c-SRC Mediates Neurite Outgrowth through Recruitment of Crk to the Scaffolding Protein Sin/Efs without Altering the Kinetics of ERK Activation*‡

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SRC family kinases have been consistently and recurrently implicated in neurite extension events, yet the mechanism underlying their neuritogenic role has remained elusive. We report that epidermal growth factor (EGF) can be converted from a non-neuritogenic into a neuritogenic factor through moderate activation of endogenous SRC by receptor-protein-tyrosine phosphatase (a physiological SRC activator). We show that such a qualitative change in the response to EGF is not accompanied by changes in the extent or kinetics of ERK induction in response to this factor. Instead, the pathway involved relies on increased tyrosine phosphorylation of, and recruitment of Crk to, the SRC substrate Sin/Efs. The latter is a scaffolding protein structurally similar to the SRC substrate Cas, tyrosine phosphorylation of which is critical for migration in fibroblasts and epithelial cells. Expression of a dominant negative version of Sin interfered with receptor-protein-tyrosine phosphatase a/EGF- as well as fibroblast growth factor-induced neurite outgrowth. These observations uncouple neuritogenic signaling in PC12 cells from sustained activation of ERK kinases and for the first time identify an effector of SRC function in neurite extension.

Most analyses that use reductionist systems such as PC12 cells to identify pathways governing neuronal process formation have focused on the Ras/ERK pathway, the requirement of which is well established. The neuritogenic effect of constitutively active MEK has been taken to indicate that ERK activation is also sufficient for neuritogenesis. Yet, physiological ERK activation, e.g. in response to EGF, often does not engender neurite extension. An attempt to resolve this paradox has been the postulate that the kinetic pattern of ERK signaling after a stimulus is what dictates the nature of the ensuing response (1). In this view, “sustained” activation of ERK (hours), such as typically seen for FGF or NGF, would be the signal that specifies a neuritogenic outcome as opposed to the “transient” (<1 h) ERK induction associated with non-neuritogenic factors such as EGF. However, such a correlation does not necessarily imply sufficiency or necessity.

Diverse and independent approaches have exposed the necessity of postulating pathways to process formation other than ERK. Recurrently, tyrosine kinases of the SRC family (SFKs) have been proposed as candidates for such “parallel pathways.” A mutation in Trk retains ERK induction but abolishes neurite outgrowth by NGF (2). Analysis of platelet-derived growth factor receptor mutants showed that sustained ERK activation per se is insufficient but that neuritogenesis requires additional signals possibly involving SRC (3). v-SRC induces neurite outgrowth in PC12 cells (4), whereas c-SRC inhibition can block outgrowth (5, 6). This crucial role of SFKs in neurite extension is not restricted to PC12 cells but is equally clearly encountered in conditionally immortalized (7) and primary neurons (8, 9). SFKs associate with adhesion molecules that facilitate process elongation (10, 11) and are enriched in nerve growth cones, where they interact with the cytoskeleton in a protein-tyrosine phosphatase (PTP)-dependent manner (12).

In fibroblasts and epithelial cells, SRC is intimately implicated in phosphorylation of focal adhesion proteins, turnover of these structures, and cell motility (13, 14). v-SRC can activate or inhibit ERK kinases, depending on the cell type and stage of transformation (15, 16). Its action as a transforming oncogene can be separated from its ability to activate Ras and ERK (17, 18). One of the major SRC substrates, Cas, in complex with the adaptor Crk, is a key mediator of cell migration in fibroblasts and epithelial cells (19–23). By contrast, in neuronal cells, the identity of signaling steps downstream of c-SRC has remained obscure. One reason for this lack of progress has been the previous extensive reliance on mutationaly activated v-SRC. Given the drastic degree of kinase activation and deregulation of the latter, this approach has not been informative regarding the function of endogenous c-SRC. Indeed, the signaling pathways downstream of oncogenic and cellular SRC proteins were recently shown to differ substantially; for instance, c-SRC-mediated activation of certain promoters relies exclusively on Rap1, whereas transforming SRC alleles also signal through Ras (24). Approaches using oncogenic SRC alleles are particularly compromised by the experimental difficulty of separating the biological effect of mutationally activated v-SRC from its ability to deregulate ERK kinases (15, 16).

SFKs are regulated by a conformational mechanism that is controlled by phosphorylation. Intramolecular interactions between the SH2 domain and a C-terminal tyrosine phosphorylation site (Tyr-527 in chicken SRC) in combination with SH3-mediated interactions stabilize a kinase-inactive conformation.
(25). In consequence, wild type SRC family kinases are reversibly activated in situ by ligands to their SH2 and SH3 domains (24, 26, 27) or by PTPs that mediate Tyr(P)-527 dephosphorylation (28). Extensive evidence identifies receptor-PTP (RPTPα) as one such physiological Tyr(P)-527 phosphatase. This PTP, which is particularly abundant in neural tissue, associates physically with SRC and Fyn (26, 29, 30), and its overexpression activates these kinases (26, 29–32); conversely, loss of RPTPα leads to dose-dependent reductions in SRC and Fyn kinase activities and generates integrin signaling deficits similar to SRC−/− cells (33, 34). RPTPα itself undergoes tyrosine phosphorylation at a residue (Tyr-798) in its C terminus. This modification leads to Grb2 recruitment (35, 36) and alters RPTPα function (36), SRC-activating ability (26), and localization (37). In the present study, we have exploited the role of RPTPα as a physiological SRC activator to identify signaling events downstream of SRC in neuronal cells.

MATERIALS AND METHODS

Cell Culture—PC12 cells were cultured in Dulbeco’s modified Eagle’s medium plus 10% fetal calf serum and 10% horse serum. Retinovirus production and infection were as described (36). For neuritogenesis, 5 × 10^4 cells were seeded per 35-mm plate, grown overnight, and starved (0.5% fetal calf serum plus 0.5% horse serum) for 16–20 h. To this medium was then added 50 ng/ml acidic FGF plus 5 or 100 ng/ml EGF.

Data Analysis—After 2 days of stimulation, neurite length was measured on photographed fields containing 100–250 cells. Data were expressed in two ways; first, as total neurite length averaged over cell number (bar diagrams; y axis = cell diameters); second, as percent neurite-bearing cells (percent cells bearing at least one neurite larger than two cell diameters). The level of statistical significance was assessed by a two-sided t test (unequal variance). In bar diagrams, error bars always indicate 95% confidence intervals. For numerical data (% neurite-bearing cells), the extent of a 95% confidence interval is indicated by the number between brackets. All key conclusions were reconfirmed on independent clones and/or pools of clones selected en masse.

Antibodies and Plasmids—Anti-RPTPα (36), and anti-Sin (38) sera have been described. Anti-SRC was from Calbiochem. For the immunoprecipitation/in vitro ERK assay and immunoblotting, anti-ERK-1 C-16 and anti-ERK-2 C-14 (Santa Cruz) were used, respectively; anti-phospho-ERK antibody was from New England Biolabs. Anti-phosphotyrosine antibody 72 was described (36); 4G10 was from Upstate Biotechnology. Anti-Cas was a gift of T. Parsons and A. Bouton (University of Virginia). Anti-CrKcL C-20 was from Santa Cruz. Anti-Nck sera were provided by E. Skolnik (New York University) or from Santa Cruz (C-19).

RPTPα constructs were described (36). The Sin deletion SinSD, lacking residues 101–256 (38), was generated using the Exsite kit (Stratagene). Two retroviral vectors were used: pLXSHD (36), and pBabeI-EG (D. Unutmaz, NYU). The latter encodes an long terminal repeat-driven di-cistronic transcript consisting of the transduced cDNA and green fluorescent protein (3’ to an internal ribosomal entry site), cDNA inserts were ligated between Xhol and BamHI of pLXSHD or into the BamHI site of pBabeI-EG.

Immunoprecipitation and Immunoblotting—Lysates in 50 mM Hepes, pH 7.5, 1% Triton-X-100, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1 mM Na3VO4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin were cleared at 16,000 × g for 15 min. They were blocked in phosphate-buffered saline plus 10% fetal calf serum for 30 min and incubated with primary antibody for 1 h followed by 10 μg/ml secondary antibody and examined under a Zeiss photomicroscope.

In Vitro Kinase Assays—For SRC assays, cells were lysed in radioimmune precipitation buffer (50 mM Hepes, pH 7.4, 1% deoxycholic acid, 1% Triton-X-100, 0.1% SDS, 150 mM NaCl, 1 mL EGTA) plus protease and PTP inhibitors. Anti-SRC precipitates were washed 3× in radioimmunoprecipitation buffer plus twice in TBS (150 mM NaCl, 20 mM Tris, pH 7.5) and split for anti-SRC immunoblotting and kinase assay. Enolase substrate was denatured in 50 mM sodium acetate at 30 °C for 5 min and neutralized with 1 mM Tris-HCl, pH 7.5. Reactions (50 μl) in kinase buffer (20 mM Tris, pH 7.5, 5 mM MnCl2) contained 12.5 μg of enolase plus 10 μCi of [γ-32P]ATP and were incubated at 30 °C for 10 min. ERK assays involved precipitation from Triton lysate and incubation in 25 μl of buffer (10 mM Tris, pH 7.5, 10 mM MgCl2) with 10 μCi of [γ-32P]ATP plus 12.5 μg of myelin basic protein for 25 min at 30 °C. Reactions were stopped by SDS-PAGE sample buffer, and gel autoradiographs were quantitated using phosphorimaging.

RESULTS

EGF Induces Neurite Outgrowth in c-SRC-overexpressing PC12 Cells—v-SRC induces growth factor-independent neuritogenesis in PC12 cells (4), but how this may relate to the physiological function of c-SRC is poorly understood. We used retroviral infection to generate a pool of PC12 cells overexpressing c-SRC (Fig. 1A). This conferred upon EGF (normally a solely mitogenic factor) (1) an ability to induce neurite formation equal to that of a bona fide neuritogenic factor (FGF) in control cells (Fig. 1C). This suggests that an SRC-dependent function can contribute to a non-neuritogenic factor (EGF) into a neuritogenic one. Increases in tyrosine phosphorylation after c-SRC overexpression were largely limited to four proteins of 130, 90, 70, and 60 kDa (the latter being itself (Fig. 1B).

RPTPα Localizes to Cell-Cell Contact Zones and Tips of Spontaneous Spikes and Activates Endogenous SRC—To ask whether endogenous levels of c-SRC could similarly contribute to conversion of EGF into a neuritogenic factor, we relied on the known c-SRC-activating function of RPTPα (26, 29–34). We previously reported generation of PC12 lines expressing wt or mutant RPTPα (36). The mutants used were RPTPαCCSS, catalytically inactive due to mutation of the active site cysteine residues in either PTP domain (Cys-442, Cys-732) to serine, and RPTPαY798F, in which Tyr-798, the site of tyrosine phosphorylation in RPTPα, was mutated to phenylalanine (resulting in loss of Grb2 recruitment but normal in vitro catalytic activity (35)). Immunofluorescence revealed RPTPα was membrane-localized and concentrated in cell-cell contact zones and at the tips of spontaneous spikes (Fig. 2A); no significant differences were seen in localization of wt versus mutant proteins (not shown).
wt RPTPa and RPTPaY798F, but not RPTPaCCSS, moderately increased c-SRC activity (by 2-fold), with the level of c-SRC protein remaining unchanged (Fig. 2B). This increase in specific activity likely reflects the well-documented ability of RPTPa to reduce the phosphorylation level of the Tyr-527 residue in c-SRC (33, 34). Contrasting with the situation for c-SRC-overexpressing cells, we observed no constitutive effects of RPTPa on cell morphology or on neurite extension in the absence of added growth factors (data not shown and Fig. 3A).

RPTPa Stimulates EGF-induced Neurite Outgrowth in a Manner That Requires Both SRC and ERK—As reported previously (36), the presence of wt RPTPa, but not catalytically inactive RPTPaCCSS, impairs the ability of FGF (and NGF; data not shown) to cause neurite outgrowth. Mutation of the Tyr-798 phosphorylation site in RPTPa not only abolishes, but inverts this effect of wt RPTPa, since RPTPaY798F behaves instead as a net potentiator of FGF-induced outgrowth (36) (Fig. 3A).

We here report that, unexpectedly, the effect of wt RPTPa on responsiveness to EGF was the opposite of that on FGF responsiveness; that is, wt RPTPa converted EGF into a promoter of neurite outgrowth (Fig. 3A, left panel). Furthermore, RPTPaY798F promoted neurite outgrowth in response to EGF even more powerfully than wt RPTPa (Fig. 3A, left panel). In contrast, RPTPaCCSS did not potentiate EGF responsiveness; this catalytically inactive protein even somewhat reduced the formation of short spikes that occurs in response to EGF. It is conceivable that this weak inhibitory effect of RPTPaCCSS reflects dominant negative-like interference with the function of endogenous RPTPa or of a related RPTP. However, to what extent C-to-S mutant PTPs can indeed be relied on to be specific and true dominant-negatives is as yet still unclear; hence, interpretation of the significance of this observation is probably premature. Assessment of mRNA levels by Northern blotting for the metalloprotease transin, a late marker for NGF-induced neuronal differentiation of PC12 cells (39, 40), revealed that the EGF-induced neurite extension correlated fully with expression of this marker. EGF treatment lead to transin induction in cells expressing wt RPTPa or RPTPaY798F but not RPTPaCCSS; moreover, this effect was stronger in RPTPaY798F than in wt RPTPa-expressing cells (supplemental data included in the on-line version of manuscript). This indicates that EGF-induced neurite outgrowth is dependent on the newly found role of SRC overexpression, although less drastic, since the effect of RPTPa remained fully EGF-dependent (Fig. 1C versus Fig. 3, A and B, and data not shown). Pharmacological SRC inhibition using PP1 abolished the neuritogenic effect of RPTPa and RPTPaY798F on stimulation with EGF, whereas cell morphology and viability remained unaffected (Fig. 3B), indicating that the stimulatory effect of RPTPa on EGF-induced neuritogenesis depends on the activity of a SFK. At the same time, EGF-induced neurite outgrowth still continued to be dependent on ERK activity, as shown by application of PD98059 (a selective MEK1 inhibitor) (Fig. 3B). We did observe a tendency of EGF-stimulated RPTPaY798F-expressing cells to still form “stumps” in the presence of 10 μM PD98059. However, <2% of cells formed protrusions longer than 2 cell diameters, and any
protrusions formed were <1 cell diameter. The number of these protrusions could be reduced significantly by raising the concentration of PD98059 to 25 μM (data not shown). Hence, these protrusions cannot be referred to as neurites, and their true biological significance is questionable at best.

**Conversion of EGF into an Outgrowth-promoting Factor by RPTPα Is Not Accompanied by Alteration in ERK Kinetics**—A widely cited model traces back the divergent effects of EGF versus FGF on normal PC12 cells to differences in their kinetics of ERK activation. It has been proposed that the relatively transient ERK activation induced by EGF is insufficient for neuritogenesis, which would require the more sustained (>2 h) activation of ERK that is observed for NGF or FGF. This hypothesis was prompted by observations that experimental induction of neurite formation by overexpression of many signaling molecules is invariably accompanied by a shift in ERK activation kinetics from a transient to a sustained mode (1). Hence, we wished to determine whether the ability of wt RPTPα and RPTPαY798F to convert EGF into a neuritogenic factor could similarly be accounted for by an alternation, from transient to sustained, in the kinetics of EGF-induced ERK activation.

We observed that wt RPTPα inhibited ERK activation in response to FGF at both the 5-min and 6-h time points, with wt RPTPα more potent at reducing ERK activation than RPTPαCCSS and RPTPαY798F (Fig. 4A), suggesting that optimal inhibition required both catalytic activity and Grb2 binding. Although reduced ERK activation might explain the ability of wt RPTPα to inhibit FGF-induced neurite formation (Fig. 3A), this correlation broke down for RPTPαY798F, which reduced ERK activation by FGF (Fig. 4A and data not shown) but enhanced neurite extension induced by this factor (Fig. 3A).

The situation in the case of EGF was analyzed in extensive detail. As expected from the literature (1), in control cells, EGF- and FGF-induced ERK activities were comparable at the 5-min time point but differed significantly at later time points, with FGF-induced activation sustained longer (Fig. 4, A–C). Strikingly, however, neither wt RPTPα nor RPTPαY798F (which both potentiate EGF-induced neurite outgrowth; see Fig. 3) potentiated the extent of ERK induction by EGF. In the short term (5 min), expression of wt or mutant RPTPα actually tended to reduce ERK activation (Fig. 4B, left panel). More importantly, none of the wt or mutant RPTPα proteins significantly altered EGF-induced ERK activation at the late time point (6 h) (Fig. 4B, right panel). As expected from the literature (1), in the control (vector) cells at late time points, EGF-induced ERK activation was significantly lower than FGF-induced activation (Fig. 4B, right panel, and C). Similar conclusions regarding the effect of RPTPα on ERK activation were reached by in vitro kinase assay (Fig. 4B) and by immunoblotting with phospho-specific antibodies for the activated state of ERK 1 and 2 (Fig. 4C).

We conclude that wt RPTPα and RPTPαY798F counteract the induction of ERK by FGF but do not alter the extent or kinetics of ERK activation in response to EGF. This breakdown of the correlation between neurite outgrowth and the extent of sustained ERK induction leaves the ability of wt RPTPα and RPTPαY798F to induce a neuritogenic response to EGF unexplained.

**RPTPα in a SRC-dependent Manner Elevates Tyrosine Phosphorylation of the Docking Proteins Cas and Sin**—In a search for alternative explanations for conversion of EGF into an outgrowth-promoting factor, we assessed how RPTPα affected cellular tyrosine phosphorylation. We found that wt RPTPα and RPTPαY798F (but not RPTPαCCSS) specifically elevated tyrosine phosphorylation of a 90-kDa protein and also caused a more modest increase in a 130-kDa species (Fig. 5A). This pattern is similar to that observed after an increase in c-SRC expression (Fig. 1B). EGF or FGF did not affect the phosphorylase content of these 90- and 130-kDa proteins, and wt or mutant RPTPα did not alter the overall patterns of tyrosine phosphorylation induced in response to EGF or FGF (not shown).

In trying to characterize these proteins, we found (data not shown) that RPTPα had not affected phosphorylation of cortactin and Cbl (both SRC substrates) or of FRS-2 (implicated in FGF-dependent PC12 differentiation). However, we successfully identified the proteins whose tyrosine phosphorylation level was elevated by RPTPα as Sin (90 kDa) and Cas (130 kDa).
Role of SRC Substrate Sin in Neurite Outgrowth

Fig. 5. RPTPα elevates tyrosine phosphorylation of Sin and Cas in a SRC-dependent manner and enhances complex formation with Crk and Nck—A, equal amounts of lysate from clones infected with control, wt, or mutant RPTPα-expressing virus were analyzed by anti-phosphotyrosine (P-Tyr) immunoblotting. Proteins migrating at 130 and 90 kDa are indicated. B, equal amounts of total lysate from vector-infected (V) or RPTPα-expressing cells (WT) were subjected to anti-Sin (left panels) or anti-Cas (right panels) immunoprecipitation. In lanes marked IP, the immune precipitates were split into two portions. One portion was immunoblotted with anti-phosphotyrosine (upper panels), and the other was immunoblotted with anti-Sin (lower panel, left) or anti-Cas (lower panel, right). In lanes marked SUP, aliquots of lysates collected before (bIP) or after immunoprecipitation (aIP) with the respective antibodies were analyzed by immunoblotting with the antibodies indicated on the left. C, lysates were subjected to immunoprecipitation with anti-CrkL or anti-Nck, and immune precipitates were analyzed by immunoblotting with anti-Cas, -Sin, -CrkL, and -Nck. D, anti-Sin immune precipitates were prepared from control cells (V) or cells expressing wt RPTPα (WT) or RPTPαY798F (YF) treated or not with the SRC inhibitor PP1 (1 μM) for 30 min before lysis. Precipitates were analyzed by anti-phosphotyrosine (top) or anti-Sin (bottom) immunoblotting. E, anti-CrkL precipitates were prepared from control cells (V) or cells expressing wt RPTPα (WT) treated or not with the SRC inhibitor PP1 (1 μM) for 30 min before lysis. Precipitates were analyzed by immunoblotting with anti-Sin (top), anti-Cas (middle), and anti-CrkL (bottom).

(Fig. 5, B and D), 2 related docking proteins and known SRC substrates (21, 22). Quantitative immunodepletion experiments demonstrated that these 2 proteins (or associated proteins of similar sizes) accounted for the bulk of the RPTPα-induced increase in tyrosine phosphorylation at 90 and 130 kDa (Fig. 5B).

Pharmacological SRC inhibition was used to determine whether the RPTPα-induced increase in tyrosine phosphorylation of Sin required SRC activity. As shown in Fig. 5D, the SRC inhibitor PP1, along with its ability to inhibit neurite outgrowth described above (Fig. 3B), also reduced tyrosine phosphorylation of Sin to undetectable levels, indicating that RPTPα-induced phosphorylation of Sin is fully SRC-dependent. RPTPα Induces Complex Formation of Sin and Cas with the Adaptors Crk and Nck—In fibroblasts, Cas associates with, and is a substrate, an effector, and an activator of c-SRC (22), controlling organization of the actin cytoskeleton, cell migration, and activation of JNK kinases (21, 41, 42). Sin (Efs), whose expression is more restricted than Cas, was isolated in independent screens as a ligand for the SH3 domains of both SRC (38) and Fyn (43). A third mammalian member of this family, HEF-1, was isolated on the basis of its ability to induce pseudohyphal growth in yeast (21). All three share a common domain structure consisting of an SH3 domain, a “substrate” domain containing tyrosine phosphorylated SH2 binding sites, a proline-rich region, and a C-terminal domain with SH3 and SH2 consensus binding sites for SFKs (Fig. 6A) (21). Their association with cytoplasmic tyrosine kinases is followed by phosphorylation of the substrate region and ensuing recruitment of adaptors and other signaling proteins, leading to assembly of higher order complexes with the potential for interactions between associated effectors (21). RPTPα enhanced such recruitment of the adaptor CrkL to Sin and Cas, as shown by analysis of anti-CrkL immune precipitations from control and wt RPTPα-expressing cells by anti-Cas or anti-Sin immunoblotting (Fig. 5C). Similar results were obtained for CrkII (which is less abundant in PC12 cells than CrkL (44); not shown) and for Nck (Fig. 5C). Consistent with the ability of the SRC inhibitor PP1 to antagonize the increase in tyrosine phosphorylation of Sin that is induced by RPTPα (Fig. 5D), PP1 also reversed the increase in RPTPα-induced complex formation between Sin or Cas and CrkL (Fig. 5E).

Sin-Crk Coupling Is a Necessary Mediator of RPTPα/SRC-dependent Neuritogenesis—To test the functional relevance of increased phosphorylation of Sin as caused by RPTPα-induced activation of SRC, we designed a mutant, SinSD, which lacks—To test the functional relevance of increased phosphorylation of Sin as caused by RPTPα-induced activation of SRC, we designed a mutant, SinSD, which lacks the substrate region that encompasses the tyrosine residues whose phosphorylation recruits Crk and Nck (Fig. 6A). By retaining the binding sites for the SH2 and SH3 domains of SRC and Fyn, we expected SinSD to compete with endogenous Sin for association with SRC and, thus, to antagonize assembly of productive complexes around endogenous Sin. An analogous mutant of Cas has been successful in elucidating the contributions of Cas to cell migration (20) and JNK activation in fibroblasts (42). Parental PC12 cells and wt RPTPα or RPTPαY798F expressors were infected with a control or SinSD-expressing retrovi-
As expected, SinSD successfully and specifically interfered with tyrosine phosphorylation of endogenous Sin (Fig. 6B). No effect of SinSD was noted on the growth rate or morphology or unstimulated cells (not shown). However, SinSD interfered with tyrosine phosphorylation of endogenous Sin. Parental cells and cells expressing wt RPTPα or RPTPαY798F were infected with control (vector) or SinSD-expressing virus. Total lysates (TL) were analyzed by immunoblotting (IB) with anti-RPTPα (upper) and with anti-Sin (second panel from top). Anti-Sin immune precipitates (IP) were analyzed by immunoblotting with anti-phosphotyrosine (third panel from top) and with anti-Sin (bottom panel). C, SinSD blocks neuritogenesis of RPTPα-expressing cells in response to EGF. Pools of cells expressing wt RPTPα or RPTPαY798F in the absence of presence of SinSD were exposed to EGF, and average neurite length (expressed in cell diameters) was measured after 48 h. Error bars denote 95% confidence intervals; Statistical significance for SinSD-infected RPTPα (WT) and SinSD-infected RPTPα (YF) cells was tested with respect to vector-infected RPTPα (WT) cells and vector-infected RPTPα (YF) cells, respectively (***, p < 0.001). D, FGF-induced neurite outgrowth in parental PC12 cells (Uninf.) or cells infected with control empty vector or a SinSD-expressing retrovirus. E, schematic representation of dominant-negative CrkIR38K, in which a lysine residue critical for SH2 domain function was mutated to arginine. F, expression of CrkIR38K. Pools of cells expressing wt RPTPα (WT) or RPTPαY798F (YF) were infected with empty (vector) or CrkIR38K-encoding retrovirus, and expression of RPTPα and CrkIR38K was monitored by immunoblotting. G, CrkIR38K blocks neuritogenesis of RPTPα-expressing cells in response to EGF. Cells expressing wt RPTPα (left) or RPTPαY798F (right) in the presence or absence of CrkIR38K were exposed to EGF for 48 h, and average neurite length was measured (**, p < 0.01). Average neurite length in control (non-Crk)-superinfected RPTPα-expressing cells was somewhat lower than in Fig. 3 due to reduced RPTPα expression as a consequence of superinfection (data not shown).
induced neuritogenesis. We introduced a CrkII mutant, CrkR38K, into wt RPTPα- or RPTPαY798F-expressing cells (Fig. 6F); this mutant adaptor contains a point mutation in its SH2 domain that abolishes its ability to associate with tyrosine-phosphorylated docking proteins (Fig. 6E). It can be expected to interact with the full range of effectors available to endogenous CrkII but to sequester them in a complex incapable of associating with endogenous docking proteins for Crk such as Cas and Sin. The experiment revealed clear interference of CrkR38K with RPTPα/SRC-dependent neurite outgrowth (Fig. 6G).

**DISCUSSION**

Correlative evidence originally gave rise to the hypothesis that the qualitative distinction between a non-neuritogenic versus a neuritogenic response is dictated by quantitative differences in ERK signaling (1). We observed here conversion of a non-neuritogenic (EGF) into a neuritogenic factor without alteration in the extent or kinetics of ERK induction. Moreover, in response to FGF, wt RPTPα and RPTPαY798F inhibited ERK activation, yet the latter elicited neurite extension. Our data thus demonstrate that the kinetic pattern of ERK activation does not constitute a necessary determinant of growth factor specificity. Potentially related observations have been made using other approaches, e.g. studying the synergism between NGF and IL-6 in a particular variant PC12 line (46), or studying the effect of stable transfection of GTPase-deficient G protein subunits (47) (however, in the latter case, the effect was constitutive, not showing the growth factor dependence observed here). In the present study, we identify SRC-mediated assembly of Sin-Crk complexes as the “alternative” pathway that contributes to formation of neuritic processes.

The latter conclusion is based on 1) the well established function of RPTPα in SRC activation (26, 29, 31–34) (Fig. 2B); 2) the similar effects of c-SRC (Fig. 1C) and RPTPα (Fig. 3) on EGF-induced neuritogenesis; 3) the fact that pharmacological SRC inhibition abolished the ability of RPTPα to alter the nature of the response to EGF (Fig. 3B); 4) the similarity between the patterns of tyrosine phosphorylation induced by RPTPα and c-SRC (Figs. 1B and 5A); 5) the observed SRC-dependent increase in phosphorylation and Crk binding of Sin and Cas, two known c-SRC substrates (Fig. 5B); and 6) the ability of both the dominant-negative SRC substrate SinSD and a dominant-negative version of the associated adaptor CRK to inhibit neuritogenesis (Fig. 6, C and F). Although each argument is by itself subject to alternative interpretations, taken together they strongly implicate the cascade SRC > Sin > Crk as a signaling cassette capable of contributing to neurite outgrowth. Notwithstanding the wealth of previous evidence implicating SRC in neurite formation, the identity of its downstream effectors had remained surprisingly enigmatic; to our knowledge, the present demonstration of the role of Sin-Crk complexes constitutes the first elucidation of the mechanism of action of SFKs in this process. We consistently noted that expression of dominant negative versions of Sin or Crk reduced RPTPα/EGF- or FGF-induced neurite outgrowth but did not altogether abolish it (Fig. 6, C, D, and G). Further study will determine whether this reflects an only partial dominant negative effect (for instance because of an inability to achieve insufficient expression levels of SinSD or Crk-R38K) or, more interestingly, reflects the existence and function of alternative effectors (other than Sin) downstream of SRC that function in neurite outgrowth.

Docking of Crk to Cas, a close homolog of Sin, is a crucial signaling step in fibroblast migration, acting in an ERK-independent manner (19–21, 23), and PTPs that dephosphorylate Cas also severely affect cell migration (28). Given the mechanistic analogies between migration and neurite extension, the contribution of related scaffolding proteins and effector pathways to the latter would thus not seem implausible, yet had thus far not been recognized.

The pathway outlined above needs to be qualified in two ways. First, it is unclear to what extent the effects observed occur through activation of SRC versus Fyn. Although RPTPα regulates both kinases (30, 33, 34), the role of Fyn in PC12 cells is a totally unexplored issue that may require further study; however, because Sin is a common substrate for both (38, 43), it may constitute a point of convergence. Second, we have not been able to dissect with reasonable certainty the respective contributions of Cas versus Sin. Given their similarity, antibody cross-reaction, common binding partners, and ability to heterodimerize (21, 48), resolution of this issue will have to rely on reagents as yet to be developed, such as cell lines that are null for either.

Strikingly, wt RPTPα potentiated the neuritogenic capacities of EGF but inhibited FGF-dependent neuritogenesis (36). The latter function likely results from a separate ability of RPTPα to impair FGF-induced ERK activation, which offsets the neuritogenesis-stimulating effect of activating the SRC-Sin-Crk pathway. Two arguments suggest that this inhibitory effect of RPTPα on FGF signaling involves a non-SRC substrate for RPTPα that is important only for FGF/NGF-mediated outgrowth. First, c-SRC overexpression mimicked the stimulating effect of RPTPα on EGF responsiveness but not its inhibitory effect on FGF responsiveness (Fig. 1C versus Fig. 3A). Hence, the effect of RPTPα on FGF signaling must diverge from that on EGF responsiveness upstream of SRC. Second, the two functions could be mutually separated; the ability of RPTPα to inhibit FGF-induced outgrowth is dependent on its Tyr-798 phosphorylation site, but its outgrowth-eliciting ability in response to EGF is not. Thus, abolishing the ability of RPTPα to be phosphorylated (the Y798F mutation) converts RPTPα from an inhibitor to a net stimulator of FGF-induced neurite outgrowth (most likely due to an additive effect of enhanced Sin-Crk signaling) and further potentiates the ability of RPTPα to induce neurite outgrowth in response to EGF. We suggest that phosphorylation of Tyr-798 in RPTPα is necessary for the ability of RPTPα to dephosphorylate a substrate in the pathway from the FGF receptor to ERK activation. Such modulation of the substrate specificity of RPTPα by Tyr-798 phosphorylation could occur in various ways, e.g. displacement (as described for the RPTPα-Tyr-798/SRC-SH2 interaction in fibroblasts (26); but see below), RPTPα-bound Grb2 acting as an adaptor mediating recruitment of a specific substrate to RPTPα, or altered RPTPα intracellular localization (37).

Zheng et al. (31), and den Hertog et al. (32) reported 3–6-fold and 4–6-fold increases in SRC kinase activity following RPTPα overexpression. The comparatively more modest (2-fold; Fig. 2) effect of RPTPα on c-SRC activity seen here in PC12 cells may reflect a more modest level of RPTPα overexpression in the present case or, alternatively, may be an intrinsic feature of PC12 cells. De-phosphorylation of Tyr-527 activates c-SRC 10–20-fold (49). However, increases of this magnitude in total cellular SRC activity are never observed. Rather, increases in overall cellular SRC activity that are of physiological significance tend to be much more modest, in all probability reflecting activation of only a distinct subpopulation of the total content of cellular SRC. EGF and NGF were reported to increase tyrosine phosphorylation of c-SRC by a few fold (50). Determining to what extent the RPTPα-activated and growth factor-activated SRC pools may be related or overlapping should be an area of detailed and careful further analysis.

Although mutation of Tyr-798 abolished the ability of RPTPα
to inhibit FGF-dependent outgrowth, we observed it to have minor effects on the ability of RPTPα to activate SRC. Thus, wt RPTPα and RPTPαY798F both enhanced in vitro Src kinase activity (Fig. 2B), in vivo phosphorylation of the SRC substrate site (Fig. 5), and EGF-dependent neurite outgrowth itself (Fig. 3). We have tried to quantitate the effect of the Y798F mutation precisely enough so as to be able to document small effects of it on SRC activation or on tyrosine phosphorylation of Sin. These experiments (not shown) suggested that the slightly lower SRC activation by RPTPαY798F as compared with wt RPTPα (Figs. 2B, and 5A) in part reflects the somewhat lower level of expression of the RPTPαY798F mutant protein. Our data differ from those of Zheng et al. (26), who observed that SRC activation by RPTPα in fibroblasts is highly dependent on the Tyr-798 phosphorylation site in RPTPα (to displace the Tyr-527 substrate site from the SRC SH2 domain) (26). Possibly, in PC12 cells, SRC SH2 displacement can be already efficiently mediated by other means (perhaps involving Sin itself (24)), rendering the contribution of tyrosine-phosphorylated Tyr-798 in RPTPα less rate-limiting.

Signaling downstream of SRC, Cas, and Sin is thought to rely on the small GTPase Rap1 (24). Although Crk-mediated Rap1 activation itself is activation implicated in sustained ERK activation (44), our data argue for a contribution of Crk separate from this role. A worthwhile avenue for further dissection of the mechanism of control of neurite outgrowth by Sin-Crk coupling may involve the contribution of Rac-like small G proteins. The controlling role of Cas-Crk complexes in fibroblast motility has been shown to depend on this pathway (20). DOCK180, recruited by Crk, activates Rac1 (51), which could thus exert localized effects on the actin cytoskeleton. Rac and Cdc42 stimulate filopodia and lamellipodia in the growth cone of neuronal cells, and a balance between Rho-like and other small G protein activities may control neurite outgrowth (52). The viral v-Crk oncogene has been shown to control Rho and to modulate axonal growth in vitro and in vivo (53, 54).

SRC activity and Cas phosphorylation are induced in response to cell-cell contact (55) and membrane depolarization (56). We suggest that neurotogenic signaling by the SRC > Sin-Cas > Crk cassette may be particularly relevant for stimuli that rely heavily on SFKs for promoting neurite outgrowth, such as cell-cell adhesion molecules (8, 9). Indeed, RPTPα localizes to areas of cell-cell contact (Fig. 2), and several cell-cell adhesion molecules interact with PTPα in neuronal cells (28, 57); particularly, glycosylphosphatidylinositol-linked contactin associates in cis with RPTPα and the SFK Fyn (11). We have thus far not observed clear effects of selected cell-cell adhesion or extracellular matrix molecules or growth factors on the total level of Sin phosphorylation (data not shown); very likely this issue will require a detailed one-by-one analysis of phosphorylation sites in Sin. At any rate, a role for SRC > Sin-Cas > Crk signaling in specifying neurite outgrowth may help solve two paradoxes associated with the concept of sustained ERK activation as necessary and sufficient for neurite outgrowth (1). First, given the complex multiplicity of neural cell types, “process extension” would not be expected to be a unitary event, making reliance on a single pathway unlikely (3, 7). Second, the ability of cells to elaborate multiple processes with different and plastic properties demands control mechanisms that can act locally. A role for SRC, whose localization and activity are tightly regulated, in assembly of localized signaling complexes around scaffolding proteins such as Sin or Cas may be relevant in this respect.

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Role of SRC Substrate Sin in Neurite Outgrowth

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