PAK1 Negatively Regulates the Activity of the Rho Exchange Factor NET1*

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Rho family small G-protein activity is controlled by guanine nucleotide exchange factors that stimulate the release of GDP, thus allowing GTP binding. Once activated, Rho proteins control cell signaling through interactions with downstream effector proteins, leading to changes in cytoskeletal organization and gene expression. The ability of Rho family members to modulate the activity of other Rho proteins is also intrinsic to these processes. In this work we show that the Rac/Cdc42hs-regulated protein kinase PAK1 down-regulates the activity of the RhoA-specific guanine nucleotide exchange factor NET1. Specifically, PAK1 phosphorylates NET1 on three sites in vitro: serines 152, 153, and 538. Replacement of serines 152 and 153 with glutamate residues down-regulates the activity of NET1 as an exchange factor in vitro and its ability to stimulate actin stress fiber formation in cells. Using a phospho-specific antibody that recognizes NET1 phosphorylated on serine 152, we show that PAK1 phosphorylates NET1 on this site in cells and that Rac1 stimulates serine 152 phosphorylation in a PAK1-dependent manner. Furthermore, coexpression of constitutively active PAK1 inhibits the ability of NET1 to stimulate actin polymerization only when serines 152 and 153 are present. These data provide a novel mechanism for the control of RhoA activity by Rac1 through the PAK-dependent phosphorylation of NET1 to reduce its activity as a guanine nucleotide exchange factor.

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Three classes of proteins known as guanine nucleotide exchange factors (GEFs),

1 GTase activating proteins, and guanine nucleotide dissociation inhibitors (5). GEFs regulate Rho GTase activation by catalyzing the release of GDP, thus allowing the binding of GTP. GTase activating proteins inactivate Rho proteins by stimulating their intrinsic GTase activities. Guanine nucleotide dissociation inhibitors preferentially bind to the inactive, GDP-bound forms of Rho proteins, thereby sequestering them from their sites of action. In this regulatory scheme it is the GEFs that control small G-protein activation in response to growth factor and mitogen stimulation.

The Rho GEF family currently consists of 25 cloned genes and is predicted to contain up to 50 family members when fully characterized (7–10). Each Rho GEF displays a unique specificity for different Rho family small G-proteins. For example, the Rho GEF Dbl efficiently catalyzes GDP release for RhoA, Rac1, and Cdc42hs (11). On the other hand, the Rho GEF Tiam1 specifically catalyzes nucleotide exchange only for Rac1 (12). The regulatory mechanisms controlling the enzymatic activities of different Rho GEFs are equally diverse. These include binding to phosphoinositides, altered protein-protein interactions, changes in subcellular localization, and site-specific phosphorylation (9, 13). The Rho GEF Vav, for example, is activated by the combined effects of phosphorylation on specific tyrosine residues and the binding of phosphoinositide 3,4,5-phosphate. This relieves the actions of an autoinhibitory domain and stimulates its GDP exchange activity toward Rho proteins (14–17). Similarly, the activity of the Rho GEFs Tiam1, Dbl, and FGR are positively regulated by phosphorylation (18–20).

The neuroepithelioma transforming gene 1 (NET1) is a Rho GEF that was first identified in a screen for transforming genes in NIH 3T3 cell focus formation assays (21). It contains a negative regulatory domain in its amino terminus, and deletion of this domain creates an oncogenic form of the protein. In addition to transforming NIH 3T3 cells, expression of oncogenic NET1 stimulates actin stress fiber formation, c-Jun NH2-terminal kinase/mitogen-activated protein kinase activation, and serum response factor activation (21, 22). Recently it was demonstrated that wild-type NET1 is localized in the nucleus, and that truncation of the amino terminus results in relocalization of a fraction of the NET1 to the cytoplasm. This was at least partially due to the elimination of two putative nuclear localization signals within the amino terminus (23). Thus, NET1

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The abbreviations used are: GEF, guanine nucleotide exchange factor; NET1, neuroepithelioma transforming gene 1; GST, glutathione S-transferase; HA, hemagglutinin; V12Rac1, Rac contain a glycine 12 to valine substitution; AMP-PNP, 5‘-adenyl-β,γ-imidodiphosphate; GTPγS, guanosine 5’-O-(thiotriphosphate); TRITC, tetramethylrhodamine isothiocyanate.
activity is regulated at least in part through subcellular localization. No other mechanisms controlling NET1 activity have been described.

In this work we show that NET1 is negatively regulated through phosphorylation by the serine/threonine protein kinase PAK1. Specifically, PAK1 phosphorylates the oncogenic form of NET1 (NET1ΔN) on serines 152, 153, and 538 in vitro. Replacement of serines 152 and 153 with glutamate residues, which mimics their phosphorylation, inhibits the GDP/GTP exchange activity of NET1 toward RhoA in vitro, as well as the ability of NET1 to stimulate actin stress fiber formation in cells. Using an antibody that specifically recognizes NET1 phosphorylated on serine 152, we show that PAK1 phosphorylates this site in cells and that expression of constitutively active Rac1 stimulates the phosphorylation of NET1 on serine 152 in a PAK-dependent manner. Furthermore, we show that PAK specifically down-regulates NET1 activity in cells only when serine 152 is intact. These data demonstrate a novel mechanism for controlling NET1 activity and provide an additional means by which Rac1 controls RhoA activity.

**Experimental Procedures**

**Cells, Plasmids, and Recombinant Proteins**—HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected using LipofectAMine Plus (Invitrogen) according to the manufacturer’s instructions. All NET1 eukaryotic expression constructs were contained in a pEFHA (22). Full-length mouse NET1 (amino acids 1–595) and NET1ΔN(122–595) were as described (22). NET1-(156–595), NET1-(156–595-HA) (22), NET1-(362–560), and NET1-(122–155) were created by PCR and sequenced to confirm correct amplification. Replacement of serines 152, 153, 538, and 557 with alanine or glutamic acid residues in any construct were also made by PCR, and the entire cDNA was sequenced to confirm correct amplification. Constitutively active PAK1 (PAK1 L107F) and the PAK1 autoinhibitory domain (amino acids 83–149) were contained in pCMV5 (24).

For the production of GST fusion proteins, all NET1 cDNAs, as well as RhoA, were subcloned into pGEXK (Amersham Biosciences). NET1 proteins and RhoA were produced in BL21 DE3 Escherichia coli (Stratagene) after transformation with the appropriate construct. One-liter cultures were grown at 37 °C to an A600nm = 0.8, and expression of fusion proteins was stimulated by addition of isopropyl β-D-thiogalactopyranoside to 400 μM, followed by incubation at 37 °C for 4 h. Cell pellets were washed twice with 10 ml of NET1 lysis buffer (50 mM HEPES, pH 7.5, 300 mM NaCl, 10 mM MgCl2, 100 μM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 80 μM β-glycerophosphate, 0.5 mM sodium orthovanadate, 10 μM β-galactosidase, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride); the DNA was sheared by passages through a 23-gauge needle; and 22-gauge needle. lyses were pelleted by centrifugation (16,000 × g for 10 min at 4 °C). Hemagglutinin (HA)-tagged NET1 proteins were then immunoprecipitated from soluble lysates using a mouse anti-HA antibody, and the immunoprecipitates were washed 3 × 1 ml with buffer A (500 mM NaCl, 20 mM Tris-HCl (pH 8.0), followed by washing 1 × 1 ml with 20 ml Tris-HCl (pH 8.0). Immunoprecipitated proteins were resuspended in 1 × Laemmli sample buffer, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Amersham Biosciences). The anti-pSer-152 antibody was used for Western blotting by diluting in Tris-buffered saline plus 0.05% Tween 20 (TBST) + 5% nonfat milk and incubating overnight at room temperature. All other antibodies were diluted in TBST + 0.025% nonfat milk and incubated with membranes for 1 h at 37 °C or overnight at 4 °C. After washing with TBST, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies (KPL) for 30 min at room temperature. After washing with TBST, the membranes were developed by enhanced chemiluminescence. Quantification of Western blots was performed by densitometry followed by analysis using NIH Image.

**Guanine Nucleotide Exchange Assays**—Exchange factor assays measuring NET1 activity toward GST-RhoA in vitro were performed essentially as described (27). Briefly, GST-RhoA was loaded with GDP for 5 min at room temperature in GDP loading buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, 1 mM GDP, 100 μM AMP-PNP). Loading was terminated by the addition of MgCl2 (10 mM final concentration), followed by 15 min on ice. Aliquots of GST-bound RhoA (85 pmol) were then mixed with GST- NET1 fusion proteins (10 pmol of fusion proteins per reaction) in GST reaction buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM MgCl2, 100 μM AMP-PNP, 5 μM GTPγS, 10 μCi of [35S]GTPγS) and incubated at 30 °C. At specific times, portions of the reaction were removed (11 pmol of RhoA per time point) and the reaction was stopped by the addition of termination buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM MgCl2). Proteins were collected by filtration through 0.2-μm pore size nitrocellulose disks. The nitrocellulose disks were then washed with 15 ml of terminating buffer and [35S]GTPγS-bound RhoA was detected by scintillation counting.

**Microinjection of NET1 Expression Plasmids**—NIH 3T3 cells plated on glass coverslips were incubated in Dulbecco’s modified Eagle’s medium with 0.05% fetal bovine serum for 24 h prior to injection. All plasmids were injected into the nuclei of cells at a concentration of 0.05 mg/ml as previously described (22). Briefly, 4 h after injection, the cells were fixed in 3% formaldehyde in phosphate-buffered saline at 37 °C for 5 min followed by permeabilization with 0.3% Triton X-100 in phosphate-buffered saline for 5 min at room temperature, and the cells were then incubated with mouse anti-HA antibody (monoclonal antibody 12CA5) to detect expression of HA-tagged NET1 proteins. Coexpressed, Myc-tagged PAK1 L107F was detected using rabbit anti-Myc tag antibody (Invitrogen). For extensive washing in phosphate-buffered saline, binding of primary antibodies was detected by incubating with donkey anti-rabbit or anti-mouse antibodies coupled to either fluorescein isothiocyanate or 7-amino-4-methylcoumarin-3-acetic acid, as indicated. F-actin morphology and accumulation were monitored by co-staining with tetramethylrhodamine-conjugated phalloidin (Molecular Probes). Coverslips were mounted on slides with Gelvatol and imaged with an Axio Imager M1 epifluorescence microscope equipped with a Spot CCD camera using fixed exposure times. Images were imported into the OpenLab software package. Relative fluorescence intensities of TRITC-phalloidin labeled actin in injected and adjacent uninjected cells were recorded on a 0- to 256-bit scale. The fluorescence intensities from 5 to 10 injected, HA-tag expressing cells per experiment are reported as the mean intensity.

**Results**

**Characterization of NET1 Phosphorylation by PAK1 in Vitro**—To date there have been no descriptions of the biochemical mechanisms controlling the activity of the Rho GEF NET1. Because Rac1 has been reported to down-regulate the activity of RhoA (28–30), we examined whether NET1 activity is regulated through phosphorylation by the Rac1 effector PAK1. We first examined whether PAK1 could phosphorylate NET1 in vitro. For these assays, wild-type NET1 and an amino-terminal truncated, transforming version of NET1 (NET1ΔN, amino acids 122–595) were expressed in bacteria as GST fusion pro-
teins and affinity purified. These proteins were then tested as PAK1 substrates in vitro using recombinant, constitutively active GST-PAK1. As shown in Fig. 1A, GST-PAK1 efficiently phosphorylated both full-length NET1 and NET1ΔN. Phosphoamino acid analysis revealed that both NET1 proteins were exclusively phosphorylated on serine residues (data not
Phosphorylation of NET1 by PAK1 Inhibits NET1 Activity

shown). We then constructed a series of deletion mutants to identify the subdomains of NET1 that were phosphorylated by PAK1. Thus, GST fusion proteins corresponding to the Dbl and pleckstrin homology domains (amino acids 156–361 and 362–501, respectively), as well as the amino- and carboxyl-terminal flanking regions (amino acids 122–155 and 502–592, respectively) were produced in bacteria and tested as PAK1 substrates. As shown in Fig. 1D, recombinant PAK1 efficiently phosphorylated flanking regions 122–155 and 502–595, but not the isolated Dbl or pleckstrin homology domains. Thus, in vitro, PAK1 phosphorylates multiple residues of NET1ΔN that are located in two distinct domains.

Alignment of the amino acid sequences surrounding a number of published PAK1 substrates demonstrates that PAK1 phosphorylation sites often contain the consensus sequence (RX)(RX)-X-S*-S/Φ, where Φ is a hydrophobic amino acid.2 Furthermore, in proteins containing two adjacent serine residues at the P2 and P1 sites, the first serine is often preferentially phosphorylated. Thus, using this consensus sequence as a guide, we mutated potential PAK1 phosphorylation sites in both NET1ΔN (122–155) and NET1ΔN (156–595) to alanine, expressed these polypeptides as GST fusion proteins in E. coli, and tested them for phosphorylation by GST-PAK1 in vitro.

The amino-terminal portion of NET1 contained two potential PAK1 phosphorylation sites, located at serines 152 and 153, with serine 152 predicted to be the preferred site. As shown in Fig. 1C, replacement of serine 152 with alanine reduced the phosphorylation of NET1ΔN (122–155) to 72% of wild-type NET1ΔN (122–155), whereas replacement of serine 153 had no effect. On the other hand, replacement of both serines with alanine (S152A/S153A) completely eliminated phosphorylation by PAK1. Thus, these data indicate that PAK1 phosphorylates NET1ΔN (122–155) mainly on serine 152 in vitro, and that if this site is mutated to alanine, serine 153 will be used as a substrate instead. Phosphorylation of the serine at the P1 site position after mutation of the P2 serine to a non-phosphorylatable residue has been observed previously for the PAK1 substrates MEK1 and Raf-1 (26, 31, 32).

We then identified the site phosphorylated by PAK1 in the C terminus of NET1. This sequence also contained two potential PAK1 phosphorylation sites, located at serines 538 and 557. However, substitution of these sites with alanines revealed that only serine 538 was phosphorylated by PAK1 in vitro (Fig. 1D). To confirm that we had identified all of the PAK1 phosphorylation sites in NET1ΔN, we then created a triple alanine mutant of NET1ΔN (S152A/S153A/S538A) and tested it as a substrate for PAK1. As shown in Fig. 1E, NET1ΔN containing the triple serine to alanine alteration was no longer phosphorylated by PAK1. Thus, PAK1 phosphorylates three sites on NET1ΔN in vitro: serines 152, 153, and 538 (Fig. 1F).

Replacement of Serines 152 and 153 with Acidic Residues Inhibits the GDP Exchange Activity of NET1ΔN in Vitro—We next tested whether replacement of the PAK1 phosphorylation sites in NET1ΔN with alanine or glutamate residues affected the exchange factor activity of NET1ΔN toward RhoA in vitro. In these experiments, replacement with alanine was expected to be neutral in effect, whereas replacement with negatively charged glutamic acid was expected to mimic phosphorylation by PAK1. We first tested the effect of replacing serines 152 and 153 with alanine or glutamate. As shown in Fig. 2A, wild-type NET1ΔN efficiently catalyzed GTPγS binding by RhoA. In addition, replacing these serines with alanines did not significantly affect NET1ΔN enzymatic activity. However, substitution with glutamates at these sites significantly reduced the ability of NET1ΔN to stimulate GTPγS binding by RhoA. These data suggest that phosphorylation of serines 152 and 153 by PAK1 negatively regulates the ability of NET1ΔN to stimulate GDP exchange for RhoA.

We then tested the effect of replacing serine 538 with alanine or glutamate. As shown in Fig. 2B, neither change significantly affected the exchange activity of NET1ΔN. This suggests that phosphorylation of serine 538 by PAK1 does not affect NET1ΔN activity toward RhoA. Lastly, we tested the effects of replacing all three PAK1 phosphorylation sites with alanine or glutamate residues. As shown in Fig. 2C, the triple alanine mutant exhibited the same GEF activity toward RhoA as wild-type NET1ΔN, but the triple glutamate mutant was much less effective at catalyzing GTPγS binding by RhoA. Similar results were observed with recombinant, full-length GST-NET1ΔN (data not shown). Thus, we conclude from these experiments that phosphorylation of either NET1ΔN or full-length NET1 by PAK1 on serines 152 and 153 may negatively regulate NET1 activity toward RhoA, and that phosphorylation of serine 538 is likely to be without effect.

Acidic Substitutions at the Amino-terminal PAK1 Phosphorylation Sites Block the Ability of NET1ΔN to Stimulate Actin Stress Fiber Forma
dit—It has been shown that expression of NET1ΔN in cells stimulates F-actin accumulation and stress fiber formation, similar to the effect of expressing constitutively active RhoA (22). In NIH 3T3 cells, the two primary RhoA effectors are thought to be the formin mDia1 and Rho kinase, which nucleate and bundle actin filaments into stress fibers, respectively (33). To test whether alteration of the amino-terminal PAK1 phosphorylation sites affected the ability of NET1ΔN to regulate cytoskeletal organization, serum-starved NIH 3T3 cells were microinjected with HA epitope-tagged NET1ΔN, or NET1ΔN containing alanine or glutamate substitutions at these sites. Four hours after injection, the cells were fixed and stained by indirect immunofluorescence for expression of NET1ΔN proteins (anti-HA) and filamentous actin (F-actin) (Fig. 3A, right and left columns, respectively). The presence of F-actin was detected by incubating the cells with TRITC-labeled phalloidin, which binds specifically to polymerized actin. The relative amounts of F-actin in each cell were then quantified by monitoring fluorescence because of the TRITC-phalloidin. In these assays, expression of NET1ΔN induced the formation of numerous actin stress fibers, consistent with previously published results (22) (Fig. 3A). Quantification showed that NET1ΔN stimulated an average 3-fold increase in mean TRITC fluorescence relative to neighboring un.injected cells, or to cells expressing a catalytically inactive variant of NET1ΔN (L321E) (22) (Fig. 3B). Substitution of serines 152 and 153 with alanines did not significantly affect the ability of NET1ΔN to stimulate stress fiber formation or F-actin accumulation (Fig. 3A, bottom panels). In contrast, glutamate substitution at the PAK1 phosphorylation sites significantly disrupted the ability of NET1ΔN to stimulate stress fiber formation and F-actin assembly (Fig. 3, middle panels). Quantification of these effects showed that NET1ΔN S152E/S153E stimulated only a slight increase in mean F-actin staining relative to the surrounding, non-injected cells (Fig. 3B). These results strongly indicate that phosphorylation of NET1ΔN on serines 152 and 153 negatively regulates its activity in cells, and are consistent with the observed effects of glutamate substitution of these sites on the enzymatic activity of NET1ΔN in vitro (Fig. 2).

NET1 Is Phosphorylated on Serine 152 in Cells Following the Expression of Constitutively Active PAK1 or Rac1—To determine whether NET1 was phosphorylated by PAK1 in cells, an antibody was produced that specifically recognized NET1 only

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2 J. A. Frost, unpublished observations.
when phosphorylated on serine 152 (anti-pSer-152). This residue was chosen rather than serine 153 because PAK1 primarily phosphorylated NET1 on serine 152 in vitro (Fig. 1C).

We first tested the anti-pSer-152 antibody for its ability to recognize NET1 when phosphorylated on serines 152 and 153. Recombinant, GST-NET1ΔN or GST-NET1ΔN fusion proteins containing alanine substitutions at serines 152 and 153, or at serine 538, were incubated in kinase buffer, with or without recombinant GST-PAK1. Following separation by SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane and immunoblotted with the anti-pSer-152 antibody. As shown in Fig. 4A, the anti-pSer-152 antibody recognized NET1ΔN only when it was phosphorylated by PAK1 (compare lanes 1 and 5). In addition, substitution of serines 152 and 153 with alanines blocked recognition by this antibody (lane 6), whereas substitution of serine 538 with alanine was without effect (lane 7). Reprobing the blot with an antibody specific for GST demonstrated that there were similar amounts of GST-NET1ΔN protein in each lane (bottom panel). Thus, this data shows that the anti-pSer-152 antibody only recognizes NET1 when phosphorylated on serines 152 or 153.

To determine whether the antibody was specific for phosphorylation on serine 152, we tested its ability to recognize NET1 with single alanine substitutions at serines 152 and 153. B, GST-NET1ΔN proteins containing alanine or glutamate substitutions at serine 152 and 153. C, GST-NET1ΔN proteins containing alanine or glutamate substitutions at serines 152, 153, and 538.

FIG. 2. Guanine nucleotide exchange activities of different GST-NET1ΔN proteins toward RhoA in vitro. GST-NET1ΔN and GST-NET1ΔN proteins containing alanine or glutamate substitutions at the PAK1 phosphorylation sites were tested for their abilities to stimulate [γ-35S]GTP•S binding by GST-RhoA in vitro. Results are the average of at least three independent experiments. Error bars are S.E. ± mean. A, GST-NET1ΔN proteins containing alanine or glutamate substitutions at serines 152 and 153. B, GST-NET1ΔN proteins containing alanine or glutamate substitutions at serine 538. C, GST-NET1ΔN proteins containing alanine or glutamate substitutions at serines 152, 153, and 538.

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To determine whether the antibody was specific for phosphorylation on serine 152, we tested its ability to recognize NET1 with single alanine substitutions at serines 152 and 153. B, GST-NET1ΔN proteins containing alanine or glutamate substitutions at serine 538. C, GST-NET1ΔN proteins containing alanine or glutamate substitutions at serines 152, 153, and 538.
the anti-pSer-152 antibody by immunoblotting. As shown in Fig. 4B, substitution of serine 152 with alanine completely eliminated recognition by this antibody (lane 4). On the other hand, substitution of serine 153 only modestly reduced recognition by the antibody (lane 5). Because serine 153 was not appreciably phosphorylated by PAK1 in vitro (Fig. 1C), we interpret these data to mean that the anti-pSer-152 antibody recognizes NET1 only when phosphorylated on serine 152, and that the unphosphorylated form of serine 153 may contribute to the epitope recognized by this antibody.

To determine whether PAK1 phosphorylated NET1 on serine 152 in cells, HEK 293 cells were transfected with HA epitope-tagged NET1ΔN, with or without constitutively active PAK1. The cells were lysed in RIPA buffer, the NET1ΔN was immunoprecipitated, and the precipitates were immunoblotted with the anti-pSer-152 antibody. As shown in Fig. 5A, NET1ΔN exhibited a low level of phosphorylation on serine 152 in serum-starved cells (lane 2). Co-expression of constitutively active PAK1 (PAK1*), on the other hand, strongly stimulated the phosphorylation of NET1ΔN on serine 152 (compare lanes 2 and 3). Furthermore, the ability of Rac1 to stimulate the phosphorylation of NET1ΔN on serine 152 was dependent on the activation of endogenous PAKs 1–3 (24, 34, 35), these data indicate that Rac1 requires the activity of one or more endogenous PAKs to stimulate the phosphorylation of NET1ΔN on serine 152. We also tested whether Rac1 would stimulate the phosphorylation of full-length NET1 on serine 152 in cells (compare lanes 2 and 3), although the degree of phosphorylation was less than that observed for NET1ΔN. It is not clear why full-length NET1 was phosphorylated less efficiently than NET1ΔN. One possible explanation is that a substantial portion of full-length NET1 is localized to the nucleus and therefore may be inaccessible to PAK1. Alternatively, the amino-terminal truncation that makes NET1ΔN oncogenic may expose serine 152 for phosphorylation by PAK1 (see “Discussion”). Nevertheless, these data clearly demonstrate that PAK1 phosphorylates both NET1ΔN and full-length NET1 in cells.

We then tested whether Rac1 could stimulate the phosphorylation of NET1 on serine 152, and whether this depended on endogenous PAK activity. Thus, HEK 293 cells were transfected with NET1ΔN, minus or plus constitutively active Rac1 (V12Rac1). The NET1ΔN was then immunoprecipitated and tested for phosphorylation on serine 152 by Western blotting. As shown in Fig. 6A, expression of V12Rac1 strongly stimulated the phosphorylation of NET1ΔN on serine 152 (compare lanes 2 and 3). Furthermore, the ability of Rac1 to stimulate the phosphorylation of NET1ΔN on serine 152 was dependent on endogenous PAK activity, because co-expression of the autoinhibitory domain from PAK1 blocked Rac1-dependent phosphorylation of NET1ΔN (compare lanes 3 and 4). Given that expression of this autoinhibitory domain will block the activation of endogenous PAKs 1–3 (24, 34, 35), these data indicate that Rac1 requires the activity of one or more endogenous PAKs to stimulate the phosphorylation of NET1ΔN on serine 152. We also tested whether Rac1 would stimulate the phosphorylation of full-length NET1 on serine 152. As shown in Fig. 6B, expression of constitutively active Rac1 also stimulated full-length NET1 phosphorylation at this site, and this was dependent on the activation of endogenous PAKs. As was the case for phosphorylation by constitutively active PAK1, the phosphorylation of wild-type NET1 stimulated by V12Rac1 was less robust than for NET1ΔN. However, these data plainly demonstrate that both full-length NET1 and NET1ΔN are phosphorylated on serine 152 by endogenous PAKs in response to Rac1 activation.

The Cellular Activity of Full-length NET1 Is Regulated through Phosphorylation of Serines 152 and 153—Overexpression of full-length NET1 also stimulates actin reorganization, albeit to a much lower extent than NET1ΔN (23). To test if
serine 152 was a critical site for regulation of the full-length NET1 protein in cells, serum-starved NIH 3T3 cells were microinjected with expression vectors for HA epitope-tagged, full-length NET1, or NET1 variants bearing alanine or glutamate substitutions at the amino-terminal PAK1 phosphorylation sites. We also tested whether co-expression of constitutively active PAK1 would affect the ability of NET1 or NET1 S152A/S153A to elevate F-actin levels. As for experiments with NET1/H9004N, the cells were fixed 4 h after injection and stained for NET1 expression (anti-HA) and polymerized actin (F-actin), and the levels of F-actin were quantified by TRITC-phalloidin staining. As shown in Fig. 7A, overexpression of full-length NET1 stimulated a moderate degree of actin polymerization, with a nearly 2-fold increase in F-actin levels compared with the neighboring uninjected cells (Fig. 7C). In these experiments, the NET1 was localized predominantly to the nucleus, although a low level of cytoplasmic staining was observed. Because RhoA must be activated at the plasma membrane to stimulate actin polymerization, this suggests that only very low levels of cytoplasmic NET1 are required for effects on the actin cytoskeleton. When we tested the phosphorylation site mutants, we observed that glutamate substitution at the PAK1 phosphorylation sites (NET1 S152E/S153E) only modestly inhibited the ability of NET1 to stimulate stress fiber formation and F-actin accumulation (Fig. 7, middle panels, and C). On the other hand, alanine substitution at these sites significantly enhanced the ability of full-length NET1 to induce stress fiber formation and F-actin accumulation (Fig. 7A, bottom panels). In fact, the ability of NET1 S152A/S153A to stimulate actin polymerization was nearly as great as NET1/H9004N (compare Figs. 3B and 7C). Because alanine substitution of these sites did not affect the enzymatic activity of NET1 in vitro (see text for Fig. 2), these data suggest that a fraction of the full-length NET1 that localizes to the cytoplasm in these cells is negatively regulated by phosphorylation of serines 152 and 153. This would not be unexpected, because NIH 3T3 cells exhibit a high level of basal PAK activity even after serum starvation. Thus, the activity of full-length NET1 may be reduced in these cells compared with what would be possible in the absence of endog-
A.  

1 2 3 4 5  Lane 

Anti-pSer-152 

Anti-HA 

Anti-Myc 

− + + + NET1ΔN 

− + + + V12Rac1 

− − + + PAK1 AID 

B.  

1 2 3 4 5  Lane 

Anti-pSer-152 

Anti-HA 

Anti-Myc 

− + + + + NET1 

− − + + V12Rac1 

− − + + + PAK1 AID 

FIG. 6. Constitutively active Rac1 stimulates the phosphorylation of NET1ΔN and full-length NET1 on serine 152 in a PAK-dependent manner. A, HEK 293 cells were transfected with HA epitope-tagged NET1ΔN, minus or plus Myc epitope-tagged, constitutively active Rac1 (V12Rac1) and the PAK1 autoinhibitory domain (PAK1 AID). After serum starvation, the cells were lysed in RIPA buffer and the NET1ΔN proteins were immunoprecipitated with an antibody against the HA epitope and tested for phosphorylation on serine 152 by Western blotting. The panels, from top to bottom, show a membrane probed with the anti-p-Ser-152 antibody, the same membrane reprobed with an anti-HA antibody, and a Western blot of the cell lysate to detect Myc epitope-tagged Rac1 expression. B, HEK 293 cells were transfected with HA epitope-tagged full-length NET1, minus or plus Myc epitope-tagged V12Rac1 and the PAK1 AID, and tested for phosphorylation on serine 152 as described in A.

PAK activity. Even so, these data still demonstrate that serines 152 and 153 are critical to the ability of full-length NET1 to stimulate actin polymerization, and suggest that phosphorylation of these sites down-regulates NET1 activity in the cell.

To determine whether PAK1 directly regulated the activity of full-length NET1, we co-injected constitutively active PAK1 with full-length NET1 or NET1 containing alanine substitutions at the NH2-terminal phosphorylation sites (NET1S152A/S153A). As shown in Fig. 7B, coexpression of active PAK1 effectively blocked the ability of NET1 to stimulate actin stress fiber formation. Quantification of this effect showed that in these cells the level of F-actin staining was comparable with the surrounding, noninjected cells (Fig. 7C). On the other hand, NET1S152A/S153A was resistant to down-regulation by PAK1 (Fig. 7B), although the formation of stress fibers (which typically traverse most of the cell) was somewhat impaired (Fig. 7B, bottom panels). Because constitutively active PAK1 stimulates the dissolution of actin stress fibers (24), this suggests that active PAK1 expression affected the assembly of F-actin into stress fibers, but did not affect the ability of NET1 to stimulate the generation of new actin filaments per se. Thus, these results demonstrate that PAK1 negatively regulates the activity of full-length NET1 only when serines 152 and 153 are intact.

DISCUSSION

In this study we have identified NET1 as a novel PAK1 substrate. Specifically, NET1 is phosphorylated by PAK1 on serines 152, 153, and 538 in vitro, and on serine 152 in cells. Furthermore, expression of constitutively active Rac1 stimulates the phosphorylation of NET1 on serine 152 in a PAK-dependent manner. Three separate findings indicate that phosphorylation of this site negatively regulates the catalytic activity of NET1 toward RhoA. First, substitution of serines 152 and 153 with glutamate residues, which act as phospho-mimetics for many proteins, negatively regulates the enzymatic activity of NET1 toward RhoA in vitro. Second, NET1S152E/S153E is impaired in its ability to stimulate F-actin accumulation. Lastly, coexpression of constitutively active PAK1 with NET1 blocks the ability of full-length NET1 to stimulate actin stress fiber formation only when serines 152 and 153 are intact, indicating that PAK1 down-regulates NET1 activity in cells through the phosphorylation of these sites.

The biochemical mechanism by which phosphorylation of serines 152 and 153 inhibits the GDP exchange activity of NET1 is not clear, but may be because of disruption of the hydrophobic core within a novel NH2-terminal extension of the NET1 Dbl domain. This extension was first identified as a region of homology between NET1 and a subset of Rho GEFs (22), and its role was recently characterized in the crystal structure of RhoA with the related Rho GEF LARG (36). In LARG this NH2-terminal extension consisted of two small α-helices (αN1 and αN2) that directly interacted with the switch 1 domain of RhoA (36). Furthermore, deletion of this extension, or mutation of a key tryptophan residue within the extension inhibited the enzymatic activity of LARG by nearly 80% in vitro. Thus, phosphorylation of this segment in NET1 may down-regulate its enzymatic activity by blocking interaction with the switch 1 domain of RhoA. In support of this hypothesis, we have observed that the region between residues 122 and 155 of NET1 is of critical importance to the catalytic activity of NET1. For example, although NET1ΔN (amino acids 122–595) is fully active as an exchange factor in vitro, a smaller protein lacking amino acids 122–155 (NET1(156–595)) is significantly impaired in its GDP exchange activity toward RhoA.

By producing an antibody that specifically recognizes NET1 phosphorylated on serine 152, we have successfully demonstrated that PAK1 stimulates its phosphorylation in cells. We have also shown that expression of constitutively active Rac1 led to serine 152 phosphorylation, and that this depended on the activation of endogenous PAKs. A requirement for endogenous PAK activity was proven by co-expressing the autoinhibitory domain from PAK1, which blocks the activation of group I PAKs (isoforms 1–3) (24, 34, 35). Because HEK 293 cells do not express PAK3, the isoforms that phosphorylate NET1 in these cells must be PAKs 1 and/or 2. Both of these kinases play important roles in growth factor-stimulated, Rac1- and Cdc42-dependent signaling to the actin cytoskeleton. In addition, activation of PAK2 by caspases is important for some of the morphological changes associated with apoptosis (reviewed by Bokoch (37)). Thus, down-regulation of NET1 activity by PAKs may be critical for ligand-stimulated morphological changes associated with a number of extracellular stimuli.

Previous work has shown that the ability of Rac1 to negatively regulate RhoA activity is important for controlling Rac1-mediated generation of new actin filaments per se. Thus, these results demonstrate that PAK1 negatively regulates the activity of full-length NET1 only when serines 152 and 153 are intact.

3 Qin, H., Carr, H. S., Wu, X., Muallem, D., Tran, N. H., and Frost, J. A. (2005) J. Biol. Chem. 280, 7603–7613.
dependent cellular differentiation and transformation (28–30),
and recently a biochemical mechanism that contributes to this
activity has been described (38). Specifically, Rac1 was shown
to stimulate the formation of reactive oxygen species which, in
turn, caused the inactivation of the low molecular weight pro-
tein tyrosine phosphatase LMW-PTP. This led to an increase in
the tyrosine phosphorylation and the activity of p190 Rho-
GTPase activating protein, which then down-regulated RhoA
activity. In addition, Dan et al. (39) have shown that expression
of PAK5 also down-regulates RhoA activity, although a mech-
anism accounting for this regulation was not identified (39). We
have not been able to demonstrate a global down-regulation of
RhoA activity following the expression of constitutively active
PAK1 (data not shown), making it unlikely that phosphoryla-
tion of NET1 by PAK1 accounts for the general decrease in
total GTP-RhoA levels observed by others. However, the down-
regulation of NET1 activity that we observe may provide for a
localized decrease in RhoA activation under conditions in
which NET1 normally regulates RhoA.

Our work also provides an added level of control to cellular
mechanisms that generate new actin filaments and regulate
their assembly into stress fibers. Previous work suggests that
in NIH 3T3 cells RhoA utilizes two key effectors in the produc-
tion of stress fibers, Rho kinase (ROCK) and the mammalian
Diaphanous-related formin mDia1 (33). ROCK is thought to
contribute to stress fiber assembly by phosphorylating pro-
tein, such as the myosin-binding subunit of protein phospha-
tase type 1, that regulate the bundling of actin filaments into
stress fibers, and its activation results in the formation of
stellate actin bundles contracted at the cell center (40). The
formin mDia1 is thought to contribute to stress fiber formation
by phosphorylating proteins, such as the myosin-binding subunit of protein phospha-
tase type 1, that regulate the bundling of actin filaments into
stress fibers, and its activation results in the formation of
stellate actin bundles contracted at the cell center (40). The
formin mDia1 is thought to contribute to stress fiber formation
by phosphorylating non-branched actin filaments, and the activities
of ROCK and mDia1 have been proposed to cooperate to pro-
duce fully functional stress fibers (41). In support of this, co-
expression of the so-called “activated” versions of ROCK and
mDia1 together can lead to a profound induction of stress fiber

![FIG. 7. Coexpression of constitutively active PAK1 blocks F-actin accumulation stimulated by expression of full-length NET1, but not by NET1 S152A/S153A.](image-url)
formication that mimics the effects of expression of artificially activated RhoA or NET1. In the present study, we observed that NET1 S152A/S153A co-expression with active PAK1 could "rescue" F-actin accumulation (quantified as TRITC-phalloidin staining), but not stress fiber assembly. This result suggests that PAK1 does not affect the ability of the formin to nucleate and elongate actin filaments but does interfere with or counteracts signals that drive filament assembly into stress fibers. Thus, the ability of PAK1 to interfere with stress fiber formation (stimulated by NET1) is likely because of disruption of ROCK-dependent effects on actin stress fiber formation.

Although NET1 potently stimulates actin polymerization in the cell, a role for NET1 in normal cell physiology has not yet been determined. Recent studies indicate that the cellular activity of overexpressed NET1 is controlled through nuclear localization, such that the full-length protein exists in the nucleus, sequestered from cytoplasmic and plasma membrane-bound pools of RhoA (23). Although the authors of this study were unable to demonstrate ligand-dependent export of full-length NET1 from the nucleus, they did show that the cytoplasmic, oncogenic form of NET1 (NET1

Plant Cell 12, 79–87 (2000)