Phosphorylation of c-Crk II on the Negative Regulatory Tyr\(^{222}\) Mediates Nerve Growth Factor-induced Cell Spreading and Morphogenesis*

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The Crk family of adaptor proteins participate in diverse signaling pathways that regulate growth factor-induced proliferation, anchorage-dependent DNA synthesis, and cytoskeletal reorganization, important for cell adhesion and motility. Using kidney epithelial 293T cells for transient co-transfection studies and the nerve growth factor (NGF)-responsive PC12 cell line as a model system for neuronal morphogenesis, we demonstrate that the non-receptor tyrosine kinase c-Abl is an intermediary for NGF-inducible c-Crk II phosphorylation on the negative regulatory Tyr\(^{222}\). Transient expression of a c-Crk II Tyr\(^{222}\) point mutant (c-Crk Y222F) in 293T cells induces hyperphosphorylation of paxillin on Tyr\(^{31}\) and enhances complex formation between c-Crk Y222F and paxillin as well as c-Crk Y222F and c-Abl, suggesting that c-Crk II Tyr\(^{222}\) phosphorylation induces both the dissociation of the Crk SH2 domain from paxillin and the Crk SH3 domain from c-Abl. Interestingly, examination of the early kinetics of NGF stimulation in PC12 cells showed that c-Crk II Tyr\(^{222}\) phosphorylation preceded paxillin Tyr\(^{31}\) phosphorylation, followed by a transient initial dissociation of the c-Crk II paxillin complex. PC12 cells overexpressing c-Crk Y222F manifested a defect in cellular adhesion and neuritogenesis that led to detachment of cells from the extracellular matrix, thus demonstrating the biological significance of c-Crk II tyrosine phosphorylation in NGF-dependent morphogenesis. Whereas previous studies have shown that Crk SH2 binding to paxillin is critical for cell adhesion and migration, our data show that the phosphorylation cycle of c-Crk II determines its dynamic interaction with paxillin, thereby regulating turnover of multiprotein complexes, a critical aspect of cytoskeletal plasticity and actin dynamics.

The Crk adaptor proteins, first described as the product of an avian oncogene, v-crk, contain Src homology 2 (SH2) and SH3 domains that serve as binding sites for a diverse set of signaling proteins (1–3). A number of studies with v-Crk have established a paradigm in which SH2 and SH3 domain-coupled signals positively regulate growth factor- (4–6) as well as integrin-dependent pathways (7, 8). Recent studies have also linked the cellular homologs of v-Crk, namely c-Crk I, c-Crk II, and CrkL (9, 10), with cellular proteins that induce Rac GDP-GTP exchange activity (11, 12), cytoskeletal reorganization (13), cellular migration (14), and anchorage-dependent cell growth (15). Although c-Crk and v-Crk may have some overlapping functions due to their SH2 and N-terminal SH3 domains, both c-Crk II and CrkL contain an additional SH3 domain in the C terminus (10, 16) as well as a conserved tyrosine (c-Crk II Tyr\(^{221}\) and CrkL Tyr\(^{207}\) in the human isoforms; c-Crk II Tyr\(^{222}\) in the avian isoform) that can be phosphorylated by the non-receptor tyrosine kinase c-Abl (17, 18). Moreover, c-Crk Tyr\(^{222}\) is flanked by an AQP5 motif in the linker region, which when phosphorylated, conforms to a consensus Crk SH2 binding site and has been hypothesized to negatively regulate c-Crk function(s) by forming an intramolecular bridge (19). Although these studies suggest a model of c-Crk regulation that is analogous to that of Src family tyrosine kinases upon phosphorylation by C-terminal Src kinase (CSK) (20), the underlying mechanism by which phosphorylated Tyr\(^{222}\) modulates c-Crk II biological functions is not well understood.

Following their activation by binding to extracellular matrix molecules, integrins couple with intracellular tyrosine kinases such as FAK, Pyk2, Src, and c-Abl, leading to the formation of complex signaling networks involving kinases, their substrates, and adaptor proteins (21–25). Two of the major tyrosine-phosphorylated proteins that orchestrate the assembly of focal adhesions are paxillin and p130\(^\text{cas}\) (26, 27). Although unrelated structurally, both genes encode multidomain proteins that can associate with and become phosphorylated by various nonreceptor tyrosine kinases (FAK, Src, CSK, and c-Abl for paxillin and FAK and Src family members for p130\(^\text{cas}\)) (25, 28). Furthermore, after tyrosine phosphorylation, both paxillin and p130\(^\text{cas}\) present multiple docking sites for the Crk SH2 domain (29–31), thereby recruiting additional signaling proteins to focal adhesions via the Crk N-terminal SH3 domain (3). Accordingly, the binding of DOCK180 to p130\(^\text{cas}\)-Crk com-

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1 The abbreviations used are: SH2, Src homology 2; FAK, focal adhesion kinase; NGF, nerve growth factor; DME, Dulbecco’s modified Eagle’s medium; GFP, green fluorescent protein; PBS, phosphate-buffered saline; BSA, bovine serum albumin.
plex has been implicated in altering cell morphology via the activation of Rac1 (32), and mutations in either the Crk SH2 domain or the Crk-binding sites of p130Cas have been shown to impair cell migration in carcinoma cells (14). Recently, it has also been shown that phosphorylation of Tyr321 and Tyr326 on paxillin regulates cell migration through an association of Crk in NBT-II cells (33), suggesting that Crk binding to paxillin and p130Cas may have redundant functions.

Although integrin-mediated tyrosine phosphorylation of paxillin and p130Cas has been best characterized, various growth factors including insulin, epidermal growth factor, and platelet-derived growth factor also induce rapid changes in cell shape that correlate with tyrosine phosphorylation of paxillin and p130Cas (34-36). Although these results clearly support a model of cross-talk between receptor tyrosine kinases and molecules residing in focal adhesions, it remains unclear whether Crk proteins might be signaling intermediates that orchestrate these interactions. Using the PC12 cells as a model system of nerve growth factor (NGF)-induced neuronal morphogenesis (37), we and others have previously shown that NGF induces rapid actin reorganization and membrane ruffling that accompany alterations in cell shape (38), with concomitant tyrosine phosphorylation of c-Crk II and paxillin (4, 39). In the present study, we describe a novel pathway in which activation of the NGF receptor TrkA leads to c-Abl-mediated phosphorylation of c-Crk II at Tyr222, which catalyzes the dissociation of the Crk SH2 domain from paxillin and the dissociation of the Crk SH3 domain from c-Abl. The significance of this mechanism is highlighted by the findings that a c-Crk II Tyr222 mutant (c-Crk Y222F), while causing constitutive complex formation between c-Crk and paxillin as well as c-Crk and c-Abl, impairs NGF-dependent cellular spreading and neurite outgrowth. Thus, although previous studies have indicated that Crk binding to paxillin and p130Cas is critical for cell adhesion and motility, we propose that the dynamic nature of substratum adhesion and detachment, a critical aspect of NGF-induced morphogenesis, is determined in part by the phosphorylation/dephosphorylation cycle of c-Crk II on Tyr222.

**EXPERIMENTAL PROCEDURES**

**Cells and Tissue Culture**— Fibroblasts immortalized from Abi (+/−) or Fak (−/−) homozygous mice were generous gifts of David Baltimore (California Institute of Technology) and Tadashi Yamamoto (Tokyo University) respectively, and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. Abi (−/−) fibroblasts engineered to stably re-express wild-type c-Abl after DNA transfection with pBabe-c-Abl were a gift from Tohru Ouchi (Mount Sinai School of Medicine). Wild-type PC12 cells were maintained in DMEM supplemented with 10% calf serum and 5% horse serum. TrkA-overexpressing PC12-615, c-Crk II overexpressing PC12 cells, and c-Crk Y222F-expressing PC12 cells were maintained in DMEM containing 10% calf serum and 5% horse serum with 200 μg/ml G418. 293T cells and Bosc23 ectopic virus-packaging cell lines were grown in DMEM supplemented with 10% fetal calf serum.

**Antibodies**— Anti-peptide polyclonal antibodies that recognize Tyr322-phosphorylated c-Crk II (pCrk) were provided by Michiyuki Matsuda (National Institute of Infectious Diseases, Tokyo, Japan) (40), and monoclonal anti-Src antibodies were from Jean Brugge (Harvard Medical School). Anti-TrkA polyclonal antibodies were generated as described previously (41). Commerically available antisera were purchased from the indicated sources: anti-phosphotyrosine (TyrP) antibodies P20 (Transduction Laboratories), 4G10 (Upstate Biotechnology, Inc.), and PT-99 (Santa Cruz Biotechnology); anti-FLAG monoclonal M2 (Eastman Kodak Co.); anti-c-Abl monoclonal 8E9 (Pharminagen) and anti-c-Abl monoclonal Ab-3 (Calbiochem); anti-paxillin monoclonal (Zymed Laboratories Inc. or Transduction Laboratories); anti-FAK, anti-c-Crk II, and anti-p130Cas (all from Santa Cruz Biotechnology).

**Plasmid Constructions and DNA Transfection**— The c-ckr II, c-abl, and trkA plasmids and respective mutants have been previously described. Briefly, avian c-ckr II and c-ckr Y222F were subcloned into the pEBG vector, driven by the human elongation factor 1-α promoter (42). Wild-type and kinase-deficient (K290M) murine type IV c-abl (43) were provided by David Baltimore as described previously (44) and were subcloned into pcDNA3 (Invitrogen). Expression plasmids for TrkA (pC-TrkA), kinase-dead c-Src (pBHR2-KDsrc), or an activated FAK (pCR K454M FAK) were provided by David Baltimore (McGill University), Hisataka Sabe (Osaka Bioscience institute), and Steven Hanks (University of Virginia), respectively. To construct a FLAG epitope-tagged paxillin expression vector, a full-length chicken paxillin cDNA in pGEX (26) was digested with BamHI and then subcloned into pFLAG-CMV2 (Eastman Kodak) using BamHI/EcoRI linkers. Site-directed mutations of the FLAG site directed mutagenesis kit (Stratagene). The following sequences (5′-3′) of the sense mutagenic oligonucleotides were used, with mismatches indicated in uppercase: for the Y31F mutation, gag gaa aca ctc tcc tca cct act g; for Y118F, gag gag caa gct gtg tgc atg ctc aac; and for Y182F, gag gag cct ccc tct tct atc cca gag. All point mutations were confirmed by DNA sequencing.

To generate clonal PC12 cell lines stably overexpressing Crk protein, cells were transfected with 18 μg of pEBG-c-Crk II or pEBG-c-Crk Y222F plasmid DNA and 2 μg of pMexEco using the LipofectAMINE reagent (Life Technologies, Inc.), and cell lines were expanded as described previously (4). We found that a high level of c-Crk II or c-Crk Y222F expression was unstable in PC12 cells over multiple passage.

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G plus-Sepharose (Santa Cruz Biotechnology). After washing with lysis buffer, immune complexes were subjected to SDS-PAGE and Western blot analysis using standard protocols. Blots were incubated with the indicated primary antibodies and the appropriate horseradish peroxidase-conjugated secondary antibodies, followed by detection using enhanced chemiluminescence (ECL) reagent (Renaisssance, NEN Life Science Products).

**Protein Kinase Assays—**Immune complex kinase assays for c-Abl were performed using γ-[32P]ATP and GST-Crk 120–225 as an exogenous substrate as described previously (17). After 20 min at room temperature, kinase reactions were terminated by boiling in SDS sample buffer before SDS-polyacrylamide gel electrophoresis. Gels fixed in 50% methanol, 10% acetic acid were subjected to autoradiography. Quantitation of kinase activity was performed using a Molecular Dynamics Phospho-Imager.

**Immunofluorescence and Confocal Microscopy—**Wild-type PC12 and c-Crk II- or c-Crk Y222F-expressing PC12 cells were plated on glass coverslips coated with collagen type IV and cultured in the presence of NGF for the indicated times (5). After rinsing with PBS, cells were fixed at room temperature for 30 min in 3% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 5 min at 37°C. Unattached cells were dislodged from the bottom of the well by three washes in PBS. Attached cells were fixed in glutaraldehyde and stained with 0.5% trypan blue. Quantitation of attached cells was performed by measuring absorbance at 590 nm of individual wells using a microtiter plate reader. Each value represents the mean and S.D. of determinations made on triplicate cultures performed in parallel and is expressed as a percentage of cell attachment on the indicated substrate relative to the cell attachment to poly-L-lysine.

**RESULTS**

**Stimulation of Cells with NGF Induces Phosphorylation of Paxillin on Tyr31 and c-Crk II on Tyr222—**When PC12 cells are stimulated to differentiate with NGF, multiple proteins, including c-Crk II and paxillin, become tyrosine-phosphorylated within minutes after growth factor stimulation (4, 39, 46). To identify the residues on paxillin and c-Crk II that are tyrosine-phosphorylated following NGF stimulation, Tyr to Phe point mutants of paxillin or c-Crk II were co-expressed with the NGF receptor TrkA in human epithelial kidney (HEK) 293T cells (Fig. 1). Mutagenesis of the three putative high affinity Crk SH2 binding motifs in the N-terminal region of paxillin (Tyr31, Ser-Tyr-Pro, Tyr118-Ser-Phe-Pro, and Tyr182-Val-Ile-Pro (26)) virtually abrogated NGF-inducible paxillin phosphorylation (Fig. 1A, lanes 2 versus 3). To map the sites of NGF-inducible paxillin phosphorylation in more detail, Tyr31, Tyr118, and Tyr182 residues were mutated singly or in combination and co-expressed with TrkA in 293T cells (Fig. 1B). Paxillin mutants containing a Y31F substitution exhibited the most significant reduction (>70%) in Trk A-dependent phosphorylation after NGF treatment (Fig. 1B), whereas a Y113F,Y182F paxillin double mutant with an intact Tyr31 was less affected. To demonstrate that Tyr31 represents the major NGF-inducible Crk binding motif in paxillin, 293T cells co-expressing TrkA and c-Crk II with either FLAG-tagged paxillin, FLAG-tagged Y31F paxillin, or FLAG-tagged paxillin Y31F,Y118F,Y182F paxillin, were immunoprecipitated with anti-Crk antibodies (Fig. 1C). As indicated, mutation of Tyr31 in paxillin significantly disrupted binding to c-Crk II, which was not further abrogated upon co-expression of Y31F,Y118F,Y182F paxillin with TrkA and c-Crk II (Fig. 1C, inset). These results indicate that paxillin Tyr31 is the major site mediating c-Crk II interaction following NGF stimulation in vivo.

Although Tyr31 is a major site for c-Crk II phosphorylation in vitro (17), we sought to verify that the same tyrosine residue becomes tyrosine phosphorylated in vivo following TrkA activation. Toward this goal, wild-type c-Crk II or the point mutant of c-Crk II at Tyr222 (c-Crk Y222F) were coexpressed with TrkA in 293T cells. Using either an anti-phosphotyrosine (Tyr(P)) antisera or phospho-c-Crk-specific Tyr31 and Tyr222 (pCrk) antisera, we found that wild-type c-Crk II, but not c-Crk Y222F, was phosphorylated upon NGF treatment (Fig. 1D, compare lanes 2 and 3 in respective panels). Together, these observations suggest that c-Crk II is phosphorylated exclusively on Tyr222 upon NGF stimulation in vivo.

**NGF-inducible Tyrosine Phosphorylation of c-Crk II and of Paxillin Requires c-Abl—**A recent study by Yano et al. (47) reports an interaction between c-Abl and TrkA in both PC12 cells and after cotransfection into 293T cells. Since c-Abl is known to interact with c-Crk II (17) and with paxillin (23), it may be a likely candidate to mediate the NGF-induced tyrosine phosphorylation of these proteins. To assess the role of c-Abl in NGF-induced c-Crk II phosphorylation, c-Crk II and TrkA were coexpressed in fibroblasts derived form Abl (−/−) mice. Whereas c-Crk Tyr31 was readily phosphorylated in NGF-stimulated PC12 cells (39) or in 293T cells co-expressing c-Crk and TrkA (Fig. 1D), NGF treatment of Abl (−/−) fibroblasts expressing TrkA and c-Crk II did not lead to detectable c-Crk II phosphorylation (Fig. 2A, lane 3). However, when wild-type c-Abl expression was reconstituted in Abl (−/−) fibroblasts by stable gene transfer (Fig. 2A, Abl kinase panel), NGF-induced c-Crk II phosphorylation was also restored (Fig. 2A, lane 4). c-Crk II Tyr222 was not readily detectable in Abl-reconstituted Abl (−/−) fibroblasts in the absence of TrkA expression and NGF stimulation (Fig. 2A, inset). These results suggest that c-Abl is likely an intermediary kinase responsible for c-Crk II tyrosine phosphorylation following NGF stimulation.

To determine whether NGF-inducible paxillin tyrosine phosphorylation is also dependent on c-Abl, FLAG-tagged paxillin and TrkA were transiently co-expressed with either wild-type c-Abl or the kinase-deficient mutant K290M c-Abl (Fig. 2B). Overexpression of wild-type c-Abl results in high levels of tyrosine phosphorylation of paxillin, which was not significantly augmented by TrkA expression (Fig. 2B, lanes 2 versus 3, Tyr(P) (anti-pTyr) blot). However, expression of K290M c-Abl abolished the phosphorylation of paxillin following Trk A activation (lane 4). Indeed, both wild-type and K290M c-Abl formed stable complexes with paxillin in co-precipitation analysis (Fig. 2B, anti-Abl blot; lanes 2–4), although as expected, no paxillin-associated kinase activity was detected when K290M c-Abl was expressed (Fig. 2B, Abl kinase assay, lane 4).

Other nonreceptor tyrosine kinases such as FAK and Src have been implicated in the integrin-dependent tyrosine phosphorylation of paxillin on Tyr31 and Tyr118 (30, 48), which in turn create Crk SH2 binding sites (29, 30). While the results in Fig. 2B demonstrate that c-Abl can also phosphorylate paxillin, it is not clear whether c-Abl also requires FAK or Src following
TrkA activation. To address this, mouse embryo fibroblasts (MEF) derived from wild-type or FAK \((2/2)\) embryos (49) were cotransfected with TrkA and wild-type paxillin (Fig. 2C). TrkA-dependent tyrosine phosphorylation of paxillin was evident in FAK \((2/2)\) cells, and the extent of phosphorylation was similar in cells expressing or lacking FAK (Fig. 2C, lanes 2 and 4). Moreover, when paxillin was co-transfected with TrkA in 293T cells in the presence of either kinase-deficient c-Abl (K290M c-Abl), kinase-deficient FAK (K454M FAK), or kinase-deficient-Src (K295M c-Src), K290M c-Abl exhibited the most significant inhibition in TrkA-induced paxillin phosphorylation (Fig. 2D). These results suggest that different activating stimuli may utilize distinct intermediate non-receptor tyrosine kinases for the tyrosine phosphorylation of paxillin; Src and FAK for integrin and c-Abl for NGF.

Complex Formation between c-Crk II and Paxillin and c-Crk II and Abl Is Regulated by c-Crk II Tyr 222 Phosphorylation—Whereas the phosphorylation of Tyr 31 in paxillin induces an association between Crk and paxillin (Fig. 1C), Tyr222 phosphorylation in c-Crk II induces an intramolecular association of the Crk SH2 domain and is expected to lead to a dissociation of Crk from paxillin (17). To examine the effect of c-Crk Tyr222 phosphorylation on the cellular turnover of Crk/paxillin complexes, 293T cells co-expressing TrkA, FLAG-tagged paxillin, and wild-type c-Crk II or Y222F c-Crk were analyzed (Fig. 3). Expression of c-Crk Y222F resulted in an increased level of cell...

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**Fig. 1. Mapping of the NGF-inducible tyrosine phosphorylation sites on paxillin and c-Crk II.**

A. Each of the three putative Crk SH2 binding sites in paxillin, Tyr31, Tyr118, and Tyr182, was mutated to phenylalanine (paxillin 3Y > 3F). 293T cells were cotransfected with 1.0 \(\mu\)g of cDNA encoding TrkA and 0.2 \(\mu\)g of either wild-type FLAG-tagged paxillin or mutant as indicated. After 48 h, cells were stimulated with 100 ng/ml NGF, and cellular lysate (500 \(\mu\)g) was immunoprecipitated with 1.0 \(\mu\)g of anti-FLAG antibody (M2). Complexes were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes, and blots were probed with either anti-FLAG, anti-paxillin, or anti-Tyr(P) (anti-pTyr) as indicated.

B. 293T cells were co-transfected as in panel A with TrkA and either wild-type paxillin or one of the paxillin mutants indicated. Subsequently, cells were stimulated with NGF, and total cellular lysate was immunoblotted (20 \(\mu\)g protein) with anti-Tyr(P) antibody to compare tyrosine phosphorylation of individual paxillin mutants. The blot was then re-probed with anti-FLAG antibody (M2) or anti-phospho-TrkA (pTrkA) to verify expression.

C. NGF-inducible Tyr31 phosphorylation on paxillin is the principal site for c-Crk II binding. 293T cells expressing TrkA and c-Crk II with either FLAG-tagged wild-type paxillin or the paxillin triple mutant (paxillin Y > F) were immunoprecipitated with an anti-Crk antibody and immunoblotted with either anti-Tyr(P) or anti-FLAG antibodies. Loading controls for the amount of paxillin, Crk, and phospho-TrkA are shown in the lower three panels. In the inset, the relative binding of c-Crk II to wild-type paxillin, Y31F paxillin, or Y31F,Y118F,Y182F paxillin is compared. Immunoblots were quantified by laser densitometric scanning of the films.

D. 293T cells were co-transfected with 1.0 \(\mu\)g of cDNA encoding TrkA and 0.2 \(\mu\)g of either wild-type c-Crk or c-Crk Y222F constructs as indicated. After 48 h, cells were treated with 100 ng/ml NGF for 5 min, and the lysates were immunoblotted with either anti-phospho-Crk (anti-pCrk) or anti-Tyr(P) antibodies. The blot was then re-probed with anti-Crk and anti-TrkA antibodies to verify expression (bottom panels). WB, Western blotting; IP, immunoprecipitation.
lular paxillin tyrosine phosphorylation (Fig. 3A, lanes 2 and 3 in anti-Tyr(P) (anti-pTyr) blot), and a much greater proportion of the total pool of c-Crk II existed as a complex with paxillin compared with the wild-type c-Crk II, as evident from co-immunoprecipitation analysis (Fig. 3B). Interestingly, only the unphosphorylated pool of c-Crk II interacted with paxillin, and the Tyr222-phosphorylated pool of c-Crk II was found exclusively in the supernatant. These results suggest that at steady state, there is an equilibrium between two separate pools of c-Crk II: (i) unphosphorylated c-Crk II, which complexes with tyrosine-phosphorylated paxillin, and (ii) tyrosine-phosphorylated c-Crk II, which is monomeric.

Given the hyperphosphorylation of paxillin in cells expressing Y222F c-Crk II and the fact that paxillin can serve as a substrate for Abl following NGF stimulation, we investigated the effect of Y222F c-Crk II on the cellular interaction between Abl and the SH3 domain of Crk (Fig. 4A). When 293T cells expressing wild-type c-Abl and c-Crk Y222F were immunoprecipitated with anti-Crk antibodies, the activity of c-Abl associated with c-Crk Y222F was more than 4-fold greater than that associated with equivalent amounts of wild-type c-Crk II (Fig. 4A and inset), and importantly, there was much more Abl phosphorylated c-Crk II, which is monomeric.

**Fig. 2.** c-Abl is required for NGF-inducible c-Crk II and paxillin tyrosine phosphorylation. A, c-Abl null (-/-) fibroblasts were transfected with TrkA and c-Crk II constructs as indicated. Abl kinase assay was performed as described (top panel; Ref. 17). In lane 4, c-Abl-deficient cells were stably transfected with pBabe-c-Abl to obtain c-Abl-reconstituted (Abl +/-) cells. In the lower two panels, lysates were immunoblotted with anti-Crk or anti-Tyr(P) (anti-pTyr) antibodies as indicated to verify c-Crk expression and tyrosine phosphorylation. In the inset, the extent of c-Crk Tyr222 phosphorylation (*) in Abl (-/-) cells in the presence or absence of TrkA is indicated. B, stable association of paxillin and c-Abl. 293T cells were cotransfected with cDNAs encoding FLAG-tagged paxillin and one of the plasmids indicated. Cell lysates were either immunoblotted with anti-Tyr(P) to compare paxillin tyrosine phosphorylation or reprobed with anti-FLAG antibody to verify paxillin expression. In the lower two panels, extracts were immunoprecipitated with anti-FLAG antibody, divided into equal aliquots, and analyzed for Abl kinase activity (Abl kinase assay) or immunoblotted with anti-c-Abl antibody 8E9 to verify expression (anti-Abl WB). C, control mouse embryo fibroblasts (MEF) (lanes 1 and 2) or FAK (-/-) fibroblasts (lanes 3 and 4) were transfected with FLAG-tagged paxillin in the presence or absence of TrkA construct as indicated. After NGF stimulation, protein extracts were immunoblotted with anti-Tyr(P) to compare paxillin tyrosine phosphorylation (top panel). The blot was then reprobed with anti-FAK and anti-FLAG antibodies to verify appropriate protein expression (lower two panels). D, c-Abl, but not FAK or Src, mediates Trk A-dependent paxillin phosphorylation. 293T cells were cotransfected with vectors encoding paxillin and TrkA and one of the kinase-deficient constructs encoding c-Abl (K290M), FAK (K454M), or c-Src (K416M) as indicated. Cell lysates were immunoblotted with anti-Tyr(P) to compare paxillin tyrosine phosphorylation. The blot was sequentially reprobed with the indicated antibodies to verify appropriate kinase expression. WB, Western blotting; IP, immunoprecipitation.
co-precipitating with c-Crk Y222F under these conditions (Fig. 4A, top panel). No Abl activity associated with the c-Crk W170K SH3 domain mutant (not shown), confirming previous studies that the interaction between Abl and Crk is dependent on the Crk SH3 domain (17). Interestingly, when replicate lysates to those used in Fig. 4A were immunoprecipitated with anti-FLAG antibodies (to precipitate paxillin), there was also a much greater pool of Abl co-precipitating with paxillin in c-Crk Y222F-expressing 293T cells, as well as a greater pool of Crk in these complexes (Fig. 4B). Collectively, the results in Figs. 3 and 4 indicate that Crk Tyr222 phosphorylation actively induces both the dissociation of the Crk SH2 domain from paxillin and the Crk SH3 domain from Abl and that mutagenesis of the Tyr222 in c-Crk potentiates Abl-dependent paxillin tyrosine phosphorylation and results in enhanced Crk/paxillin association. A, 293T cells were cotransfected with plasmid vectors encoding FLAG-tagged paxillin and TrkA together with wild-type c-Crk II or c-Crk Y222F as indicated. After NGF stimulation, protein extracts (20 μg) were immunoblotted with anti-Tyr(P) (anti-pTyr) to compare paxillin tyrosine phosphorylation (top panel). The numbers in parentheses indicate the relative intensity of the immunopositive signal as determined by densitometry. The membrane was also reprobed with antibodies to FLAG, Crk, or pTrk to verify appropriate expression. B, c-Crk Y222F increases complex formation between Crk and paxillin. Lysates from 293T cell cotransfectants (Fig. 3A, lanes 2 and 3) were immunoprecipitated with anti-FLAG antibody, divided into equal aliquots, and immunoblotted with either anti-pCrk or anti-Crk antibodies. The top panel (anti-pCrk spnt) represents the supernatant fraction recovered from the anti-FLAG immunoprecipitation. WB, Western blotting; IP, immunoprecipitation.
phosphorylation by inducing a persistent Crk/paxillin/Abl protein complex in cells.

NGF-induced Dissociation of c-Crk II-Paxillin Complex in PC12 Cells Is Also Inhibited by c-Crk Y222F—The transient transfection experiments in 293T cell system suggests a model in which c-Crk II Tyr222 phosphorylation is required for the regulated turnover of Crk-paxillin-Abl protein complexes. To test the biological importance of this model during neuronal differentiation, we first investigated the kinetics of c-Crk II and paxillin tyrosine phosphorylation in the NGF-treated PC12-615 cell line (41) (Fig. 5A). An early time course analysis revealed that whereas TrkA activation was detectable 1 min after NGF stimulation, tyrosine phosphorylation of c-Crk II was apparent by 2 min and persisted for at least 1 h, with an upward electromobility shift of c-Crk II (Fig. 5B). Although basal tyrosine phosphorylation of paxillin was observed in unstimulated cells (Fig. 5C, lane 1), NGF-augmented paxillin phosphorylation was detectable 5 min after NGF stimulation and maintained for up to 3 h after NGF treatment (Fig. 5C and data not shown).

The fact that NGF-induced c-Crk II Tyr222 phosphorylation precedes paxillin phosphorylation predicts that c-Crk II may initially dissociate from an existing pool of tyrosine-phosphorylated paxillin. To test this hypothesis, wild-type c-Crk II or c-Crk Y222F were transiently expressed in PC12-615 cells using recombinant Crk-expressing retrovirus (Fig. 5D). In the cells overexpressing wild-type c-Crk II, a complex of c-Crk II and paxillin was detectable in unstimulated cells, and this complex dissociated within 2 min of NGF addition (Fig. 5D, lanes 2 and 3). In contrast, expression of c-Crk Y222F promoted Crk/paxillin association in the basal state and resulted in hyperphosphorylation of paxillin. In agreement with the results in 293T cells, NGF stimulation of these cells failed to induce the dissociation of paxillin from the anti-Crk immunocomplex (Fig. 5D, lanes 4 and 5).

Mutation of c-Crk II Tyr222 Impairs Cellular Adhesion and NGF-induced Neurotogenesis—The enhanced interaction of hyperphosphorylated paxillin with c-Crk Y222F could either positively or negatively modulate the cytoskeletal dynamics that are critical for NGF-induced neurotogenesis. To examine the effects of c-Crk Y222F overexpression during NGF-inducible neurotogenesis, wild-type c-Crk II and c-Crk Y222F were expressed in PC12 cells by stable gene transfer. Independent PC12 cell lines overexpressing c-Crk II or c-Crk Y222F at 2–3 times the endogenous c-Crk II levels were clonally expanded (Fig. 6A). Whereas parental PC12 cells or c-Crk II-overexpressing PC12 cells demonstrated rapid c-Crk II tyrosine phosphorylation following NGF stimulation, no endogenous c-Crk II tyrosine phosphorylation was observed in NGF-treated c-Crk Y222F-expressing cells (Fig. 6A), a result confirmed in several independent clones. When NGF-stimulated neurite outgrowth was compared between the three cell types, we found that the c-Crk Y222F-expressing PC12 cells tended to form aggregates with short or no visible neuritic processes, even after 72 h of NGF treatment (Fig. 6B, panels viii). To determine if paxillin localization perturbation due to c-Crk Y222F expression, immunolocalization of paxillin was performed. As shown in Fig. 6B, punctate F-actin and paxillin staining were localized to the leading edges of the growth cones in cells expressing wild-type c-Crk II (panels ii and v for native PC12 cells and panels iii and vi for c-Crk II-expressing PC12 cells), consistent with the formation of normal focal contacts. With Y222F c-Crk-expression,
**Fig. 6. c-Crk Y222F inhibits NGF-dependent neuritogenesis and causes paxillin mislocalization.** A, Wild-type and c-Crk Y222F-expressing PC12 cells were treated for 5 min in the presence or absence of 100 ng/ml NGF as indicated. The lysates were immunoblotted with anti-Crk to verify Crk overexpression or immunoprecipitated with anti-Crk antibodies to detect phosphorylated c-Crk II (anti-TyrP) (anti-pTyr) panel. A, native PC12 cells (panels i-iii), c-Crk II-expressing PC12 cells (panels iv-vi), or c-Crk Y222F-expressing PC12 cells (panels vii-ix) were treated with 50 ng/ml NGF for 72 h (top 3 panels) or 36 h (bottom 6 panels). For immunofluorescence, cells were fixed, permeabilized, and stained with either rhodamine-conjugated phalloidin (panels ii, v, and viii) or anti-paxillin monoclonal antibody (panels iii, vi, ix). WB, Western blotting; IP, immunoprecipitation.

paxillin localized diffusely in the cytoplasm, particularly in the perinuclear region, but lacked discernable localization in the short process (Fig. 6B, panels viii and ix).

The possible mislocalization of paxillin in c-Crk Y222F-expressing PC12 cells together with the established role of paxillin and c-Crk II in focal adhesion assembly and in integrin-mediated cellular adhesion prompted us to evaluate the effects of Y222F c-Crk on cell attachment and adherence to extracellular matrix (Fig. 7). Previous studies have documented that PC12 cells readily attach to poly-D-lysine and collagen IV (45). Although attachment to collagen IV requires integrin activation, cells attach to poly-D-lysine in an integrin-independent manner (50). In a quantitative cell attachment assay, PC12 cells were plated on either BSA, poly-D-lysine, or collagen IV in the presence of 50 ng/ml NGF for 6 h (Fig. 7A). Native PC12 cells adhered to collagen IV and exhibited 85% attachment when normalized to adherence on poly-D-lysine (Fig. 7A). In contrast, c-Crk Y222F-expressing PC12 cells were significantly impaired in their ability to attach to collagen IV (approximately 25%) yet maintained ability to adhere to poly-D-lysine. Interestingly, PC12 cells overexpressing wild-type c-Crk II also exhibited a modest decrease in adhesion on collagen IV, despite apparently normal neuritogenesis. Since a decrease in substrate adhesion might have a direct effect on cellular flattening, an area/height ratio analysis was carried out as an independent criterion for assessing the effect of c-Crk Y222F on PC12 cytoskeleton (Fig. 7B). Using BSA-DiI (which stains plasma membranes) followed by optical sectioning with confocal microscopy, we found that both native and c-Crk II-expressing PC12 cells exhibit similar area/height ratios, whereas c-Crk Y222F expression changes the cell shape from flat and polygonal to round, as revealed by the decrease (>50%) in area/height ratios.

Because stable cell lines exhibiting defects in cellular adhesion may result in instability of c-Crk expression with passaging, transient overexpression of c-Crk II or c-Crk Y222F in the PC12-615 cells with the marker gene GFP was utilized for further analysis (Fig. 7C). Consistent with the results using stable PC12 transfectants, GFP-positive cells overexpressing c-Crk Y222F exhibited a round morphology compared with those expressing GFP alone (not shown). Similarly, in a quantitative cell attachment assay, the ratio of poorly adherent/attached GFP-positive cells was approximately 3-fold higher in the Crk Y222F-expressing cells compared with the vector alone-infected cells, whereas cells overexpressing wild-type c-Crk II exhibited a modest increase in detachment (a 1.6-fold increase). When GFP-positive gated cells were counter-stained with phosphatidylethanolamine-conjugated annexin V, there was no difference in staining in the GFP-positive and -negative cells, indicating that the loss in cellular adhesion does not result from apoptosis (data not shown).

**DISCUSSION**

In the present study, we have investigated the role of Tyr222 phosphorylation in the c-Crk II adaptor protein and found that c-Crk II phosphorylation is critical for normal cytoskeletal signaling and cellular adhesion. Overexpression of c-Crk Y222F in cells stabilized an association between c-Crk II and paxillin and c-Abl, resulting in persistent complex formation of these proteins and hyperphosphorylation of paxillin and led to a defect in cell adhesion and neuritogenesis in PC12 cells. We propose that sequential tyrosine phosphorylation of c-Crk II on Tyr222 and paxillin on Tyr31, induced following NGF stimulation, permits rapid turnover of multiprotein complexes that regulate cytoskeletal plasticity and actin reorganization, a strategy that may also be adopted for other cell type-specific morphogenetic events.
The mechanism by which c-Crk II promotes cell adhesion and migration has been mainly explored in fibroblasts and epithelial cells during adhesion and cell migration along the extracellular matrix (7, 12, 14, 33, 51). In such cells, integrin receptor activation results in tyrosine phosphorylation of p130Cas and paxillin, thereby recruiting c-Crk II via its SH2 domain.

**FIG. 7.** c-Crk Y222F impairs integrin-mediated cell attachment and causes detachment of PC12 cells. A, control PC12 cells, c-Crk II, or c-Crk Y222F-expressing PC12 cells were plated for 6 h on either 1.0 mg/ml BSA or 2 mg/ml collagen IV-coated surfaces as indicated and assayed for attachment by a quantitative cell attachment assay. Each value represents the mean of determinations made in triplicate wells performed in parallel and expressed as the percentage of attachment to the positive control (i.e. poly-D-lysine). Vertical error bars indicate S.D. B, serial x-y confocal sections obtained from parental PC12 cells, c-Crk II expressing PC12 cells, or c-Crk Y222F-expressing PC12 cells (from left to right). The smaller panels are cross-section representatives, and the area to height ratios are indicated by the numbers below each panel. C, PC12 cells were infected with recombinant retroviral vectors expressing GFP alone or coexpressing GFP with either wild-type c-Crk II or c-Crk Y222F as in Fig. 6. The percentage of GFP-positive cells that remained adherent versus the percentage removed in the wash were quantified by fluorescence-activated cell sorter analysis gated with a fluorescein isothiocyanate filter. Data are plotted as the average ratio of detached/attached cells plated in triplicate and are the average of two independent experiments. In the inset, GFP-positive cells were FACSorted and collected. Detergent lysates were prepared, and 20 μg of protein was immunoblotted with either anti-GFP or anti-Crk antibodies to demonstrate coexpression. WB, Western blotting.

**FIG. 8.** A model for the c-Crk II phosphorylation switch. The cycle represents a combination of features that may be attributable to dynamic cytoskeletal changes following NGF stimulation. c-Abl, which is shown in a complex with TrkA (47), transduces an intracellular signal that results in the sequential phosphorylation of c-Crk Tyr222 and paxillin Tyr31 (panel A). In this model, c-Crk Tyr222 is rate-limiting for the dissociation of cellular complexes of c-Crk and paxillin as well as c-Crk and Abl (panel B). Subsequently, a dephosphorylated Crk molecular may associate again with Abl to establish another cycle of Crk/paxillin turnover (panel C).
domain (25). c-Crk II in turn can interact with downstream effectors such as the guanine nucleotide exchange proteins C3G and DOCK180 (11, 52). When coupled, the regulated binding of c-Crk II to p130cas may serve as a regulator of stress fiber formation (13) and cell migration (12, 14), leading to the activation of both JNK- and Rac1-dependent signaling pathways (15, 32, 53, 54). The present findings that c-Crk Y222F overexpression manifests an apparent defect in cytoskeletal organization, despite leading to persistent complex formation between Crk, paxillin, and c-Abl implies that purely inductive signaling is insufficient for the effects of Crk on cellular adhesion and migration. We posit that the effects of c-Crk II on cellular adhesion are determined by a balance of positively and negatively acting signals transmitted through the differential phosphorylation of c-Crk II on Tyr222.

Following stimulation of cells with NGF, we have shown here that the non-receptor tyrosine kinase c-Abl is an intermediary for TrkA-inducible phosphorylation of c-Crk II on Tyr222 and paxillin on Tyr31. Although FAK is clearly implicated in paxillin phosphorylation and in integrin-mediated focal adhesions and cell spreading (30, 51), there is also precedent for FAK-independent pathways in such processes (55). For example, in CML cells or myeloid cells expressing BCR-Abl, CrkL has been shown to link BCR-Abl with paxillin, leading to paxillin phosphorylation on Tyr31 and Tyr118 (56). Moreover, studies in fibroblasts have shown that a pool of cytoplasmic c-Abl associates with and tyrosine phosphorylates paxillin after integrin-dependent adhesion (23). Recent results by Yano et al. shows that c-Abl can form a physical complex with Trk A (47) that may in part explain previous studies showing an association between TrkA and c-Crk II in co-immunoprecipitation studies (57, 58). Interestingly, mapping studies between TrkA and c-Abl indicated that c-Abl interacts with TrkA through the juxtamembrane region of TrkA, a region also known to be required for TrkA-mediated neurite outgrowth (59). In future studies it should be important to examine whether juxtamembrane mutants of TrkA fail to induce c-Crk II and paxillin phosphorylation following NGF stimulation.

The findings that NGF simultaneously induces Tyr222 phosphorylation in c-Crk II and Tyr31 phosphorylation in paxillin suggests that a cyclical model for Crk and paxillin association has important implications in vivo. Early changes in the association of paxillin and c-Crk II and CrkL have also been observed following stimulation of cells with insulin-like growth factor and epidermal growth factor (60, 61). Hence, we propose that the breakdown of focal contacts may occur initially (within 5 min of NGF treatment) and is followed by cyclical turnover of c-Crk II/paxillin complexes (after 5 min of NGF treatment). Even though c-Crk II and paxillin tyrosine phosphorylation appears unchanged at steady state, our model predicts the presence of (i) phosphorylated c-Crk II, which is monomeric, and (ii) dephosphorylated c-Crk II, which complexes with c-Abl and with tyrosine-phosphorylated paxillin (Fig. 8). Thus, in a dynamic equilibrium, c-Crk “regenerates” monomeric c-Crk II via Tyr222 phosphorylation, which results in the dissociation of phospho-c-Crk II from tyrosine-phosphorylated paxillin (SH2-dependent) and from c-Abl (SH3-dependent). Although a definitive proof of this hypothesis may require “real time” analysis, the enhanced association of hyperphosphorylated paxillin and c-Abl with the c-Crk Y222F mutant (this study) is consistent with the dynamic molecular interactions, as suggested by this model. Accordingly, when the pathway required for turnover is impaired upon introduction of the Y222F mutation, c-Abl-mediated phosphorylation of paxillin is not counterbalanced by the formation of phospho-c-Crk II monomer. As a result, phosphorylated paxillin remains associated with c-Crk (Y222F) and, presumably, further phosphorylated by c-Abl within the complex (Fig. 8).

The fact that c-Crk Tyr222 phosphorylation disrupts the binding of Crk to both paxillin and c-Abl suggests that such phosphorylation functions to sequester the binding surfaces of both domains in the native structure. Although NMR spectroscopy analysis on c-Crk II indicates the SH2 domain of Crk binds phosphorylated Tyr222 (19) and suggests a conformation change in the Crk SH3 domain, these studies did not address whether the binding affinity of the Crk SH3 domain was changed. The crystal structures of the Src and Hck kinases (62, 63) may also provide insight into how Tyr222 phosphorylation in Crk may function. Indeed, it has been recently shown that intramolecular binding of phosphorylated Tyr527 tail to the SH2 domain indirectly inhibits Src enzymatic activity by facilitating a second intramolecular interaction between the SH3 domain and the Src catalytic domain (SH1) (62). Given the importance of Tyr222 phosphorylation in regulating c-Crk binding affinities, detailed structural studies are warranted to ascertain how phosphorylation of Tyr222 affects the interdomain structures of the SH2 and SH3 domains.

Given the cyclical nature of c-Crk II/paxillin binding and turnover, an equally important issue pertains to the dephosphorylation of c-Crk II Tyr222 and of paxillin. Currently, however, there is no known Crk-specific phosphatase, despite the finding that treatment of cells with the tyrosyl phosphatase inhibitor sodium vanadate phosphatase increases c-Crk II tyrosine phosphorylation in vivo.2 In contrast, numerous phosphatases, including Shp2, PTP-PEST, PTP-α, LAR, and PTEN, have been implicated in the regulation of cell adhesion and migration by dephosphorylating focal adhesions proteins such as FAK, p130cas, and paxillin (67–71). For example, fibroblasts derived from PTP-PEST (−/−) mice display hyperphosphorylated paxillin and a concomitant decrease in cell adhesion and motility, suggesting that PTP-PEST plays a role in the breakdown of focal adhesions (68). In the c-Crk Y222F-expressing PC12 cells, hyperphosphorylated paxillin is unable to interact with focal contacts and actin microfilaments in the lamellipodia of advancing growth cones. Therefore, like c-Crk II, the phosphorylation status of paxillin is tightly coupled to cytoskeletal dynamics, since both hypo- and hyperphosphorylated paxillin might be disruptive for focal adhesion complex formation. In view of the phosphotyrosine-dependent on/off interaction between paxillin and c-Crk II, it will be interesting to determine whether the putative PTPases specific for c-Crk II may also play a direct role in reversing paxillin phosphorylation.

In summary, we propose a model based on the differential tyrosine phosphorylation of c-Crk II to account for rapid and dynamic cytoskeletal reorganization following growth factor stimulation. Our data are consistent with the emerging evidence that c-Crk II- and Crk-associated proteins may exert specialized functions in the generation, dissipation, and regeneration of mechanochemical force required for cell shape changes. Future research will focus on mutations that disrupt the Crk/paxillin/Abl circuit to specifically address the complexity of molecular cross-talk underscored by the present study.
c-Crk Phosphorylation and NGF Signaling

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