Characterization of the Biochemical and Transforming Properties of the Neuroepithelial Transforming Protein 1*

Received for publication, October 26, 2004, and in revised form, December 15, 2004. Published, JBC Papers in Press, December 16, 2004, DOI 10.1074/jbc.M412141200

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Rho family small G proteins are key regulators of cytoskeletal organization and oncogenic transformation whose activation is controlled by a family of proteins known as guanine nucleotide exchange factors (GEFs). In this work we have characterized the structural and biological determinants for cytoskeletal regulation and cell transformation by the neuroepithelioma transforming gene 1 (NET1), which is a GEF specific for RhoA, but not Cdc42 or Rac1. Previously it was shown that the biological activity and nuclear localization of NET1 is controlled by its amino terminus. Here we demonstrate that the amino terminus of NET1 does not function as cis-acting autoinhibitory domain, nor does it affect the ability of full-length NET1 to stimulate actin stress fiber formation. We also show that the nuclear localization of NET1 is controlled by two separate domains within its amino terminus, only one of which contains the previously identified NLS sequences. Importantly, we find that the ability of NET1 to stimulate actin stress fiber formation does not correlate with its transforming activity, because NET1 proteins that potently stimulate stress fiber formation do not transform cells. Furthermore, the presence of a potential PDZ binding site in the C terminus of NET1 is critical to its ability to transform cells, but is not required for enzymatic activity or for effects on the actin cytoskeleton. Thus, these data highlight a divergence between the ability of NET1 to stimulate cytoskeletal reorganization and to transform cells, and implicate the interaction with PDZ domain-containing proteins as critical to NET1-dependent transformation.

Rho family small G proteins play critical roles in regulating many aspects of cell physiology, including cytoskeletal organization, cell motility, vesicle trafficking, cell cycle progression, and neoplastic transformation (1–4). They do so by acting as molecular switches, cycling between their active, GTP-bound and inactive, GDP-bound states. Once activated, they stimulate signaling in multiple pathways by binding to downstream effector proteins and modulating their activities. Currently at least 21 mammalian Rho family GTPases have been identified, with the Rac1, Cdc42, and RhoA proteins being the most thoroughly characterized (5).

The activities of wild type Rho family proteins are controlled by three classes of enzymes known as Rho guanine nucleotide exchange factors (GEFs)1, Rho GTPase-activating proteins, and Rho guanine nucleotide dissociation inhibitors (Rho GDIs) (6). Rho GEFs stimulate the dissociation of GDP from inactive Rho proteins, thereby promoting their accumulation in the active, GTP-bound state. Rho GTPase-activating proteins stimulate the intrinsic GTPase activity of Rho proteins, thus causing their inactivation. Rho guanine nucleotide dissociation inhibitors are cytosolic proteins that bind to inactive, GDP-bound Rho proteins and localize them to the cytosol. Within this regulatory network, it is the Rho GEFs that mediate Rho protein activation in response to extracellular ligands. Thus, elucidating the regulatory mechanisms controlling Rho GEF activity is critical to understanding how growth factors control Rho-dependent signaling.

The human Rho GEF family contains over 50 genes, all of which contain two conserved domains known as the Dbl (diffuse B-cell lymphoma) homology (DH) and pleckstrin homology (PH) domains (7–9). The DH domain is named after the first mammalian Rho GEF identified and accounts for the enzymatic activity of these proteins. The function of the PH domain varies depending upon the Rho GEF being analyzed. Biochemical roles for the PH domain include subcellular targeting, stabilization of the DH domain and enhancement of its activity, and regulation of the interaction with substrate Rho proteins (10–16). All DH domain-containing Rho GEFs require the presence of a PH domain for activity in the cell. Rho GEFs exhibit distinct specificities for different Rho family small G proteins in vitro and in cells. For example, the Rho GEF Vav activates RhoA, Rac1, and Cdc42 equally well, whereas the Rho GEF Tiam1 is specific for Rac1 (7, 8).

The biochemical mechanisms controlling the activities of most Rho GEFs have not been characterized. However, for those few that have been studied, two common regulatory themes have emerged. First, many Rho GEFs contain autoinhibitory domains that negatively regulate their enzymatic activities in the absence of an appropriate stimulus. When these domains are deleted, the Rho GEFs are constitutively activated in vitro and are oncogenic in cell transformation assays (8, 17). Physiological mechanisms for release from autoinhibition in

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1 The abbreviations used are: GEF, guanine nucleotide exchange factor; Dbl, diffuse B-cell lymphoma; DH, Dbl homology; PH, pleckstrin homology; NET1, neuroepithelioma transforming gene 1; wt, wild type; NLS, nuclear localization signal; NES, nuclear export signal; GST, glutathione S-transferase; AMP-PNP, adenosine 5′-(α,γ-imino)triphosphate; GTPγS, guanosine 5′-3-O-(thio)triphosphate; PBS, phosphate-buffered saline; HA, hemagglutinin.

* This work was supported by Career Development Award 993008N from the American Heart Association (to J. A. F.) and Research Scholar Award RSG-02-192-01-TBE from the American Cancer Society (to J. A. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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clude altered protein-protein interactions, binding to phosphatidylinositol phosphates, and site-specific phosphorylation. For example, Vav is activated by phosphorylation on tyrosine 174 and binding to phosphatidylinositol 3,4,5-triphosphate, which together block the effects of an amino-terminal autoinhibitory domain (12, 18–21). A second common mechanism for controlling Rho GEF activity is through the regulation of intracellular localization. This often entails translocation from the cytosol to receptor complexes at the plasma membrane. For example, the Rho GEF Tiam1 is recruited to the plasma membrane upon stimulation of cells with serum growth factors (11, 22).

The Rho GEF neuroepithelioma transforming gene 1 (NET1) codes for a 595-amino acid protein that consists of tandem DH and PH domains, flanked by amino-terminal and carboxy-terminal extensions of 155 and 93 amino acids, respectively. It was originally cloned as a transforming gene in a screen for novel oncogenes using an NIH 3T3 cell focus formation assay (23). The oncogenic form of NET1 cloned from this screen lacked the first 145 amino acids of the wild type protein, which was critical to its transforming activity, because overexpression of wild type NET1 (wt NET1) was not transforming. Later it was shown that deletion of the first 121 amino acids of NET1 also produced a protein that was transforming in NIH 3T3 focus formation assays and that this form of NET1 (NET1ΔN) catalyzed GDP exchange on RhoA, but not Rac1 or Cdc42 in vitro (24). These authors also demonstrated that expression of NET1ΔN stimulated the formation of actin stress fibers, which is a hallmark of RhoA activation, and caused the phosphorylation of c-Jun by the JNK family of mitogen-activate protein kinases when co-expressed in cells. These data indicated that NET1 was a RhoA-specific GEF that was negatively regulated by its amino terminus. However, mechanisms controlling the activity of wt NET1 were not described.

Recently it was shown that wt NET1 activity in the cell is controlled through subcellular localization, such that wt NET1 is localized to the nucleus when ectopically expressed in cells (25). In this work it was demonstrated that the amino-terminal 121 residues of wt NET1 targets it to the nucleus through the actions of two nuclear localization signal (NLS) sequences contained within this region, and that mutation of these NLS sequences resulted in a partial redistribution of full-length NET1 to the cytosol (25). Furthermore, in this same study the PH domain of NET1 was shown to contain a nuclear export signal (NES) sequence. These data suggested that the ability of wt NET1 to regulate RhoA activation is negatively regulated by nuclear localization. However, despite considerable efforts by these authors, extracellular stimuli controlling the export of NET1 from the nucleus were not identified.

To better understand the regulatory mechanisms controlling NET1 activity, we have characterized the requirement for distinct domains within NET1 in controlling its enzymatic activity, subcellular localization, and ability to transform cells. Our results demonstrate that two separate domains within the NET1 amino terminus target it to the nucleus, only one of which contains the previously identified NLS sequences. The contribution of this second domain to nuclear localization is critical, because the ability of NET1 to stimulate cytoskeletal rearrangement and cell transformation is dependent upon localization to the cytosol. In addition, we find that the amino terminus of NET1 does not negatively regulate its enzymatic activity in vitro, nor does it down-regulate the ability of NET1 to control actin cytoskeletal organization. The ability of NET1 proteins to stimulate actin stress fiber formation does not correlate with transforming activity, such that NET1 proteins that are extremely potent at stimulating stress fiber formation do not transform cells. Thus, these data highlight a divergence between the ability of NET1 to stimulate cytoskeletal reorganization and to transform cells. This point is emphasized by our finding that a potential PDZ binding site in the C terminus of NET1 must be present for efficient transformation of cells, but is not required for enzymatic activity or for effects on the actin cytoskeleton.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections—**HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen) plus 100 units/ml streptomycin/penicillin (Invitrogen). Swiss 3T3 and NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (Invitrogen) plus 100 units/ml streptomycin/penicillin (Invitrogen). HEK 293 and NIH 3T3 cells were transfected using Lipofectamine Plus (Invitrogen) with 2 μg of DNA, according to the manufacturer’s instructions.

**Plasmids and Recombinant Proteins—**For eukaryotic expression all cDNAs were contained in pEFHA (24). Wild type, mouse NET1, and NET1ΔN (amino acids 122–595) were as described previously (24). For NET1 plus NES, the nuclear export sequence (NES) from PKI (LALKLAGLDI) (26) was added onto the carboxy terminus of wild type NET1. Thus, wt NET1 plus NES, the nuclear localization signal (NLS) from the SV40 large T antigen (PKKKRKV) was added onto the carboxy terminus of NET1ΔN by PCR (27). The mouse NET1ΔN cDNA was cloned from the Mammalian Gene Collection (ATCC), and was subcloned into pEFHA by PCR. NET1 156–595, NET1 156–501, NET1(122–501), NET1ΔN, and NET1ΔNaC4, and NET1ΔNaAla were amplified by PCR and ligated into pEFHA. All cDNAs amplified by PCR were sequenced in their entirety to confirm correct amplification.

Glutathione S-transferase (GST)-NET1 and GST-wild type RhoA fusion proteins were created by subcloning each cDNA into pGEXKG (Amersham Biosciences). BL21(DE3) Escherichia coli (Strategene) were transformed with the plasmids, and the proteins were expressed by addition of 400 μM isopropyl 1-thio-β-D-galactopyranoside after the culture had reached an A600 = 0.8. Incubation with isopropyl 1-thio-β-D-galactopyranoside continued for 4 h at 37 °C. Cells were pelleted by centrifugation and stored at −80 °C. Cells were lysed by addition of lysozyme followed by sonication, insoluble proteins were pelleted by centrifugation (30 min at 35,000 × g, 4 °C), and GST fusion proteins were purified by glutathione-agarose affinity chromatography. Purified fusion proteins were dialyzed into dialysis buffer (20 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol) and stored at −80 °C. For purification of GST-wt RhoA, 10 μM GDP was included in all purification and dialysis steps.

**Guanine Nucleotide Exchange Assays—**GEF assays were performed essentially as described (28), using equimolar amounts of GST-NET1 proteins. Briefly, GST-wt RhoA was preloaded for 5 min at room temperature with GDP loading buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM NaCl, 1 mM EDTA, 100 mM AMP-PNP, 10 mM GDP, 1 mM dithiothreitol). Loading was terminated by the addition of MgCl2 to a final concentration of 10 mM. Approximately 700 mM GDP-wt RhoA was then incubated with 300 nM NET1 protein in GTPγS buffer (20 mM Tris-HCl, pH 8.0, 1 μM GDP, 5 μM GTPγS, 0.5 μM [γ32P]GTPγS bound to GST-wt RhoA was recovered by filtration through BAS5 nitrocellulose filters (Schleicher and Schuell). The filters were washed with termination buffer, and bound nucleotide was quantified by liquid scintillation.

**Microinjection and Indirect Immunofluorescence—**Swiss 3T3 cells were plated on glass coverslips and allowed to grow to 30% confluence. Prior to injection the cells were incubated for 24 h in Dulbecco’s modified Eagle’s medium plus 100 units/ml penicillin/streptomycin without serum. All plasmids were injected into the nuclei of cells at 0.2–0.5 μg/ml using a Zeiss Axiovert S100 microscope. Four hours after injection the cells were fixed with 3.7% formaldehyde in PBS at 37 °C for 5 min. Cells were then permeabilized with 0.2% Triton X-100 in PBS at room temperature for 5 min. To detect expressed, HA-tagged NET1 proteins the cells were incubated with 2 μg/ml mouse anti-HA (Santa Cruz Biotechnology) in PBS plus 0.2% Tween 20 and 1 mg/ml bovine serum albumin (PBST plus bovine serum albumin) for 1 h at 37 °C. After washing 3 × 5 min with PBST,
the cells were incubated with Texas Red-conjugated donkey anti-mouse (Jackson Laboratories) and fluorescein isothiocyanate-phalloidin (Sigma) (to detect F-actin) diluted in PBS+ Plus bovine serum albumin, for 1 h at 37 °C. The cells were washed 3 × 5 min with PBS and once with distilled water, and then mounted on glass microscope slides with FluorSave reagent (Calbiochem). Fluorescent cells were visualized with either a Zeiss Axioskop with a mounted Hamamatsu C4742-95 digital camera, or a Zeiss 510 Meta confocal microscope. Images were recorded using MetaVue or MetaMorph software (Universal Imaging).

**Subcellular Fractionation**—NIH 3T3 cells in 10-cm dishes were transfected with HA epitope-tagged NET1 expression vectors using Lipofectamine Plus. Prior to harvest the cells were serum-starved in Dulbecco’s modified Eagle’s medium without calf serum for 24 h. Cells were then washed once with PBS, resuspended in 0.5 ml of hypotonic lysis buffer (50 mM Tris (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 80 μM β-glycerophosphate, 1 mM sodium orthovanadate), and incubated on ice for 10 min. The cells were then lysed using 20 strokes in a Dounce homogenizer, and nuclei and unbroken cells were pelleted by centrifugation for 10 min at 1,500 × g, 4 °C. The supernatant was centrifuged for 30 min at 100,000 × g, 4 °C to isolate the cytosol. The nuclear pellets were washed once with 1 ml of hypotonic lysis buffer, and nuclei were pelleted again by centrifugation at 14,000 × g, 10 min at 4 °C. The nuclear pellets were then solubilized by resuspension in 2% SDS buffer (20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 2% SDS, 50 mM NaF, 80 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride), and DNA was sheared by drawing through a 20-gauge needle 10 times. Insoluble material was pelleted by centrifugation for 10 min at 16,000 × g at room temperature, and the supernatant was saved. The concentration of proteins present in the nuclear and cytoplasmic fractions was determined by bichinchoninic acid assay (Pierce), and equal amounts of each fraction were resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane (Amersham Biosciences), and HA-epitope-tagged NET1 proteins were detected by Western blotting using mouse or rabbit anti-HA (Santa Cruz Biotechnology), followed by HRP-conjugated donkey anti-mouse or anti-rabbit (KPL). Western blots were developed using enhanced chemiluminescence and visualized with x-ray film (Kodak). The purity of the nuclear and cytoplasmic fractions was determined by Western blotting with rabbit anti-lamin B1 (Santa Cruz Biotechnology) and rabbit anti-superoxide dismutase-1 (Santa Cruz Biotechnology), which are localized to the nuclear and cytoplasmic compartments, respectively.

**Foci Formation Assays**—Foci formation assays were performed essentially as described previously (29). Briefly, low passage NIH 3T3 cells were transfected in six centimeter dishes (BD Biosciences) with 2 μg of DNA using Lipofectamine Plus. All transfections were performed in duplicate. Cultures were refed every 3–4 days with growth media for a total of 16 days. Cells were then fixed with 10% acetic acid in water for 10 min at room temperature and then stained with 0.4% crystal violet (Sigma) in 10% ethanol in water for 10 min at room temperature. After staining, the cells were washed several times with water, allowed to air dry, and foci were counted.

**RESULTS**

**The Amino Terminus of NET1 Does Not Regulate Its Enzymatic Activity**—The enzymatic activity of many Rho GEFs is regulated through the actions of a cis-acting autoinhibitory domain (7, 8). Because deletion of the amino terminus of NET1 creates a protein that is transforming, we examined whether the enzymatic activity of NET1 was also regulated by an amino-terminal autoinhibitory domain. Thus, these data indicate that NET1 does not contain a negative regulatory domain in its amino terminus that directly controls its enzymatic activity, and sets NET1 apart from other Rho GEFs such as Vav, and Dbl, which do contain autoinhibitory domains (30, 31).

**The Splice Variant NET1A Displays a Subcellular Localization and Cellular Activity That Is Distinct from wt NET1**—Given that NET1 localization in the cytosol is critical to its ability to regulate the actin cytoskeleton (25), we tested whether NET1A, which lacks the amino-terminal NLS sequences present in wt NET1, was cytosolic and thus behaved as a hyperactive form of NET1. To test this, we examined the ability of NET1A to elicit changes in the actin cytoskeleton as an indicator of NET1 activity. Thus, serum-starved Swiss 3T3 cells were microinjected with eukaryotic expression vectors for a control protein (β-galactosidase), wt NET1, NET1ΔN, or NET1A. Four hours later the cells were fixed and stained for expression of these proteins and for F-actin. As shown in Fig. 2A, serum-starved Swiss 3T3 cells are usually flat, stain positive for cortical F-actin, and exhibit few actin stress fibers. Injection of a control plasmid that drives the expression of β-galactosidase did not significantly affect this phenotype (panels a and b). Similarly, expression of wild type NET1 did not stimulate actin stress fiber formation (panels c and d). The wt NET1 was predominantly nuclear, although some cytoplasmic staining was observed in cells expressing very high amounts of NET1 (data not shown). In these cells some increase in F-actin staining was noted, as previously observed (25). On the other hand, expression of NET1ΔN caused the formation of numerous actin stress fibers in every injected cell (panels e and f). These cells typically were less spread out than the surrounding cells, and the NET1ΔN protein was localized in both the nucleus as well as the cytoplasm. Importantly, when we tested for effects of NET1A expression, we found that it also potently stimulated actin stress fiber formation (panels g and h). In fact, NET1A was as efficient as NET1ΔN at stimulating activity toward purified GST-RhoA, which was measured through the binding of the nonhydrolyzable GTP analog GTP-γS. As shown in Fig. 1, RhoA alone exhibited a modest ability to bind to GTP-γS that was greatly enhanced by the addition of each NET1 protein. Furthermore, each NET1 protein exhibited a nearly equivalent activity toward RhoA in vitro. Thus, these data indicate that NET1 does not contain a negative regulatory domain in its amino terminus that directly controls its enzymatic activity, and sets NET1 apart from other Rho GEFs such as Vav, and Dbl, which do contain autoinhibitory domains (30, 31).

**FIG. 1.** The NET1 amino terminus does not contain an autoinhibitory domain. The NET1 proteins shown were tested for their ability to stimulate [γ-32P]GTPγS binding by wt RhoA in vitro in GDP exchange assays. NET1 proteins and wt RhoA were purified from E. coli as GST fusion proteins. Shown is the average of three independent experiments. Errors are mean ± S.E.
the polymerization of actin stress fibers, although NET1A expression did not cause cells to round up in the way that NET1AN did. Staining for the expressed NET1A protein demonstrated that there were slightly higher levels of cytoplasmic expression as compared with wt NET1; however, the majority of the NET1A was localized to the nucleus.

We confirmed the subcellular localization of these NET1 proteins by subcellular fractionation. For these experiments NIH 3T3 cells were transfected with the HA-epitope-tagged NET1 constructs shown. The cells were serum-starved, lysed in a hypotonic buffer, and then separated into nuclear and cytoplasmic fractions. Equal amounts of these fractions were resolved by SDS-PAGE, and the presence of NET1 proteins was detected by Western blotting using an antibody specific for the HA-epitope. We confirmed the validity of our fractionation procedure by blotting for lamin B1 and superoxide dismutase-1, which are localized to the nuclear and cytoplasmic compartments, respectively. This analysis confirmed that the majority of wt NET1 was localized to the nucleus (Fig. 2B). NET1A was also largely contained in the nuclear fraction, although there was slightly more NET1A in the cytosolic fraction as compared with wt NET1. The majority of NET1AN, on the other hand, was localized to the cytoplasm. Thus, taken together these data indicate that NET1A tends to localize to the cytoplasm to a slightly higher degree than wt NET1, and this leads to an increased ability of NET1A to stimulate actin polymerization in the cell.

Because NET1A lacks the NLS sequences present in wt NET1 and does not contain an identifiable NLS in the unique portion of its amino terminus, it is unclear as to how NET1A localizes to the nucleus. The DH, PH, and C-terminal regions of wt NET1 do not contribute to its nuclear localization (25), suggesting that the amino-terminal region conserved between wt NET1 and NET1A may be important in targeting both forms of NET1 to the nucleus. Therefore, we examined which regions in the amino terminus of wt NET1 were required to direct its nuclear localization. This was examined by indirect immunofluorescence in microinjected cells, as well as by subcellular fractionation of transfected cells. In this analysis we tested wt NET1, the amino terminus of wt NET1 (NET1-(1–155)), the portion of the amino terminus lacking in NET1AN (NET1-(1–121)), and the unique portion of wt NET1 not found in NET1AN (NET1AN-(1–85)). This region of NET1 (NET1-(1–85)) contains both of the NLS sequences previously identified (25). We also injected or transfected cells with vectors coding for the unique amino terminus of NET1AN (NET1AN-(1–31)), as well as portions of the region common to both wt NET1 and NET1AN (NET1AN-86–121 and NET1AN-86–155). Unfortunately, we could not detect the expression of these proteins either by indirect immunofluorescence or Western blots, so we were unable to analyze their localization.

We first determined the ability of the microinjected constructs to localize to the nucleus by indirect immunofluorescence. As shown in Fig. 3A, wt NET1, NET1-(1–155), and NET1-(1–121) were largely contained within the nuclei of the expressing cells (panels a–c, respectively). However, NET1-(1–85) was localized to both the nucleus and cytoplasm of expressing cells (panel d). These results, which are quantified in Fig. 3B, show that in >80% of the expressing cells, wt NET1 and NET1-(1–155) were found exclusively in the nucleus. NET1-(1–121) was slightly less efficient at nuclear localization than NET1-(1–155), but the difference was not statistically significant. On the other hand, the vast majority of cells injected with the NET1-(1–85) plasmid showed both nuclear and cytoplasmic localization. We confirmed these results by subcellular fractionation. As shown in Fig. 3C, NET1-(1–85) was evenly distributed between the nuclear and cytoplasmic fractions, whereas the majority of NET1-(1–155) and NET1-(1–121) were present only in the nuclear fraction. Thus, these results demonstrate that the NLS sequences present in the domain unique to wt NET1 (amino acids 1–85) are not sufficient to direct NET1 exclusively to the nucleus, and that the amino-terminal region common to both wt NET1 and NET1AN (amino acids 86–121) is necessary for efficient nuclear localization.

Cytoplasmic Localization of Full-length NET1 Stimulates Actin Stress Fiber Formation in Cells but Is Insufficient for Cell Transformation—Our finding that wt NET1 and NET1AN exhibit similar GEF activities toward RhoA in vitro suggests that
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A.

![Images](http://www.jbc.org/)

**B.**

| NET1 Protein | Exclusively Nuclear Staining (%) |
|--------------|----------------------------------|
| wt           | 0                                |
| 1-155        | 80                               |
| 1-121        | 60                               |
| 1-85         | 40                               |

**C.**

![Images](http://www.jbc.org/)

**Fig. 3.** Nuclear localization of wt NET1 requires sequences contained within two distinct domains of the amino terminus. A, quiescent Swiss 3T3 cells were microinjected with expression vectors for HA-epitope tagged wt NET1 (a), NET1-(1–155) (b), NET1-(1–121) (c), or NET1-(1–85) (d). 4 h later the cells were fixed and stained for NET1 expression using an antibody to the HA-epitope. Shown are representative images. B, quantification of the results from Fig. 3A. Results are shown as the percentage of cells expressing the NET1 proteins exclusively in the nucleus. Errors are mean ± S.E. C, subcellular fractionation of NIH 3T3 cells transfected with the NET1 proteins shown. Equal amounts of nuclear and cytoplasmic fractions were analyzed. The panels are Western blots for HA-tagged NET1 proteins present in the nuclear and cytoplasmic fractions (top and bottom panels, respectively).

the ability of NET1ΔN to stimulate actin cytoskeletal rearrangement and to transform cells is the result of an inappropriate localization to the cytoplasm. To determine whether the amino terminus negatively regulates the ability of NET1 to stimulate cytoskeletal reorganization or cell transformation, we examined the effect of redirecting full-length NET1 to the cytoplasm. We also tested whether relocation of NET1ΔN to the nucleus blocked its effects on cells. To induce the expression of full-length NET1 in the cytoplasm, we added a nuclear export signal (NES) sequence, derived from the NES of the PKI protein (26), to the C terminus of NET1 (NET1 plus NES). We used this approach rather than mutation of the NLS sequences in wt NET1 because of the concern that these mutations might alter the regulation of NET1 activity apart from effects on nuclear localization. Similarly, the nuclear localization of NET1ΔN was induced by the addition of a nuclear localization signal (NLS) sequence, derived from the SV40 large T antigen (27), to the carboxyl terminus of NET1ΔN (NET1ΔN plus NLS).

We first determined whether addition of the NES or NLS sequences altered the enzymatic activities of each NET1 protein in vitro. To resolve whether the activity of NET1 plus NES was intact, HEK293 cells were transfected with HA-epitope tagged, wt NET1 or NET1 plus NES. The NET1 proteins were then immunoprecipitated with an anti-HA antibody and examined for their relative abilities to stimulate the binding of GTP-γS by RhoA. These proteins were immunoprecipitated from transfected cells rather than purified as GST fusion proteins from E. coli, because the GST-NET1 plus NES protein was insoluble in bacteria. In these assays we found that wt NET1 and NET1 plus NES stimulated GTP-γS binding by RhoA to similar extents (Fig. 4A). The stimulation of GTP-γS binding by RhoA was dependent upon the presence of the NET1 proteins, because immunoprecipitates from nontransfected cell lysates had no effect (data not shown). In addition, roughly equivalent amounts of wt NET1 and NET1 plus NES were immunoprecipitated, as determined by Western blotting (data not shown). Thus, this experiment indicates that addition of the NES to wt NET1 did not adversely affect its activity toward RhoA in vitro. To test whether addition of the NLS to NET1ΔN affected its activity, NET1ΔN and NET1ΔN plus NES were produced in E. coli as GST fusion proteins and purified by glutathione-agarose affinity chromatography. As shown in Fig. 4B, addition of the NLS to NET1ΔN did somewhat decrease its ability to stimulate GTP-γS binding by RhoA. However, this protein still exhibited significant activity toward RhoA in vitro, thus allowing further analysis of its activity in cells.

We then examined the relative abilities of each protein to stimulate actin stress fiber formation, as well as their localization within the cell. For these assays each construct was microinjected into quiescent Swiss 3T3 cells, and its effect on cell morphology was monitored 4 h later. As shown in Fig. 4C, expression of the NET1 plus NES protein resulted in the formation of numerous actin stress fibers irrespective of the level of protein expressed. Similar to cells expressing NET1ΔN, NET1 plus NES-expressing cells were also somewhat shrunken in size. A greater degree of cytoplasmic localization was apparent for NET1 plus NES as compared with wt NET1, although the majority of the protein was still nuclear. This was not unexpected, because NET1 plus NES contains the two NLS sequences present in wt NET1. The level of NET1 plus NES redirected to the cytoplasm was apparently very low, because we were unable to demonstrate a clear increase in cytoplasmic NET1 plus NES protein as compared with wt NET1 by subcellular fractionation (Fig. 4E). Nevertheless, this experiment shows that a modest relocation of full-length NET1 to the cytoplasm is sufficient to stimulate actin stress fiber formation, and that the ability of NET1 to stimulate actin cytoskeletal reorganization is not affected by the presence of its amino terminus.
We then tested whether addition of the NLS sequence to NET1ΔN affected its ability to stimulate actin reorganization. Thus, cells were microinjected with NET1ΔN or NET1ΔN plus NLS expression vectors, and tested for localization of the NET1 proteins and for effects on the actin cytoskeleton. As shown in Fig. 4D, the majority of the NET1ΔN plus NLS protein was...
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Fig. 5. Cytoplasmic localization of NET1 is not sufficient for cellular transformation. NIH 3T3 cells were transfected with expression vectors coding for the NET1 proteins shown to examine focus formation activity. Sixteen days later the cells were fixed and stained with crystal violet to identify cell foci. Values represent the number of foci formed per dish expressed as a percentage of those observed with transfection of NET1ΔN, and represent the average of four independent experiments. Errors are mean ± S.E.

localized to the nucleus, although a small amount of cytoplasmic expression was still observed (panel c). This localization was quite distinct from the NET1ΔN protein, which was expressed throughout the cell (panel a). The increase in nuclear localization of NET1ΔN plus NLS as compared with NET1ΔN was confirmed by subcellular fractionation (Fig. 4E). Importantly, the cells expressing NET1ΔN plus NLS did not exhibit an increase in actin stress fiber formation (Fig. 4D, panel d). The lack of stress fiber formation is unlikely to be due to the reduced exchange activity of NET1ΔN plus NLS, because its enzymatic activity was similar to that of the NET1 truncation mutants 156–595 and 156–501, which were effective at stimulating actin stress fiber formation (see Figs. 6B, 6C, and 7A). Thus, these experiments confirm previous results indicating that the ability of NET1 to stimulate actin stress fiber formation is determined primarily by its localization to the cytosol (25).

We then tested whether subcellular localization was the only determinant of the transforming activity of NET1. As a measure of transforming activity, we monitored the ability of NET1 proteins to stimulate focus formation in NIH 3T3 cells. For evaluative purposes, the ability of each NET1 protein to stimulate focus formation was compared with NET1ΔN, which was previously shown to be oncogenic in this assay (24). As shown in Fig. 5, both wt NET1 and NET1ΔN plus NLS were ineffective at stimulating focus formation in these cells. This correlates with their nuclear localization and their inability to stimulate actin cytoskeletal reorganization. Unexpectedly, NET1ΔA and NET1 plus NES were also ineffective at stimulating focus formation. This was surprising, because both proteins were as effective as NET1ΔN at stimulating actin stress fiber formation. Thus, these data indicate that the transforming activity of NET1 cannot be correlated with its ability to stimulate actin stress fiber formation.

Regions Outside the DH and PH Domains Are Required for Full Activity of NET1 in Vitro and in Cells—The amino terminus of NET1 targets it to the nucleus. However, additional contributions of the amino- and carboxyl-terminal domains to NET1 function are not known. Furthermore, these domains do not exhibit homology to any known protein interaction domains and are not conserved among other Rho GEFs. Therefore, we examined whether these regions contributed to the enzymatic activity of NET1 in vitro, or to its ability to stimulate cytoskeletal rearrangement or cellular transformation. To test the contribution of these domains to the enzymatic activity of NET1 in vitro, we produced as GST fusion proteins in E. coli truncation mutants containing the isolated DH and PH domains (NET1-(156–501)), the DH/PH domains plus the carboxyl terminus (NET1-(156–595)), and the DH/PH domains plus the segment of the amino terminus contained in NET1ΔN (NET1-(122–501)) (Fig. 6A). Equimolar amounts of each protein were then assayed for their relative abilities to stimulate GTPγS binding by RhoA in vitro. As shown in Fig. 6B, both NET1-(156–501) and NET1-(156–595) were less efficient at stimulating GTPγS binding by RhoA than wild type NET1. NET1-(122–501), on the other hand, was as effective as wild type NET1 at stimulating GTPγS binding by RhoA than wild type NET1. These assays also demonstrate that the C-terminal portion of NET1 (amino acids 502–595) is not required for NET1 activity toward RhoA in vitro.

We then examined the intracellular distribution of these proteins and their relative abilities to stimulate actin stress fiber formation when microinjected into quiescent Swiss 3T3 cells. As shown in Fig. 7A, each NET1 truncation mutant was localized to both the cytoplasm and the nucleus of the cell. Cell fractionation demonstrated that NET1-(156–595) was preferentially localized to the cytoplasmic fraction, whereas NET1-(156–501) and NET1-(122–501) were localized mainly to the nucleus (Fig. 7B). The nuclear localization of these two NET1 proteins may reflect the size of these proteins, which are small enough to diffuse freely through the nuclear pore, or to the lack of possible cytoplasmic targeting sequences in the C terminus of NET1. In keeping with their reduced abilities to stimulate GTPγS binding by RhoA in vitro, NET1-(156–501) and NET1-(156–595) were only moderately effective at stimulating actin stress fiber formation in cells (Fig. 7A, panels d and f). On the other hand, NET1-(122–501) was as efficient as NET1ΔN at stimulating actin stress fiber formation (compare panels b and h). This correlated well with the maximal enzymatic activity of this protein toward RhoA observed in vitro. Thus, these data indicate that residues 1–121 and 502–595 (the amino- and carboxyl-terminal ends of NET1) are not required for NET1-dependent effects on the actin cytoskeleton.

We then examined the transforming activity of each protein in NIH 3T3 focus formation assays. As shown in Fig. 8, none of the truncation mutants appreciably stimulated focus formation in NIH 3T3 cells. Because NET1-(122–501) was as efficient as NET1ΔN at stimulating actin stress fiber formation, these results reinforce the notion that effectiveness at stimulating actin polymerization does not correlate transformation activity. This also indicates that the carboxyl-terminal end of NET1 (amino acids 502–595) is important to its ability to transform cells, since this domain is present in NET1ΔN but not in NET1-(122–501).

NET1ΔN Requires a Putative PDZ Binding Site in Its C Terminus to Efficiently Transform Cells—PDZ domains are protein-protein interaction domains found in a wide range of signaling proteins. They are named after the first three proteins in which this domain was identified (PSD-95, DLG, and ZO-1) and mediate interaction with proteins containing PDZ binding sites. These binding sites are typically contained within the C terminus of the target protein and can be grouped into three classes according to their consensus sequences. Class I PDZ binding domains consist of the consensus sequence X(S/
where \( H_9021 \) is a hydrophobic residue (32). Because the C terminus of NET1 contains the sequence ETLV-COOH, we reasoned that this may encode a class I PDZ binding domain, and thus may target NET1 for interaction with one or more proteins that mediate its effects in the cell. Therefore we tested whether deletion or inactivation of this binding site would affect the activity of NET1 in vitro or in cells.

Interaction of PDZ binding sites with their cognate PDZ domain-containing proteins can be eliminated either by deleting the PDZ binding site, or by adding an alanine onto the C terminus of the binding site (33). Thus, we created NET1 mutants lacking the last four amino acids (NET1\_C4) or containing an alanine added onto its C terminus (NET1\_Ala) and tested for effects on enzymatic activity in vitro, ability to stimulate cytoskeletal reorganization, and ability to transform cells. To test for effects on enzymatic activity, each protein was expressed in E. coli as a GST fusion protein and assayed for activity toward RhoA in vitro. As shown in Fig. 9A, NET1\_N, NET1\_N\_C4, and

\[
\text{T}_\Phi\text{COOH}, \text{ where } \Phi \text{ is a hydrophobic residue } (32). \text{ Because the C terminus of NET1 contains the sequence ETLV-COOH, we reasoned that this may encode a class I PDZ binding domain, and thus may target NET1 for interaction with one or more proteins that mediate its effects in the cell. Therefore we tested whether deletion or inactivation of this binding site would affect the activity of NET1 in vitro or in cells.}

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FIG. 6. Identification of regions amino- and carboxyl-terminal to the DH and PH domains of NET1 required for catalytic activity in vitro. A, schematic depicting wt NET1 and the NET1 deletion mutants that were tested. The light gray boxes represent the Dbl homology (DH) domains, the darker gray boxes represent the Pleckstrin homology (PH) domains, and the solid bars represent nuclear localization signals (NLS). Numbers represent amino acid residues according to the sequence of wt NET1. B and C, GDP exchange assays measuring the ability of NET1 deletion mutants to stimulate \( ^{35}\text{S}\text{GTPyS} \) binding by wt RhoA in vitro. All proteins were purified from E. coli as GST fusion proteins. Shown is the average of three independent experiments. Errors are mean ± S.E.
net1 transforming activity of net1

net1 pdz binding site of net1 had a dramatic effect on the

by focus formation assay. as shown in fig. 9

alteration of the net1 constructs shown and tested for transformation

for cell transformation. thus, nih 3t3 cells were transfected

with the net1 constructs shown. sixteen days later the cells were fixed and

stained with crystal violet to visualize cell foci. values represent the

number of foci formed per dish expressed as a percentage of those

observed with transfection of net1an, and represent the average of

four independent experiments. errors are mean ± s.e.

net1anala exhibited nearly equivalent activities toward rhoa

in vitro. thus, alteration of the putative pdz binding site in

net1an did not affect its enzymatic activity.

we then examined whether this site was required for sub-

cellular localization or for effects on the actin cytoskeleton. serum-starved swiss 3t3 cells were microinjected with

expression vectors for ha-tagged net1an, net1anac4, or

net1anala, fixed 4 h later and stained for net1 expression

and for f-actin. as shown in fig. 9b, each net1 protein was

localized to both the cytoplasm and nucleus. some cells showed

less punctate staining for net1anac4 and

net1anala than for net1an, but this was not a consistent

effect. in addition, cell fractionation showed that each protein

was distributed in roughly the same proportions between the

nucleus and cytoplasm (fig. 9c). similarly, each protein

stimulated the formation of numerous actin stress fibers with

similar efficiency (fig. 9b). thus, alteration of the pdz binding

site had little effect on the ability of net1an to stimulate
cytoskeletal rearrangement.

we then tested whether the pdz binding site was required for cell transformation. thus, nih 3t3 cells were transfected with the net1 constructs shown and tested for transformation by focus formation assay. as shown in fig. 9d, alteration of the pdz binding site of net1an had a dramatic effect on the transforming activity of net1an, such that net1anac4 and net1anala exhibited only 28 and 33% of the transforming activity of net1an. thus, these experiments indicate that the putative pdz binding site of net1an is required for cell transformation, but is not required for effects on the actin cytoskeleton or for enzymatic activity in vitro.

discussion

rho family small g proteins control many aspects of cell

proliferation, including cell cycle progression and cytokinesis. they do so by acting as molecular switches, cycling between their active, gtp-bound, and inactive gdp-bound states. their ability to bind gtp in the cell is stimulated by a family of proteins known as rho geFs. thus, there has been a great deal of emphasis in the field on identifying regulatory mechanisms that control the activation of individual rho geFs. in this study we have explored the regulatory mechanisms governing the rho geF net1 both in vitro and in cells.

net1 was first cloned by virtue of its transforming activity

in nih 3t3 cell focus formation assays and later as a rhoa-

interacting protein in a yeast two-hybrid screen (23, 24). the original transforming version of net1 lacked the first 145 amino acids of the wild type protein, indicating that net1 activity may be negatively controlled by its amino terminus. precedent had been set previously for this type of regulation, because rho geFs such as dbl and vav had been shown to contain cis-acting autoinhibitory domains adjacent to their dh domains (21, 34, 35). however, when we examined whether net1 also contained an autoinhibitory domain, we found that the enzymatic activities of recombinant wt net1 and onco-
genetic net1 (net1an) were identical (fig. 1). this finding indicates that net1 does not contain a cis-acting autoinhibitory domain that directly regulates its enzymatic activity and supports a model in which the ability of net1 to control rhoa activation in the cell is regulated primarily by the release of net1 from the nucleus to the cytoplasm (25).

however, the mechanism controlling the nuclear localization of net1 appears to be more complex that previously suggested (25). this statement is based on two separate observations. first, we observed that the splice variant net1a, which lacks the nls sequences present in wt net1 and does not contain an identifiable nls sequence of its own, was still largely targeted to the nucleus (fig. 2). second, we observed that a polypeptide corresponding to the unique amino-terminal region of wt net1 that contains the nls sequences (amino acids 1–85) was localized throughout the cell when transiently expressed and that a larger polypeptide containing sequences conserved between wt net1 and net1a (amino acids 1–121 of wt net1) was localized exclusively to the nucleus (fig. 3). thus, the region corresponding to amino acids 86–121 of wt net1 must participate in targeting both wt net1 and net1a to the nucleus, even though this region lacks an identifiable nls sequence. the mechanism by which this region contributes to the nuclear localization of net1 isoforms is unclear.

previously it was shown that mutation of the two nls sequences in wt net1 resulted in the partial redistribution of net1 to the cytoplasm and an increase in actin stress fiber formation (25). however, one caveat of these experiments was that mutation of the nls sequences may have blocked negative regulation by the amino terminus directly, or by proteins that interact with the amino terminus. to avoid these limitations we examined whether relocational of wt net1 to the cyto-

plasm through the addition of a nuclear export signal to the c terminus was sufficient to allow it to stimulate cytoskeletal reorganization or to transform cells. we also tested whether it was essential for net1an to localize to the cytoplasm to stimulate actin stress fiber formation or cellular transformation. net1an relocation to the nucleus was brought about by addition of a nuclear localization signal to its c terminus.

these experiments demonstrated that relocational of full-

length net1 to the cytoplasm was sufficient to stimulate actin

stress fiber formation but was not enough for cellular transforma-

tion. on the other hand, relocation of net1an to the

nucleus completely blocked its ability to stimulate actin reor-

ganization and to transform cells (figs. 4 and 5). thus, net1 proteins

must be localized to the cytoplasm to stimulate actin stress fiber formation and cellular transformation. these experiments also show that the amino terminus of wt net1 does not block its ability to stimulate actin polymerization. furthermore, because very little of the net1 plus nes was actually localized to the cytoplasm, these data demonstrate that only modest amounts of cytoplasmic net1 are required to stimulate cytoskeletal reorganization. this observation was supported by our finding that expression of net1a profoundly stimulated actin stress fiber formation, even though the expressed net1a

fig. 8. net1 requires its carboxyl-terminal domain to trans-

form cells. nih 3t3 cells were transfected with expression vectors for

the net1 proteins shown. sixteen days later the cells were fixed and

stained with crystal violet to visualize cell foci. values represent the

number of foci formed per dish expressed as a percentage of those

observed with transfection of net1an, and represent the average of

four independent experiments. errors are mean ± s.e.

\[ Foci \text{ Formation} \]

\[ \left( \frac{\% }{\text{Percent of NET1AN}} \right) \]

\[ \begin{array}{c}
\text{NET1AN} \\
150-595 \\
156-501 \\
122-501
\end{array} \]
was also largely restricted to the nucleus (Fig. 2). Thus, these data emphasize the point that the tight control of cytoplasmic levels of NET1 isoforms is critically important to the cell, because mislocalization of even small amounts of NET1 to the cytosol will result in actin cytoskeletal rearrangement.

We were also interested in defining regions within NET1 other than the DH and PH domains that were important to its activity in vitro and in cells. Apart from the NLS sequences present in the amino terminus of wt NET1, the amino and carboxyl termini of wt NET1 do not display homology to other genes or known protein-protein interaction domains. Our experiments showed that the combined DH and PH domains of NET1 were sufficient to stimulate actin stress fiber formation in cells. However, full enzymatic activity in vitro required the presence of amino acids 122–155, which are directly amino-terminal to the DH domain (Figs. 7 and 8). Furthermore, residues 1–121 and 502–595 were dispensable for enzymatic activity toward RhoA in vitro, and for the stimulation of actin stress fiber formation in cells. The contribution of residues 122–155 to the enzymatic activity of NET1 may be due to the formation of an α-helical extension of the Dbl domain recently identified in the related Rho GEF LARG (36). In the crystal

Fig. 9. Analysis of the requirement for the PDZ binding site of NET1 for enzymatic activity, effects on the actin cytoskeleton, and cellular transformation. A, GDP exchange assays were performed to measure the effect of alteration of the PDZ binding site on enzymatic activity toward RhoA in vitro. All proteins were produced as GST fusions in E. coli, and purified by affinity chromatography. B, serum-starved Swiss 3T3 cells were microinjected with plasmids coding for the NET1 proteins shown. 4 h later the cells were fixed and stained for NET1 expression (panels a, c, and e) and for F-actin (panels b, d, and f). NET1 proteins were detected using an antibody to the amino-terminal HA-epitope. C, subcellular fractionation of NIH 3T3 cells transfected with HA-epitope tagged NET1 proteins. Equal amounts of nuclear and cytoplasmic fractions were analyzed by Western blotting using an antibody to the HA-epitope. The top and bottom panels are Western blots of the nuclear and cytoplasmic fractions, respectively. D, effects of alteration of the PDZ binding site on cellular transformation. NIH 3T3 cells were transfected with vectors coding for the NET1 proteins shown. Sixteen days later the cells were fixed and stained to reveal cell foci. Values represent the number of foci formed per dish expressed as a percentage of those observed with transfection of NET1ΔN, and represent the average of three independent experiments. Errors are mean ± S.E.
structure of LARG this extension folded into a hydrophobic core that formed contacts with the switch 1 domain of RhoA. Mutation of this extension reduced the enzymatic activity of LARG by nearly 80%, thus demonstrating its importance to the catalytic mechanism of this protein. Because the residues comprising this extension are conserved in NET1, it may be that this region plays a similar role in regulating NET1 activity.

The inability of NET1-(122–501) to stimulate transformation in NIH 3T3 cells demonstrated the importance of the C-terminal domain of NET1 to its transforming activity. This is most likely due to the presence of a putative PDZ binding domain in the C terminus, because inactivation of this site either by deletion or through the addition of an alanine onto the C terminus of NET1A severely inhibited its focus formation activity (Fig. 9D). Importantly, the presence of the PDZ binding site was not required for the enzymatic activity of the PDZ binding site mutants in vitro, or for their effects on the actin cytoskeleton (Fig. 9, A–C). The identity of the protein that interacts with the PDZ binding site of NET1 that is required for transformation is unclear. There are over 320 PDZ-domain-containing proteins in the human genome, 70% of which are predicted to bind to class I PDZ binding domains such as that present in NET1 (32, 37). However, there is precedent for the transforming activity of oncogenes being dependent upon interaction with PDZ domain containing proteins. For example, the E6 protein from the type 16 human papilloma virus and the Tax protein from the type 1 human T cell leukemia virus each interact with the human homolog of the Drosophila discs large protein to efficiently transform cells (38, 39). We are currently screening for proteins that interact with the NET1 PDZ binding site.

An important finding of these studies is the apparent disconnect between the ability of NET1 proteins to stimulate actin cytoskeletal reorganization and to transform cells. For example, NET1 plus NES, NET1-(122–501), and NET1A were each as effective NET1A at stimulating actin stress fiber formation but were not transforming in focus formation assays (Figs. 2 and 5). For NET1 plus NES and NET1-(122–501), this inability is most likely due to a loss of interaction with PDZ domain-containing proteins that aid in NET1-dependent transformation. For NET1A, this cannot be the case, because the PDZ binding site is intact. The lack of transforming activity by NET1A may be due to a number of reasons, including the possibility that cellular transformation requires very high levels of cytoplasmic NET1 that are not achieved by NET1A. Alternatively, it is also possible that NET1A is subject to some form of negative control that is lacking in NET1A. At any rate, it is clear that the ability of NET1 to transform cells does not correlate with its ability to stimulate actin polymerization. Such a distinction has been noted by other groups when studying the actions of constitutively active Rho family small G proteins (4). Thus, the signaling cascades stimulated by NET1A leading to cell transformation must diverge from those that are required to effect cytoskeletal reorganization. A key to this oncogenic signaling must be the interaction with PDZ domain-containing proteins through the PDZ binding site of NET1. Future experiments will be directed at identifying the PDZ domain-containing proteins that interact with NET1 and characterizing their contributions to NET1-dependent transformation.

Acknowledgments—We are thankful to Art Alberts for providing the wt NET1 and NET1ΔN expression vectors, and to Carmen Dessauer for advice with GDP exchange assays.

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Characterization of the Biochemical and Transforming Properties of the Neuroepithelial Transforming Protein 1
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J. Biol. Chem. 2005, 280:7603-7613.  
doi: 10.1074/jbc.M412141200 originally published online December 16, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M412141200

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