Nerve Growth Factor Stimulates the Tyrosine Phosphorylation of Endogenous Crk-II and Augments Its Association with p130Cas in PC-12 Cells*

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The cellular homologs of the v-Crk oncogene product consist primarily of Src homology region 2 (SH2) and 3 (SH3) domains. v-Crk overexpression causes cell transformation and elevation of tyrosine phosphorylation in fibroblasts and accelerates differentiation of PC-12 cells in response to nerve growth factor (NGF). To further explore the role of Crk in NGF-induced PC-12 cell differentiation, we found that both NGF and epidermal growth factor stimulate the tyrosine phosphorylation of endogenous Crk II. Moreover, hormone stimulation enhanced the specific association of Crk proteins with the tyrosine-phosphorylated p130Cas, the major phosphotyrosine-containing protein in cells transformed with v-Crk. This interaction is mediated by the SH2 domain of Crk and can be inhibited with a phosphopeptide containing the Crk-SH2 binding motif. Furthermore, the Crk-SH2 domain binds tyrosine-phosphorylated paxillin, a cytoskeletal protein, following treatment of PC-12 cells with NGF or epidermal growth factor. These data suggest that Crk functions in a number of signaling processes in PC-12 cells.

Many receptors for growth and differentiation factors possess intrinsic protein-tyrosine kinase activity, which is increased following ligand binding, resulting in tyrosine autophosphorylation (1). These tyrosine-phosphorylated proteins serve as high affinity binding sites for Src homology 2 (SH2)-domain-containing signaling proteins (2-4). SH2 domains have been identified in a wide range of proteins. Some of these proteins are enzymes with defined catalytic activity (5-8), while others serve as adapters or activators for other proteins. SH2 proteins can also contain src homology 3 (SH3) domains, regions that bind to proline-rich sequences in other proteins (9, 10).

The Crk proteins, originally isolated as an oncogene product in an avian sarcoma virus (11), belong to the adapter-type SH2-SH3-containing molecules. Other members of this group include Grb2 (12) and Nck (13). These bifunctional proteins are thought to couple tyrosine-phosphorylated receptors or their substrates via the SH2 domain to downstream effectors via SH3 binding. v-Crk encodes a viral Gag protein fused to one SH2 and one SH3 domain. Three cellular homologs of v-Crk have been identified, Crk-I, a 21-kDa protein with only one SH2 and SH3 (14), Crk-II, a 40/42-kDa protein consisting of one SH2 and two SH3 domains (15), and Crk-L, a 36-kDa Crk-like protein with one SH2 and two SH3 domains (16). Despite their lack of tyrosine kinase activity, expression of v-Crk or Crk-I but not Crk-II leads to cell transformation and increased tyrosine phosphorylation (11, 15, 17). Furthermore, v-Crk binds directly to the major tyrosine-phosphorylated proteins in v-Crk-transformed cells, presumably via its SH2 domain (18). These phosphoproteins include paxillin, a focal-adhesion-associated protein (19), and the newly identified p130Cas (20). p130Cas has no catalytic domain, but it contains a cluster of predicted high affinity Crk-SH2 binding motifs and forms a stable complex in vivo with v-Crk. The Crk-SH3 domain has been shown to interact with several proteins including Abl (21) Sos (22), and more recently, C3G (22-24). Since Sos and C3G contain a guanine-nucleotide exchange activity, Crk proteins have been hypothesized to play a role in the regulation of p21ras-GTP formation. In this regard, Crk mutants with dysfunctional SH2 or SH3 domains inhibited nerve growth factor (NGF)-induced differentiation (22, 25). Moreover, expression of v-Crk in PC-12 cells enhanced differentiation produced by both NGF and epidermal growth factor (EGF) (26).

Although the mechanism by which neurotrophic factors support the survival and differentiation of sympathetic neurons remains elusive, tyrosine phosphorylation is known to play an important role in signal initiation (27, 28). We report here that both NGF and EGF rapidly stimulated the tyrosine phosphorylation of endogenous Crk-II in PC-12 pheochromocytoma cells and induced its association with a variety of intracellular signaling proteins.

EXPERIMENTAL PROCEDURES

Cell Lines—PC-12 rat pheochromocytoma cells were grown as described (29). Before hormonal treatment, subconfluent cultures of cells grown in 100- or 150-mm dishes were serum-deprived for 12-24 h. Unless otherwise indicated, 100 nM NGF (Bioproducts for Science) or 100 ng/ml EGF (Collaborative Research) were added directly to the medium, and the incubation continued for the indicated times at 37 °C.

Immunoprecipitations and Immunoblotting—After hormonal treatment, cells were washed twice with ice-cold phosphate-buffered saline before the addition of lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EDTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 100 mM NaF, 10 µg/ml each aprotinin and leupeptin, and 1 mM phenylmethylsulfonyl fluoride and incubated on ice for 15 min. After centrifugation at 10,000 × g for 15 min at 4 °C, equal protein concentrations of lysates were immunoprecipitated with 2 µg of anti-Crk-II, anti-p130Cas (20), or anti-paxillin antibodies (Transduction Laboratories, Lexington, KY). After 12 h at 4 °C, immunoprecipitates were mixed with protein G protein A-agarose (Oncogene Science) for 2 h, and the immunocomplexes were solubilized at 100 °C for 5 min in 25 µl of Laemmli sample buffer.
Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. Individual proteins were detected with the specified antibodies, and phosphotyrosine was detected using a mixture of the anti-phosphotyrosine antibodies RC20H (Transduction Laboratories) and 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY). Bound antibodies were detected with horseradish peroxidase-linked secondary antibodies followed by the enhanced chemiluminescence (ECL) system (Amersham Corp.). To reprobe immunoblots, the nitrocellulose membranes were incubated for 30 min at 60°C with 62.5 mM Tris-HCl, pH 6.8, containing 2% SDS and 0.7% 2-mercaptoethanol and then were washed extensively with 10 mM Tris-HCl, pH 8, and 150 mM NaCl.

Denaturing immunoprecipitation of tyrosine-phosphorylated Crk-II was performed by first immunoprecipitating Crk-II from untreated or treated cell lysates with anti-Crk-II antibodies as described above. After the immune complexes had been washed 3 times with lysis buffer, they were denatured by boiling in 50 μl of denaturing buffer (1% SDS, 50 mM Tris-HCl, pH 7.7) for 5 min. The agarose beads were pelleted by centrifugation, and the supernatants were saved. To 25 μl of the supernatants were added 1 ml of the lysis buffer and 2 μg of the anti-phosphotyrosine antibodies. The immune complexes were mixed with protein G/protein A-agarose, and the proteins were resolved by SDS-PAGE as described above. Autoradiographs were quantified by computer-assisted video densitometry using the Bio Image system (Imaging Systems, Millipore Corp., Ann Arbor, MI).

In Vitro Binding Assays and Phosphopeptide Competition—The glutathione S-transferase (GST) fusion protein containing the SH2 domain of Crk was the generous gift of Drs. R. B. Birge and H. Hanafusa (19). The GST-Grb2 fusion protein was described previously (30), and the generation and production of GST-Grb2-SH2 fusion protein was as described elsewhere (31). For in vitro association experiments, GST fusion proteins bound to glutathione-agarose beads or GST alone were incubated for 90 min at 4°C with 250 μl of cell lysate (equivalent to 5 × 10⁶ cells in lysis buffer). After washing the beads 3 times with lysis buffer, the adsorbed proteins were eluted and analyzed as described above for immunoprecipitates. The phosphotyrosine-containing peptides derived from the major phosphorylation sites of the EGFR receptor, DAEpYp1LIpQG (pY992), VPEpYINQVPK (pY1068), and NpVPpYNQpLN (pY1086) were synthesized, purified, and characterized as described previously (32). For phosphopeptide competition experiments, peptides (final concentration 100 μM) were preincubated with the GST-SH2 domain for 30 min prior to the addition of cell lysates, and the incubation continued for 90 min. After washing with lysis buffer, the SH2 bound proteins were separated by SDS-PAGE and identified by immunoblotting with anti-phosphotyrosine or anti-p130Cas antibodies.

RESULTS

NGF and EGF Stimulate the Tyrosine Phosphorylation of Crk in PC-12 Cells—The binding of NGF and EGF to their tyrosine kinase receptors in PC-12 cells induces their association with a variety of SH2-containing proteins. In some cases, recruitment of these proteins to the receptors can result in their tyrosine phosphorylation (33, 34). v-Crk overexpression in PC-12 cells markedly enhanced both NGF- and EGF-induced phosphorylation. Moreover, v-Crk is tyrosine-phosphorylated in response to both NGF and EGF in these cells (26). Because of the proposed role for Crk in NGF signaling pathways in PC-12 cells, we first examined the growth factor-dependent tyrosine phosphorylation of endogenous Crk proteins in these cells. PC-12 cells were treated with NGF or EGF for 1 min, and lysates were precipitated with anti-Crk-II antibodies. The resulting immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and blotted with anti-phosphotyrosine antibodies (Fig. 1A). Both NGF and EGF stimulated the tyrosine phosphorylation of an immunoprecipitated protein migrating with an apparent molecular mass of 42 kDa (Fig. 1A). When the membrane used for the experiment shown in panel A was stripped of the antibodies and reprobed with the anti-Crk-II antisemur, we detected predominately the 40-kDa form of Crk-II with only a trace of the 42-kDa form (Fig. 1B). This pattern was identical in both unstimulated and stimulated cells, as was reported (22). Thus, the growth factors appear to selectively produce the phosphorylation of the higher molecular weight form of Crk-II, in agreement with previous studies demonstrating that the extended 42-kDa form of Crk-II is modestly tyrosine-phosphorylated by EGF in human carcinoma A431 cells overexpressing this form of Crk (35).

The proteins in the anti-Crk-II immunoprecipitates were subjected to second round of immunoprecipitation under denaturing conditions with anti-phosphotyrosine antibodies, followed by immunoblotting with anti-Crk-II antibodies (Fig. 1C). A single band was recognized by the anti-Crk-II antibodies in the anti-phosphotyrosine immunoprecipitates. This band appears to comigrate with the 42-kDa form of Crk-II in the control loading. On the basis of comparison of the Crk-II signals present in the loading control (Fig. 1C, lane 4, 50% of the material used in the first round) and the anti-phosphotyrosine immunoprecipitates (lanes 2 and 3), we estimated that a significant proportion of the Crk-II (about 30–40%) is tyrosine-phosphorylated. Furthermore, it appears that the more slowly migrating species of Crk-II, which is phosphorylated on tyrosine (Fig. 1A), preferentially bound to the anti-phosphotyrosine antibodies. These results further established the fact that Crk-II undergoes an increase in tyrosine phosphorylation upon NGF or EGF stimulation.

NGF and EGF Stimulate the Tyrosine Phosphorylation of p130Cas in PC-12 Cells—In previous studies, a 130-kDa tyrosine-phosphorylated protein was detected in fibroblasts...
Lysates were prepared and incubated with anti-p130Cas antibodies in PC-12 cells. Tyrosine phosphorylation of p130Cas. PC-12 cells were treated with growth factors could produce the phosphorylation sites with proline in the 42-kDa form of Crk-II is very low compared with the 40-kDa form (Fig. 1B). It is therefore possible that the relative amount of this form of Crk-II immunoprecipitated with the anti-p130Cas antibodies is substantially lower than that immunoprecipitated with anti-Crk-II antibodies and cannot be detected under these conditions.

NGF and EGF Augment the Formation of a Crk-p130Cas Complex in PC-12 Cells—To further explore the growth factor-dependent interaction of endogenous Crk with p130Cas, PC-12 cells were treated with NGF or EGF for 1 min, and lysates were incubated with anti-Crk-II, anti-p130Cas, or anti-Syp antibodies. The resulting immunoprecipitates were subjected to immunoblotting with anti-p130Cas antibodies. Although v-Crk overexpressed in 3Y1 cells could be immunoprecipitated with anti-p130Cas antibodies (20), we were unable to detect Crk-II proteins by blotting with anti-Crk-II antibodies the anti-p130Cas immunoprecipitates from NGF- or EGF-treated cells. It appears that the level of expression of the 42-kDa form of Crk-II is very low compared with the 40-kDa form (Fig. 1B). It is therefore possible that the relative amount of this form of Crk-II immunoprecipitated with the anti-p130Cas antibodies is substantially lower than that immunoprecipitated with anti-Crk-II antibodies and cannot be detected under these conditions.

NGF and EGF stimulate the tyrosine phosphorylation of two proteins with molecular masses between 115 and 125 kDa that specifically immunoprecipitated with anti-Syp antibodies (36). However, no p130Cas immunoreactive species were detected in anti-Syp immunoprecipitates, indicating that Syp-associated phosphoproteins are not related to p130Cas. Moreover, p130Cas did not associate with other SH2/SH3-containing proteins including Nck and Shc (data not shown), demonstrating a specific interaction between Crk-II and p130Cas.

The concentration dependence of the effect of NGF and EGF on the appearance of the Crk-p130Cas complex in PC-12 cells was also evaluated (Fig. 4). Increasing concentrations of NGF or EGF were added to PC-12 cells for 1 min. Lysates were immunoprecipitated with anti-Crk-II antibodies and cannot be detected under these conditions.

**Figure 3.** NGF and EGF promote increased association of endogenous Crk with p130Cas in PC-12 cells. The lysates from PC-12 cells unstimulated (C) or stimulated with NGF (100 nM) or EGF (100 ng/ml) for 1 min at 37°C, were immunoprecipitated (IP) with anti-Crk-II, anti-p130Cas, or anti-Syp antibodies. The resulting immunoprecipitates were subjected to immunoblotting with anti-p130Cas antibodies. The arrow indicates the position of p130Cas. Molecular mass markers, indicated at the left, are given in kDa.

p130Cas is also detected as a broad band, perhaps due to different phosphorylation states. The blot was completely stripped of the anti-phosphotyrosine antibodies, and the region between the 68- and 200-kDa prestained molecular size markers was excised and reprobed with anti-p130Cas antibodies (Fig. 2B). p130Cas was precipitated equally from all samples and comigrated with the major phosphotyrosine-containing protein detected in this size range. In order to identify the 42-kDa phosphotyrosine-containing protein, the region between the 29- and 68-kDa prestained molecular size markers was immunoblotted with anti-Crk-II antibodies. Although v-Crk overexpressed in 3Y1 cells could be immunoprecipitated with anti-p130Cas antibodies (20), we were unable to detect Crk-II proteins by blotting with anti-Crk-II antibodies the anti-p130Cas immunoprecipitates from NGF- or EGF-treated cells. It appears that the level of expression of the 42-kDa form of Crk-II is very low compared with the 40-kDa form (Fig. 1B). It is therefore possible that the relative amount of this form of Crk-II immunoprecipitated with the anti-p130Cas antibodies is substantially lower than that immunoprecipitated with anti-Crk-II antibodies and cannot be detected under these conditions.
 inhibited with anti-Crk-II antibodies followed by immunoblotting with anti-phosphotyrosine, anti-Crk-II, or anti-p130Cas antibodies. Immunoblotting with anti-phosphotyrosine antibodies revealed an increase in tyrosine-phosphorylated 125–135-kDa protein associating with endogenous Crk (Fig. 4A). This increase was observed with as little as 1 nM NGF and 1 ng/ml EGF. The samples contained equal amounts of Crk-II as detected with anti-Crk-II antibodies immunoblotting (Fig. 4B). To test whether the 125–135-kDa phosphoprotein is p130Cas, the same blot was stripped and reprobed with anti-p130Cas antibodies. Fig. 4C shows that p130Cas comigrated on SDS-PAGE with the 125–135-kDa phosphotyrosine-containing protein.

p130Cas Associates with Crk via Its SH2 Domain—Experiments with peptide libraries suggested that the SH2 domain of Crk specifically binds to phosphotyrosine residues containing the primary sequence pYXXP (9), of which there are 15 in p130Cas (20). The interactions of phosphotyrosine-containing proteins with the SH2 domain of Crk were further examined in PC-12 cells stimulated with NGF or EGF using the SH2 domain of Crk expressed as a GST fusion protein immobilized on glutathione-agarose beads. Following treatment of PC-12 cells with NGF or EGF for 1 min, lysates were precipitated with GST-fusion proteins of Crk-SH2 or Grb2-SH2 or GST. The precipitates were washed, and the bound proteins were separated by SDS-PAGE and detected by immunoblotting with anti-phosphotyrosine antibodies. As shown in Fig. 5A, both GST-Crk-SH2 and GST-Grb2-SH2 but not GST alone precipitated a 170-kDa tyrosine-phosphorylated protein present in EGF-treated PC-12 cells that was shown to be the EGF receptor (data not shown and Refs. 26 and 34). Additionally, both NGF and EGF stimulated the interaction of 125–135-kDa and 70-kDa phosphotyrosine-containing proteins with GST-Crk-SH2 but not with GST-Grb2-SH2 or GST alone. GST-Grb2-SH2 precipitated a tyrosine-phosphorylated protein migrating at approximately 52 kDa, previously identified as Shc (34).

To test whether the 125–135-kDa phosphotyrosine-contain-
ing protein that bound to GST-Crk-SH2 is p130Cas, the same protein from PC-12 cells stimulated with NGF or EGF. As evident from the anti-phosphotyrosine immunoblotting of the bound proteins (Fig. 6A), binding of the EGF receptor and p130Cas to GST-Crk-SH2 was quantitatively inhibited by the phosphopeptide DADEpYLIPQQG (pY992) which contains proline in the +3 position relative to tyrosine, indicating a Crk-SH2 binding motif. The peptide NPVpYNQPLN (pY1086) only partially inhibited the binding of GST-Crk-SH2, while the phosphopeptide VEPepYINQSVPK (pY1068) had no effect. Reprobing the same immunoblot with anti-p130Cas antibodies revealed a concordant decrease in the amount of p130Cas-associated with the Crk-SH2 domain in the presence of pY992 and pY1086 (Fig. 6B).

The SH2 region of Grb2 is predicted to bind to a consensus sequence pYXNY (38). The high affinity binding site for Grb2 on the EGF receptor has been mapped to the autophosphorylation site Tyr1068 (39). Both phosphotyrosine peptides, pY1068 and pY1086, which contain potential Grb2-SH2 recognition sites, completely inhibited GST-Grb2-SH2 binding to the tyrosine-phosphorylated EGF receptor from PC-12 cells treated with EGF. However, the pY992 peptide that does not contain this motif had no effect on GST-Grb2-SH2 binding (Fig. 6A).

**DISCUSSION**

Cellular overexpression of the oncogenic form of Crk leads to cell transformation and elevation of intracellular phosphotyrosine levels of specific proteins (11, 15, 17). Moreover, overexpression of v-Crk in PC-12 cells accelerates their differentiation in response to both NGF and EGF (26). Although this protein apparently lacks catalytic activity, both its transforming and differentiating functions require intact SH2 and SH3 domains (14, 18, 25). Crk has been shown to associate with two guanine nucleotide exchange proteins, Sos and C3G, via its SH3 domain (22–24), suggesting a role in regulating the Ras signaling pathway. Additionally, Crk proteins can undergo tyrosine phosphorylation in transformed cells or in response to growth factors (21, 26, 35, 40). Although transfection studies with v-Crk have suggested that this protein plays a major role in tyrosine kinase-initiated signals, little is known about the interactions of endogenous Crk family members.

We report here that treatment of PC-12 cells with NGF or EGF causes the rapid tyrosine phosphorylation of the 42-kDa form of endogenous Crk-II. In addition to Crk, the NGF- and EGF-dependent tyrosine phosphorylation of another SH2 and SH3 domain-containing adapter, Nck, has been reported (41, 42), although Grb2 is not tyrosine-phosphorylated after exposure to any growth factor (12).

The role of the tyrosine phosphorylation of Crk is not clear, but this modification may modulate interactions of the Crk-SH3 domain. Alternatively, the tyrosine phosphorylation of Crk may induce its interaction with other SH2-containing signaling proteins, or it may produce an intramolecular interaction. In this regard, the c-Abl kinase binds to the Crk-SH3 domain and tyrosine-phosphorylates c-Crk on a single tyrosine, which creates a binding site for Crk-SH2 domain (21, 43).

In addition to its tyrosine phosphorylation, Crk is also known to associate with certain tyrosine-phosphorylated proteins via its SH2 domain (17–19). The major phosphotyrosine-containing protein detected in v-Crk-transformed cells has recently been cloned (20). This 130-kDa protein, called Cas, associates directly with v-Crk in these cells (20). We report here that...
tyrosine phosphorylation and enhanced its association with endogenous Crk-II. Because p130Cas contains 15 potential Crk-SH2 recognition sites, we suspected that p130Cas might associate with the SH2 domain of the later protein. This interaction was directly demonstrated using a GST-fusion protein containing the SH2 domain of Crk. These data suggest that upon stimulation of PC-12 cells with NGF or EGF, p130Cas undergoes tyrosine phosphorylation, further inducing its binding to Crk. Although the significance of this association is not known, Crk may be constitutively associated with the nucleotide exchange factor C3G. Thus, one possible physiological role for the p130Cas-Crk-C3G complex in tyrosine kinase-induced signaling may involve targeting the activation of p21ras and its homologs. However, the growth factor-dependent interactions that result from tyrosine phosphorylation. Thus, Crk proteins belong to a family of bifunctional adapter molecules that link tyrosine phosphorylation to a multitude of downstream cellular processes.

A low but detectable level of tyrosine-phosphorylated p130Cas that is already associated with Crk could be detected in nonactivated PC-12 cells (Figs. 2A and 4A). While this work was under review, several reports have demonstrated adhesion-induced tyrosine phosphorylation of p130Cas suggesting a role for p130Cas in integrin-mediated signal transduction (44, 45). It is therefore possible that the low level of p130Cas tyrosine phosphorylation seen in unstimulated PC-12 cells is due to the adhesion of the cells to the dishes. However, it is possible that even a 3-4-fold increase in the tyrosine phosphorylation of p130Cas in response to NGF or EGF and its interaction with Crk further amplify and propagate NGF and EGF signaling.

Following NGF or EGF treatment of PC-12 cells, GST-Crk-SH2 also binds the tyrosine-phosphorylated paxillin, a focal adhesion-associated protein. Paxillin becomes phosphorylated on tyrosine in response to a number of stimuli (26, 46, 47) and is thought to be a substrate of the focal adhesion kinase p125FAK (37). It has been shown that the tyrosine phosphorylation of paxillin by p125FAK creates binding sites for the Crk-SH2 domain (37). Interestingly, we detected p125FAK with Crk-SH2-associated proteins. However, the presence of p125FAK in this complex may be mediated through its interaction with paxillin. Moreover, p125FAK may also phosphorylate other proteins present in this complex, including p130Cas or Crk itself.

Despite the relatively high binding affinity of Crk to phosphorylated paxillin in vitro, so far we have detected neither paxillin nor p125FAK in anti-Crk immunoprecipitates. Similarly, we did not observe the communoprecipitation of the EGFr receptor with Crk in PC-12 cells. This may reflect the relative instability of such interactions or may indicate that the association is below the limit of detection. Consistent with this later possibility, both the EGFr receptor and paxillin are communoprecipitated with v-Crk in PC-12 cells expressing v-Crk. On the other hand, the association of Crk with tyrosine-phosphorylated p130Cas is easily detected in PC-12 cells in a growth factor-dependent manner.

The multitude of interactions described here indicate that Crk is indeed a versatile signaling molecule that is likely to participate in a number of pathways. Its interactions with p130Cas and C3G via SH2 and SH3 domains suggest that Crk may play a key role as an adapter targeting nucleotide exchange factors for Ras and its homologs. However, the growth factor-dependent interaction of Crk with paxillin and therefore the potential interaction with p125FAK suggests that it may play a separate role in regulating cytoskeletal interactions that result from tyrosine phosphorylation. Thus, Crk proteins belong to a family of bifunctional adapter molecules that link tyrosine phosphorylation to a multitude of downstream cellular processes.

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