Cell, Vol. 103, 919–930, December 8, 2000, Copyright ©2000 by Cell Press

PIKE: A Nuclear GTPase that Enhances PI3Kinase Activity and Is Regulated by Protein 4.1N

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Summary

While cytoplasmic PI3Kinase (PI3K) is well characterized, regulation of nuclear PI3K has been obscure. A novel protein, PIKE (*PI3K*inase *E*nhancer), interacts with nuclear PI3K to stimulate its lipid kinase activity. PIKE encodes a 753 amino acid nuclear GTPase. Dominant-negative PIKE prevents the NGF enhancement of PI3K and upregulation of cyclin D1. NGF treatment also leads to PIKE interactions with 4.1N, which has translocated to the nucleus, fitting with the initial identification of PIKE based on its binding 4.1N in a yeast two-hybrid screen. Overexpression of 4.1N abolishes PIKE effects on PI3K. Activation of nuclear PI3K by PIKE is inhibited by the NGF-stimulated 4.1N translocation to the nucleus. Thus, PIKE physiologically modulates the activation by NGF of nuclear PI3K.

Introduction

Phosphoinositide 3-kinase (PI3K), which phosphorylates phosphoinositides on the D-3 position, regulates a variety of cellular functions (Auger et al., 1989; Majerus et al., 1990; Cantley et al., 1991; Krugmann and Welch, 1998). PI3K is activated by growth and differentiation proteins such as nerve growth factor (NGF) (Ohmichi et al., 1992; Ashcroft et al., 1999; Neri et al., 1999; Tanaka et al., 1999), platelet derived growth factor (PDGF) (Auger et al., 1989; Joly et al., 1994; Franke et al., 1995), insulin receptor substrate-1 (IRS-1) (Ruderman et al., 1990; D'Mello et al., 1997) and CD28 (Ward et al., 1993). These growth factors also activate GTPases, predominantly of the *ras* family, which in turn activate PI3K by binding to its p110 subunit (Rodriguez-Viciana et al., 1994, 1996, 1997; Stoyanov et al., 1995). The direct pathway from tyrosine phosphorylated receptors to PI3K and activation of PI3K by GTPase proteins function synergistically (Stephens et al., 1993; Klinghoffer et al., 1996; Vanhaesebroeck et al., 1997).

PI3K also occurs in the nucleus (Neri et al., 1994; Kim, 1998; Lu et al., 1998; Marchisio et al., 1998; Bavelloni et al., 1999). Stimulation of cells with NGF activates nuclear PI3K with nuclear accumulation of 3-phosphorylated phosphoinositide lipids (Neri et al., 1994; Tanaka et al., 1999). Ras proteins regulating nuclear PI3K have not been established. Ran is one predominantly nuclear *ras* protein (Rush et al., 1996), implicated in RNA export from the nucleus, protein import, mitotic regulation, and cell cycle progression. MxB, a large GTPase homologous to dynamin and induced by interferon, occurs in the nucleus, but is predominantly cytoplasmic (Melen et al., 1996; Melen and Julkunen, 1997).

Our studies of protein 4.1N (Walensky et al., 1999; Ye et al., 1999) led to an interest in nuclear effects of cytoskeleton proteins via plasma membrane associated tyrosine kinase receptors. Protein 4.1N is a neuronal selective isoform of the erythrocyte membrane cytoskeleton protein 4.1R. Protein 4.1N binds the nuclear mitotic apparatus protein (NuMA), a nonhistone nuclear protein that leaves the nucleus at mitosis and is associated with poles of the mitotic spindle (Lydersen and Pettijohn, 1980; Price and Pettijohn, 1986; Compton et al., 1992; Yang and Snyder, 1992). 4.1N mediates the antimitotic antiproliferative actions of NGF (Ye et al., 1999).

In the yeast two-hybrid analysis that identified interactions of 4.1N and NuMA, we also observed interactions with another protein, which we have shown to be a nuclear GTPase that binds to PI3K and activates it. We have designated this protein the *p*hospho*i*nositide *k*inase *e*nhancer (PIKE).

Results

Binding of 4.1N to PIKE

We conducted yeast two-hybrid analysis using the C-terminal domain (679–879 amino acids) of 4.1N as bait. Eleven of the thirteen clones include a variety of overlapping fragments of the protein, which we have designated PIKE. We observe interactions between the C-terminal portion of 4.1N(4.1N-CTD) and PIKE (1–318) regardless of which protein is used as bait or prey. By contrast, the N-terminal portion of 4.1N(4.1N-NTD) fails to interact with PIKE. In HEK 293 cells, transfected HA-PIKE binds to 4.1N-CTD but not to 4.1N-NTD, consistent with our yeast two-hybrid findings (Figure 1A).

Coimmunoprecipitation studies demonstrate interactions between 4.1N and PIKE in intact cells. Two different length fragments of PIKE bind to 4.1N, whereas Elk1, employed as a control, fails to bind to 4.1N (Figure 1B). Robust binding also occurs between full-length PIKE and the 4.1N-CTD. (Figure 1C). Interactions are the same regardless of whether 4.1N or PIKE contain HA or Myc tags.

Of the 11 yeast two-hybrid fragments of PIKE that bind 4.1N, the smallest one that interacts robustly comprises the N-terminal 173 amino acids. Using truncations of this fragment, we find that the first 23 amino acids of PIKE may be sufficient for binding 4.1N (Figure 1D). Experiments with GST-fusion proteins also show that the 1–23 amino acid fragment of PIKE interacts robustly with 4.1N, as do the other fragments at the extreme N-terminal portion of PIKE (Figure 1E).

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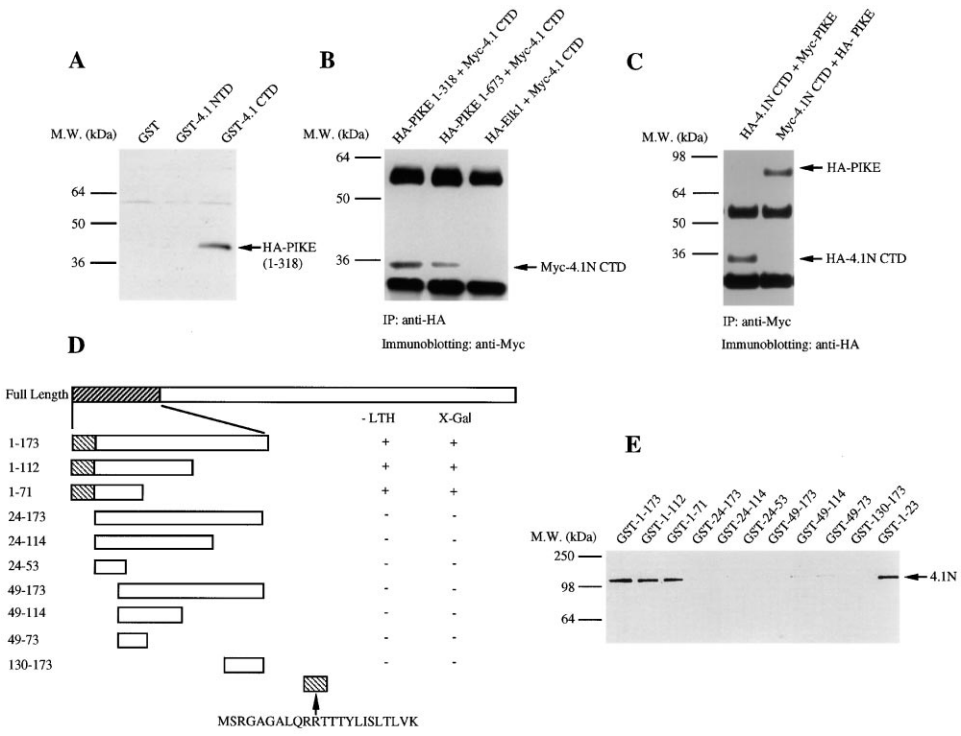

Figure 1. 4.1 and PIKE Associate In Vitro and In Vivo

(A) In vitro binding of HA-PIKE (1–318) to GST-4.1-CTD. Lysates from HEK 293 cells transfected with HA-PIKE (1–318) cDNA were incubated with GST, GST-4.1N-NTD, or GST-4.1N-CTD. Bound proteins were visualized by Western immunoblotting with anti-HA antibody.

(B) Coimmunoprecipitation of HA-PIKE with Myc-4.1N-CTD. HEK293 cells were cotransfected with Myc-4.1N CTD and HA-tagged PIKE (1–318, 1–673), HA-Elk1 respectively. After immunoprecipitation with anti-HA antibody, bound proteins were visualized by Western blotting with anti-Myc antibody.

(C) Coimmunoprecipitation of full-length PIKE with 4.1N CTD. HEK293 cells were respectively cotransfected with Myc- or HA-tagged PIKE and 4.1N CTD. After immunoprecipitation with anti-Myc antibody, bound proteins were visualized by Western blotting with anti-HA antibody.

(D) The N-terminal 23 amino acids of PIKE associate with 4.1N CTD in the yeast two-hybrid system. Truncations of the N terminus of PIKE were constructed in pPC86 and cotransfected with pPC97 4.1N CTD plasmids into the Y190 yeast. Only the 1–73, 1–112, and 1–173 constructs show both histidine prototrophy and β-gal activity. Other constructs are negative in either assay.

(E) The N-terminal 23 amino acids of PIKE bind to 4.1N in vitro. Lysates from HEK 293 cells transfected with 4.1N cDNA were incubated with GST-PIKE N-terminal truncations. The bound protein 4.1N was visualized by Western immunoblotting with anti-4.1N antibody.

Structure of PIKE

Screen of a lambda phage cDNA library of rat brain reveals a 2.3 kb open reading frame encoding a 753 amino acid sequence comprising the full length of PIKE (Figure 2A). We observe substantial homology in the C-terminal portion of PIKE to several GTP binding proteins including centaurin γ-a, Rab7, and R-Ras (Figure 2B). Besides the *ras*-like domain (aa 402–686), PIKE contains a PH domain (aa 670–704) immediately to the C-terminal side of the *ras*-like domain. In the N-terminal portion of PIKE, we observe three proline-rich domains (aa 28–34; 182–189; and 353–362) (Figure 2C).

Amino acid residues 387–752 of PIKE are virtually identical to residues 56–420 of human cDNA KIAA0167 (accession number NM_014770). Over this same region, PIKE and KIAA0167 have 89% nucleic acid identity. Unlike PIKE, KIAA0167 contains an extension that includes the C-terminal half of the PH domain indicated in PIKE, an ARF-GAP domain, and ankyrin repeats. KIAA0167 has the same domain structure as *D. melanogaster* Centaurin γ-1a and *C. elegans* hypothetical protein Y39A1A.15b, indicating that KIAA0167 is the human ortholog of Centaurin-γ-1a. Conceivably, PIKE might be an alternatively spliced form of Centaurin γ-1a in rat. However, we identified from database a gene on human chromosome 12 (human cosmid clone 6e5, accession number 4,001,539) that has 91% identity over the entire PIKE cDNA. This human gene contains the three proline-rich domains that human KIAA0167 lacks, indicating that human cosmid clone 6e5 contains human PIKE. Thus, KIAA0167 and PIKE have homologous GTPase/PH domains but appear to be distinct genes. On the other hand, we found a rat EST clone (EST202125; accession number AI007674), which is identical to the 250 nucleotides at the 3′ end of PIKE, including the same stop codon. This provides confirmation of the sequence determined for our clone. We have replicated our own sequence determination 6 times.

Tissue Distribution and Intracellular Localization of PIKE

Northern blot analysis of various rat tissues reveals a prominent band in the brain at about 5.0 kb (Figure 3A). Besides the 80 kDa PIKE, Western analysis shows a 65 kDa band that is prominent in brain, and faint in lung, heart, and liver. Both the 80 kDa and the small-size bands are blocked by preincubating the anti-serum with recombinant PIKE fragment. Both bands are evident

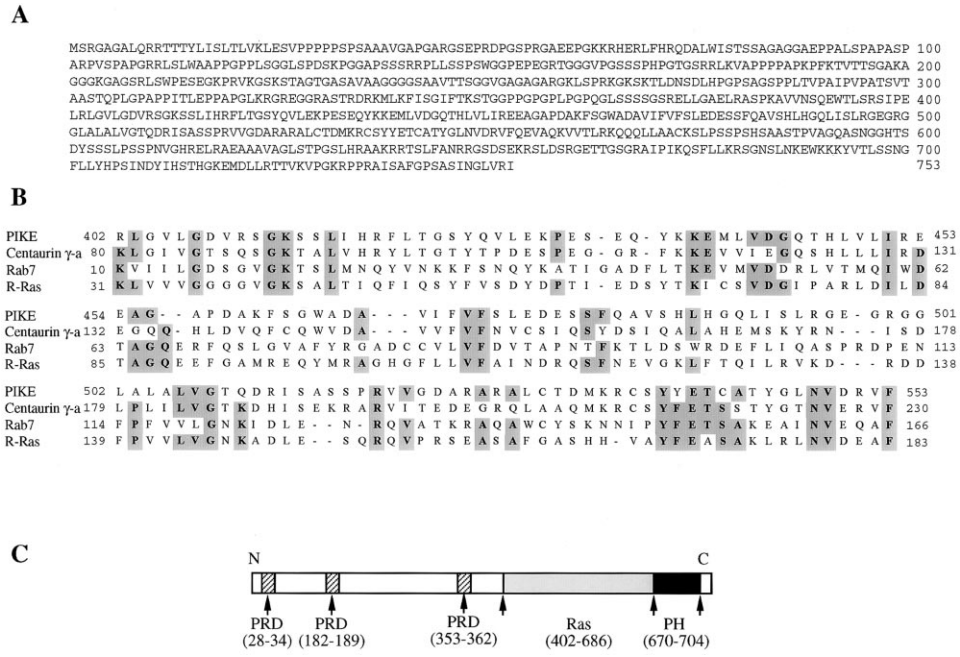

Figure 2. PIKE Encodes a Novel GTPase

(A) Full length amino acid sequence of PIKE.

(B) PIKE is a 753 amino acid protein with sequence homology to the defined GTP binding domain of Centaurin γ-a, Rab7, and R-Ras.

(C) PIKE has three proline rich domains (PRD) in the N terminus, and in the C terminus, a *Ras*-like domain and a Pleckstrin homology (PH) domain.

with different antibodies raised against distinct fragments of PIKE N terminus (data not shown).

In situ hybridization shows PIKE exclusively in neurons, with the highest levels in the hippocampus, in CA1, and the dentate gyrus. In the cerebellum, signal is low in the molecular layer, but enriched in granule and Purkinje cells.

Subcellular fraction reveals PIKE exclusively in the nuclear fraction (Figure 3D). The lower molecular weight immunoreactive bands are not evident in nuclei but are enriched in endoplasmic reticulum/Golgi and synaptosomal fractions. The identity of the fractions is substantiated by the exclusive appearance of the nuclear protein NuMA in the nuclear fraction, the mitochondrial protein cytochrome C in the mitochondrial fraction, and the Golgi protein GM130 in the endoplasmic reticulum/Golgi fraction.

In transfected HEK293 cells, HA-tagged PIKE staining occurs exclusively in nuclei, whose identity is confirmed by DAPI staining (Figure 3E). The nuclear localization of PIKE is the same whether its lysine 413 and serine 414 are mutated to alanine and asparagine respectively, or HA is tagged at the N or C terminus. By contrast, staining for α-tubulin is extranuclear. Thus, the lower molecular weight immunoreactive band, which is cytosolic, does not reflect a smaller form of nuclear PIKE protein but is instead a cross-reactive band.

NGF Augments the GTP Loading of PIKE and Leads to Its Interactions with 4.1N in the Nucleus

The consensus GTP binding domain in the C-terminal portion of PIKE suggests that it possesses GTPase activity. We have directly demonstrated binding of [^{32}P]ATP and [^{32}P]GTP to PIKE (Figure 4A). The C-terminal portion of PIKE, which contains the consensus GTP binding sequence, binds both [^{32}P]ATP and [^{32}P]GTP, while the N-terminal portion of the protein fails to display such binding. Binding is saturable, being almost completely displaced by 10 mM unlabeled nucleotide. Both GTP and ATP bind to PIKE with K_d values of 16 nM and 41 nM, respectively, and PIKE binds about 0.6 mol of GTP-γ-S/mol and 0.9 mol of ATP-γ-S/mol of protein, respectively. Unlabeled ATP-γ-S reduces [^{35}S]GTP-γ-S binding to PIKE with half-maximal inhibition at about 30–40 nM ATP, similar to its K_d for direct binding to PIKE.

PIKE possesses GTPase activity. Like GST-Ras, PIKE hydrolyzes bound GTP into GDP, while GST-4.1N CTD fails to do so (Figure 4B). Though 4.1N binds to PIKE, it fails to stimulate PIKE GTPase activity. Using estimations from the release by PIKE of ^{32}Pi from [γ-^{32}P]GTP, the turnover number of PIKE for GTPase activity is 0.018 min^{-1}, compared to 0.007 min^{-1} for GST-Ras.

PIKE is detected in the homogenate and P1 (unbroken cells and nuclei) fractions (Figure 4C). As PIKE occurs in nuclear fractions, we examined the influence of NGF treatment of PC12 cells on the activation of nuclear PIKE. NGF treatment augments PIKE binding to GTP 5-fold at about 0.5 hr. Activity declines at 4 hr, and by 24 hr has returned to baseline levels (Figure 4D).

4.1N translocates to the nucleus of PC12 cells following NGF treatment, but with a slower time course than what we have observed for activation of PIKE. 4.1N appears in the nucleus only in very small levels one hr after NGF treatment, with peak levels at 24–48 hr (Ye et al., 1999). We examined the influence of NGF treatment of PC12 cells on the association of 4.1N with PIKE in

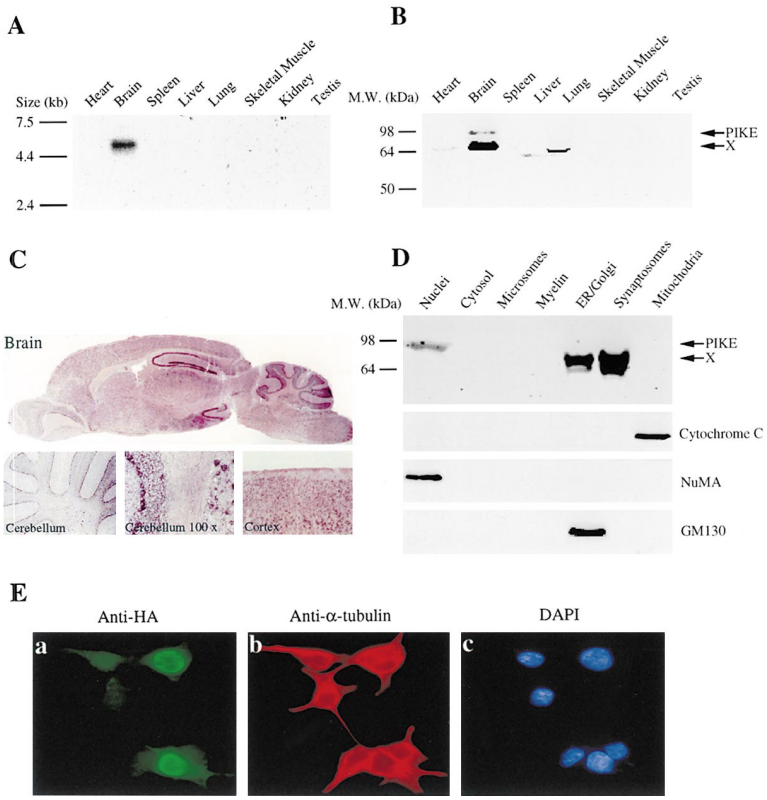

Figure 3. Tissue Distribution of PIKE

(A) Rat multiple tissue Northern blot hybridized with a probe derived from the N-terminal portion of PIKE. A 5 kb transcript is enriched in brain.

(B) Rat multiple tissue Western blot probed with affinity-purified mouse anti-PIKE antibody. PIKE is expressed as an 80 kDa protein only in brain. An unidentified 65 kDa band cross-reacts with PIKE antibody in brain, heart, lung, and liver.

(C) Neuronal distribution of PIKE. In situ hybridization shows PIKE enriched in cerebellum, cortex, and hippocampus. Under high magnification, PIKE is expressed only in neurons with virtually no signal evident in glial cells. In the cerebellum, signal is very low in the molecular layer but enriched in the granule cell and the Purkinje cell layers. At high magnification, intense signal is evident in most or all Purkinje cells and in most or all granule cells.

(D) Subcellular localization of PIKE. The 80 kDa band reflecting PIKE occurs exclusively in the nuclear fraction. The lower molecular weight immunoreactive bands are not evident in nuclei but are enriched in endoplasmic reticulum/Golgi and synaptosomal fractions. The identity of the fractions is substantiated by the exclusive appearance of the nuclear protein NuMA in the nuclear fraction, the mitochondrial protein cytochrome C in the mitochondrial fraction, and the Golgi protein GM130 in the endoplasmic reticulum/Golgi fraction.

(E) Immunofluorescent staining of HA-PIKE in HEK 293 cells. HA-tagged PIKE was transfected into HEK 293 cells, which were then stained with rabbit polyclonal anti-HA antibody and mouse monoclonal anti-α-tubulin antibody. Nuclei were stained with DAPI.

the nucleus (Figure 4E). NGF treatment produces a time-dependent increase in levels of PIKE associated with 4.1N. PIKE is first demonstrable at 1 hr in the immunoprecipitate with substantially higher levels at 24 hr and peak values at 48 hr. The activation of PIKE by NGF prior to nuclear translocation of 4.1N indicates that these two events are not likely to be causally linked. NGF does not alter the total concentration of PIKE (Figure 4E) or 4.1N in PC12 cell lysates (data not shown). Equal amounts of 4.1N are immunoprecipitated with 4.1N antibody at all time intervals after NGF treatment (Figure 4E).

To assess whether the interaction of PIKE and 4.1N is dependent upon guanine nucleotides in PC12 cells, we incubated glutathione beads bound to GST-4.1N with lysates of HEK293 cells transfected with HA-PIKE (Figure 4F). Binding of PIKE 4.1N is not influenced by the presence of GTP-γ-S or GDP-β-S.

PIKE Binds to PI3Kinase

To look for direct interactions between PIKE and PI3K, we isolated His-PIKE on nickel beads, loaded the beads with GTP-γ-S or with GDP, then incubated them with lysates of HEK293 cells that had been transfected with PI3K with an HA tag for the p85 subunit and a myc tag for the p110 subunit (Figure 5A). Western blot analysis reveals robust binding of PIKE to p85 for PIKE preparations preloaded with GTP-γ-S but not for preparations preloaded with GDP. PI3K activity is demonstrable for preparations preloaded with GTP-γ-S but not for preparations preloaded with GDP (Figure 5B).

To ascertain the portion of PIKE that binds to PI3K, we first cotransfected HEK 293 cells with various fragments of PIKE together with p85 (Figures 5C–5E). p85 binds robustly to PIKE and binding is the same for wild-type PIKE as PIKE with the GTP binding lysine-413 mutated to alanine and serine-414 to asparagine. Binding is dependent on the N-terminal 23 amino acids of PIKE, as it is substantially reduced in forms of PIKE lacking these 23 amino acids. Deletion of the N-terminal 261 amino acids of PIKE abolishes binding. Deletion of the PH domain at the C terminus of PIKE does not interfere with binding. However, deletion of the C-terminal 40% of PIKE, eliminating the GTP binding domain, abolishes binding.

Structural requirements for PIKE binding to p110 differ somewhat from binding to p85 (Figures 5E and 5G). As with p85, p110 binding is not affected by the K413AS414N mutation or by deletion of the C-terminal PH domain from PIKE. Whereas removal of the GTP binding area of PIKE abolishes binding to p85, this deletion only causes a modest reduction in binding to p110. While deletion of the N-terminal 23 amino acids of PIKE greatly reduces binding to p85, the same deletion does not influence binding to p110. Like p85, p110 binding to PIKE is abolished by deletion of the N-terminal third of the molecule. In control experiments, we have demonstrated that all the transfected proteins are expressed to a similar extent (Figures 5D and 5F).

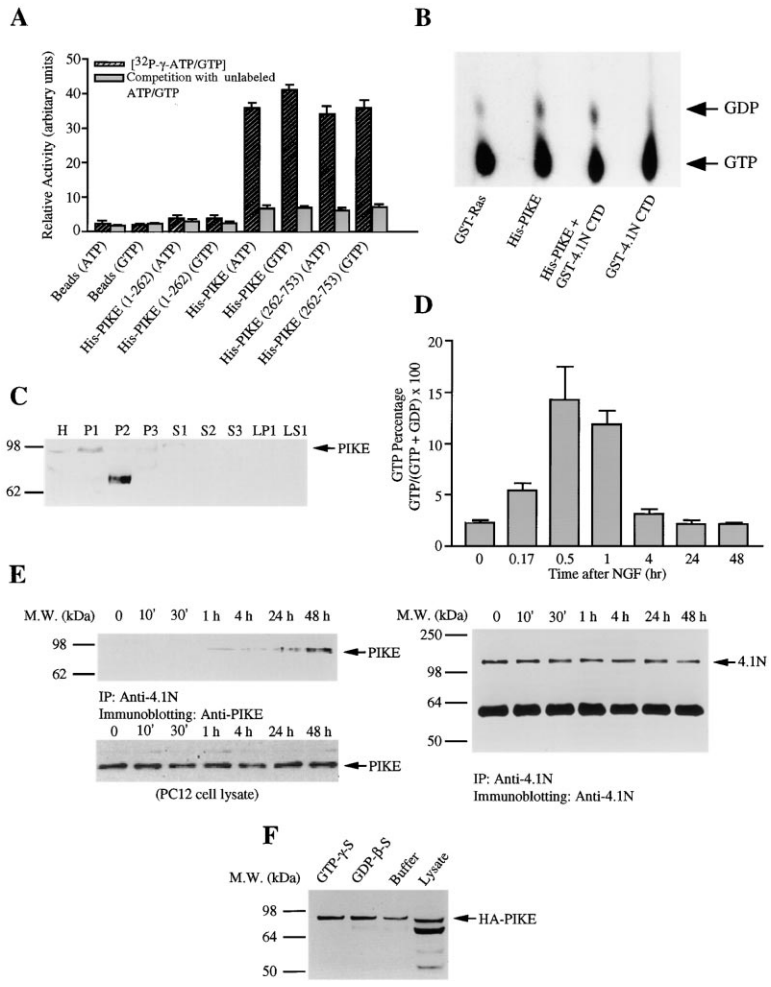

Figure 4. NGF Augments the GTP Loading of PIKE and Leads to Its Interactions with 4.1N in the Nucleus

(A) PIKE selectively binds to [^{32}P]ATP/GTP. Purified His-PIKE (1–262), His-PIKE (263–753), His-PIKE (full-length), and Ni^{2+} beads (control) were incubated with [^{32}P]ATP or [^{32}P]GTP respectively in the presence or absence of excess unlabeled ATP or GTP. After extensive washing, bound radioactivity was counted in a scintillation counter. Binding is saturable, being almost completely displaced by 10 mM unlabeled nucleotide.

(B) PIKE is a GTPase. Purified His-PIKE (0.5 μg) was incubated with [α-^{32}P]GTP for 1 hr at 37°C. The extent of GTP hydrolysis was assessed by thin layer chromatography. GST-Ras (0.5 μg) was used as a positive control, and 2 μg GST-4.1N CTD as a negative control. Although GST-4.1N CTD binds to His-PIKE, it fails to stimulate PIKE's GTPase activity.

(C) Subcellular distribution of PIKE in PC12 cells. PIKE is found in the pellet fractions of PC12 cells. H, homogenate; P, pellet; and S, supernatant. P1 fraction contains unbroken cells and nuclei, while P2 is composed of ER/Golgi and mitochondria.

(D) NGF increases PIKE's GTP loading in PC12 cells. PIKE was immunoprecipitated from the nuclear fraction of PC12 cells, which were metabolically labeled with [^{32}P]H_3PO_4 for 4 hr and treated with NGF for varying periods of time. The bound GTP and GDP were separated on TLC. The bound GTP increases 5 fold around 30 min after NGF treatment.

(E) 4.1N coimmunoprecipitates with PIKE in response to NGF treatment. PC12 cells were treated with 50 ng/ml NGF and, at the indicated times, cells were lysed and immunoprecipitated with anti-4.1N antibody. Coprecipitated PIKE was detected by Western blotting. NGF treatment does not alter overall PIKE expression in whole cell lysates. The same amount of the 4.1N protein was immunoprecipitated in each lane.

(F) HA-tagged PIKE association with 4.1N in vitro is not GTP dependent. One mM GTP-γ-S, GDP-β-S, or buffer alone was added into HA-PIKE-transfected lysate before incubating with purified GST-4.1N CTD. The bound protein was visualized with anti-HA antibody by Western blotting.

To clarify whether PIKE directly associates with both p85 and p110, we cotransfected HEK293 cells with p85 (aa 1–515) that lacks the p110 binding domain and various fragments of PIKE. We observe the same interactions between p85 (aa 1–515) and various fragments of PIKE as with full-length p85. We also cotransfected HEK293 cells with p110 (aa 123–1023) that lacks the p85 binding domain and various fragments of PIKE, and observe the same associations as with full-length p110 (data not shown).

PIKE Activates PI3K and 4.1N Blocks This Activation

To assess the functional relevance of interactions between PIKE and PI3K, we cotransfected HEK293 cells with p110, p85, PIKE, and 4.1N in various combinations (Figure 6A). Catalytic activity occurs in cells transfected with both p110 and p85 but not with p110 alone. Cotransfection with PIKE leads to a quadrupling of enzyme activity. The p85 regulatory subunit of PI3K is critical, as we observe no activity in cells transfected with p110 and PIKE, but lacking P85. Cotransfection of 4.1N into cells transfected with wild-type PIKE abolishes the PIKE induced activation of PI3K activity (Figure 6A). This loss of activation is associated with the failure of PIKE to coprecipitate with PI3K in cells that have been cotransfected with 4.1N, indicating that 4.1N competes with PI3K for binding to PIKE (Figure 6B).

To directly test the notion that 4.1N competes with PI3K for binding to PIKE, we transfected varying amounts of 4.1N into cells that were also transfected with p110, p85 and PIKE (Figure 6C). Robust binding of p110 to PIKE is abolished by progressively increased levels of 4.1N transfection.

PIKE Mediates Stimulation by NGF of Nuclear PI3K Activity and Upregulation of Cyclin D1

NGF influences various events in PC12 cells with markedly different temporal features. Within a few minutes after treatment with NGF, the GTPase activity of *ras*-like proteins and of PI3K is augmented in the cytoplasm (Ridley et al., 1992; Rodriguez-Viciana et al., 1994, 1997;

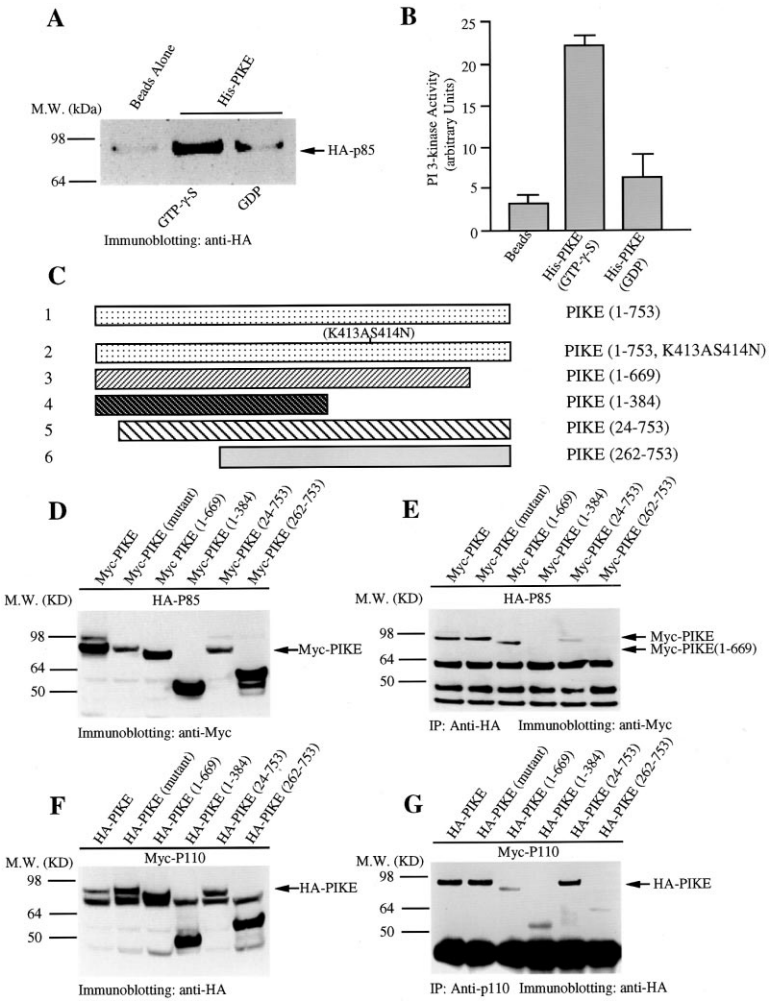

Figure 5. PIKE Associates with PI3K

(A) PIKE binding to PI3K is GTP dependent. The purified His tagged PIKE or Ni^{2+} beads alone (control) were loaded with 1 mM GTP-γ-S or GDP, then incubated with the lysate of HEK293 cells, which were transfected with HA-p85/Myc-p110. The bound proteins were visualized with anti-HA antibody.

(B) Association of PI3K activity with PIKE in vitro. The amount of activity stably associated with the beads is shown. PI3K activity bound to the GTP-loaded PIKE is substantially higher than on the GDP loaded protein.

(C) Diagram of PIKE constructs used in the coimmunoprecipitation experiments.

(D and E) Both the N and C terminus are required for PIKE association with p85. HA-p85 and Myc- tagged PIKE constructs were cotransfected into HEK293 cells. p85 was immunoprecipitated with anti-HA antibody, and bound proteins were visualized by Western blot with anti-Myc antibody. Similar levels of all Myc-PIKE constructs were expressed in all experiments (E).

(F and G) PIKE associates with p110. Myc-p110 and HA-tagged PIKE constructs were cotransfected into HEK293 cells, and p110 was immunoprecipitated by anti-p110 rabbit polyclonal antibody. The bound proteins were visualized by Western blot with anti-HA antibody. Similar levels of all HA-PIKE constructs were expressed in all experiments (F).

Altun-Gultekin and Wagner, 1996). Antimitotic influences and neurite extension occur at about 12–24 hr (Hagag et al., 1986; Milbrandt, 1988; Baetge and Hammang, 1991). If PIKE mediates the NGF activation of nuclear PI3K, then activation of PIKE and of nuclear PI3K should display similar temporal properties. Nuclear PIKE is activated at about 0.5 hr (Figure 4B). We observe maximal activation of nuclear PI3K activity in the immunoprecipitate complex of p110 at about 0.5 hr following NGF treatment of PC12 cells, the same as peak activation of PIKE (Figure 7A). By 4 hr, enzyme activity has returned almost to baseline levels.

If the activation of PI3K in the nucleus is through the association with nuclear PIKE, then NGF should activate PI3K associated with immunoprecipitates of endogenous PIKE. Accordingly, we immunoprecipitated PC12 cells with PIKE antibody at various times after NGF treatment and monitored PI3K activity in the immunoprecipitates. PI3K activity is maximally activated at 30 min with a time course identical to that of PI3K immunoprecipitates. Selectivity for the nuclear translocation of 4.1N and p110 is evident by the absence of translocation for α-tubulin following NGF treatment (Figure 7C).

The temporal correlation for PIKE and PI3K activation by NGF suggests a link between the two events. To establish a casual relationship, we utilized a dominant-negative form of PIKE for retroviral infection of PC12 cells. We developed the dominant-negative construct by making point mutations in PIKE's well-conserved GTPase domain.

PIKE's lysine 413 and serine 414, thought to bind to GTP, were mutated to alanine and asparagine respectively (Feig and Cooper, 1988). Activation of PI3K by PIKE is GTP-dependent (Figure 5B). PIKE does bind to PI3K in the presence of GDP, though less well than with GTP (Figure 5A). To examine the PIKE mutants' activation of PI3K, we cotransfected HEK293 cells with p110, p85, PIKE (K413A), PIKE (S414N), and PIKE (K413AS414N) in various combinations. Cotransfection with wild-type PIKE leads to a quadrupling of enzyme activity. However, PIKE (S414N) and PIKE (K413AS414N) fail to activate PI3K activity (Figure 7B). We utilized PIKE (K413AS414N) as a dominant-negative incorporated into a retrovirus, which was infected into PC12 cells. Following NGF treatment, PI3K activity was monitored at various time points. In contrast to the quadrupling of PI3K activity in control cells 0.5 hr after NGF, no increase occurs at any time point in the dominant-negative infected cells (Figure 7A).

PC12 cells treated with NGF undergo a G1 cell-cycle

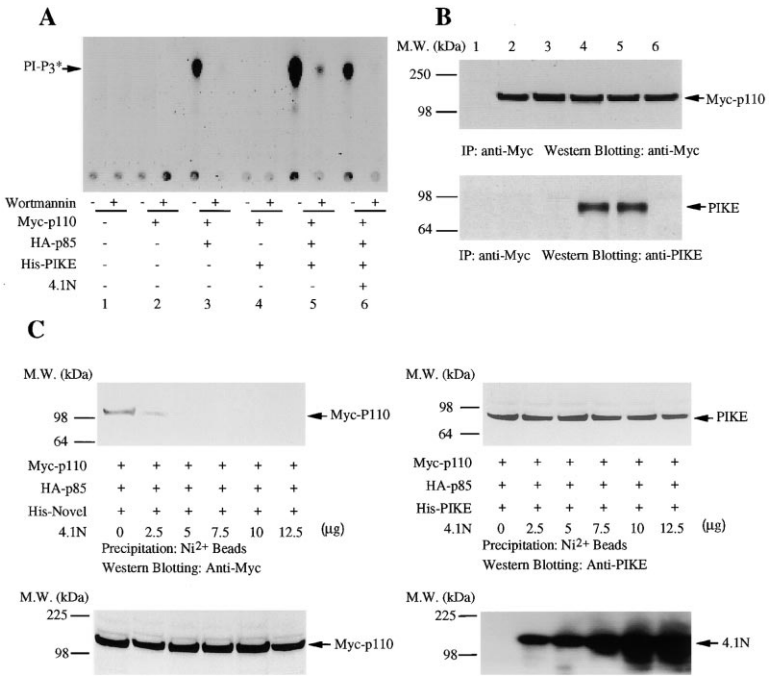

Figure 6. PIKE Enhances PI3K Activity and 4.1N Blocks This Activation

(A) TLC for PI3K activity assays. HEK293 cells were transfected with the indicated expression constructs. PI3K was immunoprecipitated by anti-Myc antibody and assayed for in vitro lipid kinase activity. Wortmannin (20 nM) inhibition shows that the kinase activity is specific.

(B) 4.1N abrogates PIKE association with PI3K. The Myc-p110 immunoprecipitates were probed by Western blot with anti-PIKE antibody. Equal amounts of Myc-p110 were immunoprecipitated from each condition (top). 4.1N inhibits PIKE binding to p110 (bottom).

(C) 4.1N competes with PI3K for binding to PIKE. Myc-p110, HA-p85, and His-PIKE were cotransfected into HEK 293 cells with 0, 2.5, 5, 7.5, 10, or 12.5 μg 4.1N plasmid. His-PIKE was isolated on Ni^{2+} beads. Coprecipitated Myc-p110 was visualized by Western blot with anti-Myc antibody. Equal amounts of Myc-p110 and PIKE were confirmed with Myc and PIKE antibodies. Progressive increases in 4.1N expression occur with transfection of increasing amounts of plasmids.

arrest and differentiate with extension of neurites. This G1 arrest is well documented by the G1-phase-specific cyclin D1, which is dramatically increased in the nuclei of PC12 cells by NGF treatment (Yan and Ziff, 1995; van Grunsven et al., 1996; Ye et al., 1999). PI3Kinase activity is required for the expression of cyclin D1 in many types of cells (Gille and Downward, 1999; Takuwa et al., 1999). To examine the role of PIKE in these events, we infected PC12 cells with PIKE (K413AS414N) retrovirus, then treated the cells with NGF, and analyzed cyclin D1's expression level (Figure 7D). In control cells, cyclin D1's concentration is enhanced 2.5- and 4-fold, respectively, after 2 and 4 days NGF treatment; however, in virus-infected cells, cyclin D1 is not altered by NGF. NGF treatment does not affect cyclin B1 protein level. To further evaluate how the PIKE mutant influences nuclear cyclin D1, we employed immunocytochemistry. About 20%, 45%, and 65% of nuclei, respectively, stain for cyclin D1 after 0, 2, and 4 days NGF treatment, compared to only 20% to 25% in the virus-infected cells (Figure 7E).

To ascertain whether PIKE is required for neurite outgrowth in PC12 cells, we monitored neurite extension in PC12 cells infected with the dominant-negative PIKE (K413AS414N) retrovirus. PC12 cells in the presence of NGF (50 ng/ml) for 4 days develop a network of neurite outgrowth, with 80% ± 3% of control and 75% ± 4% in the virus-infected cells elaborating neurites at least two cell bodies in length. Thus, PIKE and nuclear PI3K seem to be required for NGF-induced cell-cycle arrest, but not for neurite outgrowth.

Discussion

In the present study, we have discovered a novel protein, PIKE, which possesses GTPase activity and is exclusively localized to the nucleus where it binds 4.1N and PI3K, activating the latter. PIKE has three proline-rich domains and a PH domain. The SH3 domain of P85 might bind to the proline-rich domain of PIKE, as SH3 and proline-rich domains of proteins are frequently associated (Prasad et al., 1993; Kapeller et al., 1994; Pleiman et al., 1994; Yu et al., 1994). PH domains bind to lipids and so might interact with the lipid substrates attached to PI3K.

We clarified how PIKE activates PI3K depending on the presence of its two subunits with PIKE being able to bind independently to each of the two subunits p85 and p110. Transfected by itself, p110 lacks catalytic activity but acquires it following cotransfection with p85. PIKE transfection quadruples this activity. PIKE can activate PI3K only when both p110 and p85 are expressed, not with p110 alone. This suggests that PIKE alone can not prevent p110 denaturation but may facilitate the stabilizing effect of p85 on p110.

NGF treatment of PC12 cells activates PIKE and nuclear PI3K with peaks at 30 min, whereas NGF activates the cytoplasmic GTPases of the *ras* family as well as cytoplasmic PI3K much more rapidly with peak activity in 5–10 min (Carter and Downes, 1992; Rodriguez-Viciana et al., 1994, 1997). These observations suggest that PIKE mediates the NGF activation of nuclear PI3K, a conclusion established by experiments in dominant-negative PIKE (K413AS414N) retrovirus–infected PC12 cells, where activation by NGF of nuclear PI3K is abolished.

Cytoplasmic PI3K activation requires activated receptor tyrosine kinases (e.g., PDGFR, EGFR, CD28, etc.) or GTPase proteins such as Ras. However, none of these known PI3K activators are present in nucleus. Our discovery that the nuclear GTPase, PIKE, enhances nuclear PI3K activity indicates that PIKE may be the nuclear counterpart of Ras. These findings provide a molecular basis for the regulation of nuclear PI3K.

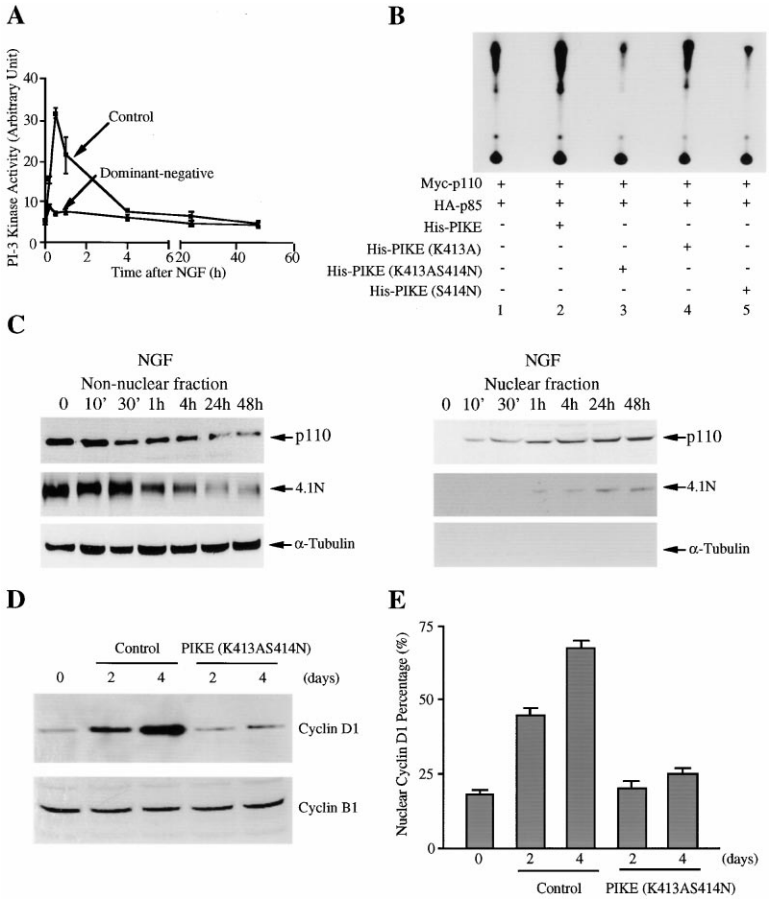

Figure 7. NGF Stimulates Nuclear PI3K Activity with a Similar Time Course as Its Activation of PIKE

(A) PIKE is required for the activation by NGF of nuclear PI3K. PC12 cells (control or dominant-negative PIKE retrovirus infected) were treated with 50 ng/ml NGF for the indicated times, nuclei were isolated and lysed. p110 was immunoprecipitated from the lysate with rabbit polyclonal anti-p110 antibody. PI3K activity assays were performed using the immunoprecipitate. Maximal activation of nuclear PI3K activity occurs at 0.5 hr in control cells, while activation is abolished in the dominant-negative retrovirus infected cells.

(B) Mutations in the GTPase domain of PIKE abolish its activation of PI3K. HEK293 cells were transfected with the indicated expression constructs. PI3K was immunoprecipitated by anti-Myc antibody and assayed for in vitro lipid kinase activity. Transfection with wild-type PIKE augments PI3K activity 3- to 4-fold, while no activation is evident with different forms of PIKE with point mutations in the GTPase domain.

(C) NGF treatment elicits nuclear translocation of PI3K and 4.1N with different time courses. Maximal increases in nuclear 4.1N are not evident until 24–48 hr, but occur at 1–4 hr for PI3K. α-tubulin levels do not change with time in nonnuclear and nuclear fractions.

(D) PIKE regulates the cyclin D1 expression in PC12 cells. PC12 cells were infected with dominant-negative PIKE (K413AS414N) retrovirus, then treated with 50 ng/ml NGF for 2 and 4 days. The protein expression levels of cyclin D1 and cyclin B1 were analyzed with Western blot. The PIKE dominant-negative mutant inhibits the upregulation of cyclin D1 by NGF.

(E) Immunofluorescent staining PC12 cells with cyclin D1. About 20%, 45%, and 65% of nuclei, respectively, stain for cyclin D1 after 0, 2, and 4 days NGF treatment, compared to only 20% to 25% in the virus-infected cells.

NGF treatment activates PIKE in the nucleus. Several proteins translocate to the nucleus following NGF treatment and thus are candidates to activate PIKE. Examples include the NGF receptor TrkA itself as well as MAP kinase (Yankner and Shooter, 1979, Sano et al., 1995; Salehi et al., 1996; Boglari et al., 1998). Stimulation of cells with NGF activates nuclear PI3K associated with an accumulation in the nucleus of 3-phosphorylated phosphoinositide lipids (Neri et al., 1994; Tanaka et al., 1999). It has been shown before that phosphoinositide lipids bind to PH domain and activate dynamin GTPase activity (Melen et al., 1996). In our studies, PI3K translocates to the nucleus following NGF treatment with a time course that resembles the activation of PIKE. In preliminary experiments, we treated PIKE with different phospholipids, including phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2) and phosphatidylinositol 3,4,5-trisphosphate (PI-3,4,5-P_3), but failed to detect PIKE's activation (data not shown).

The binding of 4.1N to PIKE prevents its interactions with nuclear PI3K, which may influence the regulation of PI3K by NGF. Thus, NGF causes 4.1N to translocate to the nucleus over a period of hours, lagging behind the translocation of PI3K and the peak activation of PIKE elicited by NGF. The decline of activated nuclear PI3K, which coincides with the appearance of nuclear 4.1N, might involve 4.1N sequestering PIKE away from nuclear PI3K. The decline of PIKE's NGF-induced GTPase activation takes place at about the same time and so also may participate in the decline of nuclear PI3Kinase.

Our findings suggest the following model for the temporal features of NGF's differential regulation of cytoplasmic and nuclear PI3K. Within a few minutes following NGF treatment, cytoplasmic PI3K is activated and translocated from the cytoplasm to the plasma membrane. This activation involves direct binding of SH2 domains of p85 to the phosphotyrosine portion of the growth factor receptor (Whitman et al., 1985; Kaplan et al., 1987; Klinghoffer et al., 1996; Hallberg et al., 1998; Ming et al., 1999) as well as binding of p110 to members of the *ras* family. Somewhat later, with a peak at about 30 min, PIKE is activated and PI3K is translocated to the nucleus and activated, presumably by PIKE. Still later, 4.1N translocates to the nucleus where it binds to PIKE and inactivates it. This binding interferes with interactions of PIKE and PI3K, and we speculate that this may account in part for diminution of the upregulated PI3K activity. At about the same time, PIKE's GTPase activity declines, which may also contribute to the fall in nuclear PI3K activity.

Targets of cytoplasmic PI3K have been studied extensively. The lipid products of PI3K in the cytoplasm activate a variety of kinases including Akt and PDK1 (Alessi et al., 1997; Frech et al., 1997). These activities influence cytoskeletal rearrangements, vesicle transport, and apoptosis, which are largely cytoplasmic events. Some cytoplasmic targets of PI3K, such as Akt, can translocate to the nucleus, but whether they remain targets of PI3K in the nucleus is unknown. Thus, nuclear processes regulated by the PI3K signaling system have not yet been established. In the nervous system, we suggest that PIKE may be a major, if not the sole mediator of PI3K activation in the nucleus. Ran is the one other GTPase that occurs exclusively in the nucleus, but whether or not it interfaces with PI3K is not known.

It has been reported that PI3K activity is required for the expression of cyclin D1 in many types of cells (Gille and Downward, 1999; Takuwa et al., 1999). To ascertain the role of PIKE and nuclear PI3K in cellular actions of NGF, we infected PC12 cells with dominant-negative PIKE (K413AS414N) retrovirus. The PIKE mutant blocks the upregulation of cyclin D1 elicited by NGF, but does not affect NGF-induced neurite extension. Though some studies have suggested a role for cytoplasmic PI3K activity in NGF-mediated neurite outgrowth (Kimura et al., 1994; Jackson et al., 1996; Ashcroft et al., 1999), others report only a minor potential link of neuronal extension and PI3K (Kobayashi et al., 1997; Ashcroft et al., 1999). Our dissociation of NGF-mediated cell cycle arrest and neurite outgrowth is consistent with other observations (van Grunsven et al., 1996; Xiong et al., 1997).

Experimental Procedures

Cells and Reagents

PC12 cells were maintained in DMEM with 10% fetal bovine serum (FBS), 5% horse serum, and 100 units penicillin-streptomycin at 37°C with 5% CO_2 atmosphere in a humidified incubator. Differentiation was initiated by addition of 50 ng/ml nerve growth factor (NGF) with culture medium changed to DMEM with 2% horse serum and 1% FBS. NGF, X-Gal, and IPTG were purchased from Boehringer Mannheim. Mouse monoclonal anti-HA, anti-Myc, anti-NuMA, and rabbit polyclonal anti-HA antibodies were supplied by Calbiochem. Rabbit polyclonal anti-p85 and p110 antibodies were purchased from Santa Cruz Biotechnology, Inc. Mouse monoclonal anti-GM130 antibody was supplied by Transduction Laboratories Inc. (Lexington, KY). -Leu DO supplement, -Leu/-Trp/-His DO supplement, Minimal SD Agar Base, and Minimal SD Base for the yeast two-hybrid screen were from Clontech. Retrovirus cloning vector pDON-A1 was from Takara Shuzo Co., LTD/Biomedical Group, Shiga, Japan.

Yeast Two-hybrid Screen

Two-hybrid screening was conducted using the Y190 yeast strain containing the HIS3 and β-galactosidase (β-gal) reporter genes and the pPC97 and pPC86 expression vectors. This was performed essentially as described (Ye et al., 1999).

Cloning of Full-length PIKE cDNA and Northern Blot Analysis

An adult rat brain cDNA library in λZAPIII vector (Stratagene) was screened using the two-hybrid PIKE fragment, labeled with ^{32}P in a nick translation system (Boehringer Mannheim). A total of 1.06×10^6 clones were screened, yielding 7 overlapping inserts. A 2.2 kilobase open reading frame was identified. A commercial rat multiple tissue Northern blot (Clonetech) was hybridized according to the manufacturer's recommendations with the N-terminal PIKE probe. The membrane was washed three times at 65°C in 0.2 × SSC and 0.1% SDS solution.

Coimmunoprecipitation and In Vitro Binding Assays

The experimental procedures for coimmunoprecipitation and in vitro binding assays are the same as described (Ye et al., 1999). For the GTP-dependent association between His-PIKE and PI3K, assays were performed as described (Vojtek et al., 1993).

Isolation of Subcellular Fractions and Immunohistochemistry

PC12 cell nuclei were isolated, and immunofluorescent staining was performed essentially as previously described (Ye et al., 1999). The subcellular fractions of PC12 cells were prepared as described (Burnett et al., 1998). Subcellular fractions from rat brain were prepared as described (Ueda et al., 1979).

In Situ Hybridization

RNA probes for PIKE were generated by subcloning each open reading frame from pPC86 or pPC97 into the SalI and NotI sites of pBS (SK)II+ (Stratagene). Brain slices for in situ hybridization were prepared and probed as described (Blackshaw and Snyder, 1997).

ATP, GTP Binding, and GTPase Assay

Assays of [^{32}P]-γ-ATP and [^{32}P]-γ-GTP binding to the His-tagged PIKE, and to its N-terminal and C-terminal fragments were performed as described (de Rooij et al., 1998). The [^{35}S] GTP-γ-S and ATP-γ-S binding to PIKE was determined by use of the nitrocellulose filter method under the conditions specified previously (Brandt and Ross, 1985). The GTPase assays were performed essentially as described (Der et al., 1986).

GTP Loading Assays

The assays were performed essentially as described (Rosen et al., 1994) with minor modification. PC12 cells were treated with 50 ng/ml NGF for the indicated times before or after metabolic labeling. PC12 cells were metabolically labeled in phosphate-free Dulbecco's modified Eagle's medium with 0.5 mCi/ml [^{32}P]H_3PO_4 for 3–4 hr at 37°C. Nuclei were isolated as described above, and lysed in 0.5 ml lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM $MgCl_2$, and 1% Triton X-100) containing 5 μg anti-PIKE antibody, and 1:10 vol of PBS/1% BSA/10% charcoal slurry was added. Extracts were rocked at 4°C for 30 min and centrifuged for 5 min at 12,000 × g. Supernatant fluid (0.5 ml) was added to 10 ml of protein A Sepharose and rocked for 30 min at 4°C, then washed 3 times with lysis buffer and once with PBS. Succeeding steps were the same as described.

In Vitro PI3K Assay

Epitope- or His-tagged proteins were precipitated from cell lysates as described above, and washed with the following buffers: 3 times with buffer A (PBS, 1% NP-40, and 1 mM DTT); 2 times with Buffer B (PBS, 0.5 M LiCl, and 1 mM DTT); 2 times with Buffer C (10 mM Tris-HCl [pH 7.4], 0.1 M NaCl, and 1 mM DTT). In experiments using His-tagged proteins, the DTT was omitted. The following steps were exactly as described (Ruderman et al., 1990).

Infection of PC12 Cells with p-DON-A1-PIKE (K413AS414N) Retrovirus

The experiments were performed essentially as described (Mulligan, 1993; Kim et al., 1998). p-DON-A1 is a 5682 kb plasmid driven by 5′ HCMV IE (Immediate Early) promoter followed by intron SD/SA sequences, as well as retrovirus caspid packaging signals. PIKE (K413AS414N) dominant-negative mutant was cloned into SalI/HpaI of the SA sequences. The vector contained neomycin and ampicillin resistance genes as well as bacterial ori. The packaging cell-line Phoenix P293T contained stable transfected and constitutively expressed retroviral *gag* and *pol* genes. Ten micrograms of p-DON-A1-PIKE mutant DNA and 10 μg plasmid DNA expressing vesicular stomatitis virus G-protein (VSV-G) were cotransfected into Phoenix P293T cells by the calcium phosphate method. After days, the culture medium was carefully collected and centrifuged at 6000 × g for 30 min to pellet cell debris. The supernatant was filtered with a 0.45 μm filter and centrifuged at 25,000 × g for 100 min at 4°C to pellet virions. After removal of the supernatant, PC12 cell culture

medium (21 ml) was added to the pellet, which was maintained overnight at 4°C. The pellet was then gently resuspended, and 3 ml aliquots were maintained at −80°C for 24 hr. PC12 cells were infected with 3 ml virus and 1X Polybrene (4 μg/ml) for 10 hr in the incubator (plates were swirled every 2 hr). Medium (8 ml) was then added to the plates, and cells were grown to 80% confluence. NGF (50 ng/ml) was then added to the PC12 cells.

Acknowledgments

This work is supported by USPHS grants DA00266 (S. H. S.) and Research Scientist Award DA-00074 (S. H. S.). The authors are indebted to Drs. Michael M. Lai, Masaaki Takahashi, Patrick E. Burnett, and Xiaodong Li for valuable suggestions. We also thank Dr. Harish C. Joshi for thoughtful discussion and advice.

Received June 15, 2000; revised November 3, 2000.

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GenBank Accession Number

The GenBank accession number for the novel PIKE nuclear GTPase reported in this paper is bankit343027.