevity of the signals derived from the B-Raf pathway that explains the kinetics of MAPK activation in this cell line. The activation of B-Raf is likely regulated by both Ras and Rap1 (9, 49). Both we and York et al. (9) found that B-Raf associates with activated Ras, a finding that is consistent with previous observations (46, 47, 50, 51). In EGF-stimulated PC12 cells, both the transient activations of Ras and Rap1 contribute to the transient B-Raf activities, whereas B-Raf activation by NGF is supported initially by Ras and then by persistent Rap1 activation.

The longevity of the signals driven by Ras and Rap1 are regulated by their GEFs, SOS and C3G, respectively. We have shown that EGF and NGF both stimulated SOS activation with the same kinetics (Fig. 4). The mechanisms regulating the Crk-C3G complex are less well understood. Tyrosine phosphorylation of C3G on Tyr-504 has been suggested to positively regulate its activity (15). We were unable to detect any tyrosine phosphorylation or mobility changes of the C3G protein upon EGF or NGF stimulation (data not shown). C3G appears to be regulated through its association with the adaptor Crk, as the dissociation of the Crk-C3G complex accompanies the inactivation of Rap1 (31, 32). We demonstrated that EGF induced a dissociation of the Crk-C3G complex, consistent with the transient Rap1 activation. In contrast, NGF stimulated the formation of a stable and sustained Crk-C3G complex, supporting the prolonged Rap1 activation (Fig. 4). These data provide support for the view that the stability of the Crk-C3G complex critically regulates Rap1 activation.

Previous studies have shown that Crk is tyrosine-phosphorylated upon NGF and EGF stimulation (34, 35). Tyrosine phosphorylation of Crk on Tyr-222 positioned between the two SH3 domains is thought to function as a substrate switch such that when Crk is not phosphorylated its SH2 domain is able to interact with the cytoskeletal protein paxillin. When Crk is phosphorylated on Tyr-222, paxillin is dissociated from the Crk complex, and the SH2 domain of Crk is free to interact with other signaling proteins, such as p130Cas (52). This quick turnover of the Crk protein complex is thought to be important for regulating cytoskeletal dynamics. In the present study, we found that EGF stimulated the transient tyrosine phosphoryl-
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The nature of the molecular linkage between Crk and growth factor receptors remains unclear. Some studies suggest that c-Cbl and She are candidate molecules linking Crk to upstream receptors, whereas others indicate that Crk is able to bind to TrkA and the EGF receptor directly (19, 34, 54–58). The interaction of Crk with the EGF receptor is likely to be direct as there is a high basal level of association in the absence of Shc or Cbl. Studies by Ribon and Saltiel (35) reported that Tyr-992 of the EGF receptor composed a Crk-binding site, and mutation of Tyr-992 interfered with the binding of the Crk SH2 domain to the EGF receptor, demonstrating the direct interactions between Crk and the EGF receptor. We were unable to detect TrkA stably associated with Crk in growth factor-stimulated cells, likely due to the relatively low expression levels of the endogenous proteins. The detection of these interactions in the previous studies was limited to studies in which TrkA was overexpressed. These data indicate that in wild type PC12 cells, if Crk and TrkA do exist in the same protein complex, their association must be unstable or separated by additional adaptor proteins.

We found it interesting that EGF induced the loss of the EGF receptor from the Crk protein complex. This is likely to be a result of recruitment of the ubiquitin ligase c-Chat to the EGF receptor complex and extensive ubiquitination of the receptor (Fig. 5) (36). The ubiquitination of the EGF receptor targets this molecule to the proteasomes. Indeed, protein levels of the EGF receptor fell below basal levels after an hour of EGF treatment (Fig. 7). We were surprised by the observation that the ubiquitination of the EGF receptor occurred rapidly and robustly within 2 min of EGF stimulation and that ~80% of the ubiquitin was lost after 5 min of EGF stimulation. Since the protein levels of the EGF receptor did not drop accordingly, these findings suggest the regulated de-ubiquitination of the EGF receptor. This fast turnover of ubiquitination and de-ubiquitination has been suggested to be involved in the trafficking and endocytosis of the receptor (59). This type of regulation was restricted to the EGF receptor, as we did not find c-Cbl in the NGF-stimulated Crk complex. We did not detect the ubiquitination or degradation of TrkA. Our data are consistent with the previous findings that c-Cbl does not bind to TrkA, and TrkA is not rapidly degraded after NGF stimulation (60, 61).

Many signaling proteins have been implicated in promoting neuronal differentiation in PC12 cells. Both the docking protein FRS2 and the tyrosine phosphatase SHP-2 have been implicated in the supported MAPK activation in response to NGF and appear to be required for inducing neurite outgrowth in these cells (18). Upon NGF stimulation, tyrosyl-phosphorylated TrkA recruits FRS2 to its receptor complex through binding to the phosphotyrosine binding domain of FRS2. FRS2 is then phosphorylated on multiple tyrosine residues (16, 62). These tyrosyl-phosphorylated residues serve as docking sites for Grb2 and SHP-2, transducing signals into the MAPK pathway. Studies by Schlessinger and co-workers (17, 18) have demonstrated that the incorporation of SHP-2 in the FRS2 complex is more important than that of Grb2 in eliciting sustained MAPK activation and neurite outgrowth. In the present study, we have provided evidence showing that Crk was recruited into an FRS2 protein complex in response to NGF stimulation (Fig. 8). Furthermore, the recruitment of Crk and the activation of Rap1 were impaired in PC12 cells overexpressing FRS2 mutated at the SHP-2-binding motif (Figs. 9 and 10). Since there is no apparent Crk consensus binding motif (YYXP) on FRS2 and SHP-2, the interaction between FRS2 and Crk is likely to be indirect and possibly mediated through SHP-2 and other adaptor molecule(s).

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REFERENCES

1. Marshall, C. (1995) Cell 80, 179–185
2. Hoff, K., Endl, D., and Gurevich, G. (1981) J. Cell Biol. 88, 189–198
3. Greene, L. A., and Tischler, A. S. (1976)Proc. Natl. Acad. Sci. U. S. A. 73, 2424–2428
4. Nguyen, T. T., Scimemi, J. C., Filouzou, C., Perinaldi, P., Carpentier, J. L., and Van Obberghen, E. (1993)J. Biol. Chem. 268, 9803–9810
5. Qui, M. S., and Green, S. H. (1992)Neuron 9, 705–717
6. Traver, S., Gomez, N., Patrickson, H., Marshall, C., and Cohen, P. (1992)Biochem. J. 288, 351–355
7. Marshall, C. J. (1998)Nature 392, 553–554
8. Segal, R., and Greenberg, M. (1996)Annu. Rev. Neurosci. 19, 463–489
9. York, R. D., Yao, H., Dillon, T., Ellig, C. L., Eckert, S. P., Mescliesley, E. W., and Stork, P. J. S. (1998)Nature 392, 622–626
10. Bourne, H. R., Sanders, D. A., and McCormick, F. (1991)Nature 349, 117–127
11. Dong, C., Waters, S. B., Holt, K. H., and Pessin, J. E. (1996)J. Biol. Chem. 271, 6328–6332
12. Waters, S. B., Holt, K. H., Ross, S. E., Suy, L. J., Guan, K. L., Saltiel, A. R., Koretzky, G. A., and Pessin, J. E. (1995) J. Biol. Chem. 270, 20883–20886
13. Zwartkruis, F. J., and Bos, J. L. (1999)Exp. Cell Res. 253, 157–165
14. Gotto, T. (1995)Mol. Cell. Biol. 15, 6746–6753
15. Ichiba, T., Kuriashi, Y., Sakai, O., Nagata, S., Groffen, J., Kurata, T., Hattori, S., and Matsuda, M. (1997)J. Biol. Chem. 272, 22115–22220
16. Taylor, N. G., Guy, G. R., and Fields, S. (1998)J. Biol. Chem. 273, 22151–22154
17. Kouhara, H., Hadari, Y. R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., Lax, L., and Schlessinger, J. (1997)Cell 89, 693–702
18. Hadari, Y. R., Kouhara, H., Lax, I., and Schlessinger, J. (1998) Mol. Cell. Biol. 18, 3966–3973
19. Lesoon, M. L., J., Androniou, C. E., Bonita, D., Miyake, S., and Band, H. (1998)Int. J. Biochem. Cell Biol. 30, 439–444
20. Waterman, H., Levkovitz, G., Abroy, I., and Yarden, Y. (1999)J. Biol. Chem. 274, 22151–22154
21. Yoshimura, K., Kondo, T., Houghton, A., Bartkiewicz, M., Horne, W. C., Zhang, Y., H., Yoshimura, A., and Baron, R. (1999)J. Biol. Chem. 274, 31707–31712
22. Levkovitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Teigyanakov, A. Y., Abroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999) Mol. Cell. Biol. 19, 1029–1040
23. Lu, I., Anneren, C. E., Reederquist, K. A., Bos, J. L., and Welsh, M. (2000)Exp. Cell Res. 259, 570–577
24. Zwartkruis, F. J., Wolthus, R. M. M., Naben, N. M., Franke, B., and Bos, J. L. (1998)EMBO J. 17, 5905–5912
25. Jaissal, R., Weissinger, E., Kolch, W., and Landreth, G. (1996)J. Biol. Chem. 271, 23626–23632
26. Mann, T. M. (1976)Anal. Biochem. 72, 248–254
27. Jaissal, R., Murphy, M., and Landreth, G. (1993)J. Biol. Chem. 268, 7055–7063
28. Wixler, V., Smola, U., Schuler, M., and Reiss, U. (1996)FEBS Lett. 385, 131–137
29. De Roodt, J., and Bos, J. L. (1997)Gene 14, 623–625
30. De Roodt, J., and Sarlowkey, G. (1996)EMBO J. 17, 2654–2660
33. Feller, S. M., Posern, G., Voss, J., Kardinal, C., Sakkab, D., Zheng, J., and Knudsen, B. S. (1998) *J. Cell. Physiol.* **177**, 535–552
34. Hempstead, B. L., Birge, R. B., Fajardo, J. E., Glassman, R., Mahadeo, D., Kraemer, R., and Hanafusa, H. (1994) *J. Biol. Chem.* **271**, 7375–7380
35. Fukazawa, T., Miyake, S., Band, V., and Band, H. (1996) *J. Biol. Chem.* **271**, 14554–14559
36. Khwaja, A., Hallberg, B., Warne, P. H., and Downward, J. (1996) *Oncogene* **12**, 2491–2498
37. Ribon, V., and Saltiel, A. R. (1996) *J. Biol. Chem.* **271**, 7375–7380
38. Songyang, Z., Shoelson, S., Chaudhuri, M., Gish, G., Pawson, T., Roberts, T., Hanafusa, H., and Cantley, L. (1993) *Cell* **72**, 767–778
39. Wood, K. W., Sarnecki, C., Roberts, T. M., and Blenis, J. (1992) *Cell* **68**, 1041–1050
40. Meakin, S. O., MacDonald, J. I., Gryz, E. A., Kubu, C. J., and Verdi, J. M. (1999) *J. Biol. Chem.* **274**, 9861–9870
41. Lange-Carter, C. A., and Johnson, G. L. (1994) *Science* **265**, 1458–1461
42. Vossler, M., Yao, H., York, R., Pan, M., Rim, C., and Stork, P. (1997) *Cell* **89**, 73–82
43. Jaiswal, R., Moodie, S., Wolfman, A., and Landreth, G. (1994) *Mol. Cell. Biol.* **14**, 6944–6953
44. Moodie, S. A., Paris, M. J., Kolch, W., and Wolfman, A. (1994) *Mol. Cell. Biol.* **14**, 7153–7162
45. Papin, C., Denouel-Galy, A., Laugier, D., Calothy, G., and Eychene, A. (1996) *J. Biol. Chem.* **271**, 24939–24947
46. Torre, M., and Bogenmann, E. (1996) *Oncogene* **12**, 77–86
47. Carthew, R. W., and Xu, C. (2000) *Curr. Biol.* **10**, R532–R534
48. Galisthen, L., Dikic, I., Batzer, A. G., Langdon, W. Y., and Schlessinger, J. (1999) *J. Biol. Chem.* **274**, 20242–20245
49. Sommerfeld, M. T., Schweigreiter, R., Barde, Y. A., and Hoppe, E. (2000) *J. Biol. Chem.* **275**, 8982–8990
50. Gryz, E. A., and Meakin, S. O. (2000) *Oncogene* **19**, 417–430