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Expression of the v-*crk* Oncogene Product in PC12 Cells Results in Rapid Differentiation by both Nerve Growth Factor- and Epidermal Growth Factor-Dependent Pathways

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The transforming gene of the avian sarcoma virus CT10 encodes a fusion protein (p47$^{gag\text{-}crk}$ or v-Crk) containing viral Gag sequences fused to cellular sequences consisting primarily of Src homology regions 2 and 3 (SH2 and SH3 sequences). Here we report a novel function of v-Crk in the mammalian pheochromocytoma cell line, PC12, whereby stable expression of v-Crk induces accelerated differentiation, as assessed by induction of neurites following nerve growth factor (NGF) or basic fibroblast growth factor (bFGF) treatment compared with the effect in native PC12 cells. Surprisingly, however, these cells also develop extensive neurite processes after epidermal growth factor (EGF) stimulation, an event which is not observed in native PC12 cells. Following EGF or NGF stimulation of the v-CrkPC12 cells, the v-Crk protein itself became tyrosine phosphorylated within 1 min. Moreover, in A431 cells or TrkA-PC12 cells, which overexpress EGF receptors and TrkA, respectively, a GST-CrkSH2 fusion protein was indeed capable of binding these receptors in a phosphotyrosine-dependent manner, suggesting that v-Crk can directly couple to receptor tyrosine kinase pathways in PC12 cells. In transformed fibroblasts, v-Crk binds to specific tyrosine-phosphorylated proteins of p130 and paxillin. Both of these proteins are also complexed to v-Crk in PC12 cells, as evidenced by their coprecipitation with v-Crk in detergent lysates, suggesting that common effector pathways may occur in both cell types. However, whereas PC12 cellular differentiation can occur solely by overexpression of the v-Src or oncogenic Ras proteins, that induced by v-Crk requires a growth factor stimulatory signal, possibly in a two-step process.

The reciprocal and highly regulated processes of cellular proliferation, cellular differentiation, and progression to a postmitotic state during embryogenesis generates the cellular diversity in the developing nervous system. Growth factors, in part, can regulate these proliferative or differentiative processes by analogous mechanisms that involve activation of transmembrane receptors with inducible tyrosine kinase activity (4). Furthermore, numerous receptor tyrosine kinases, including the epidermal growth factor (EGF) receptor, the basic fibroblast growth factor (bFGF) receptor, and the neurotrophin receptors, Trk A, B, and C, are abundantly expressed in both the central and peripheral nervous systems during development (8, 9, 25, 30a, 32, 41, 50). Intracellular signaling proteins utilizing Src homology 2 (SH2) domain interactions, such as phospholipase C-γ and p85 subunit of phosphatidylinositol 3-kinase, associate with these tyrosine-phosphorylated receptors to allow multimeric protein complexes to form and lead to the activation of specific signal transduction pathways (4, 26).

Given the complexity and extreme cellular diversity of the nervous system, the study of transformed neural crest-derived cell lines which can recapitulate many of the growth factor signaling events has been instrumental in defining the pathways resulting in neuronal differentiation and proliferation. The rat pheochromocytoma cell line, PC12 (12), which expresses receptors important for mitogenic signaling, such as the EGF and insulin-like growth factor 1 (IGF-1) receptors (7, 19), and those mediating cell differentiation and cessation of cell division, the NGF and FGF receptors (12, 51), has uncovered many common pathways resulting in opposing biological effects (5, 47). In particular, NGF induction of PC12 cell differentiation involves activation of numerous enzymes that have been shown to be important for mitogenesis and transformation in other cell types, including the NGF receptor (termed Trk A receptor), Src, Ras, Raf, phospholipase C-γ, phosphatidylinositol 3-kinase, and ERK-1 proteins (13, 16, 22–24, 28, 48, 54, 55). Furthermore, the SH2 and SH3 domain-containing adaptor proteins, Nck and Grb2, have been implicated in NGF differentiation and have been postulated to connect intracellular proteins within the signaling pathway (37, 43).

Since v-Crk is a member of this class of adaptor proteins and has potent transforming activity in chicken embryo fibroblasts (35), we sought to express v-*crk* in PC12 cells to delineate its role in a test system capable of undergoing defined mitogenic and differentiative signaling pathways. The v-*crk* oncogene encodes a 47-kDa fusion protein, p47$^{gag\text{-}crk}$, of viral Gag sequences fused to an SH2 and SH3 domain (35). These domains are derived from the c-*crk* gene encoding a protein composed of SH2-SH3-SH3 domains, with the latter SH3 domain deleted in the v-Crk product (40). Although the domains in Crk proteins are noncatalytic in nature, transformation by v-*crk* is accompanied by elevation in cellular phosphotyrosine status, and hence Crk probably modulates a kinase-phosphatase interplay in transformed cells (2, 36). Here we report that v-*crk* expression in mammalian PC12 cells not only accelerates cellular differentiation by NGF but promotes neurite outgrowth after EGF treatment, suggesting that v-*crk* expression may activate a differentiative-specific repertoire in PC12 cells.

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MATERIALS AND METHODS

Cell culture and transfection. Rat pheochromocytoma cells (PC12) were provided by David Kaplan and maintained, as described previously (22), in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and 5% horse serum. Prior to transfection, cells were plated on rat tail collagen-coated plates (12) and each 100 mM plate was incubated with a lipofectin (50 μl)-DNA (20 μg) mixture for 18 h. The v-*crk* cDNA was under the control of the murine sarcoma virus long terminal repeat in the expression vector pMEX^{neo} (2). After 48 h, cells were placed under selection in medium containing G418 (500 μg/ml). Following 5 weeks of culture, surviving colonies were subcloned and expanded. 3T3 fibroblasts overexpressing rat *trk*A (a gift of Luis Parada) were maintained in DMEM containing 10% fetal calf serum and 500 μg of G418 per ml (21). 3T3 fibroblasts stably overexpressing the human low-affinity NGF receptor, p75 (a gift of Moses Chao), were generated by cotransfection with human p75 cDNA (20) under the control of the cytomegalovirus (CMV) promoter (15) and a Rous sarcoma virus (RSV) plasmid containing the cDNA encoding hygromycin resistance and were maintained in DMEM containing 10% fetal calf serum and 0.2 μg of hygromycin per ml. A431 cells overexpressing the EGF receptor were maintained as previously described (1a, 31). For all cells, the medium was replaced with fresh medium every 48 h. If cells were treated with growth factor additions, medium changes contained freshly prepared growth factor supplementation at the following concentrations: mouse 2.5S NGF (Bioproducts for Science), 50 ng/ml; recombinant human EGF (Bioproducts for Science), 50 ng/ml; recombinant human bFGF (R and D Systems), 10 ng/ml or bovine insulin (Sigma), 50 ng/ml.

Western immunoblot analysis. To assess the level of expression of several proteins in native PC12 cells and PC12 cell transfectants, cells were lysed in RIPA buffer (1a) in the presence of 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, and 5 μg of leupeptin per ml. Protein concentrations in the lysates were normalized by the Bio-Rad reagent with bovine serum albumin as a standard. Equivalent concentrations of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. After blocking the membranes in 1% bovine serum albumin containing Tris-buffered saline, we incubated the blots with the indicated antibodies for approximately 3 h at room temperature prior to washing and further incubation with peroxidase-conjugated secondary antibody. All blots were developed by using the ECL detection system (Amersham). Anti-EGF receptor antibodies were purchased from UBI, and anti-paxillin antibody was from Zymed. Specific rabbit polyclonal antisera generated to the cytoplasmic domain of human p75 (amino acids 248 to 399) (20) or the cytoplasmic domain of human TrkA (C-terminal 367 amino acids) (33) were used to immunoprecipitate and probe for p75 and Trk A receptors, respectively. Affinity-purified anti-phosphotyrosine antibodies (anti-PY) were prepared against tyrosine-phosphorylated v-Abl proteins as described previously (2).

Detection of NGF receptors by affinity cross-linking. Mouse NGF (2.5S) was radioiodinated as described previously (17) and used within 2 weeks. Cells (2×10^6/ml) were incubated with 0.2 nM ^{125}I-NGF at 22°C for 30 min and then cross-linked with a combination of 5 mM 1-ethyl-3(3-dimethylaminopropyl) carbodiimide · HCl (EDC) and 25 μM disuccinimidyl suberate (DSS) (16). Following lysis in RIPA buffer, complexes were immunoprecipitated with the polyclonal antibodies described above. Immune complexes were washed as described previously, and radiolabeled proteins were detected by SDS-PAGE and autoradiography (21).

Northern (RNA) analysis of VGF mRNA levels. Cells were cultured for 24 h in DMEM containing 10% calf serum and 5% horse serum and supplemented or not supplemented with growth factors as described above. RNA was isolated by guanidinium extraction and separation by centrifugation over a cesium chloride cushion. Total RNA (10 μg) was separated by agarose gel electrophoresis, blotted onto nitrocellulose, and probed with a radiolabeled cDNA insert encoding rat VGF as described previously (16, 29). Agarose gels were stained with ethidium bromide prior to transfer to demonstrate equivalent amounts of rRNA.

Analysis of cell doubling time, and [^{3}H]thymidine studies. To determine cell doubling time, cells were treated with trypsin-EDTA to achieve a single-cell suspension and counted by Coulter analysis, and 7,000 cells were plated in 60-mm dishes. Cells were cultured for 14 days in DMEM containing 10% calf serum and 5% horse serum, treated with trypsin-EDTA, and again counted by Coulter analysis. Cell counts were performed in triplicate in three independent experiments, and mean counts varied by less than 10%. For thymidine incorporation studies, 7,000 cells per well were plated in 24-well plates and maintained in DMEM containing 10% calf serum and 5% horse serum (high serum) or DMEM containing 2% calf serum and 1% horse serum (low serum) supplemented or not supplemented with growth factors as described above. Cells were fed with gentle medium changes every 48 h, with addition of fresh growth factor. After 14 days, [^{3}H]thymidine (2.0 μCi per well) was added for 18 h, and then the cells were washed and harvested as described previously (27). Experiments with each condition were performed in triplicate. For analysis of the kinetics of neurite outgrowth in response to NGF and EGF, 7,000 cells were plated in 24-well plates and treated with the indicated growth factor in medium containing 2% calf serum and 1% horse serum.

Preparation of GST-CrkSH2 proteins and immunoprecipitations. Glutathione *S*-transferase (GST)–CrkSH2 was cloned from a *Sfi*I-*Eco*NI fragment of c-*crk* as described previously (2). The construct encodes a fusion protein of GST followed by the first 129 amino acids of c-Crk. For coimmunoprecipitation studies with activated receptors, 5 μg of fusion protein was added to 1 mg of cellular lysate for 30 min at 4°C on a rotary shaker. Protein complexes were collected by the addition of GSH-Sepharose beads and washed five times in RIPA buffer prior to SDS-PAGE. For immunoprecipitations, cells lysed in either RIPA buffer or nonionic lysis buffer (1% Triton X-100, 10% glycerol, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 150 mM NaCl [pH 7.5]) were incubated with either anti-Gag monoclonal antibody (1:200), anti-Crk polyclonal antibody (1:500), anti-EGFR monoclonal antibody (1:200), or anti-paxillin monoclonal antibody (1:200) for 3 h at 4°C. Immune complexes were collected with either goat anti-mouse passified protein A-Sepharose beads (mouse monoclonal antibodies) or protein A-Sepharose beads alone (rabbit polyclonal antibodies).

RESULTS

Stable expression of v-Crk in PC12 cells. PC12 cells were stably transfected with v-*crk* cDNA in the expression vector pMEX^{neo}. Following selection in G418, resistant colonies were subcloned and expanded. Of 18 clones, 6 demonstrated high levels of v-Crk expression, approximately 7- to 10-fold higher than those of endogenous c-Crk (Fig. 1A). These six indepen-

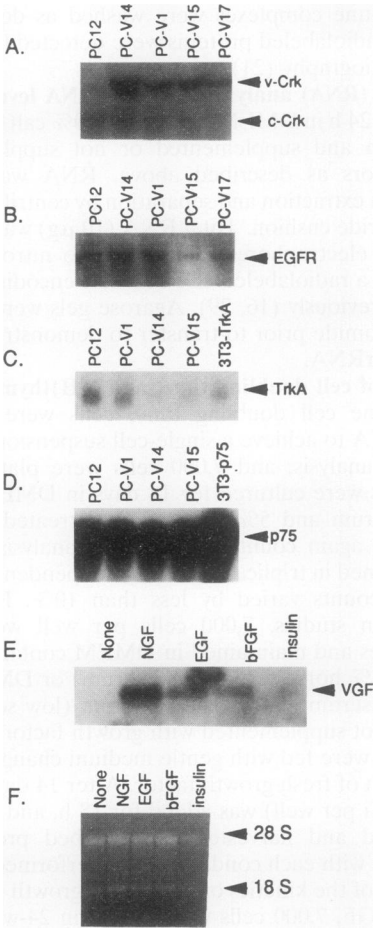

FIG. 1. Characterization of v-Crk PC12 cell subclones. (A) Expression of v-Crk in PC12 cell lines. PC12 cells were transfected with v-*crk* cDNA under the control of the murine sarcoma virus long terminal repeat in the expression vector pMEX^{neo}. Following selection in G418, surviving colonies were subcloned and the level of v-Crk expression was assessed by Western blot analysis with anti-Crk antibodies. All lanes contained equivalent amounts of cellular lysate (40 μg). (B) Expression of EGF receptors (EGFR) in v-CrkPC12 cells. Untreated cells (2×10^6) were subjected to detergent lysis, immunoprecipitated with anti-EGF receptor polyclonal antibody (UBI), and analyzed by Western blotting with anti-EGF receptor antibody. (C and D) Expression of NGF receptors in v-CrkPC12 cells. Cells expressing NGF receptors were incubated with ^{125}I-NGF and cross-linked with EDC and DSS. Following detergent lysis, receptors were immunoprecipitated with antisera specific for TrkA or p75 NGF receptor and then subjected to SDS-PAGE and autoradiography. (E) Northern analysis of VGF levels in v-CrkPC12 cells. RNA was harvested from cells cultured for 24 h in medium supplemented with either NGF (50 ng/ml), EGF (50 ng/ml), bFGF (10 ng/ml), insulin (50 ng/ml), or no additions. RNA was separated by electrophoresis, transferred to nitrocellulose, and probed with a radiolabeled insert for rat VGF. Equivalent loading of rRNA can be confirmed by ethidium bromide staining (F).

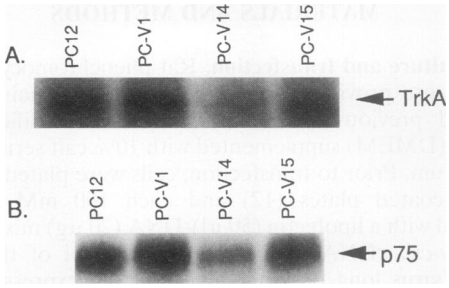

FIG. 2. Expression of NGF receptors by Western blot analysis. Cells from the indicated clones were extracted in RIPA buffer, and lysates containing 1.0 mg of protein were subjected to immunoprecipitation with anti-Trk (A) or anti-p75 (B) antiserum. Following SDS-PAGE separation and transfer to nitrocellulose, receptors were detected by probing with anti-Trk (panel A) or anti-p75 (panel B) antiserum.

dent clones of v-Crk-expressing PC12 cells (v-CrkPC12) appeared morphologically similar to parental PC12 cells but with slight flattening of the cell bodies. After NGF treatment (50 ng/ml), however, all six v-CrkPC12 clones displayed accelerated neurite outgrowth, with 30% of the cells bearing neurites of more than two cell bodies in length at 24 h and 70% of the cells expressing such neurites at 54 h. In contrast, the parental PC12 cell line and transfectants resistant to G418 but not expressing v-Crk displayed fewer than 7% of cells with neurites of two cell bodies in length at 24 h and 35% at 54 h following NGF treatment (see Fig. 4A). In addition, v-CrkPC12 cells responded to lower levels of NGF than did native PC12 cells, as assessed by neurite outgrowth, suggesting that v-Crk expression potentiates NGF-induced differentiation (see Fig. 4B). Since overexpression of the NGF receptor, TrkA, can result in a similar augmentation in the rate of NGF-induced neurite outgrowth (16), the level of expression of NGF receptors was quantitated by affinity cross-linking with ^{125}I-NGF (Fig. 1C and D) or by Western blot analysis with antisera specific for TrkA and p75 (Fig. 2). The v-CrkPC12 cells displayed similar or reduced levels of TrkA and p75 in comparison with the parental PC12 cells.

EGF induces neurite formation in v-Crk PC12 cells. To determine whether v-Crk expression could influence the signaling by other receptors expressed on PC12 cells, we treated v-CrkPC12 lines with EGF, bFGF, and insulin. As shown in Fig. 3, both EGF and bFGF treatment of v-CrkPC12 cells resulted in the acquisition of a morphologically differentiated phenotype. Over 60% of the v-CrkPC12 cells express neurites of two cell bodies in length following treatment with EGF or bFGF for 54 h (Fig. 3 and 4C). These results contrast directly with those obtained with parental PC12 cells, in which EGF did not induce neurite formation and bFGF resulted in neurite formation but at a significantly lower rate than NGF did (Fig. 2) (19, 51). The effect of EGF upon these v-Crk-expressing cells is not a result of over- or underexpression of the EGF receptor, since levels of this receptor are similar in native and v-Crk-expressing PC12 cells, as detected by Western blot analysis (Fig. 1B). However, both v-CrkPC12 and native PC12 cells treated with insulin, which is known to interact with the IGF-1 receptor in PC12 cells, failed to differentiate (Fig. 3) (6, 7). Similar morphological responses to growth factor stimulation were obtained with all six v-Crk-expressing clones.

To confirm that differentiation along a neuronal pathway was occurring, Northern analysis with the differentiation-specific probe for the secretory granule protein VGF (29, 45) was undertaken with RNA from growth factor-stimulated v-CrkPC12 cells (Fig. 1E). Previous studies have confirmed that VGF expression is induced in native PC12 cells following NGF or bFGF treatment but not following treatment with EGF, insulin, or retinoic acid (45). RNA from growth factor-stimulated v-CrkPC12 cells was probed with the cDNA encoding rat VGF, and the VGF mRNA species was induced following both EGF and NGF treatment, with comparable

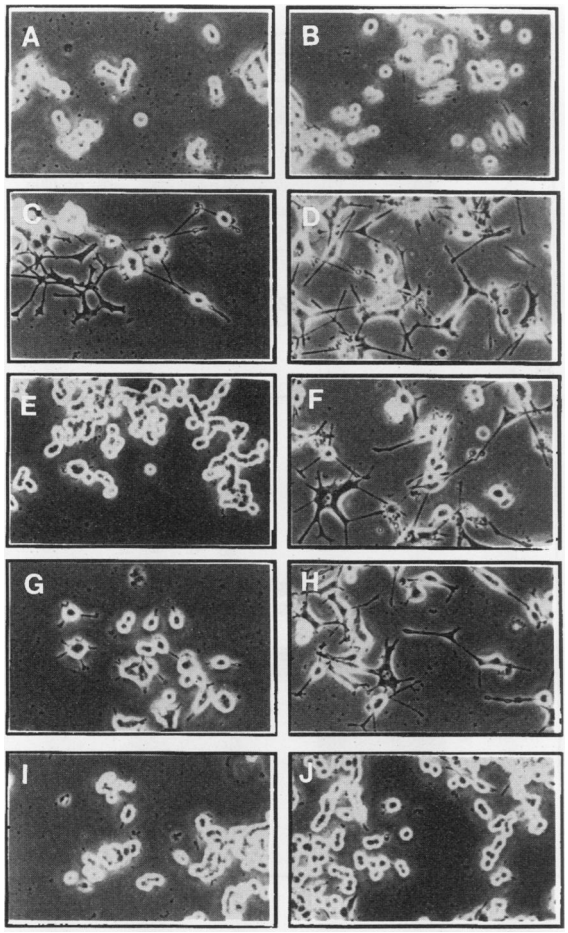

FIG. 3. Neurite outgrowth by growth factor treatment of PC12 (A, C, E, G, and I) and v-CrkPC12 (B, D, F, H, and J) cells. Cells were maintained as described in Fig. 1E except that the duration of the incubation with growth factors was 90 h. (A and B) Untreated; (C and D) NGF treated; (E and F) EGF treated; (G and H) bFGF treated; (I and J) insulin treated. Magnification, ×200.

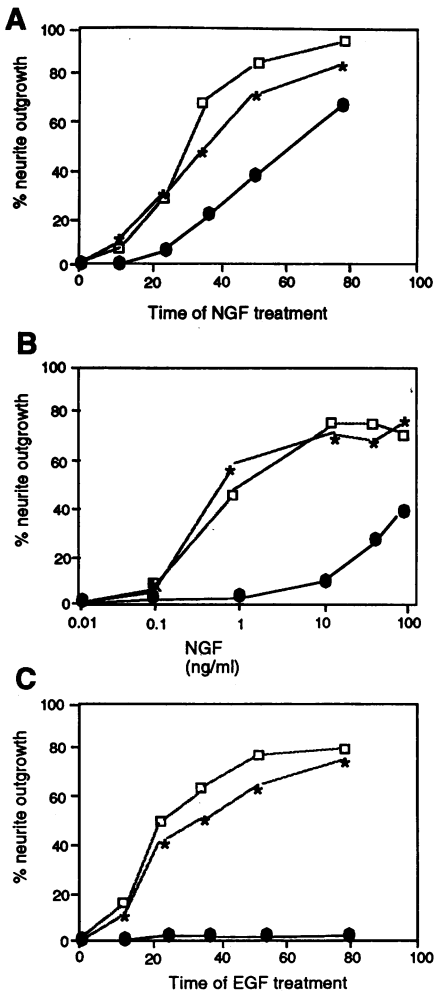

FIG. 4. Response of native and v-CrkPC12 cells to growth factor treatment. Cells were maintained in medium containing 2% calf serum and 1% horse serum plus growth factor, with addition of fresh medium and growth factor every 48 h. At the indicated times, 150 to 300 cells were counted and scored for the expression of neurites longer than one cell body. Symbols: ●, PC12; □, PC-V1; *, PC-V15. (A) Kinetics of NGF-induced neurite-induced neurite outgrowth in response to 50 ng of NGF per ml. (B) Dose response to NGF. Cells were counted after 54 h of treatment. (C) Kinetics of EGF-induced neurite outgrowth in response to 50 ng of EGF per ml.

levels of expression. No VGF mRNA was detected in native PC12 cells treated with EGF (data not shown).

Effects of v-Crk expression on PC12 cell proliferation. Since NGF and EGF normally play opposing roles with respect to cell proliferation (5, 47), we examined the mitogenic response in both PC12 and v-CrkPC12 cells treated with serum, NGF, EGF, bFGF, and insulin (Fig. 5). In the native PC12 cells, continuous exposure to NGF for 2 weeks results in a marked reduction in cell proliferation as indicated by the low levels of [^{3}H]thymidine incorporated into DNA. This is consistent with the previous results in which NGF treatment results in accumulation of PC12 cells in a G_1 state (44). In contrast, proliferation of native PC12 cells is enhanced in cells maintained in the presence of EGF and insulin (Fig. 5) (7, 19). The v-CrkPC12 cells proliferate more slowly than PC12 cells in the absence of exogenous growth factor addition, with a cell doubling time of 50 to 55 h, compared with parental PC12 cells, with a doubling time of 42 to 44 h. The v-CrkPC12 cells, however, exhibit a modest proliferative response after long-term treatment with EGF, NGF, and bFGF, suggesting that v-Crk expression can concomitantly affect both mitogenic and differentiative signaling pathways upon growth factor treatment. At present, we do not know the relationship between cellular differentiation and cellular proliferation on an individual cell basis.

Analysis of tyrosine-phosphorylated proteins expressed in v-CrkPC12 cells. The above results strongly suggest that v-Crk can modulate intracellular pathways critical in growth factor receptor-dependent signaling. Analysis of the tyrosine-phosphorylated proteins in v-CrkPC12 cells (Fig. 6A) treated with EGF or NGF for 1 min demonstrated prominent constitutive tyrosine phosphorylation of proteins of 130 and 70 kDa, as well as ligand-inducible tyrosine phosphorylation of a 47-kDa protein. To determine whether this latter band is indeed v-Crk, we immunoprecipitated replicate lysates with anti-Gag monoclonal antibody and performed anti-PY Western blot analysis. As observed in Fig. 6C, the v-Crk protein was tyrosine phosphorylated after a 1-min stimulation with either NGF or EGF. This band was confirmed to be v-Crk by reprobing the Western blot

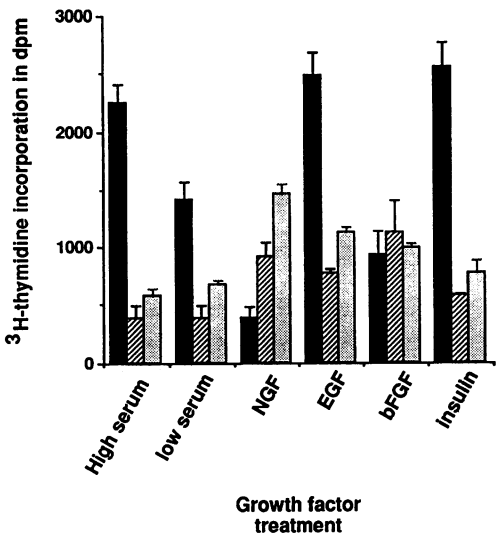

FIG. 5. Effects of serum and growth factors on [^{3}H]thymidine incorporation in v-CrkPC12 and PC12 cells. Cells were plated in 24-well plates (7,000 cells per well) and maintained as described in Fig. 1E. After 2 weeks of treatment, [^{3}H]thymidine was added for 18 h. Error bars denote the standard deviation of triplicate samples. Data are representative of three independent experiments. Symbols: ■, PC12; ▨, PC-V1; ▩, PC-V15.

with anti-Crk polyclonal antibody (results not shown). Furthermore, v-Crk became phosphorylated after bFGF but not insulin stimulation, and hence v-Crk phosphorylation on tyrosine correlated with induction of neurite outgrowth.

To investigate whether v-Crk can interact directly with activated tyrosine kinase receptors, we immunoprecipitated lysates of PC12 and v-CrkPC12 cells with anti-Gag antibodies. Although we did observe ligand-inducible stable association of v-Crk with a 180-kDa tyrosine-phosphorylated band after EGF (Fig. 6B), which was confirmed to be EGF receptor by immunostaining with anti-EGF receptor antibodies, we were unable to detect a stable association of v-Crk with TrkA in vivo. Given the relatively low level of TrkA receptors present in native PC12 cells (estimated to be less than 10,000 molecules per cell), it is possible that an interaction between v-Crk and the tyrosine-phosphorylated TrkA receptor is below the limit of detection. This is supported by the demonstration in Fig. 7 that a GST-CrkSH2 fusion protein could precipitate both activated TrkA receptors from PC12 cells overexpressing TrkA and activated EGF receptors from A431 cells. However, more studies are required to determine the nature of a v-Crk–TrkA interaction in vivo.

Transformation of fibroblasts by v-*crk* is accompanied by the binding of v-Crk to specific tyrosine-phosphorylated intracellular proteins including p130 and paxillin (2, 36). Although the function of p130 has not yet been identified, paxillin is thought to be involved in the assembly of focal adhesions and may mediate integrin signaling (52). To determine whether similar proteins were tyrosine phosphorylated and complexed to v-Crk in PC12 cells, detergent extracts of cells were subjected to immunoprecipitation with anti-Crk antisera and analyzed by Western blotting procedures. As shown in Fig. 6A, v-CrkPC12 cells express major tyrosine-phosphorylated proteins of 130 and 70 kDa. Both of these proteins are complexed to v-Crk in the v-CrkPC12 cells as evidenced by their coimmunoprecipitation with anti-Crk antibodies (Fig. 6B). The 70-kDa tyrosine-

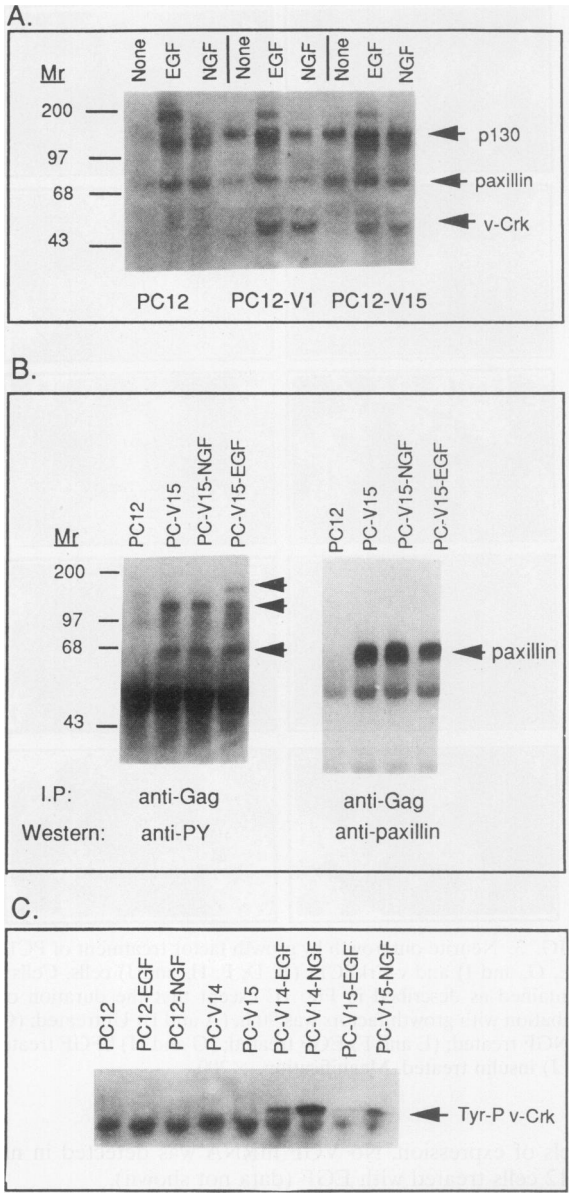

FIG. 6. Anti-phosphotyrosine analysis of PC12 and v-CrkPC12 cells. (A) Western blotting of total cell lysate with anti-PY. Cells were treated for 1 min with 50 ng of NGF or EGF per ml, total cellular lysate (50 μg) was separated by SDS-PAGE, and Western blots were analyzed with anti-PY antibody. The positions of the major tyrosine-phosphorylated proteins are indicated. (B) Analysis of v-Crk-associated cellular proteins. Approximately 1.0 mg of cellular lysate from native PC12 or v-CrkPC12 clone PC-V15 was immunoprecipitated in nonionic lysis buffer (HNTG buffer containing 1% Triton X-100) with anti-Gag antibodies followed by Western blotting with anti-PY antibody or anti-paxillin antibody. From top to bottom, the localization of the major tyrosine-phosphorylated proteins, EGF receptor, p130, and p70 paxillin, are indicated by arrows. The anti-pY immunoblot shown on the left of panel B was stripped with 2% SDS and 0.7% mercaptoethanol to release bound antibodies and subsequently reprobed with anti-paxillin monoclonal antibody. The position of paxillin is indicated. Experiments with two independent clones, PC-V1 and PC-V14, gave similar results. (C) Tyrosine phosphorylation of v-Crk following EGF or NGF stimulation. Cells from the indicated clones were treated with growth factor for 1 min, and then approximately 1.0 mg of cellular lysate was immunoprecipitated with anti-Gag antibody. Detection was with ^{125}I-conjugated protein A. The position of the tyrosine-phosphorylated v-Crk is indicated.

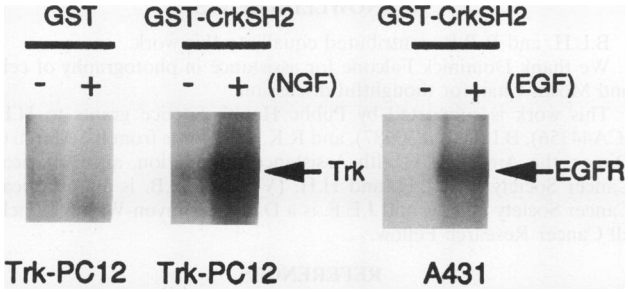

FIG. 7. Association of GST-CrkSH2 with tyrosine-phosphorylated TrkA and tyrosine-phosphorylated EGF receptors. TrkA-overexpressing PC12 cells or A431 human carcinoma cells overproducing EGF receptor were stimulated for 1 min with 50 ng of either NGF or EGF per ml. Lysates were precipitated with GST-CrkSH2 Sepharose beads. Coprecipitating proteins were analyzed with either anti-Trk or anti-EGF receptor antibodies in Western blot analysis.

phosphorylated protein was confirmed to be paxillin by reprobing the Western blot from the anti-Crk immunoprecipitation with an anti-paxillin monoclonal antibody (Fig. 6B), and the identity of the p130 was confirmed after partial proteolytic V8 mapping of ^{32}P-labeled proteins obtained from either CT10-CEF or v-CrkPC12 cells (not shown). It is noteworthy that similar v-Crk binding proteins to those observed in transformed fibroblasts (2) are also observed in v-CrkPC12 cells, although v-CrkPC12 cells display an opposing cellular phenotype.

When lysates of v-CrkPC12 cells which had been treated with NGF or EGF for long periods (2 weeks) were examined, tyrosine phosphorylation of p130 and paxillin were constitutively elevated in these cells and neither protein was hyperphosphorylated after long-term growth factor treatment (Fig. 8A and C). This result contrasts with that obtained with native PC12 cells, where tyrosine phosphorylation of paxillin was detectable only in untreated cells and on long-term exposure to NGF; by contrast, long-term treatment of native PC12 cells with EGF resulted in a loss of tyrosine-phosphorylated paxillin (Fig. 8C). Moreover, in native PC12 cells, paxillin levels were significantly reduced after EGF treatment compared with those in similarly treated v-CrkPC12 cells, suggesting that the amount and/or level of phosphorylation of paxillin may correlate with the ability of PC12 cells to differentiate.

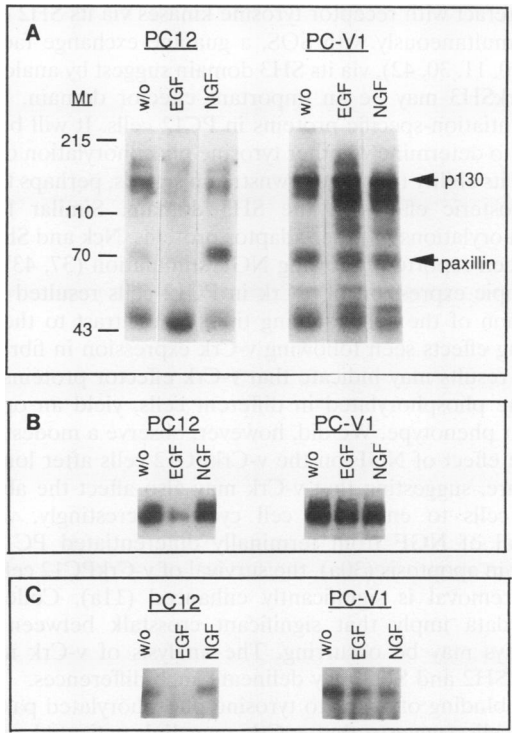

FIG. 8. Analysis of paxillin tyrosine phosphorylation in v-CrkPC12 and native PC12 cells following long-term treatment with NGF or EGF. Native PC12 cells (left side) or v-CrkPC12 clone PC-V1 (right side) were maintained in medium containing NGF (50 ng/ml) or EGF (50 ng/ml) or no additions (lanes w/o) for 2 weeks and extracted in RIPA buffer. (A) Total cellular protein (100 μg) was separated by SDS-PAGE, and Western blots were probed with anti-PY antiserum. (B) Approximately 1.0 mg of lysate was immunoprecipitated with anti-paxillin antibody followed by Western blotting with anti-paxillin. (C) The Western blot depicted in panel B was stripped and reprobed with anti-PY antibody to reveal the level of phosphotyrosine.

DISCUSSION

The differentiation of PC12 cells by NGF involves striking morphological and biochemical changes including the induction of numerous proteins required for the acquisition of a differentiated phenotype similar to that of a sympathetic neuron (14). By contrast, EGF stimulation of PC12 cells results in entry into the cell cycle and cellular proliferation (19). However, despite these opposing phenotypes, the signal transduction pathways by NGF and EGF appear to be remarkably similar, including the activation of receptor-type tyrosine kinases and the involvement of Ras proteins (5, 55). In the present study we have demonstrated that ectopic expression of the oncogenic v-Crk protein in PC12 cells eliminates the morphological distinctions between NGF and EGF treatment without modulating the levels of the EGF or TrkA receptors. Moreover, EGF induces the expression of neuron-specific mRNA for the secretory granule protein VGF, suggesting that growth and differentiative potential of PC12 cells by growth factors may be controlled by the recruitment of SH2/SH3 adaptor proteins to these activated tyrosine kinase receptors.

The mechanism by which v-Crk potentiates downstream pathways of growth factor receptor signaling remains speculative. The fact that we did not see either the activation of the EGF or TrkA receptors or neurite outgrowth in the absence of ligand suggests that expression of v-Crk cannot substitute for ligand-mediated receptor activation. This is in contrast to the situation with v-Src or oncogenic Ras, in which expression alone results in PC12 cell differentiation (1, 50a). While this work was in progress, studies involving microinjection of human c-Crk protein into PC12 cells reported that microinjection induced neurite outgrowth by the involvement of both SH2 and SH3 domains and in the absence of growth factor addition (49). It should also be pointed out that human c-Crk was reported to transform rat fibroblasts (34) whereas chicken c-Crk or v-Crk is not capable of inducing transformation by itself (39). We cannot discount the possibility that extremely high levels of v-Crk also induce spontaneous neurite outgrowth, since our selection procedure would not favor the cloning of cells with a very long doubling time. Nonetheless, the findings described above, in which each of the growth factors that potentiated PC12 cell differentiation also induced v-Crk tyrosine phosphorylation, strongly suggest that v-Crk is acting through a growth factor receptor pathway. In addition, the recent demonstration that another adaptor protein, Grb2,

can interact with receptor tyrosine kinases via its SH2 domain and simultaneously with SOS, a guanine exchange factor for Ras (10, 11, 30, 42), via its SH3 domain suggest by analogy that the CrkSH3 may be an important effector domain, binding differentiation-specific proteins in PC12 cells. It will be interesting to determine whether tyrosine phosphorylation of v-Crk affects its ability to target downstream signals, perhaps through an allosteric effect on the SH3 domain. Similar tyrosine phosphorylations of other adaptor proteins, Nck and Shc, have also been reported following NGF stimulation (37, 43).

Ectopic expression of v-Crk in PC12 cells resulted in prolongation of the cell doubling time, in contrast to the transforming effects seen following v-Crk expression in fibroblasts. These results may indicate that v-Crk effector proteins, when tyrosine phosphorylated in different cells, yield an opposing cellular phenotype. We did, however, observe a modest proliferative effect of NGF on the v-CrkPC12 cells after long-term exposure, suggesting that v-Crk may also affect the ability of these cells to enter the cell cycle. Interestingly, whereas removal of NGF from terminally differentiated PC12 cells results in apoptosis (30a), the survival of v-CrkPC12 cells after NGF removal is significantly enhanced (11a). Collectively, these data imply that significant crosstalk between signal pathways may be occurring. The analysis of v-Crk mutants within SH2 and SH3 may delineate such differences.

The binding of v-Crk to tyrosine-phosphorylated paxillin in PC12 cells suggests that v-Crk may link activated receptor tyrosine kinases to the cytoskeleton. The cytoskeletal attachment of the NGF receptor, following ligand binding, has long been recognized (46, 53). In fibroblasts, paxillin localizes to focal adhesions, specialized regions of the plasma membrane that link the actin cytoskeleton to the extracellular matrix via integrin receptors (52). Tyrosine phosphorylation of paxillin increases as cells bind to fibronectin, during cell spreading, and with the assembly of focal adhesions (3). Therefore, it is possible that tyrosine phosphorylation of paxillin plays a dynamic role in regulating actin organization characteristic of filopodia and lamellipodia at the leading edge of the neurite growth cone (18). Recently, we have demonstrated that the binding of v-Crk to paxillin can prevent tyrosine dephosphorylation by cellular phosphatase activity (2). It is hence possible that such v-Crk binding in v-CrkPC12 cells functions to accelerate their differentiation response to growth factors by a priming mechanism, perhaps by changing the cell shape or reorganizing the cytoskeleton. The absence of tyrosine-phosphorylated paxillin and the reduction in the total amount of paxillin in native PC12 cells stimulated to proliferate by EGF support this idea. Molecular cloning of paxillin and further characterization of its function in neural tissue should shed more light on this question.

Although these studies clearly implicate v-Crk in modulating receptor-type tyrosine kinase pathways in PC12 cells, the findings that v-CrkPC12 cells appear to remain uncommitted to either a proliferative or differentiative phenotype argue for the involvement of multiple v-Crk-transduced signaling pathways. However, the ability of v-Crk to aberrantly induce PC12 cell differentiation in response to EGF suggests that it can influence multiple pathways downstream of receptor kinases. Moreover, since Ras activation appears to be a requisite of both EGF and NGF signaling, it is likely that v-Crk effector proteins also lie on the Ras pathway. These cells provide a unique experimental system to study the interplay between differentiative and proliferative phenotypes and their mechanisms of signal transduction.

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B.L.H. and R.B.B. contributed equally to this work.

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