PKA phosphorylation of Src mediates Rap1 activation in NGF and cAMP signaling in PC12 cells

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Summary

Recent studies suggest that the tyrosine kinase Src plays an important role in the hormonal regulation of extracellular signal-regulated kinases (ERKs) via cyclic AMP (cAMP). Src has also been proposed to mediate signals downstream of nerve growth factor (NGF). Here, we report that the cAMP-dependent protein kinase A (PKA) induced the phosphorylation of Src at residue serine17 (S17) in multiple cell types including PC12, Hek293, AtT-20 and CHO cells. In PC12 cells, Src phosphorylation on S17 participates in the activation of the small G protein Rap1 by both cAMP and NGF. In these cells, Rap1 is required for cAMP/PKA signaling to ERKs and also for the sustained activation of ERKs by NGF. The activation of Rap1 by both cAMP and NGF was blocked by PP2, an inhibitor of Src family kinases, and by a Src mutant incapable of being phosphorylated by PKA (SrcS17A), consistent with the requirement of PKA phosphorylation of Src at S17 in these actions. PP2 and SrcS17A also inhibited the Rap1-dependent activation of ERKs by both agents. These results strongly indicate that PKA phosphorylation of Src at S17 is essential for cAMP and NGF signaling in PC12 cells and identify PKA as an important downstream target of NGF. PKA phosphorylation of Src may therefore be required for Rap1 activation in PC12 cells.

Key words: PKA, Src, ERK, Rap1, PC12 cells

Introduction

The crosstalk between the cyclic AMP (cAMP)-dependent protein kinase (PKA) and the cytoplasmic tyrosine kinase Src has recently received much attention (Stork and Schmitt, 2002). PKA phosphorylation of the β_2-adrenergic receptor promotes the activation of Src via its association with β-arrestin to trigger endocytic signaling to the extracellular signal-regulated kinase ERK (Luttrell et al., 1999). PKA can also negatively regulate Src through direct phosphorylation and activation of the carboxy terminal of Src kinase in cells (Csk) (Abrahamsen et al., 2003; Vang et al., 2001), although Csk may also be inactivated by PKA in vitro (Sun et al., 1997). Here we explore another potential mechanism of crosstalk between cAMP and Src that involves the phosphorylation of Src itself.

PKA is known to phosphorylate Src at residue serine17 (S17) within the amino terminus (Collett et al., 1979). We have previously reported a role for PKA phosphorylation of this site in the activation of the small G protein Rap1 (Schmitt and Stork, 2002b). One of the effectors of Rap1 is the MAP kinase kinase kinase B-Raf, and a role for Src in the activation of Rap1/B-Raf by PKA has been proposed (Schmitt and Stork, 2002a). Src function was inferred from these studies using Src-deficient cells and mutant Src molecules lacking putative PKA sites. In both conditions, PKA activation of Rap1 was diminished. These studies largely utilized overexpression of transfected Src and Src mutants to identify potential regulation and function of Src phosphorylation by PKA. In this study, we directly examined the phosphorylation of S17 in endogenous Src protein in several cell types. We found a significant increase in Src phosphorylation at S17 in response to stimuli that increased cAMP levels.

The regulation and function of the S17 phosphorylation was further examined in detail utilizing both PC12 and Hek293 cells. In PC12 cells, both NGF and cAMP activate ERKs in a Rap1-dependent manner. In the case of NGF, stimulation of both Ras and Rap1 are needed for ERK activation, with Ras contributing to the early phase of ERK activation and Rap1 to the late phase of ERK activation (York et al., 1998). Interestingly, we report here that both cAMP and NGF induce S17 phosphorylation and activation of endogenous Src, coinciding with the late phase of ERK activation. Furthermore, we find that phosphorylation of Src by PKA mediates the action of NGF and cAMP on Rap1 and ERKs.

Materials and Methods

Materials

PC12 cells (PC12-GR5) were kindly provided by Rae Nishi (University of Vermont, Burlington, VT). PC12-nnr5 cells that lack NGF TrkA receptor were a gift of Susan Meakin (The John P. Robarts Research Institute, London, Canada). CHO cells, Hek293 cells and AtT-20 cells were provided by the Vollum Tissue Culture Core (Oregon Health Sciences University, Portland, Oregon). Antibodies to Rap1 (also called Krev-1), ERK2, c-Src and agarose-conjugated antibody to myc were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Ras antibody and anti-Src clone GD11 were purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies specific to phosphorylated ERK (pERK) that recognize

phosphorylated ERK1 (pERK1) and ERK2 (pERK2) at residues threonine183 and tyrosine185 in the activation loop, phospho-(Ser/Thr) PKA substrate antibody (PSAb) and phospho-Src (pY416) and phospho-TrkA(Y490) antibody were purchased from Cell Signaling Technologies (Beverly, MA). Agarose-coupled v-Src antibody was purchased from Oncogene Research Product (San Diego, CA). Nerve growth factor (NGF) was from Boehringer Mannheim (Mannheim, Germany). FLAG (M2) antibody, agarose-coupled FLAG (M2) antibody, isoproterenol, Rp-cAMP, dibutyryl cAMP and epidermal growth factor (EGF) were purchased from Sigma. Forskolin, 3-isobutyl-1-methylxanthine (IBMX), 8-CPT-cAMP, PP2 (AG1879) [4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyazolo(3,4-d)pyrimidine], PD98059 [2-(2′-amino-3′-methoxyphenol)-ox-anaphthalen-4-one], SU6656 and H89 {*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide} were purchased from Calbiochem (Riverside, CA). V8 protease was purchased from Roche Applied Sciences (Indianapolis, IN). 8-(4-chloro-phenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate (8CPT-2Me-cAMP) was purchased from Biology Life Sciences (Bremen, Germany).

Cell culture

PC12-GR5 and PC12-nnr5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% horse serum, 5% fetal calf serum (FCS) and antibiotics at 37°C in 5% CO_2. For Hek293 (human embryonic kidney) cells and AtT-20 (mouse corticotroph) cells, DMEM medium was supplemented with 10% FCS. For CHO (Chinese hamster ovary) cells, F12 medium was supplemented with 10% FCS. The cells were serum-starved for 16 hours prior to treatment with various reagents. The following drug concentrations were used for treatment: 100 ng/ml NGF, 100 ng/ml EGF, 10 μM forskolin, 100 μM IBMX, 10 μM isoproterenol, 10 μM H89, 200 μM Rp-cAMP, 500 μM dibutyryl cAMP, 30 μM PD98059 and 10 μM PP2. All inhibitors were added 15 minutes prior to treatment unless otherwise indicated. SU6656 was used at 10 μM. 8-CPT-2Me-cAMP was used at 50 μM.

Plasmids and transfections

The Flag-Src, and Flag-SrcS17A plasmids were all generated as previously described (Schmitt and Stork, 2002b). pEnhanced green fluorescent protein (pEGFP-C1) was purchased from BD Biosciences, Clontech. The control vector, pcDNA3 (Invitrogen, Carlsbad, CA), was included in each set of transfections to ensure that each plate received the same amount of DNA. Lipofectamine 2000-mediated transfections were carried out according to instructions provided by the manufacturer (Invitrogen, Carlsbad, CA). Following transfection, cells were allowed to recover in medium with serum for 24 hours. Cells were then starved overnight in serum-free DMEM before treatment and lysis.

Western blotting

Protein concentrations were assessed by the Bradford method (Bio-Rad, Herkules, CA). For examination of Src phosphorylation at S17 and Y416, Src was immunoprecipitated prior to western blotting. For all other western blotting experiments, cell lysates containing equal amounts of protein per treatment condition were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer onto PolyScreen PDVF membranes (PerkinElmer Life Sciences, Boston, MA). Membranes were blocked in 5% non-fat dry milk and probed with primary antibodies according to the manufacturer's instructions, then washed and incubated with the appropriate horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ). Proteins were detected by enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA). Representative gels are shown for all experiments.

Immunoprecipitation

For immunoprecipitation of myc-ERK2, cells were lysed in RIPA buffer [1% Triton X 100, 1% sodium deoxycholate, 0.2% SDS, 125 mM NaCl, 50 mM Tris (pH 8.0), 10% glycerol, 1 mM EDTA, 25 mM glycerophosphate, 1 mM PMSF, 1 μM leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 mM NaF, 0.5 mM aprotinin, 1 mM Na_3VO_4], followed by three rounds of sonication for 20 seconds. For Src and FLAG-Src, the following lysis buffer was used: 10% glycerol, 1% NP-40, 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5 mM $MgCl_2$, 1 mM PMSF, 1 μM leupeptin, 10 μg/ml of soybean trypsin inhibitor, 10 mM NaF, 0.5 mM aprotinin, 1 mM Na_3VO_4. Equal amounts of cell lysate per condition (500-1000 μg/tube) were precipitated at 4°C for 3-4 hours. Beads were rinsed three times with the respective lysis buffers, and protein was eluted from the beads with 2× Laemmli sample loading buffer.

V8 digestion

Hek293 cells were grown to 70% confluency in ten 100-mm dishes for transfection with Flag-Src, recovered and serum-starved as described above. Five dishes were stimulated with forskolin/IBMX for 10 minutes. Each dish was lysed in 200 μl ice-cold lysis buffer [10% glycerol, 1% NP-40, 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5 mM $MgCl_2$, 1 mM PMSF, 1 μM leupeptin, 10 μg/ml of soybean trypsin inhibitor, 10 mM NaF, 0.5 mM aprotinin, 1 mM Na_3VO_4]. Cell lysates from identical conditions were combined and assayed for protein concentration. A total of 14 mg protein from each treatment was combined with 150 μl Flag-Agarose and rocked for 5 hours at 4°C. After three washes with ice-cold lysis buffer, proteins were eluted in 150 μl 2× Laemmli sample loading buffer. 30 μl per lane was loaded on a 7.5% SDS-PAGE gel. Flag-Src was visualized with Biosafe Coomassie stain (Bio-Rad, Herkules, CA) and equal pieces containing the Flag-Src band were excised from the gel. The gel slices were transferred into the wells of a new SDS-PAGE gel and overlaid with varying amounts of V8 protease. V8 limited digestion was carried out as described (Cleveland et al., 1977).

Rap1 and Ras activation assays

Activated Rap1 was isolated from cell lysates using an adapted protocol (Franke et al., 1997). Treated cells were lysed in 300 μl ice-cold lysis buffer. Lysates were clarified by low-speed centrifugation, and supernatants containing 500-1000 μg total protein were incubated with 40 μg glutathione S-transferase (GST)-RalGDS fusion protein (gift of J. L. Bos, Utrecht University, Utrecht, The Netherlands) for 40 minutes. This was followed by incubation with glutathione agarose beads for additional 40 minutes at 4°C. Beads were rinsed three times with lysis buffer and protein was eluted with Laemmli sample loading buffer. Activated Ras was isolated from stimulated cell lysates using agarose-coupled GST-Raf1-Ras binding domain (RBD) provided in the Ras Activation Assay Kit (Upstate Biotechnology) following the manufacturer's protocol. The amount of Rap1 or Ras bound to the beads was detected by western blotting using antisera to Rap1 or Ras, respectively. For the detection of Flag-Rap1, antibodies to Flag were used.

Morphological assessment of neurite outgrowth

PC12 cells were treated with NGF or dbcAMP for 24 hours. Processes greater than one cell body length were scored as neurites. Representative photomicrographs are shown of more than 200 cells examined for each condition, in three to five independent experiments.

Results

Monitoring phosphorylation of Src at S17

Phosphorylation of S17 within Src occurs within a RRXS motif (where R represents arginine, X is any amino acid and S is serine) that is targeted by PKA (Kemp and Pearson, 1990). The phosphorylation of serine within this motif can be monitored using a phospho-(Ser/Thr) PKA substrate antibody (PSAb). As a wide range of cellular proteins contains this motif and are substrates of PKA, it is necessary to immunoprecipitate endogenous Src prior to detection of phosphorylation of S17 (pS17) in Src with PSAb.

In previous studies, the amino terminus of Src was identified as the PKA phosphorylation site using limited V8 protease digestion (Collett et al., 1979; Roth et al., 1983). To confirm that PSAb recognizes the PKA phosphorylation site at the amino terminus of Src, we performed limited V8 protease digestion of Flag-Src isolated from Hek293 cells that were left untreated or stimulated with forskolin for 10 minutes (Fig. 1A). After digestion with V8 protease, the PSAb reactivity was contained entirely within the two amino terminal fragments that contain S17 (Fig. 1Ac,d). Treatment with forskolin increased the phosphorylation of these peptide fragments. We have demonstrated recently that mutant Src that contained an alanine in place of serine at position 17 could no longer be recognized by PSAb, further confirming the specificity of the antibody (Schmitt and Stork, 2002b).

cAMP induces phosphorylation of endogenous Src at S17 in multiple cell lines

In earlier studies, we examined phosphorylation of Src by PKA in cells transfected with Flag-Src. However, we wanted to confirm that endogenous Src is also a target of PKA phosphorylation and to determine whether this phosphorylation was regulated. Hek293 cells maintained a low basal level of phosphorylation at S17, as measured by Src immunoprecipitation and western blotting with PSAb (Fig. 1B). However, stimulation by forskolin, an activator of adenylate cyclases, and IBMX, an inhibitor of phosphodiesterases (Fig. 1B, left panel) or isoproterenol, an activator of β_2-adrenergic receptors that are coupled to cAMP in these cells, (Fig. 1B, right panel) increased the level of S17 phosphorylation. Examination of endogenous Src in CHO, PC12 (rat pheochromocytoma) and AtT-20 cells with PSAb also showed low basal levels of pS17 that increased upon forskolin/IBMX stimulation (Fig. 1C). We did not detect elevated basal levels of S17 phosphorylation in any of the cell

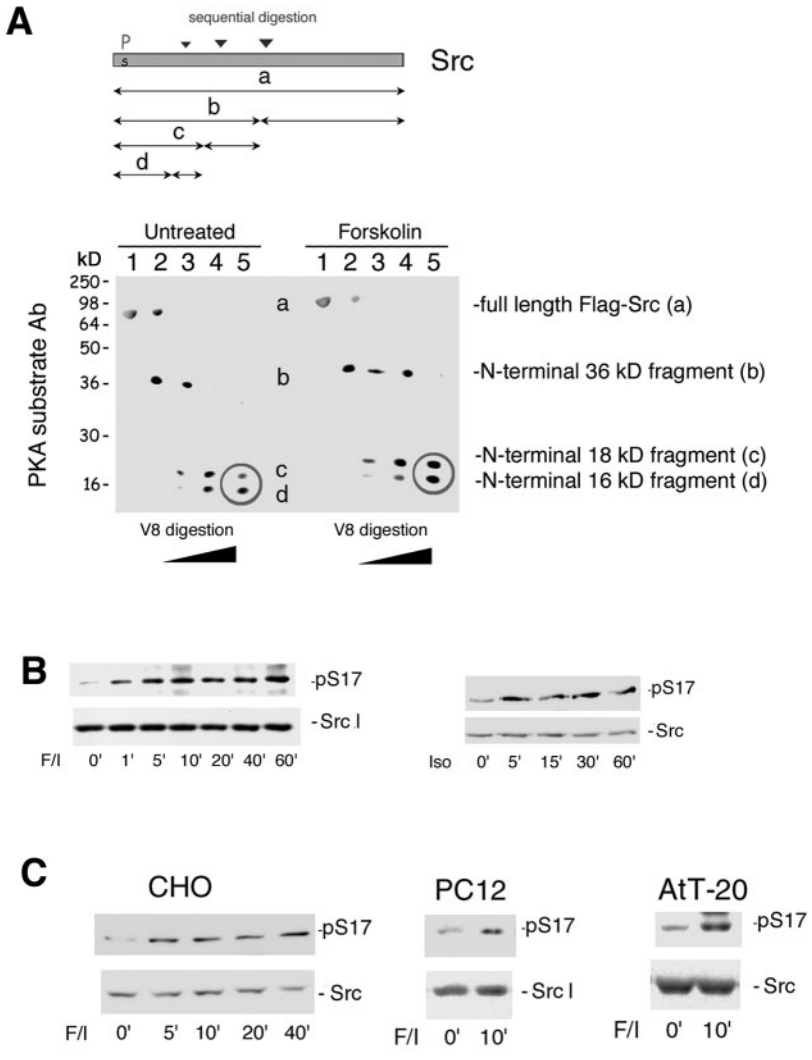

Fig. 1. Detection of phosphorylation of Src at S17. (A) Limited V8 proteolysis of Flag-Src. A diagram of the published digestion pattern for Src is shown (Collett et al., 1979; Roth et al., 1983). The migrations of the fragments a-d are provided in the gel below. Hek293 cells were transfected with Flag-Src and treated with forskolin for 10 minutes or left untreated. Flag-Src was immunoprecipitated and subjected to limited V8 proteolysis as described in Materials and Methods. SDS-PAGE gel pieces were overlaid with the following amounts of V8 protease per well: 1, 0 μg; 2, 0.005 μg; 3, 0.02 μg; 4, 0.05 μg; 5, 0.1 μg. Phosphorylation of Flag-Src at S17 was visualized by immunoblotting with PKA substrate antibody. The migration of protein standards is shown on the left of the blot (kD). The two smallest detectable fragments represent the 16 kD (d) and 18 kD (c) N-terminal fragments, which are circled in both panels. (B) cAMP and isoproterenol induce phosphorylation of endogenous Src in Hek293 cells. Hek293 cells were treated with forskolin/IBMX (F/I) or Isoproterenol (Iso) for the times indicated. Phosphorylation at S17 (pS17) was detected by western blotting with PSAb following immunoprecipitation of endogenous Src. Total Src is shown as a loading control (lower panels). (C) cAMP-induced phosphorylation of endogenous Src in multiple cell types. CHO, PC12 and AtT-20 cells were treated with forskolin/IBMX (F/I) for the times indicated. Phosphorylation at S17 (pS17) was detected following Src immunoprecipitation by western blotting with PSAb. Total Src was detected as a loading control (lower panels).

lines, as has been previously reported for cells infected with retroviral v-Src (Sefton et al., 1982) or transfected with Flag-Src (Abrahamsen et al., 2003).

Regulation of endogenous Src by PKA in PC12 cells

Having established that PKA phosphorylation of Src occurs in multiple cell types, we studied cAMP signaling in PC12 cells in detail. We first examined the consequence of S17 phosphorylation on both Rap1 and ERK activation in PC12 cells. We have previously shown in PC12 cells that cAMP activates ERKs in a Rap1- and PKA-dependent manner (Vossler et al., 1997). As shown in Fig. 2, forskolin plus IBMX and 8-CPT, a non-hydrolyzable analog of cAMP, induced ERK activation in PC12 cells (Fig. 2A,B). Activation by both agents reached a maximum at 15 minutes and was blocked by H89, an inhibitor of PKA, and PP2, an inhibitor of Src family kinases (SFKs) (Fig. 2A,B). The actions of PP2 were selective, with no effect on the autophosphorylation of TrkA itself (data not shown). Interestingly, SU6656, a presumptive Src inhibitor (Blake et al., 2000), also inhibited TrkA autophosphorylation (data not shown) (Bain et al., 2003). Therefore, only PP2 was used in subsequent studies.

Similarly, Rap1 activation by forskolin/IBMX was also inhibited by H89 and PP2 (Fig. 2C), consistent with a requirement for Src family kinases and PKA in the activation of Rap1 and ERKs by cAMP. In some cells, cAMP can trigger the PKA-independent activation of Rap1 via the cAMP-activated guanine nucleotide exchanger Epac (de Rooij et al., 1998). However, stimulating these PC12 cells with the selective Epac activator 8-CPT-(2Me)-cAMP (Enserink et al., 2002) did not activate Rap1 or ERKs (data not shown). Forskolin/IBMX did not activate Ras in these cells (Fig. 2D), although ERKs were activated in a PKA-dependent manner within the same lysates, confirming that Ras was not involved in the activation of ERKs by cAMP in PC12 cells. 8-CPT was also unable to activate Ras. EGF was used as a positive control for Ras activation (Fig. 2E). These data demonstrate that Rap1 and ERKs can be activated by a PKA/SFK-dependent mechanism in PC12 cells, as has been shown for other cell types (Schmitt and Stork, 2002a).

PKA phosphorylation of Src has been implicated in the activation of the small G protein Rap1 in selected cell types (Schmitt and Stork, 2002b), but has not been examined in PC12 cells. Inhibition of Rap1 activation by PP2 could reflect the actions of any SFK, however, only Src can be phosphorylated by PKA (Schmitt and Stork, 2002b). To address the possible role of Src phosphorylation in Rap1 activation, we first examined a time course of S17 phosphorylation using PSAb. Forskolin/IBMX-induced phosphorylation of S17 was seen at both 15 and 30 minutes and was inhibited by H89 (Fig. 3A). To assess Src activity, we used an antibody [phospho-Src (pY416)] recognizing phosphorylated Y416, the autophosphorylation site of Src. Phosphorylation on S17 was associated with Src activation, as measured by pY416. Similar to S17, the phosphorylation of Y416 was H89-sensitive (Fig. 3A).

To determine whether pS17 was required for Rap1 activation, we expressed the mutant SrcS17A in PC12 cells. SrcS17A has been shown to selectively block the ability of

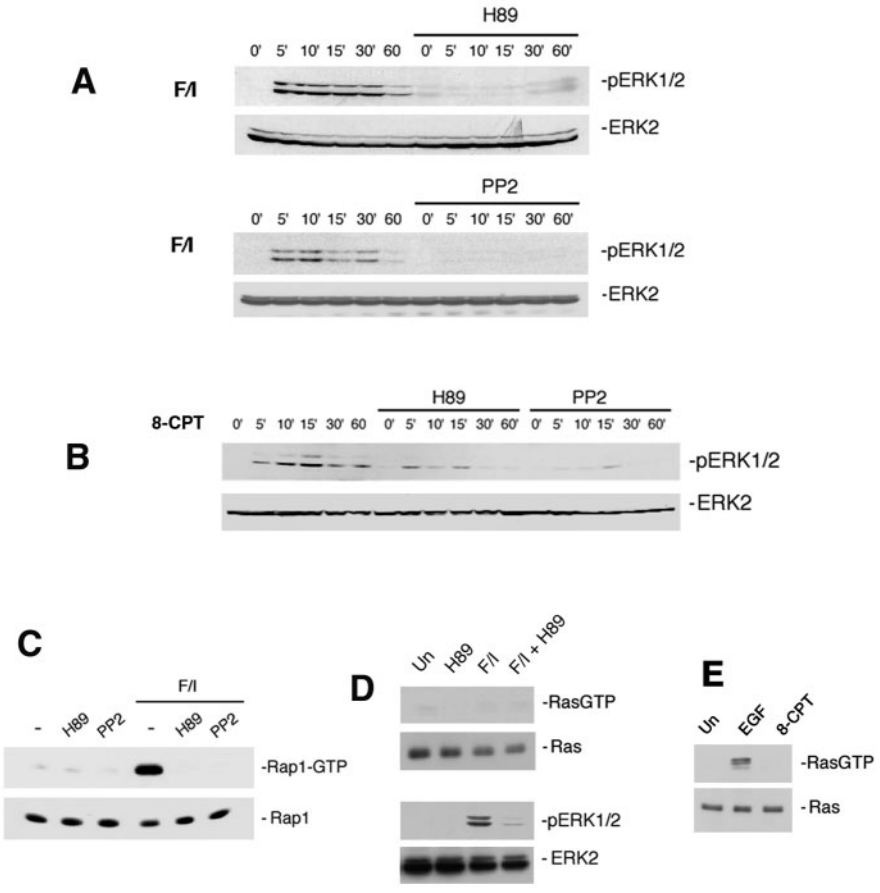

Fig. 2. cAMP activates Rap1 and ERKs in a PKA and Src tyrosine kinase-dependent manner. (A) Time course of ERK activation by forskolin/IBMX. PC12 cells were stimulated with forskolin/IBMX (F/I) for the indicated times in the presence or absence of H89 or PP2. Activation of endogenous ERKs was determined by western blot (pERK1/2). Total ERK2 levels are shown as a loading control. (B) Time course of ERK activation by 8-CPT. PC12 cells were stimulated with 8-CPT for the indicated periods in the presence or absence of H89 or PP2. Activation of endogenous ERK1/2 was determined by western blot (pERK1/2) as above. Total ERK2 levels are shown as a loading control. (C) cAMP activation of Rap1 via PKA and Src. PC12 cells were stimulated with forskolin/IBMX (F/I) for 20 minutes in the presence or absence of H89 or PP2, and lysates were analyzed for activation of endogenous Rap1 (Rap1-GTP). Total Rap1 levels are shown as a loading control. (D) cAMP does not activate Ras. PC12 cells were stimulated with forskolin/IBMX (F/I) for 10 minutes and lysates were analyzed for activation of endogenous Ras (Ras-GTP) or ERKs (pERK1/2). Total Ras and ERK2 levels are shown as loading controls. (E) 8-CPT does not activate Ras. PC12 cells were stimulated with 8-CPT (15 minutes) or EGF (5 minutes) and lysates were analyzed for activation of endogenous Ras (Ras-GTP). Total Ras levels are also shown.

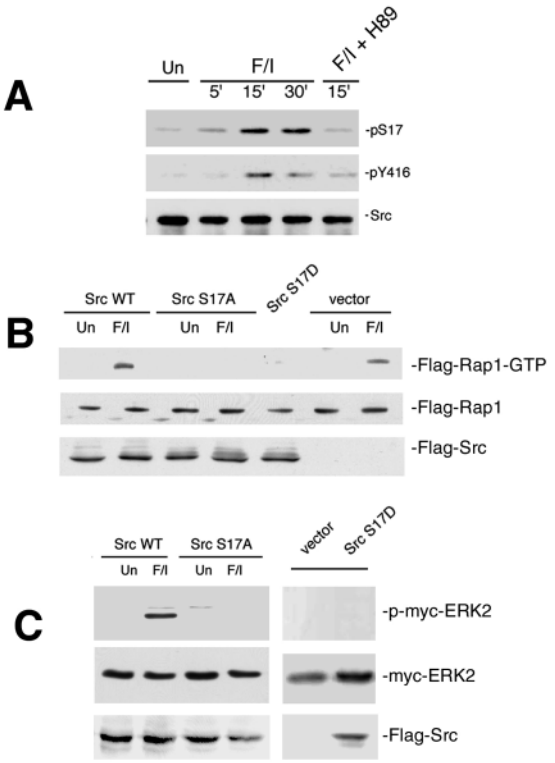

Fig. 3. Phosphorylation of endogenous Src by PKA is required for Rap1 and ERK activation in PC12 cells. (A) cAMP induction of Src phosphorylation at S17 and Y416 via PKA by cAMP. PC12 cells were stimulated with forskolin/IBMX (F/I) at various time points in the presence or absence of H89 or left untreated (Un) as indicated. Src was immunoprecipitated and analyzed for phosphorylation at S17 and Y416 by using PSAb (pS17) and phospho-Src (pY416) antibody, respectively. Total Src levels are shown as a loading control. (B) Involvement of S17 phosphorylation in Rap1 activation by cAMP. PC12 cells were cotransfected with Flag-Rap1 and pcDNA3 (vector), Flag-Src wild type, Flag-SrcS17A or Flag-SrcS17D, then stimulated with forskolin/IBMX (F/I) for 20 minutes or left untreated (Un). Lysates were analyzed for activation of Rap1 (Flag-Rap1-GTP). Total Flag-Rap1 and Flag-Src are shown as transfection and loading controls. (C) Involvement of S17 phosphorylation in ERK activation by cAMP. PC12 cells were cotransfected with myc-ERK2 and vector, Flag-Src wild type or Flag-SrcS17A (left panels). Cells were then stimulated with forskolin/IBMX (F/I) for 20 minutes or left untreated (Un) and mycERK2 was immunoprecipitated. Cells were also transfected with vector or Flag-SrcS17D as indicated in the right panels. Activation of mycERK2 was determined by western blot (p-myc-ERK2) and total myc-ERK2 levels are shown as a loading control. Total Flag-Src is shown as a transfection control (lower panels).

cAMP to activate Src in other cell types (Schmitt and Stork, 2002a). Cells were transfected with Flag-tagged wild-type Src or SrcS17A along with Flag-Rap1 and assayed for Rap1 activation following forskolin/IBMX treatment. Expression of SrcS17A, but not wild-type Src, blocked activation of both Rap1 (Fig. 3B) and ERKs (Fig. 3C) by forskolin/IBMX. These results suggest that S17 phosphorylation was required for the activation of Rap1 and ERK by forskolin. Expression of SrcS17D was not sufficient for Rap1 and ERK activation in the absence of additional stimuli (Fig. 3B,C).

Phosphorylation of Src S17 is involved in NGF activation of Rap1 and ERK

The finding that Src is required in the cAMP activation of Rap1 and ERKs in PC12 cells is novel. However, Src has previously been suggested to participate in NGF signaling to ERKs in PC12 cells (Kao et al., 2001). Here we show that PP2 blocked ERK activation by NGF at both 30 and 60 minutes (Fig. 4A), implying a selective role for Src in the late phase of ERK activation. The late phase of ERK activation by NGF in PC12 cells requires the prior activation of Rap1 (Kao et al., 2001; Meng and Casey, 2002; Wu et al., 2001; York et al., 2000; York et al., 1998), whose activation by NGF is sustained (Fig. 4B). Furthermore, activation of Rap1 by NGF at 30 minutes was both Src- and PKA-dependent (Fig. 4C), placing Src upstream of Rap1 during this late phase. In contrast, rapid NGF signaling to ERKs as well as EGF signaling to ERKs are thought to utilize Ras (Kao et al., 2001; York et al., 1998). PP2 modestly blocked EGF activation of ERKs at 5 minutes and had no effect at 10 and 15 minutes (Fig. 4A). PP2 decreased ERK activation by EGF to a limited degree at 5 minutes (Fig. 4A), perhaps reflecting the Src-dependence of the transient Rap1 activation by EGF at 5 minutes, as shown in Fig. 4B. A similar transient activation of Rap1 by EGF has been reported in PC12 cells (Kao et al., 2001). It is possible that this mechanism of transient Rap1 activation by EGF is also shared by NGF. Ras activation by either NGF or EGF was insensitive to inhibition of Src or PKA (Fig. 4D), suggesting that Src was not required for Ras-dependent signaling to ERKs by either NGF or EGF.

The data presented establish a role of Src in the late phase of ERK activation by NGF via Rap1. Therefore, we asked whether NGF could induce phosphorylation of S17 on endogenous Src. Indeed, NGF, but not EGF, induced the phosphorylation of S17 in Src (Fig. 5A). NGF also induced the phosphorylation of Y416 on Src and phosphorylation of both S17 and Y416 was sensitive to H89 (Fig. 5B). To confirm the role of PKA in the induction by NGF of phosphorylation of S17 on Src, we utilized Rp-cAMP, an inhibitor of PKA regulatory subunit (Fig. 5C). EGF, however, stimulated the phosphorylation of Y416 modestly without phosphorylating S17 (Fig. 6A), as previously shown in other cell types (Schmitt and Stork, 2002b).

Next, we used SrcS17A to test the requirement of S17 in the action of NGF on Rap1 and ERK. Expression of SrcS17A blocked NGF activation of both Flag-Rap1 and myc-ERK2 at 30 minutes (Fig. 6A,B). SrcS17A did not block either NGF or EGF activation of myc-ERK at 5 minutes (Fig. 6C). These results suggest that the PKA-dependent phosphorylation of Src on S17 is required for NGF activation of Rap1 and consequently the late phase of ERK activation. The mechanism by which NGF induces PKA-dependent phosphorylation of SrcS17 remains to be elucidated. Recently, coupling between PKA and the low affinity NGF receptor ($p75^{NTR}$) has been proposed (Higuchi et al., 2003). To determine whether the high affinity NGF receptor (TrkA) or $p75^{NTR}$ was involved, we utilized PC12-nnr5 cells, which lack the TrkA receptor (Green et al., 1986). In the absence of TrkA, NGF was unable to stimulate phosphorylation of S17, or activation of ERKs. As expected, the ability of forskolin/IBMX to activate ERKs or stimulate S17 phosphorylation did not require TrkA (Fig. 6D). These studies suggest that TrkA and cAMP utilize overlapping

signaling pathways to activate Rap1 via PKA phosphorylation of Src.

To determine the role of Src on neurite outgrowth, we treated cells with NGF or forskolin in the presence of PP2. PP2 blocked cAMP-induced neurites, but had no effect on neurites induced by NGF (Fig. 7A). In contrast, the MEK inhibitor PD98059 blocked neurite outgrowth induced by both agents. The percentage of cells bearing neurites for each condition is shown in Fig. 7B. We also examined the role of SrcS17 in neurite outgrowth. Expression of SrcS17A blocked neurite outgrowth by cAMP but not by NGF (Fig. 7C). Cells were transfected with EGFP to identify transfected cells. The percentage of EGFP-positive cells bearing neurites for each condition is shown in Fig. 7D. These results are consistent with the requirement for Rap1 in PKA activation of neurites and the lack of requirement for either PKA or Rap1 in NGF activation of neurites (Vossler et al., 1997; Yao et al., 1998).

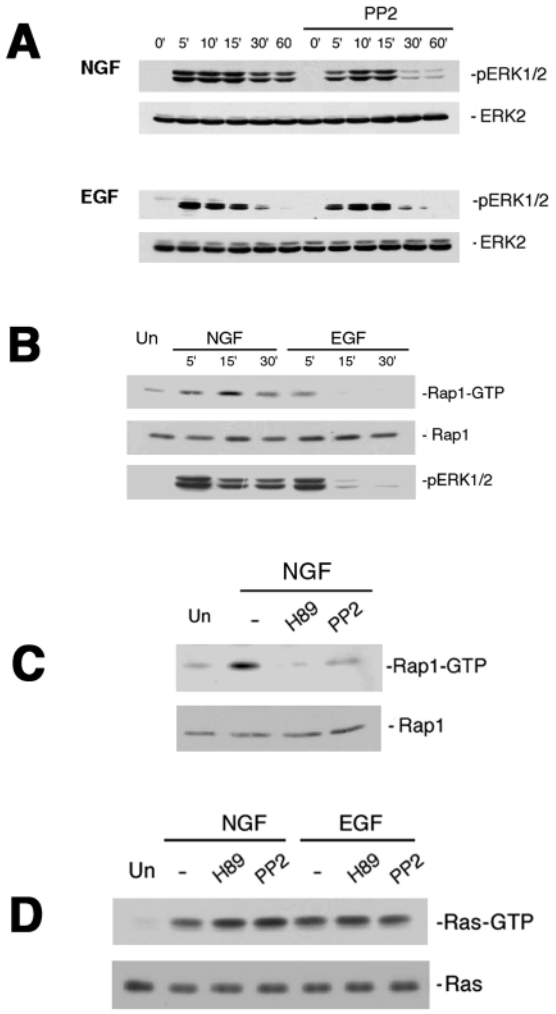

Fig. 4. NGF activates Rap1 and ERKs in a PKA and Src family kinase-dependent manner. (A) NGF activation of ERKs at late time points is Src-dependent in PC12 cells. PC12 cells were stimulated with NGF or EGF for the indicated periods in the presence or absence of PP2. Activation of endogenous ERKs (pERK1/2) was determined by western blotting. Total ERK2 levels are shown as loading controls. (B) Time course of Rap1 activation by NGF. PC12 cells were stimulated with NGF or EGF for the indicated times or left untreated (Un) and activation of endogenous Rap1 (Rap1-GTP) (upper panel) or ERK1/2 (pERK1/2, lower panel) was determined by western blot. Total Rap1 levels are shown as a loading control (middle panel). (C) PKA and Src are involved in Rap1 activation by NGF. PC12 cells were stimulated with NGF for 30 minutes in the presence or absence of H89 or PP2, or left untreated (Un). Activation of endogenous Rap1 (Rap1-GTP) was determined by western blot. Total Rap1 levels are shown as a loading control (lower panel). (D) PKA and Src-independent activation of Ras by NGF. PC12 cells were stimulated with NGF or EGF for 5 minutes in the presence or absence of H89 or PP2, or left untreated (Un). Lysates were analyzed for activation of endogenous Ras (Ras-GTP). Total Ras levels are shown as a loading control (lower panel).

Discussion

S17 is the major site of serine phosphorylation in viral Src (v-Src) (Collett et al., 1979), and the cellular homologue c-Src (Src) (Gould et al., 1985). In both proteins, S17 is a consensus PKA site. v-Src is phosphorylated at this site by cAMP in vivo (Roth et al., 1983) and by PKA in cell-free extracts (Collett et al., 1979), and c-Src is phosphorylated by PKA in vitro (Purchio et al., 1981). Early studies identified significant basal phosphorylation of S17 in v-Src-infected cells (Collett et al., 1979; Patschinsky et al., 1986; Sefton et al., 1982). An upper limit of S17 phosphorylation comes from an early study estimating the percentage of basal serine phosphorylation within v-Src at 30-60% (Sefton et al., 1982). Other studies

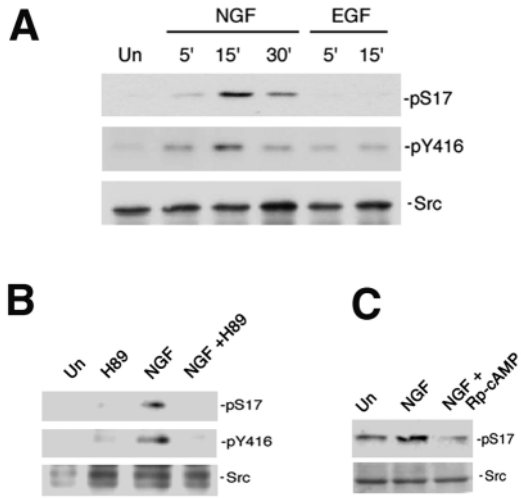

Fig. 5. S17 within Src is phosphorylated by NGF in a PKA-dependent manner in PC12 cells. (A) Time course of Src phosphorylation by NGF at S17 and Y416. PC12 cells were stimulated with NGF or EGF for the indicated times, or left untreated (Un). After Src immunoprecipitation, precipitates were analyzed for phosphorylation of Src at S17 and Y416. Total Src levels are shown as a loading control (lower panel). (B) PKA-dependent Src phosphorylation by NGF. PC12 cells were stimulated with NGF for 15 minutes in the presence or absence of H89 and Src phosphorylation was analyzed at S17 and Y416 as described in A. Total Src levels are shown as a loading control (lower panel). (C) Inhibition of NGF induction of Src phosphorylation at S17 by Rp-cAMP. PC12 cells were stimulated with NGF for 15 minutes in the presence or absence of Rp-cAMP, and Src phosphorylation was analyzed at S17 (upper panel) as described in A. Total Src levels are shown as a loading control (lower panel).

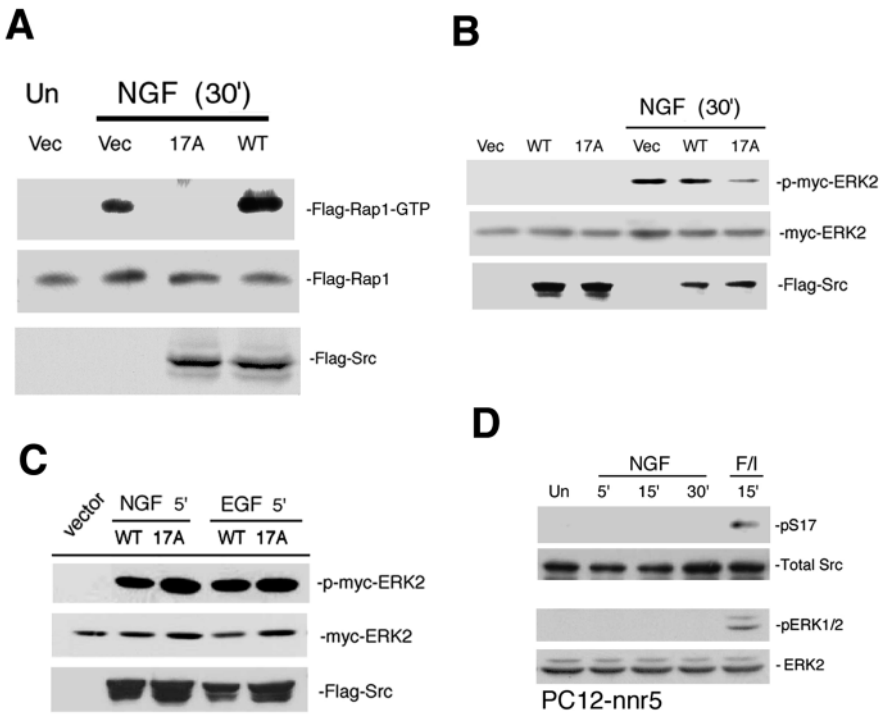

Fig. 6. S17 phosphorylation of Src is required for NGF activation of Rap1 and ERKs in PC12 cells. (A) Involvement of S17 phosphorylation in Rap1 activation by NGF. PC12 cells were cotransfected with Flag-Rap1 and pcDNA3 vector (Vec), wild-type Flag-Src (WT), or Flag-SrcS17A (17A) as indicated. Cells were stimulated with NGF for 30 minutes or left untreated (Un), and activation of Rap1 (Flag-Rap1-GTP) was determined by western blot. Total Flag-Rap1 and Flag-Src levels are shown. (B) Involvement of S17 phosphorylation in ERK activation at late time points by NGF in PC12 cells. PC12 cells were transfected with myc-ERK2 and pcDNA3 vector (Vec), wild-type Flag-Src (WT), or Flag-SrcS17A (17A) as indicated. After stimulation with NGF for 30 minutes, activation of myc-ERK2 was determined by western blot (p-myc-ERK2, upper panel). Total myc-ERK2 levels and Flag-Src levels are shown. (C) Lack of involvement of S17 phosphorylation in ERK activation at early time points by NGF. PC12 cells were transfected with myc-ERK2 and pcDNA3 vector (vector), wild-type Flag-Src (WT) or Flag-SrcS17A (17A) as indicated. After stimulation with NGF or EGF for 5 minutes, activation of myc-ERK2 was determined by western blot (p-myc-ERK2, upper panel). Total myc-ERK2 levels and Flag-Src levels are shown. (D) Requirement of TrkA in NGF stimulation of S17 phosphorylation and late ERK activation. PC12-nnr5 cells were serum-starved and stimulated with NGF or forskolin/IBMX (F/I) for the indicated times or left untreated (Un). Phosphorylation of Src at S17 was determined following Src immunoprecipitation by western blotting with PSAb. Lysates were also analyzed for activation of ERK (pERK1/2). Total Src and ERK2 levels are shown as loading controls.

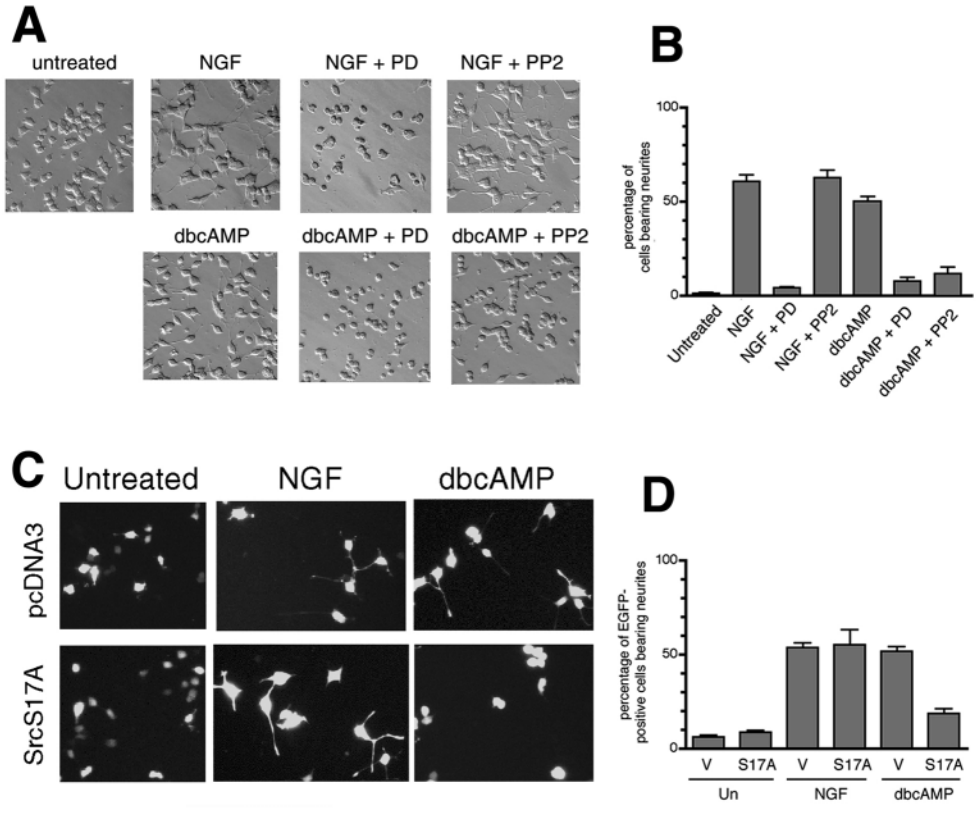

Fig. 7. Src S17 phosphorylation is required for neurite outgrowth induced by cAMP in PC12 cells. (A) ERK mediates neurite outgrowth induced by NGF and dbcAMP, but SFKs are involved only in neurite outgrowth induced by cAMP. PC12 cells were incubated with NGF or dibutyryl cAMP (dbcAMP) for 24 hours in the presence or absence of PD98059 or PP2, and neurite outgrowth was evaluated microscopically. PD98059 blocked neurite outgrowth induced by both NGF and dbcAMP, whereas PP2 only blocked outgrowth induced by dbcAMP. (B) Bar graph of mean percentage±s.e. of cells bearing neurite outgrowths when treated as described in A (n=3-5). (C) Src S17A blocks dbcAMP-induced neurite outgrowth, but not NGF-induced neurite outgrowth. PC12 cells were cotransfected with either pcDNA3 (vector) or Src S17A and with EGFP as a marker for transfected cells. The cells were incubated with NGF or dbcAMP for 24 hours. Neurite outgrowth was observed under fluorescence microscopy to identify transfected cells. (D) Bar graph of mean percentage±s.e. of EGFP-positive cells bearing neurite outgrowths when treated as described in C (n=3-5).

using transfected reporters for Src have shown high basal levels of pS17 within transfected Src that could not be further phosphorylated by cAMP elevation in vivo or by PKA in vitro (Abrahamsen et al., 2003). It is possible that high basal levels of pS17 seen previously were secondary to the high levels of expression of Src achieved in those studies. Here, we show a low basal level of pS17 within endogenous Src, which could be induced in a variety of cell lines. The physiological significance of PKA phosphorylation of Src has been a subject of debate for over twenty years (Cross and Hanafusa, 1983). However, recent studies utilizing expression of SrcS17A, which selectively interferes with PKA-dependent Src function (Schmitt and Stork, 2002a), identified potential roles of pS17 in both Rap1 (Schmitt and Stork, 2002a; Schmitt and Stork, 2002b) and ERK regulation (Klinger et al., 2002).

In PC12 cells, PKA phosphorylation of Src on S17 was associated with Src activation (pY416). These results are consistent with previous studies that showed that cAMP could modestly increase Src activity (Roth et al., 1983; Schmitt and Stork, 2002b). It is clear that pS17 is not required for Src activation by all stimuli (Cross and Hanafusa, 1983; Schmitt and Stork, 2002b). A different model of PKA regulation of Src has recently been proposed (Abrahamsen et al., 2003), where PKA activation of Csk inhibits Src activity through phosphorylation of Y527 at the C-terminus of Src. In PC12 cells, we were unable to detect regulation of Y527 phosphorylation by forskolin/IMBX (data not shown).

Forskolin stimulates the activation of Rap1 in many cell types. In PC12 cells, this action of cAMP requires PKA, although in other cells, PKA-independent activation of Rap1 has been identified. The PKA-independent activation of Rap1 by cAMP appears to require guanine nucleotide exchangers of the Epac family (Bos, 2003). However, pharmacological activation of Epac with 8CPT-2Me-cAMP did not activate Rap1 in PC12 cells, suggesting that this class of exchangers may not be expressed in these cells, although others have reported effects of 8CPT-2Me-cAMP in these cells (Christensen et al., 2003). It has also been reported that cAMP activates Ras via transactivation of the EGF and TrkA receptor in PC12 cells (Piiper et al., 2002). Ras-dependent ERK activation by cAMP was shown in hippocampal neurons (Iida et al., 2001), cortical neurons (Ambrosini et al., 2000) and melanocytes (Busca et al., 2000). However, in the present study, Ras was not activated by cAMP in PC12 cells.

NGF stimulates the activities of both Ras and Rap1 in PC12 cells, to activate ERKs and target genes (York et al., 1998). Studies shown here suggest that NGF requires PKA phosphorylation of Src to activate Rap1. In one early study, activation of Src by platelet-derived growth factor was PKA-dependent (Walker et al., 1993), although the site of PKA phosphorylation was not determined. Another study showed that insulin induced the phosphorylation of S17 tenfold (Luttrell et al., 1989). Here we demonstrate that NGF also induced the phosphorylation of S17. Both pS17 and Src activity were required for the activation of Rap1 by NGF, suggesting that NGF activation of Src may require PKA. This is supported by studies determining that the PKA inhibitor H89 blocked the induction of Y416 phosphorylation by NGF.

The PKA-dependence of the ability of NGF to stimulate Rap1 and ERK activation is consistent with earlier reports identifying the requirement of PKA for NGF action (Ginty et al., 1992; Kalman et al., 1990). We previously were unable to show increased PKA activity in total cell lysates following NGF treatment of PC12 cells (Yao et al., 1998). Using PSAb, we observed that NGF stimulated the phosphorylation of a limited set of proteins at PKA consensus sites that were sensitive to H89, suggesting that NGF induced the PKA-dependent phosphorylation of these proteins (data not shown). How PKA is activated by NGF is unknown. In particular, although components of G protein-coupled receptor (GPCR) signaling cascades can associate with TrkA (Lou et al., 2001; Moughal et al., 2004), the stimulation of GPCR signaling to cAMP by NGF has not been established. Recent studies have shown that cAMP concentration was elevated ~1.6-fold by NGF in cerebellar neurons expressing only the low affinity NGF receptor $p75^{NTR}$ and which activated PKA associated with $p75^{NTR}$ (Higuchi et al., 2003). However, we show that the capacity of NGF to induce these PKA-dependent phosphorylations in general and phosphorylation of S17 in particular was dependent on the expression of TrkA receptors. We cannot rule out the possibility that TrkA might be permissive for $p75^{NTR}$ signaling to PKA. We have previously shown that PC12 cells utilize calcium/calmodulin-sensitive adenylate cyclases to mediate the calcium-dependent signaling to Rap1 and ERK in PC12 cells (Grewal et al., 2000). Because neurotrophins can increase the intracellular Ca^{2+} concentration through phospholipase C-γ (Yamada et al., 2002), the involvement of calcium-sensitive adenylate cyclases also remains a possible mechanism.

Src activation by NGF has been proposed to function in concert with other signals to regulate neuronal differentiation (D'Arcangelo and Halegoua, 1993; Kaplan and Miller, 1997; Kremer et al., 1991; Rusanescu et al., 1995; Yang et al., 2002) and v-Src can induce neurite outgrowth in PC12 cells (Thomas et al., 1991). In PC12 cells, Src is thought to be constitutively associated with the scaffold protein FGF receptor substrate-2 (FRS-2) via its SH3 domain and FRS-2/Src is recruited to the TrkA NGF receptor upon NGF stimulation (Meakin et al., 1999). FRS-2 is highly phosphorylated by TrkA in response to NGF and serves to anchor the adaptor molecule Crk and the Crk binding partner C3G, a guanine nucleotide exchanger for Rap1, to the TrkA complex (Meakin et al., 1999). The ability of NGF, but not EGF, to induce sustained activation of ERKs depends on Rap1 activation (York et al., 1998), which reflects the ability of NGF, but not EGF, to induce a stable interaction between Crk and C3G (Kao et al., 2001). Other proteins that may participate in this recruitment by NGF include Chat (Sakakibara et al., 2002), ARMS (Arevalo et al., 2004), Cas and the Src-interacting protein Sin. Both Cas and Sin have been shown to mediate Src activation of Rap1 in other cell types (Xing et al., 2000) and a requirement for Sin in FGF-induced neurite outgrowth of PC12 cells has been shown (Yang et al., 2002). ARMS (ankyrin-rich membrane-spanning protein) has recently been identified as a potential scaffold protein coupling TrkA to Rap1/ERK activation (Arevalo et al., 2004). It is possible that PKA phosphorylation of Src by NGF participates in the assembly of this or related complexes.

Examination of the functional consequences of Src phosphorylation on neurite outgrowth revealed that blocking Rap1 activation by pharmacological inhibition of Src or by expression of SrcS17A reduces neurite outgrowth induced by cAMP but did not reduce neurite outgrowth induced by NGF.

This is consistent with previous studies demonstrating that Rap1 is required for neurite outgrowth of PC12 cells induced by cAMP, but neither Rap1 (Vossler et al., 1997) nor PKA (Yao et al., 1998) was required for neurite outgrowth induced by NGF.

The mechanism by which pS17 contributes to Src function is not well understood. Phosphorylation at S17 could provide a binding site for an unidentified protein(s) that participate in Src activation. In addition, S17 might provide a phosphorylation-dependent docking site for potential Src substrates such as Cbl (Schmitt and Stork, 2002a; Schmitt and Stork, 2002b), Sin (Kao et al., 2001; Xing et al., 2000) or Cas (Kao et al., 2001) that participate in coupling to Rap1. These two mechanisms may be interrelated: the putative binding partner may also be a substrate of Src. A third mechanism by which pS17 could alter the choice of Src substrate is by altering the subcellular localization of Src by interfering with the electrostatic interactions that anchor Src to the lipid bilayer. S17 is proximal to the farnesyl moiety of Src that provides a membrane anchor (Schultz et al., 1985) and is adjacent to a polybasic domain that may also link Src to phospholipid-rich domains (Murray et al., 1998). It has been shown that membrane attachment of chimeric proteins containing the amino-terminal membrane localization domain of Src can be disrupted by PKA phosphorylation (Murray et al., 1998).

One study examining the density dependence of basal Src activity suggested that Src activation seen at high densities was associated with a loss of Src within membranes and the increase in Src-dependent Cas phosphorylation (Kobayashi et al., 1997). Interestingly, translocation of Src away from the plasma membrane has been demonstrated following growth factor activation of Src (Walker et al., 1993; Weernink and Rijksen, 1995) and a role for PKA phosphorylation of Src was proposed (Walker et al., 1993). The demonstration that S17 phosphorylation of Src can mediate both Rap1 and ERK activation by diverse stimuli including growth factors and hormones suggests that this example of crosstalk between PKA and Src may be more widespread.

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