APRO4 negatively regulates Src tyrosine kinase activity in PC12 cells

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Summary
The Src nonreceptor tyrosine kinase plays an important role in multiple signalling pathways that regulate several cellular functions including proliferation, differentiation, and transformation. The activity of Src is tightly regulated in vivo and can be modulated by interactions of its SH2 and SH3 domains with high-affinity ligands. APRO4 (anti-proliferative 4) belongs to a new antiproliferative gene family involved in the negative control of the cell cycle. This report shows that APRO4 associates with Src via its C-terminal proline-rich domain, and downregulates Src kinase activity. Moreover, overexpression of APRO4 leads to inhibition of neurite outgrowth and Ras/MAP kinase signalling in PC12 cells. Furthermore, the kinetics of endogenous Src inactivation correlates with an increase in endogenous APRO4 co-immunoprecipitation in FGF-stimulated PC12 cells. Finally, downregulation of endogenous APRO4 by expression of antisense RNA induces the activation of Src and spontaneous formation of neurites in PC12 cells. Therefore, by controlling the basal threshold of Src activity, APRO4 constitutes an important negative regulatory mechanism for Src-mediated signalling.

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Introduction
The Src tyrosine kinase is implicated in a variety of signal transduction pathways leading to proliferation or differentiation depending on the cell type (reviewed in Brown and Cooper, 1996; Thomas and Brugge, 1997). For instance, the constitutively active, oncogenic form of c-Src, v-Src, induces differentiation of PC12 cells into neuron-like cells (Alema et al., 1985) whereas it blocks differentiation of chondroblasts into chondrocytes (Muto et al., 1977). Moreover, elevated Src tyrosine kinase activity is associated with cellular transformation (Hunter and Sefton, 1980). Src also plays important roles in cell cycle control, cell adhesion, survival, and angiogenesis (reviewed in Thomas and Brugge, 1997; Schlessinger, 2000). Therefore, Src is critical for normal cellular function and its activity needs to be tightly controlled. The Src protein is composed of two peptide-binding modules known as Src-homology (SH)2 and SH3 domains, a kinase domain that consists of two lobes: the N-lobe, and the C-lobe that contains the activation loop autophosphorylation site required for full catalytic function (Y416 in c-Src), and a regulatory C-tail containing a negative regulating tyrosine residue (Y529). The enzymatic activity of Src is controlled by intramolecular associations between the SH2 domain and the phosphorylated tyrosine and between the SH3 domain and a short region forming a left-handed polyproline type II (PPII) helix that links the SH2 domain to the kinase domain (SH2-kinase linker) resulting in a ‘closed’ inactive enzyme conformation (Cooper and Howell, 1993; Murphy et al., 1993; Okada et al., 1993; Superti-Furga et al., 1993). In the inactive state, the activation loop adopts a conformation that blocks access of substrates to the catalytic domain and prevents Y416 autophosphorylation (Gonfloni et al., 2000). Src catalytic activity is modulated by three factors: phosphorylation/dephosphorylation of the tail; competition for the SH2 domain by a high-affinity phosphotyrosine-containing ligand; and competition for the SH3 domain by a high-affinity proline-rich ligand (Cooper and Howell, 1993).

The APRO4 protein, previously named ANA (for abundant in the neuroepithelium area) belongs to an antiproliferative protein family which, in vertebrates, consists of at least eight members: APRO1 (BTG2/TIS21/PC3), APRO2 (BTG1), APRO3 (PC3B), APRO4 (BTG3/ANA), APRO5 (Tob2), APRO6 (Tob), B9.10, and B9.15 (Bradbury et al., 1991; Fletcher et al., 1991; Rouault et al., 1992; Rouault et al., 1996; Matsuda et al., 1996; Guéhenneux et al., 1997; Yoshida et al., 1998; Ikematsu et al., 1999; Zhu et al., 1999; Buanne et al., 2000; Guardavaccaro et al., 2000; Yoshida et al., 2000; Matsuda et al., 2001; Tsachanis et al., 2001; Suzuki et al., 2002). They all contain two short conserved domains in their N-terminal part (box A and box B), separated by a spacer sequence of 20-25 non-conserved amino acids, which are involved in protein-protein interactions (reviewed in Matsuda et al., 2001). The biological functions of the APRO family of proteins remain unclear. However, several reports indicate that their overexpression leads to inhibition of cell proliferation (Rouault et al., 1992; Matsuda et al., 1996; Montagnoli et al., 1996; Guéhenneux et al., 1997; Yoshida et al., 1998; Ikematsu et al., 1999; Buanne et al., 2000; Matsuda et al., 2001) and suggest that APRO proteins may be involved in negative control of the cell cycle.
The *APRO4* gene encodes a protein of 252 amino acids whose N-terminal half is homologous to the previously characterized *APRO* proteins. The C-terminal part is unique and contains a proline-rich region not conserved with other members of the *APRO* family (Guéhenneux et al., 1997; Yoshida et al., 1998). Although ubiquitously expressed, elevated levels of *APRO4* are observed in the ventricular zone of the developing central nervous system where neuroepithelial cells commit themselves to differentiate into specific cell types. *APRO4* is also highly expressed in embryonic cartilages. These observations suggest that *APRO4* may play a role in neurogenesis and chondrocytes proliferation and/or differentiation (Yoshida et al., 1998). Src, like *APRO4*, is not only involved in chondrocytic differentiation (Muto et al., 1977) but it is also highly expressed in the developing mammalian nervous system (Maness, 1992). During neurogenesis, Src is expressed during two phases of development: first in neuroectodermal cells of gastrulating embryos, then later, in committed neuroepithelial cells near the onset of terminal differentiation (reviewed in Ingraham et al., 1989). Given the similar expression profile of Src and *APRO4* and their potential role in neurogenesis and osteogenesis, the possibility that *APRO4* might interact with Src via its proline-rich region seemed reasonable and was addressed.

This report shows that *APRO4* interacts with Src and downregulates its tyrosine kinase activity via its C-terminal proline-rich domain. Moreover, overexpression of *APRO4* inhibits PC12 cell neurite outgrowth by negatively regulating Ras/MAP kinase signalling. Furthermore, in FGF-stimulated PC12 cells, the kinetics of endogenous Src inactivation correlates with an increase in endogenous *APRO4* co-immunoprecipitation. Finally, inhibition of endogenous *APRO4* expression in PC12 cells by antisense RNA leads to the activation of Src and spontaneous formation of neurites. Taken together, these results demonstrate an important role for *APRO4* in the negative regulation of Src-mediated signalling.

**Results**

*APRO4* associates with Src in vitro

To determine if *APRO4* interacted with c-Src, the SH3/SH2 domains of c-Src were expressed as bacterial fusion proteins with GST (glutathione S transferase), purified to homogeneity on glutathione-agarose beads, and tested for their ability to bind [35S]methionine labelled in vitro translated Flag-*APRO4*. 7.8±0.8% (means ± s.e.m., n=3) of the total [35S]methionine labelled in vitro translated *APRO4* used in the assay associated with both Src SH3 and SH2 domains but not with the individual Src SH2 domain. Note that GST-SH3 appeared to pull down a very small amount of Flag-*APRO4* (Fig. 1B, top panels). A truncated form of *APRO4* lacking the C-terminal proline-rich domain (Flag-*APRO4*<sub>C</sub>) did not bind to the SH3-SH2 domains whereas 5.2±0.6% (means ± s.e.m., n=3) of the total amount used for the mutated form of *APRO4* lacking the N-terminal domain (X-*APRO4*<sub>N</sub>) did (Fig. 1A,B middle and bottom panels). This result indicates that the interaction between *APRO4* and Src is mediated by the C-terminal proline-rich region of *APRO4*. Lyn and Blk SH3 and SH2 domains, two members of the Src family kinases, did not bind to in vitro translated *APRO4*. However, 6.49±0.7% (means ± s.e.m., n=3) of the total amount used for *APRO4* interacted with Fyn SH3-SH2 domains but not with Lyn, Blk, and various GST-SH3-SH2 fusion proteins: GAP (GTPase activating protein), PLC (Phospholipase C<sub>ε</sub>). (B,C, right panels) Coomassie staining of SDS-PAGE of purified GST-fusion proteins. Input: 100% of the respective [35S]-labelled proteins used in binding reactions; GST: GST-beads. Data are representative of three independent experiments.

![Fig. 1. In vitro interaction of Flag-*APRO4* with Src<sub>wt</sub>.](image)

(A) The various *APRO4* mutants used are represented schematically and the amino-acid sequence of the C-terminal region of *APRO4* (145-152) is also shown. Proline residues that were mutated to alanine are represented on the amino-acid sequence. (B, left panels) In vitro translated [35S]-labelled Flag-*APRO4*, and X-*APRO4*<sub>N</sub> associated with Src GST-SH3-SH2 whereas Flag-*APRO4*<sub>C</sub> did not. (C, left panel) Flag-*APRO4* interacted with Fyn SH3-SH2 domains but not with Lyn, Blk, and various GST-SH3-SH2 fusion proteins: GAP (GTPase activating protein), PLC (Phospholipase C<sub>ε</sub>). (B,C, right panels) Coomassie staining of SDS-PAGE of purified GST-fusion proteins. Input: 100% of the respective [35S]-labelled proteins used in binding reactions; GST: GST-beads. Data are representative of three independent experiments.

with Fyn SH3 and SH2 domains. Moreover, other proteins containing SH3 and SH2 domains such as GAP (GTPase activating protein) and PLC<sub>γ</sub> (Phospholipase C<sub>γ</sub>) did not bind to *APRO4* (Fig. 1C). These data indicate that *APRO4* interacts specifically with Src and Fyn.
APRO4 associates with Src in vivo

This interaction was also analysed in NIH 3T3 cells by co-expression of APRO4 and Src wt (wild type) followed by co-immunoprecipitation and protein immunoblotting with specific antibodies to tagged APRO4 constructs and Src. Co-expression of an Xpress-tagged APRO4ΔN construct (X-APRO4ΔN) with Src wt in NIH 3T3 cells confirmed the in vitro data showing that the C-terminal proline-rich region of APRO4 was required for the interaction with Src (Fig. 2A). As expected, Flag-APRO4ΔC did not bind to Src (Fig. 2B).

To delineate further which forms of Src were interacting with APRO4, several Src mutants were analysed by co-immunoprecipitation and protein immunoblotting with specific antibodies for APRO4 or Src. Results indicated that APRO4 interacted with Src wt (Fig. 2C,D, left panels). The amount of total cellular Src protein that was immunoprecipitated from cell lysates of transfected cells (Fig. 2A) was not significantly different from the amount of untransfected cells. Whole-cell lysates of transfected cells were analysed by western blotting with the appropriate antibody to verify equal expression of all the constructs. Data are representative of three independent experiments.
expressing Src wt and Flag-APRO4 using anti-Src antibody was estimated to be ~15% (data not shown). Quantification of the data shown in Fig. 2D (left panels) showed that only 4.7±0.8% (means ± s.e.m., n=3) of total cellular Flag-APRO4 protein co-precipitated with Src wt. Approximately 10% of total cellular Flag-APRO4 protein was immunoprecipitated from cell lysates of transfected cells expressing Src wt and Flag-APRO4 (data not shown). The amount of total cellular Src protein that co-precipitated with Flag-APRO4 was estimated to be 2.77±0.6% (means ± s.e.m., n=3) (Fig. 2C, left panels). A kinase-defective mutant of Src (K297M), a constitutively activated form of Src (Y529F), and a dominant-negative form of Src (K297M Y529F) also associated with APRO4 to the same extent (Fig. 2C,D, left panels). This latter form is mutated in the ATP-binding site of Src (K297M) and in the C-terminal phosphotyrosine, causing the protein to adopt an ‘open’ conformation with defective kinase activity. These data suggest that APRO4 is able to bind to Src even when the protein is in the ‘open’ state. However, APRO4 did not associate with a Src W118R R175 K double mutant (Fig. 2C,D). In this double mutant, the W118R mutation abolishes the SH3-binding site of the Src SH3 domain and the R175K mutation totally disrupts the phosphotyrosine-binding pocket within the Src SH2 domain. Src W118R and Src R175K single mutants were tested for their interaction with Flag-APRO4. Results showed that the amount of the interaction between Src W118R and Flag-APRO4 was significantly reduced when compared to the one observed with Src wt and Flag-APRO4 (30.8±9.5%, n=4). The interaction between Src R175K and Flag-APRO4 was also reduced when compared to the interaction between Src wt and Flag-APRO4 although to a lesser extent than with Src W118R (45.7±12.1%, n=4) (Fig. 2C,D, right panels).

Three putative SH3-domain-binding sites (defined by the consensus motif PXXP) are present in the C-terminal region of APRO4 (Fig. 1A). An APRO4 mutant in which proline residues of the three SH3-binding sites were mutated into alanine (Fig. 1A) was generated (Flag-APRO4-6PA) and tested for its ability to associate in NIH 3T3 cells by co-immunoprecipitation and protein immunoblotting with Src and anti-Flag antibodies. Flag-APRO4-6PA interacted less with Src wt than the wild-type Flag-APRO4 (23.7±15.2%, n=4) (Fig. 2C,D, right panels). These observations suggest that the Src SH3 domain is necessary but not sufficient for the interaction of Src with APRO4 and that the Src SH2 domain may be required to stabilize the interaction.

**Endogenous APRO4 interacts with endogenous Src**

To determine whether this interaction could be shown between endogenous mammalian proteins, rat pheochromocytoma PC12 cells were used because, unlike NIH 3T3 cells, expression levels of APRO4 protein could be detected by immunoblotting. Endogenous Src and normal mouse antiserum immunoprecipitates from PC12 lysates were analysed by immunoblotting with a specific affinity-purified antibody raised against a GST-APRO4 fusion protein containing the C-terminal domain of the human APRO4 protein. Both Src and APRO4 were present in the Src immunoprecipitates whereas normal mouse antiserum did not immunoprecipitate APRO4 (Fig. 3A). Around 5% of total endogenous APRO4 was found to co-precipitate with endogenous Src (Fig. 3A and data not shown). Furthermore, confocal microscopy revealed extensive cytoplasmic co-localization between Src and APRO4 in PC12 cells (Fig. 3B). Control experiments demonstrated that cross-reactivity did not occur between the two secondary antibodies (data not shown). This protein-protein interaction data demonstrates that APRO4 associates with Src and suggests that APRO4 may have an effect on Src catalytic activity.

**APRO4 inhibits Src kinase activity in vitro**

To assess the effect of APRO4 on Src kinase activity, in vitro kinase assays were carried out. To this end, GST-tagged APRO4, GST control or GST-tagged APRO4ΔC were expressed in HEK 293 cells and purified to homogeneity by using GST beads (Fig. 4A). GST-APRO4 and GST-APRO4ΔC recombinant proteins purified from transfected cells were used because, unlike in bacteria, the fusion proteins were not partially degraded. Increasing amounts of purified GST-APRO4 protein, 5 μM purified GST-APRO4ΔC or 5 μM GST control were added to a constant amount (5 units) of purified active Src kinase, and Src activity was measured in the presence of [γ-32P]ATP and enolase. Enolase is an exogenous substrate commonly used for measuring Src activity. Src activity was reduced in a dose-dependent manner, with more than 90% reduction in activity at 2 μM of GST-APRO4. However, GST-APRO4 was not phosphorylated by Src (supplementary material Fig. S1). Src kinase activity was not inhibited by addition of GST alone, demonstrating that the negative regulation is specific to APRO4 (Fig. 4B,C). A similar inhibition of Src activity was observed when GST-APRO4 produced from Cos-7 transfected cells was used (data not shown). Moreover, GST-APRO4ΔC did not reduce Src kinase activity thereby confirming that the C-terminal region of APRO4 was required for its interaction with Src (Fig. 4D,E). These results
APRO4 interacts with Src and inhibits its kinase activity.

**APRO4 downregulates Src kinase activity and Ras/MAP kinase signalling**

To determine whether APRO4 inhibited Src activity in mammalian cells, PC12 cells were transfected with either an empty vector or increasing amounts of APRO4, APRO4ΔN or APRO4ΔC expression vectors followed by stimulation with fibroblast growth factor (FGF). Endogenous Src was immunoprecipitated and assayed for in vitro kinase activity using enolase as an exogenous substrate. APRO4 and APRO4ΔN dramatically reduced Src kinase activity while APRO4ΔC did not (Fig. 5A). Autophosphorylation of Y416 at the activation loop is necessary to lead to full activation of Src tyrosine kinases (Thomas and Brugge, 1997; Xu et al., 1999). The activation loop adopts an α-helical configuration when Src is in the suppressed form but becomes extended when Src is activated, and phosphorylation of Y416 seems to stabilize this extended conformation (Xu et al., 1999). Thus, the phosphorylation state of Y416 was examined following FGF stimulation of PC12 cells transfected with either an empty vector or increasing amounts of APRO4 expression vector. As shown in Fig. 5B, APRO4 reduced the phosphorylation of Y416, confirming that Src catalytic activity was inhibited.

FGF-mediated PC12 cell differentiation has been correlated with a sustained activation of Ras and MAP kinase pathway (Togari et al., 1985; Qui and Green, 1992). Therefore, the effect of APRO4 on Ras/MAP kinase signalling output was assayed indirectly by looking at the activation of ERKs. This was carried out by measuring the transactivation of a 5×-Gal4-TATA/luciferase reporter gene by a Gal4-Elk1 construct (Elk1gal) containing the transactivation domain of Elk1. Elk1 is a member of the Ets family of transcription factors, and an important physiological substrate of ERKs. Transfected PC12 cells treated with FGF for 6 hours were assayed for Elk1 activity. Src-dependent Elk1 transcriptional activation was inhibited by APRO4 in PC12 cells treated with FGF in a dose-dependent manner. As expected, APRO4ΔC did not inhibit Elk1 activation whereas APRO4ΔN did, thereby confirming that the inhibition was mediated by the C-terminal proline-rich domain of APRO4. This inhibition was rescued when the constitutive active form of MEK1 (MEK1 S218/222D) was co-expressed (Fig. 5C). Since the Src W118R and Src R175K mutations impair Src-mediated signalling pathways (Broome and Hunter, 1996; Erpel et al., 1996), it was not possible to use these Src mutants to test whether the observed APRO4 inhibitory effect on Elk1 transactivation was due solely to its interaction with Src. Altogether, these data indicate that overexpression of APRO4 inhibits the FGF-mediated Ras/MAP kinase signalling pathway, and suggest that APRO4 may have an effect on PC12 cell differentiation.

**Overexpression of APRO4 inhibits FGF-mediated PC12 neurite formation**

PC12 cells have served as a model system for the differentiation of neuronal cells. This cell line responds to FGF by extending neurites and developing many properties similar to those of sympathetic neurons (Togari et al., 1985). To analyse the effect of APRO4 on FGF-mediated neurite outgrowth, PC12 cells were transfected with either a control
empty vector, Flag-APRO4, X-APRO4ΔN or Flag-APRO4ΔC. Transfected cells were serum starved for 20 hours before being treated with FGF for 48 hours. Fixed transfected cells were then immunostained with the indicated antibodies. After 2 days of exposure to FGF, PC12 cells transfected with the empty control vector (data not shown), and Flag-APRO4ΔC (Fig. 6C, white arrows) showed extensive neurite outgrowth whereas cells transfected with either Flag-APRO4 (Fig. 6A, white arrows) or X-APRO4ΔN (Fig. 6B, white arrows) did not. The inhibitory effect of APRO4 on FGF-mediated PC12 cell neurite outgrowth was rescued when the constitutive active form of MEK1 was co-expressed along with APRO4 (Fig. 6D, black arrows).

Neuronal differentiation of PC12 cells is characterized not only by morphological changes (neurite outgrowth) but also by synthesis of neuronal proteins (Greene and Tischler, 1976). To analyse the effect of APRO4 on neuronal differentiation of PC12 cells, the expression of a neuron-specific marker of neuronal differentiation was examined. β-tubulin isoform III (Tubulin-βIII) is synthesized exclusively by neurons and increases in conjunction with the rate of neuronal differentiation (Greene and Tischler, 1976). After two days of FGF treatment, a marked decrease in the expression of Tubulin-βIII protein was detected in PC12 cells transfected with either Flag-APRO4 or X-APRO4ΔN expression plasmids compared to the control transfected PC12 cells. Cells transfected with Flag-APRO4ΔC plasmid expressed similar Tubulin-βIII protein expression levels than the control cells. Co-expression of the constitutive active form of MEK1 (HA-MEK1 S218/222D) with Flag-APRO4 rescued the inhibitory effect of APRO4 on Tubulin-βIII protein expression (Fig. 7A). Moreover, the inhibitory effect observed with APRO4 on Tubulin-βIII protein expression was rescued by co-expressing a constitutively active form of Src (Src Y529F) or a constitutive active form of Fyn (Fyn Y531F) (Fig. 7B). However, when PC12 cells were transfected with Src Y529F or myc-tagged Fyn Y531F and assayed for in vitro kinase activity in the presence of enolase and either purified GST-APRO4 protein or GST control protein, both Src Y529F and Fyn Y531F kinase activities were inhibited by GST-APRO4 but not by GST alone (see supplementary material Fig. S2). Thus, despite the fact that GST-APRO4 inhibited the kinase activity of Src Y529F and Fyn Y531F proteins in vitro, both proteins were able to restore Tubulin-βIII protein expression even when co-expressed with Flag-APRO4 in PC12 cells. A possible explanation for this is that a substantial fraction of APRO4 may already interact with the endogenous Src present in PC12 cells. Thus, the level of APRO4 protein overexpressed in the transfected cells may not be sufficient to inhibit the kinase activity of both the endogenous Src as well as the overexpressed Src Y529F protein. The same explanation may apply for Fyn Y531F. This possibility does not exclude the fact that a fraction of the overexpressed APRO4 protein may also be associated with other endogenous signalling targets. Moreover, since Src Y529F and Fyn Y531F are constitutively activated, both proteins have a higher level of kinase activity than the wild-type Src. Therefore, higher concentrations of co-expressed APRO4 may be required to inhibit Src Y529F and

![Fig. 5. Inhibition of Src kinase activity, Ras/MAP kinase signalling in PC12 cells by overexpression of APRO4.](image_url)
Fyn Y531F in vivo. Nevertheless, since APRO4 interacts with
Fyn in vitro (Fig. 1C), these data suggest that overexpression of
APRO4 affects the FGF-mediated signalling cascade by
downregulating not only Src kinase activity but also Fyn kinase
activity.

APRO4 is required for tightly regulating Src activity
APRO4 endogenous expression was first examined in PC12
cells stimulated with FGF for several days. As shown in Fig.
8A, APRO4 protein expression increased kinetically to reach
a peak after 4 days of FGF treatment (~fourfold). To test
whether the association between APRO4 and Src changed
during neurogenic stimulation, the presence of APRO4 in Src
immunoprecipitates prepared from PC12 cells at different
times after stimulation with FGF was monitored. As shown in
Fig. 8B, the inhibition of Src kinase activity reflected by the
phosphorylation status of Y416 closely correlated with an
increase in APRO4 protein level in Src immunoprecipitates
(~fourfold increase after 4 days of FGF treatment compared to
the unstimulated state). These data indicate that stimulation of
PC12 cells with FGF results in an upregulation of APRO4,
which consequently leads to a progressive downregulation of
Src activity and suggest that APRO4 is required for tightly
regulating Src kinase activity during the FGF-mediated
differentiation process.

To further investigate the physiological relevance of the
APRO4-Src interaction in PC12 cells, endogenous expression of
APRO4 was inhibited by expression of an antisense vector
(pAS-APRO4). The specificity of pAS-APRO4 antisense vector
toward APRO4 was addressed by co-transfecting PC12 cells with
either Flag-APRO4 and HA-MEK1 S218/222D, cells expressing only Flag-APRO4
(red) did not develop neurites whereas cells expressing Flag-APRO4
(red) as well as HA-MEK1 S218/222D (green) expression plasmids showed extensive neurite
outgrowth (panel D, black arrows). Polyclonal anti-Flag antibody, monoclonal anti-Xpress
and anti-HA antibodies showed no signal with untransfected PC12 cells (panels A-C, black
arrows). More than 300 transfected PC12 cells were analysed per condition in four
independent experiments. Bars, 10 μm.
Negative regulation of Src by APRO4

APRO4 antisense vector was specifically downregulating APRO4 (data not shown). PC12 cells were transiently transfected with either an empty control vector or pAS-APRO4 followed by immunoblotting of lysates with a specific affinity-purified APRO4 antibody. The antisense vector reduced APRO4 levels without affecting the expression of Src (Fig. 9A). To analyse the effect of reduced APRO4 expression, Src kinase activity reflected by the phosphorylation status of Y416 was examined in antisense treated PC12 cells. The phosphorylation of Y416 was increased in cells transfected with pAS-APRO4 compared to control PC12 cells. When PP1, a specific Src inhibitor, was added to the growth medium of pAS-APRO4 transfected PC12 cells, the phosphorylation of Y416 was nearly abolished (Fig. 9B). Similar results were obtained from four independent experiments to exclude the possibility that this effect might be due to a population variation of PC12 cells. It should be noted that the anti-Y416 antibody does cross-react with other members of the Src family and recognizes the autophosphorylation site of other Src members. Because Fyn interacts with APRO4 (Fig. 1A), this experiment does not exclude the possibility that Fyn activity may also be upregulated in cells transfected with pAS-APRO4.

To test further the effect of endogenous APRO4 on the Ras/MAP kinase signalling pathway, PC12 cells were co-transfected with an empty control vector or pAS-APRO4, Gal4-Elk1 transactivation construct, and 5×-Gal4-TATA/luciferase reporter gene and assayed for Elk1 transcriptional activity as described earlier. Elk1 activity was increased in pAS-APRO4 transfected PC12 cells relative to PC12 control cells (Fig. 9C). Activation of the Ras/MAP kinase cascade leads to the simultaneous phosphorylation of ERK on T202 and Y204 residues. This simultaneous phosphorylation results in the activation of the ERK protein. Thus, the phosphorylation state of ERK was examined in PC12 cells transiently transfected with pAS-APRO4 or an empty control vector. As shown in Fig. 9D, ERK phosphorylation was increased in pAS-APRO4 transfected cells relative to the control cells. Furthermore, this increased phosphorylation was abolished when cells were treated with the Src specific inhibitor PP1.

![Figure 7](image-url) Inhibition of FGF-mediated PC12 cell differentiation by overexpression of APRO4. (A,B) PC12 cells were transiently transfected with either an empty control vector, Flag-APRO4, X-APRO4ΔN, Flag-APRO4ΔC, or Flag-APRO4 and the indicated expression plasmids. After 24 hours, cells were serum starved for 20 hours, then either left untreated or treated with FGF for 2 days and analysed for expression of the differentiation marker Tubulin-βIII by immunoblotting (IB). Actin was used as an internal control to verify equal loading of proteins. Data are representative of three independent experiments.

![Figure 8](image-url) Downregulation of Src kinase activity correlated with increased endogenous APRO4 expression in FGF-stimulated PC12 cells. (A) Uptregulation of endogenous APRO4 protein expression in FGF stimulated cells. PC12 cells were serum starved for 20 hours and then stimulated with FGF (25 ng/ml) for the indicated times before being collected. Whole-cell lysates were immunoblotted with the indicated antibodies. Actin was used as internal control to verify equal loading of proteins in each condition. Densitometric quantitation of relative APRO4 expression is indicated underneath each lane of the top panel. (B) Serum starved PC12 cells were stimulated with FGF (25 ng/ml) for the indicated times, and analysed by co-immunoprecipitation (IP) followed by immunoblotting (IB) with anti-phospho-Y416, anti-Src and anti-APRO4. Immunoblotting with Src antibody confirmed that equivalent amounts of Src were present in immunoprecipitates reactions. Densitometric quantitation of relative Src phosphorylation is indicated underneath each lane of the top panel. Data are representative of three independent experiments.
These data indicate that downregulation of endogenous APRO4 expression by antisense APRO4 results in the activation of the Ras/MAP kinase signalling pathway.

Finally, to assess the effect of endogenous APRO4 on PC12 cell neurite outgrowth, cells were transfected with an empty control vector or pAS-APRO4 together with pEGFP-N3 plasmid. Transfected cells were positively identified by their GFP fluorescence. Cells were then serum starved for three days before being fixed and immunostained with APRO4 antibody. PC12 cells transfected with pAS-APRO4 developed neurites after 3 days (Fig. 9E, top panels, white arrows) while none of the PC12 cells transfected with the empty vector did (data not shown). Moreover, the pAS-APRO4 transfected PC12 cells treated with PP1 did not extend neurites (Fig. 9E, bottom panels, white arrows). These data indicate that, at least in PC12 cells, APRO4 is associated with Src and required for tightly regulating Src tyrosine kinase activity.

**Discussion**

Src tyrosine kinase plays a pivotal role in many important cellular events such as cell proliferation, differentiation, transformation, survival, adhesion and migration (Brown and...
Negative regulation of Src by APRO4

Cooper, 1996; Thomas and Brugge, 1997; Schlessinger, 2000). Src kinase activity is normally tightly controlled through intramolecular interactions between the SH2 domain and the C-terminal phosphotyrosine and between the SH3 domain and a linker region between the SH2 domain and the N-terminal kinase lobe (Cooper and Howell, 1993; Murphy et al., 1993; Okada et al., 1993; Superti-Furga et al., 1993). The structure of the inactive form of Src shows that both SH3 and SH2 mediated intramolecular interactions function to inhibit the enzyme (Sicheri and Kuriyan, 1997; Williams et al., 1997; Xu et al., 1997). Disruption of these interactions with high affinity ligands for the Src SH2 domain, the Src SH3 domain or both domains results in the activation of the enzyme.

Many interacting proteins that upregulate Src activity have been isolated, but besides phosphatases (Yokoyama and Miller, 2001) and Csk (Nada et al., 1991), only a few proteins that downregulate Src activity have been identified. Caveolin, a 21-24 kDa integral membrane protein (Li et al., 1996), and RACK1 (receptor for activated C kinase), a homolog of the 24 kDa integral membrane protein (Li et al., 1996), and downregulate Src activity have been identified. Caveolin, a 21-2001) and Csk (Nada et al., 1991), only a few proteins that interact with Src SH3 and/or SH2 domains and downregulate its kinase activity by holding it in an inactive conformational state. Recently, DOC-2/DAB2 (for differentially expressed in ovarian carcinoma-2/disabled-2) has been shown to interact with Src SH3 domain via a proline-rich domain and inhibit its kinase activity by an unknown mechanism (Zhou et al., 2003). While the mechanism by which APRO4 exerts its negative effects on Src remains unknown, APRO4 may also belong to this emerging group of inhibitory proteins that interact with Src SH3 and/or SH2 domains and downregulate its kinase activity by holding it in an inactive conformational state. Further investigation will be required to address this issue and dissect the mechanism(s) involved.

Several lines of evidence indicate that APRO4 interacts with and downregulates Src activity. First, APRO4 directly inhibits Src activity in vitro. Second, overexpression of APRO4 downregulates Src activity in FGF stimulated PC12 cells. Third, inhibition of endogenous Src activity correlates with overexpression of endogenous APRO4 expression in FGF stimulated PC12 cells. Fourth, downregulation of endogenous APRO4 by expression of antisense RNA leads to spontaneous formation of neurites in PC12 cells. While the mechanism by which overexpressed APRO4 downregulates Src activity in vivo remains unknown, one possibility is that, by stably associating with Src, APRO4 may block the access of both Src SH3 and SH2 domains to Src binding partners. Indeed, overexpression of APRO4 and EGFR (epidermal growth factor receptor) in NIH 3T3 cells shows that APRO4 can partially compete with the EGFR for binding to Src (Z.R., unpublished observation). Since the Src SH3 domain is required for EGFR mitogenic signalling (Wilson et al., 1989; Broome and Hunter, 1996; Tice et al., 1999), APRO4 may partially dissociate Src from the EGFR and contribute to the downregulation of Src activity. In support to this, overexpression of APRO4 inhibits mitogenesis in NIH 3T3 cells (Z.R., unpublished observation). Another possibility is that by binding to Src SH3 and SH2 domains, APRO4 may allosterically mask Y416 and impedes its phosphorylation, which is required for the full activation of Src. Further investigation will be required to address this issue in more detail.

This report shows that during the process of FGF-mediated PC12 neuronal differentiation, endogenous Src activity is activated. This activation which lasts around 12 hours is consistent with previously reported data showing that stimulation of Src and Ras/MAP kinase signalling cascade is an early event in FGF signal transduction pathway (Kremer et al., 1991) and that a sustained MAP kinase activity is required for FGF-mediated PC12 neuronal differentiation (Qui and Green, 1992). During this time, the amount of endogenous APRO4 protein co-immunoprecipitated with Src is very low. However, when the activity of the ERK pathway returns to basal levels at later times following FGF stimulation (Qui and Green, 1992), Src activity decreases as the amount of endogenous APRO4 protein co-immunoprecipitated with Src increases. These data indicate that endogenous APRO4 titrates the activity of Src during FGF-mediated PC12 cell differentiation. Moreover, downregulation of endogenous APRO4 in PC12 cells by expression of antisense RNA leads to spontaneous formation of neurites due to a ‘de-repression’ of Src kinase activity. These data suggest a model where, at least in PC12 cells, APRO4 binds to Src and protects it from being improperly activated by holding it repressed or unavailable to Src ligands. Therefore, APRO4 acts like a conformational regulator that controls the basal threshold for the activation of Src. Overexpression of APRO4 raises this threshold, whereas downregulation of APRO4 lowers it. Taken together, these data provide evidence that APRO4 constitutes an important negative regulatory component of Src activity.

Little is known about the regulation of endogenous APRO4 expression. Its protein levels are regulated by FGF in PC12 cells (this report). As for NIH 3T3 cells, endogenous APRO4 protein cannot be detected by immunoblotting even after stimulating the cells with EGF (Z.R., unpublished observation). However, it cannot be excluded that EGF may regulate APRO4 expression. Conversely, APRO4 expression is induced by redox changes in several cell types (Seo et al., 1999) suggesting that APRO4 may play an important role during oxidative stress responses. APRO4 expression is cell cycle-dependent and peaks at the G1 phase (Guéhenneux et al., 1997; Yoshida et al., 1998). APRO4 is also induced along with several antiproliferative genes in primary mouse embryonic fibroblast by overexpression of p19ARF, a tumour suppressor gene involved in cell-cycle arrest (Kuo et al., 2003). These observations indicate that APRO4 may also be involved in the negative control of the cell cycle. Finally, its high expression in the ventricular zone of the developing central nervous system and in embryonic cartilages (Yoshida et al., 1998) suggests that APRO4 may be involved in neurogenesis and osteogenesis. Since Src is also highly expressed in neurons during development and in osteoblasts (reviewed in Thomas and Brugge, 1997), APRO4 may play a role in these events by regulating Src kinase activity. Further studies on the regulation of APRO4 expression will provide interesting clues on the biological significance of Src/APRO4 interaction.

In conclusion, the data presented here show that APRO4 constitutes a novel negative regulatory mechanism for Src-mediated signalling. Since Src is involved in many biological responses such as transformation, mitogenesis, and differentiation, APRO4 may exert profound effects on these physiological processes.
Materials and Methods

Cell culture
NIH 3T3, HEK 293, and Cos-7 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. PC12 cells were grown in DMEM supplemented with 10% horse serum and 5% fetal bovine serum on poly-D-Lysine-coated plates.

Plasmids and protein expression
APRO4 cDNA was isolated by PCR from a human brain cDNA library using a 5' primer flanked with an EcoRI restriction site: 5'-GCGAATTCCTTCCAATGTGGCACCCCTTG-3' and a 3' primer flanked with a SalI restriction site: 5'-ACGGCTGGACTGGGATGCTGCAAATAGGGT-3' (APRO-3PRIME). The cDNA was then cloned into the EcoRI-Xhol sites of the pSS-188 vector (derived from pCR3.1 vector, Invitrogen) carrying a CMV promoter and in which a N-terminal Flag epitope tag has been introduced to make Flag-APRO4 construct. X-APRO4ΔN encompasses the C-terminal domain (amino acids 145 to 252) of APRO4 and was cloned into the EcoRI-BamHI sites of the pcDNA3.1/His vector containing an Xpress tag (Invitrogen). Flag-APRO4ΔC (amino acids 1 to 134) was isolated by excising an Apal restriction site DNA fragment from the Flag-APRO4 construct. Flag-APRO4 (Δ1-181) was obtained by using a 5' primer flanked with an EcoRI restriction site: 5'-GCGAATTCCTTCCAATGTGGCACCCCTTG-3' and the 3' primer flanked with a SalI restriction site (APRO-3PRIME), and cloned into the EcoRI-Xhol sites of the pSS-188 vector. SacII, Y29F, 297M (provided by T. Hunter), K29M Y29F (provided by S. Roche), MEK1 S218/222D (provided by M. J. Weber), GST-GAP, GST-PLC (provided by A. Kazlauskas), GST-Fyn, GST-Lyn, GST-Bik SH3-SH2 constructs (provided by J. C. Campher), myc-ToB (provided by E. Nishida), pSCT-PC3 (provided by F. Tirone), and Fyn Y531F (provided by P. J. Lombroso) have been described previously (Kazlauskas et al., 1991; Pleiman et al., 1993; Catling et al., 1995; Broome and Hunter, 1996; Guardavaccaro et al., 2000; Maekawa et al., 2002; Nguyen et al., 2002). An expression vector encoding the enhanced variant of the green fluorescent protein (pEGFP-N3) was purchased from Clontech (Mountain View, CA). The following antibodies were used: anti-Flag (M2) (Sigma), anti-Xpress (Invitrogen), anti-Myc (Roche Diagnostics), anti Src Mab 327 (Calbiochem), anti-Src (SRC2) polyclonal antibody (Santa Cruz Biotechnology), anti-phospho Tyr416, anti-phospho-ERK, anti-ERK (Cell Signaling Technology), anti-GST polyclonal antibody (Santa Cruz Biotechnology), anti-phospho-tyrosine (4G10) monoclonal antibody (Upstate cell signalling), a polyclonal rabbit antibody was also raised against two combined APRO4 peptides (aa 14-28: RLVRKHDKLKKEA VE, and aa 238-252: CDRNHWINPHMLAPH) and purified on protein A-coupled resin. Proteins were detected by enhanced chemiluminescence assay (ECL Plus, Amersham).

Immunoprecipitation and immunoblotting
48 hours post-transfection, NIH 3T3 or PC12 cells were incubated in lysis buffer L (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM EDTA, 2 mM Na3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF) for 30 minutes at 4°C. Lysates were clarified by centrifugation at 15,000 g for 15 minutes and preincubated with protein G-sepharose for 1 hour at 4°C. Supernatants were then incubated overnight with the relevant antibody and protein G-sepharose, washed three times with buffer L and then resolved by SDS-PAGE followed by immunoblotting on a polyvinylidene difluoride (PVDF) membrane (Biorad) and processed as described by the manufacturer's instructions.

Site-directed mutagenesis
Site-directed mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene) following instructions provided by the manufacturer. All mutations were verified by nucleotide sequencing using an ABI373 automatic sequencer.

In vitro translation and GST-fusion binding assay
Flag-APRO4, Flag-APRO4ΔC, and X-APRO4ΔN and APRO4 mutant plasmids were subcloned into the pEX51 vector (Invitrogen) using a 5' primer (APRO-3PRIME), and a 3' primer (APRO-3PRIME) for the construction of the pEX-GST-APRO4 cDNA fragment. The cDNA was then cloned into the EcoRI-Xhol sites of the pGEX-5X1 vector. The following constructs were obtained: a 3' primer flanked with a BamHI restriction site (GEXH5): 5'-CGGGATCCCTTCCAATGTGGCACCCCTTG-3' and a 5' primer (APRO-3PRIME), the GST-APRO4 cDNA fragment was amplified by PCR, subcloned in the BamHI-SalI sites of the pcDNA3.1/His vector, and sequenced. pcDNA-GST-APRO4 encompasses APRO4 nucleotides 1-252 from Clontech. Flag-PC3 was obtained by subcloning the PC3 cDNA fragment by E. Nishida), pSCT-PC3 (provided by F. Tirone), and Fyn Y531F (provided by P. J. Lombroso) were described previously (Kazlauskas et al., 1991; Pleiman et al., 1993; Catling et al., 1995; Broome and Hunter, 1996; Guardavaccaro et al., 2000; Maekawa et al., 2002; Nguyen et al., 2002). An expression vector encoding the enhanced variant of the green fluorescent protein (pEGFP-N3) was purchased from Clontech (Mountain View, CA). The following antibodies were used: anti-Flag (M2) (Sigma), anti-Xpress (Invitrogen), anti-Myc (Roche Diagnostics), anti Src Mab 327 (Calbiochem), anti-Src (SRC2) polyclonal antibody (Santa Cruz Biotechnology), anti-phospho Tyr416, anti-phospho-ERK, anti-ERK (Cell Signaling Technology), anti-GST polyclonal antibody (Santa Cruz Biotechnology), anti-phospho-tyrosine (4G10) monoclonal antibody (Upstate cell signalling), a polyclonal rabbit antibody was also raised against two combined APRO4 peptides (aa 14-28: RLVRKHDKLKKEA VE, and aa 238-252: CDRNHWINPHMLAPH) and purified on protein A-coupled resin. Proteins were detected by enhanced chemiluminescence assay (ECL Plus, Amersham).

HEK 293 and Cos-7 cells were transiently transfected with either pcDNA-GST, pcDNA-GST-APRO4 or pcDNA-GST-APRO4ΔC plasmids. 48 hours post-transfection, cells were lysed in buffer L and 0.5 mM DTT for 30 minutes at 4°C. After centrifugation at 15,000 g for 15 minutes, cleared lysates were incubated with glutathione-agarose beads overnight at 4°C. Beads were washed four times with lysis buffer L and 0.5 mM DTT and eluted according to the manufacturer's instructions.

In vitro kinase assay
Anti-endogenous Src immunoprecipitates from transfected PC12 cells were washed twice with kinase reaction buffer (40 mM HEPEs (pH 7.4), 10 mM MgCl2, 3 mM MnCl2) and then incubated with 5 μg of acid-denatured rabbit muscle enolase (Sigma) as an exogenous substrate. 100 μM ATP, 1 mM DTT and 10 μCi of [γ-32P]ATP for 15 minutes at 30°C. Proteins were resolved by SDS-PAGE and visualized by autoradiography with an intensifying screen.

Assay for neurite formation and immunofluorescence staining
PC12 cells were cultured on cover glasses coated with poly-D-Lysine. Cells were transiently transfected with the indicated plasmids, then serum starved for 20 hours before being stimulated with FGF for 48 hours. Cells were then fixed in 3% paraformaldehyde in 1× phosphate buffered saline (PBS) for 30 minutes at room temperature and were permeabilized in 0.5% Triton X-100 in PBS for 1 minute. Src was detected with anti-Src monoclonal antibody MAb 327 and a goat anti-mouse Alexa Fluor 488-conjugated secondary antibody. Flag-APRO4 and Flag-APRO4ΔC were detected with a polyclonal anti-Flag antibody (Sigma) and a goat anti-rabbit Texas Red-conjugated secondary antibody (Jackson Immunolaboratories). Xpress-APRO4ΔN (X-APRO4ΔN) was detected with a monoclonal anti-Xpress antibody (Invitrogen) and a goat anti-mouse Texas Red-conjugated secondary antibody (Jackson Immunolaboratories) in a final dilution of 1:100 and 1:50 for Flag-APRO4 and anti-Flag antibody and a goat anti-rabbit Texas Red-conjugated secondary antibody. HA-MEK1 S218/222D was detected with a monoclonal anti-HA antibody (Roche Diagnostics) and a goat anti-mouse Alexa 488-conjugated secondary antibody (Molecular Probes). Confocal microscopy was performed on a Zeiss LSM510 using
a Zeiss 100X oil immersion lens. Images were collected using appropriate excitation and emission filters sets.

Statistical analysis

Results were analysed by Student’s t-test. Differences between two means with a P<0.05 were regarded as significant. All values were expressed as means ± s.e.m. from at least three independent experiments carried out in triplicate.

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