The Rho Exchange Factor Net1 Is Regulated by Nuclear Sequestration*

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Net1 is a guanine nucleotide exchange factor specific for the small GTPase Rho. Oncogenic activation of Net1 occurs by truncation of the N-terminal part of the protein, which functions as a negative regulatory domain. Here, we have investigated the mechanism of Net1 regulation via its N terminus. We find that Net1 localizes to the nucleus, whereas oncogenic Net1 is found in the cytoplasm. Nuclear import of Net1 is mediated by two nuclear localization signals present in the N terminus of the protein, and forced cytoplasmic localization of Net1 is sufficient to activate Rho. In addition, the pleckstrin homology (PH) domain of Net1 acts as a nuclear export signal. Because an amino acid substitution in the PH domain that inhibits guanine nucleotide exchange factor activity does not inhibit nuclear export, we conclude that this PH domain has at least two functions. Together, our results suggest that Net1 can shuttle in and out of the nucleus, and that activation of Rho by Net1 is controlled by changes in its subcellular localization.

Rho GTPases, including Rho, Rac, and Cdc42, are key molecules in inducing changes in the organization of the actin cytoskeleton and in gene transcription that drive a large variety of biological responses following the addition of extracellular stimuli (1–3). Not surprisingly, therefore, the activity of Rho GTPases needs to be tightly controlled, and aberrant Rho GTPase signaling has been implicated in a variety of human conditions including faciogenital dysplasia and Wiskott-Aldrich syndrome, as well as in cellular transformation and tumor progression (4–7).

Like all members of the Ras superfamily, Rho GTPases function as binary switches that cycle between an inactive, GDP-bound state and an active, GTP-bound state (2, 3). Activation is mediated by guanine nucleotide exchange factors (GEFs) that stimulate the exchange of GDP for GTP, but this is still a poorly understood aspect of Rho GTPase signaling. More than 50 mammalian RhoGEFs have been identified, and they share a domain of about 200 amino acids designated the Dbl homology (DH) domain, which is necessary to bind to the GTPase and to stimulate nucleotide exchange activity, as well as a pleckstrin homology (PH) domain, located C-terminally adjacent to the DH domain (8–12). In addition, most GEFs contain other functional domains including Src homology domains 2 and 3, Ser/Thr or Tyr kinase, Ras-GEF, Rho-GTPase-activating protein, and PDZ, which are likely to be involved in linking GEFs to upstream receptors and signaling molecules (11, 13).

Several of the characterized GEFs, including Dbl, Net1, Lbc, Lfc, Lsc, Dbs, Ost, Vav, Ect2, and Tim, were originally isolated as oncogenes in experimental transformation assays, and their transforming activity was shown to be tightly linked with their ability to activate Rho GTPases and their downstream effectors (6, 11–13). Although for most GEFs there is no direct evidence that they play a role in cancer, mutations in Tiam-1 and leukemia-associated Rho GEF (LARG) have been found in human tumors (14, 15). It remains to be seen whether mutations in GEFs are a more widespread phenomenon in human tumor formation and progression.

Little is known about the molecular mechanisms that regulate GEF activity. Oncogenic activation of GEFs is often associated with truncation of the N terminus, suggesting that this might provide a negative regulatory domain (11, 13). To date, the best understood example of this is Vav. It has been demonstrated that the N terminus of Vav binds directly, via an intramolecular interaction, to its DH domain, thereby blocking interaction with GTPases (16). Phosphorylation of Tyr-174 in the Vav N terminus by Src-family tyrosine kinases relieves the autoinhibition. Similar mechanisms of activation have been suggested for Dbl, which, like Vav, is activated by phosphorylation of the N terminus, and for p115RhoGEF, which is activated upon binding of Ga13 to an N-terminal RGS domain (17–19).

Activation of GEFs has also been reported to involve the DH domain. Binding of phosphatidylinositol-3-kinase products to the DH domain of Vav, for example, disrupts an intramolecular interaction between the PH and DH domains (20). For other GEFs, such as Lbc, Lfc, or Dbs, the PH domain appears to be required to target the protein to the plasma membrane, probably by binding to phosphorylated phosphoinositides (PIPs) or perhaps proteins at the membrane (21–23). Deletion or mutation of a conserved tryptophan residue within the PH domain results in loss of transforming activity, and in some cases this can be restored by addition of a CAAX motif to target the protein to the plasma membrane (21–23).

The net1 gene, which encodes a specific GEF for Rho, was originally isolated in a tissue culture screen for novel oncogenes using the focus formation assay in NIH 3T3 fibroblasts (24, 25). Experimental constitutive activation of human and mouse Net1 can be achieved through truncation of the N-terminal 145 and 121 amino acids, respectively (24, 25). Here, we have
investigated the mechanism by which N-terminal sequences negatively regulate the activity of Net1. We find that the N terminus of Net1 harbors nuclear localization signals, which, when removed or mutated, cause relocalization of Net1 to the cytoplasm leading to activation of Rho. Furthermore, we find that the PH domain has at least two activities, one for export of Net1 from the nucleus and the other for activation of Rho once in the cytoplasm. Together, our data suggest that Net1 regulates Rho activity through changes in its intracellular localization.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Leptomycin B (LMB), a gift from M. Yoshida (University of Tokio, Tokyo, Japan), was dissolved in EtOH at a concentration of 10 μg/ml and stored at −20 °C. Antibodies to NF-κB, CBP, Erk2, and human Net1, as well as the corresponding Net1 block sequences, were from Santa Cruz Biotechnology. Anti-Flag antibody was from Sigma.

**Plasmids used in this study**

| Plasmid | Characteristics and source |
|---------|-----------------------------|
| Net1    | pAS357, encodes for mouse Net1. Made by subcloning from pEFmNet1 (25) into pRK5myc. |
| Net1ΔN  | pAS356, encodes for Net1 lacking the first 121 aa. Made by subcloning from pEFmNet1ΔN (25) into pRK5myc. |
| Net1ΔN2 | pAS362, encodes for Net1 lacking the first 21 aa. Made by PCR using pRK5myc::Net1 as a template and subcloning into pRK5myc. |
| Net1ΔN3 | pAS363, encodes for Net1 lacking the first 74 aa. Made by PCR using pRK5myc::Net1 as a template and subcloning into pRK5myc. |
| Net1 N  | pAS353, encodes for the first 231 aa of Net1. Made by subcloning from pEFmNet1 into pRK5Flag. |
| Net1C   | pAS373, encodes for the first 502 aa of Net1. Made by PCR using pRK5myc::Net1 as a template and subcloning into pRK5myc. |
| Net1ΔNΔC| pAS377, encodes for aa 122–502 of Net1. Made by subcloning from pAS373 into pAS356. |
| Net1C   | pAS380, encodes for Net1 lacking the first 448 aa. Made by PCR using pRK5myc::Net1ΔN as a template and subcloning into pRK5myc. |
| Net1ΔNΔPH| pAS384, encodes for Net1ΔN lacking the PH domain (aa 378–502). Made by PCR using pRK5myc::Net1ΔN as a template and subcloning into pRK5myc. |
| Net1ΔNω492L| pAS387, encodes for Net1ΔN containing a Trp→Leu point mutant at aa 492. Made by subcloning from pEFmNet1ΔNω492L (25) into pRK5myc. |
| Arhgef3 | pAS417, encodes for N-terminally Myc-tagged Arhgef3. Made by PCR using clone CS0DF028YC06 (Invitrogen) as a template and subcloning into the pRK5myc-variant pTB399. |
| Arhgef3ΔN| pAS418, encodes for N-terminally Myc-tagged Arhgef3 lacking the first 105 aa. Made by PCR using clone CS0DF029YC06 (Invitrogen) as a template and subcloning into the pRK5myc-variant pTB399. |
| N17Rac  | pHR5Flag containing Flag-tagged dominant-negative Rac. |
| C3 transferase | pAS404, pHR5Flag containing Flag-tagged C3 transferase. |
| Rev-NES-GFP | pRev(1.4)NES3-GFP (50). |
| Rev-GFP  | pRev(1.4)GFP (50). |
| Rev-PH-GFP| pAS391, expresses PH domain of Net1 fused to Rev-GFP. Made by PCR using pRK5myc::Net1ΔN as template and subcloning into pRev1-GFP. |
| Rev-W492L-GFP| pAS392, expresses PH domain of Net1 containing the W492L point mutant fused to Rev-GFP. Made by PCR using pRK5myc::Net1ΔNω492L as template and subcloning into pRev1-4GFP. |
| Net1 NLS*| pAS426, expresses full-length Net1 containing R12,14,15,16A and R67,69,73A, K68A point mutations in NLS1 and NLS2, respectively. pAS426 was reconstituted from two PCR fragments generated with primers 5′-cga tgc gaa gtc ccg gcc ggc gca cag aag cag ccc and 3′-cct aga gaa gtc ccg gcc ggc gca cag aag ccc tct aga gaa tgc ttc act ttt ctc tgc tgc tgc tgc ttc ctt cca aag gac gca ccc cct tct cct atc ata ttt ctc tgc tgc tgc tgc tgc ttc ctt cca aag gac gca ccc cct cct atc atc ttt ctc tgc tgc tgc tgc tgc ttc ctt cca aag gac gca ccc cct cct cct cct cct cct cct pRK5myc. |

Eukaryotic expression vectors (0.1 μg/ml, unless stated otherwise) together with biotin-dextran were injected into the nucleus of 50–100 cells over a period of 15 min. Cells were returned to the incubator for 1–3 h for optimal expression. To assay the effect of blocking nuclear export, LMB was added 30 min after injection for 1.5 h at a concentration of 40 ng/ml. **Immunofluorescence Staining Protocols**—Microinjected Swiss 3T3 cells were fixed with 4% paraformaldehyde/PBS for 10 min, permeabilized in 0.2% Triton X-100/PBS for 5 min, incubated with NH4/C1/PBS (2.7 mg/ml) for 10 min to remove free aldehyde groups and then stained as previously described (26). Coverslips were rinsed in PBS between each step of the staining procedure. Primary antibodies were diluted in PBS and left on the coverslip for 30 min. After washing, the coverslips were incubated for 30 min with fluorescently conjugated secondary antibodies and AMCA-coupled streptavidin (to identify biotin-dextran-injected cells) diluted in PBS. Where necessary the cells were further incubated with rhodamine-phalloidin (200 ng/ml) for 10 min to visualize the actin cytoskeleton. Coverslips were mounted on Mowiol mountant containing p-phenylenediamine as an anti-bleaching agent. After 30 min at 37 °C, the coverslips were examined and the cells analyzed on a Zeiss Axiopt microscope using Zeiss 40× 1.4 oil immersion objectives. Pictures were taken with a Hamamatsu C9885-10 video camera. Cells injected with constructs expressing GFP-tagged proteins were fixed and permeabilized as described above, and coverslips were then directly mounted onto slides without further staining. Representative pictures are shown, and the data are presented as the means ± standard deviation. **Transfection of COS-7 Cells**—Cells were seeded in six-well plates at a density of 2 × 10^5 cells/well in 2 ml of medium, incubated overnight, and transfected using LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. A total of 1 μg of DNA was used for each transfection. After transfection the cells were incubated at 37 °C for 16 h before harvesting. **Nuclear and Cytoplasmic Fractionation**—Nuclear-cytoplasmic fractionation of Swiss 3T3 or transfected COS-7 cells was performed using the NE-PER® nuclear and cytoplasmic extraction reagents from Pierce following the manufacturer’s instructions. Swiss 3T3 cells were seeded at a density of 2 × 10^5 cells/10-cm dish for 24 h before lysis. COS-7 cells were plated in DMEM containing 10% fetal calf serum and penicillin/streptomycin. Confluent quiescent, serum-starved Swiss 3T3 cells for microinjection were prepared as follows. Cells were plated in DMEM containing 5% serum at a density of 5 × 10^5 on 10-cm culture dishes. Seven to 10 days after seeding, the cells became quiescent, at which time they were serum-starved for 16 h in DMEM containing 2% calf serum and 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 μg/ml amphotericin B.
were transfected as described above. After fractionation the protein concentrations were measured, and ~20 μg of protein extracts were used from each fraction. Samples were denatured in Laemli buffer at 95 °C for 5 min. SDS-PAGE and Western analysis were performed by standard methods.

RESULTS

Net1, but Not Oncogenic Net1, Localizes to the Nucleus—As a first step toward analyzing the regulation of Net1, we microinjected cDNA constructs encoding Myc-tagged, murine Net1 or an oncogenic version of Net1 (Net1ΔN, lacking the first 121 amino acids) into quiescent, serum-starved Swiss 3T3 fibroblasts and examined the cells for changes in the actin cytoskeleton. As described before, expression of Net1ΔN, but not Net1, strongly induced the formation of actin stress fibers (25) (Fig. 1A, panels II and IV). Interestingly, when we monitored the expression of the injected constructs, we found a striking difference in their subcellular distributions; Net1 localized exclusively to the nucleus, whereas Net1ΔN was mainly found in the cytoplasm (Fig. 1A, panels I and III).

To examine the subcellular distribution of Net1 and Net1ΔN biochemically, we performed Western analysis using nuclear and cytoplasmic fractions of COS-7 cells transiently transfected with Net1 or Net1ΔN. In agreement with our observations in injected fibroblasts, more than 95% of Net1 is present in the nuclear fraction, whereas ~50% of Net1ΔN is found in the cytoplasmic fraction (Fig. 1B).

To determine whether the nuclear localization of overexpressed Net1 is a true reflection of the subcellular distribution of the endogenous protein, we examined the levels of endogenous Net1 in the nuclear and cytoplasmic fractions of growing Swiss 3T3 fibroblasts using a commercial antibody directed against a peptide in the N terminus of human Net1. As shown in Fig. 1C, the antibody is not highly specific but it does recognize a protein of ~68 kDa, which is the same size as transfected Net1 (data not shown), and is competed away with excess Net1 peptide. This protein is almost exclusively present in the nuclear fraction.

Together, these results indicate that Net1 localizes to the nucleus, whereas Net1ΔN is present in the cytoplasm.

Cytoplasmic Localization of Net1 Is Sufficient to Induce Stress Fiber Formation—The above results raise the possibility that Net1 is unable to activate Rho and stimulate stress fiber formation because it is sequestered in the nucleus, whereas Net1ΔN is able to activate Rho simply because it is localized in the cytoplasm. We therefore asked whether forced cytoplasmic localization of Net1 is sufficient to induce stress fibers. To investigate, we microinjected high concentrations of Net1 cDNA into quiescent, serum-depleted Swiss 3T3 cells. As seen in Fig. 1D, in cells expressing high levels of Net1, some Net1 protein was detected in the cytoplasm (compare panels I and III). The cells that showed some cytoplasmic localization of Net1 also exhibited stress fibers, suggesting that localization of Net1 in the cytoplasm is sufficient to activate Rho (Fig. 1D, panels II and IV).

The N Terminus of Net1 Contains Two Functional Nuclear Localization Sequences—Nuclear import of proteins with a molecular mass of >40–50 kDa is dependent on the presence of a nuclear localization signal (NLS), which is recognized by the import machinery that mediates the translocation of protein into the nucleus (27). Because Net1 localizes to the nucleus, it is likely that one or several NLS sequences are present in the Net1 sequence. A search through the entire Net1 sequence reveals three potential NLS sequences. Two simple NLS sequences, consisting of a short stretch of basic amino acids, are present in the N terminus of Net1 (NLS1 = amino acids 12–19 and NLS2 = amino acids 66–72), and a bipartite NLS, consisting of a short stretch of basic amino acids preceded by an essential doublet of basic residues 5–14 amino acids upstream, is located in the C terminus (NLS3 = amino acids 536–552).

To investigate whether these sequences are functional NLS sequences that contribute to the nuclear localization of Net1, we constructed a series of deletion mutants of Net1 in which one or several of the NLS sequences were removed (Fig. 2A). CDNs expressing the Net1 deletion mutants were microin-
jected into quiescent, serum-starved Swiss 3T3 fibroblasts, and the localization of the mutant proteins and their ability to induce stress fiber formation were assessed by immunofluorescence and by staining of the actin cytoskeleton. In addition, to confirm the localization of the Net1 deletion mutants biochemically, we transiently transfected these cDNAs into COS-7 cells and performed nuclear-cytoplasmic fractionation. As seen in Fig. 2B, Net1ΔN2, lacking NLS1, is able to stimulate stress fiber formation, suggesting that some Net1ΔN2 is cytoplasmic. Although this is not obvious in Fig. 2B, immunofluorescence is not a very sensitive way to detect low levels of cytoplasmic protein and so biochemical fractionation of transfected COS-7 cells was performed. Fig. 2C confirms that a small but significant amount of Net1ΔN2 is in the cytoplasm (~15%) compared with full-length Net1. Removal of both NLS1 and NLS2 (Net1ΔN3) causes the relocalization of a major fraction of the protein to the cytoplasm seen both by immunofluorescence and fractionation, leading to strong induction of stress fibers, similar to that observed with Net1ΔN (Fig. 2B panels III and IV) and C. An N-terminal fragment of Net1 (Net1N) that bears both N-terminal NLS sequences is found exclusively in the nucleus (Fig. 2B, panel V) and C. This suggests that Net1 is imported into the nucleus via two NLS sequences in its N terminus.

To demonstrate that localization is the only property that is altered in the N-terminal deletion mutants of Net1, we made a Net1 construct (Net1 NLS*) in which we mutated NLS1 and NLS2 by replacing the basic residues with alanine residues. Mutating NLS1 and NLS2, like removal of the N terminus, leads to relocalization of Net1 into the cytoplasm and to the induction of stress fibers (Fig. 2, B (panels VII and VIII) and C).

Removal of the C-terminal NLS3 does not lead to relocalization of Net1 to the cytoplasm nor to the formation of actin stress fibers (Fig. 2, B (panels IX and X) and C). Furthermore, a C-terminal fragment of Net1 (Net1C) containing NLS3 is not imported into the nucleus (Fig. 2, B (panels XIII and XIV) and C). In addition, the cytoplasmic fraction and activity of Net1ΔN are not altered when the C terminus is removed (Fig. 2, B (panels XI and XII) and C). This suggests that NLS3 is not a functional NLS and that the C terminus of Net1 is not required for its function.

The Net1 Homolog Arhgef3 Is Cytoplasmic and Active—We next examined whether NLSs are also present in other Net1 family members. Human Net1, which is 82% identical to murine Net1, also possesses NLS2, suggesting that human Net1 is also localized to the nucleus (Fig. 3A). A splice variant of mouse Net1, which unlike full-length Net1 induces transformation, is identical to Net1 except for the N-terminal 31 amino acids and

![Fig. 2. Net1 contains two NLSs. A, schematic representation of Net1 constructs used in this study. For more details see Table I. B, quiescent, serum-starved Swiss 3T3 cells were microinjected with cDNA constructs encoding Myc-tagged Net1ΔN2 (pAS362), Net1ΔN3 (pAS363), Net1 NLS* (pAS426), Net1ΔC (pAS373), Net1ΔNΔC (pAS377) or Net1C (pAS380), or Flag-tagged Net1 N (pAS353). Cells were fixed and stained with anti-Myc (I, III, VII, IX, XI, and XIII) or anti-Flag (V) antibodies and rhodamine-phalloidin (II, IV, VI, VIII, X, XII, and XIV). C, COS-7 cells were transfected with cDNA constructs encoding Myc-tagged Net1ΔN2 (pAS362), Net1ΔN3 (pAS363), Net1 NLS* (pAS426), Net1ΔC (pAS373), Net1ΔNΔC (pAS377) or Net1C (pAS380), or Flag-tagged Net1 N (pAS353). Cells were fractionated into cytoplasmic (C) and nuclear (N) extracts, and extracts were subjected to SDS-PAGE and Western analysis using anti-Myc or anti-Flag antibodies.]
Swiss 3T3 fibroblasts. As in COS-7 cells, Arhgef3 and Arhgef3ΔN proteins are localized to the cytoplasm, and both proteins are active and strongly induce the formation of actin stress fibers (Fig. 3C). The induction of stress fibers by Arhgef3 is blocked by C3 transferase, which specifically inhibits Rho (28), but is unaffected by dominant-negative Rac (N17Rac), suggesting that, like Net1, Arhgef3 is a Rho-specific GEF (Fig. 3D). However, unlike Net1, Arhgef3 is cytoplasmic, and its activity is not regulated by its N terminus.

**Nuclear Export of Net1 Requires Its PH Domain**—To investigate if and how Net1 is exported from the nucleus, we made use of the finding that some Net1ΔN protein, although it lacks the N-terminal NLS sequences, is found in the nucleus. We assessed whether Net1ΔN shuttles between the nucleus and the cytoplasm. Swiss 3T3 cells were microinjected with Net1ΔN and then treated with LMB, an inhibitor of CRM1-dependent nuclear export (27).

Cells were stained for Net1ΔN and with anti-NF-κB antibody as a control. As seen in Fig. 4A, endogenous NF-κB rapidly accumulates in the nucleus upon LMB treatment (Fig. 4A, panels II and IV). Nuclear localization of Net1ΔN is also increased in the presence of LMB, although the effect is less dramatic than for NF-κB, possibly because Net1 is overexpressed (Fig. 4, A (panels I and III) and B). This suggests that Net1ΔN shuttles between the nucleus and the cytoplasm and that its export is mediated by the CRM1 export machinery.

We next asked which part of Net1 is required for nuclear export. To investigate this we microinjected various Net1ΔN deletion mutants into quiescent, serum-starved Swiss 3T3 cells. One deletion mutant, Net1ΔNAPH, which lacks the PH domain, strongly accumulates in the nucleus compared with Net1ΔN (Fig. 4C, panel II), endogenous NF-κB, possibly because Net1 is overexpressed (Fig. 4, A (panels I and III) and B). This suggests that Net1ΔN shuttles between the nucleus and the cytoplasm and that its export is mediated by the CRM1 export machinery.

The above results indicate that the PH domain of Net1 is crucial for nuclear export. However, in contrast to Net1ΔN, Net1ΔNAPH, this mutant was still exported from the nucleus (data not shown). This suggests that this leucine-rich sequence found in the Net1 PH domain is not a functional NES. It is therefore possible that nuclear export involves the interaction with a NES-containing protein. Alternatively, the PH domain of Net1 may contain a previously unidentified NES.

**PH Domains Have Been Shown to Bind to PIPs as Well as to Proteins, such as β-subunits of heterotrimeric G proteins or protein kinase C (29, 30). A point mutation in a conserved tryptophan residue (W492L) in the PH domain of Net1 has been shown to inhibit the activity of Net1ΔN (25). To determine whether the W492L mutation also inhibits nuclear export, we microinjected a Net1ΔN construct containing the W492L mutation into quiescent, serum-starved Swiss 3T3 cells. Interestingly, in contrast to Net1ΔNAPH, Net1ΔN W492L was still exported from the nucleus (Fig. 4C, panel V). However, as previously reported, Net1ΔN W492L did not induce stress fiber formation (Fig. 4C, panel VI) (25). This suggests that the PH domain of Net1 has two functions; 1) it mediates the export of the protein from the nucleus, and 2) it is required for the GEF activity of the Net1.

**The PH Domain of Net1 Is Sufficient to Drive Nuclear Export**—The above results indicate that the PH domain of Net1 is required for nuclear export. To determine whether the Net1 PH domain is sufficient to promote nuclear export, we fused the PH domain as well as the PH domain containing the W492L point
mutation to an export-deficient, GFP-tagged mutant of the nuclear protein Rev and asked whether the presence of the PH domain would lead to export of Rev (Fig. 5A). Rev-PH-GFP, Rev-W492L-GFP, as well as Rev-GFP and Rev-NES-GFP, containing the original NES of Rev, were each microinjected into quiescent, serum-depleted Swiss 3T3 fibroblasts. Cells were returned to the incubator for 30 min for expression of the construct and then treated with LMB (40 ng/ml) (III and IV) or the empty vehicle (I and II) for 1.5 h. Cells were fixed, stained with anti-Myc (I and III) or anti-NF-κB (II and IV) antibodies. B, the percentage of expressing cells showing a clear nuclear staining of Net1ΔN was determined. C, the PH domain of Net1 is required for export of Net1ΔN from the nucleus. cDNAs encoding Myc-tagged Net1ΔN (pAS356), Net1ΔNPH (pAS384), and Net1ΔNW492L (pAS387) were microinjected into quiescent, serum-starved Swiss 3T3 cells. Cells were fixed and stained with anti-Myc antibody (I, III, and V) and rhodamine-phalloidin (II, IV, and VI).

FIG. 5. The PH domain of Net1 is sufficient to drive nuclear export of Rev. A, schematic presentation of Rev-GFP constructs. See Table I for more details. B, quiescent, serum-starved Swiss 3T3 fibroblasts were microinjected with cDNA constructs encoding GFP-tagged Rev, Rev-NES containing the original Rev nuclear export sequence, Rev-PH (pAS391), and Rev-W492L (pAS392).

The mechanism of Net1 regulation is distinct from that of Vav or Dbl, where it has been shown that the sequences in the N-terminal region of these proteins are required for their localization and activity. The close relative of Net1, Arhgef3, which lacks the N-terminal NLS sequences, is cytoplasmic and constitutively active after expression in Swiss 3T3 cells. These results suggest that Net1-mediated activation of Rho is controlled by its cellular localization (Fig. 6).

FIG. 4. Net1ΔN shuttles between the nucleus and the cytoplasm. A, cDNA encoding Myc-tagged Net1ΔN was microinjected into quiescent, serum-depleted Swiss 3T3 fibroblasts. Cells were returned to the incubator for 30 min for expression of the construct and then treated with LMB (40 ng/ml) (III and IV) or the empty vehicle (I and II) for 1.5 h. Cells were fixed, stained with anti-Myc (I and III) or anti-NF-κB (II and IV) antibodies. B, the percentage of expressing cells showing a clear nuclear staining of Net1ΔN was determined. C, the PH domain of Net1 is required for export of Net1ΔN from the nucleus. cDNAs encoding Myc-tagged Net1ΔN (pAS356), Net1ΔNPH (pAS384), and Net1ΔNW492L (pAS387) were microinjected into quiescent, serum-starved Swiss 3T3 cells. Cells were fixed and stained with anti-Myc antibody (I, III, and V) and rhodamine-phalloidin (II, IV, and VI).
Regulation of the Rho Exchange Factor Net1

N terminus bind directly to the DH or PH domain, respectively, thereby preventing their interaction with GTPases (16, 17). However, Net1 regulation is similar to that of CDC24, a GEF for CDC42, in yeast (31, 32); CDC24 is imported and kept in the nucleus by association with FAR1. Nuclear export and thus activation of CDC42 are triggered either by entry into the cell cycle, when FAR1 is degraded, or by mating pheromone, which stimulates export of the FAR1-CDC24 complex. Nuclear-cytoplasmic shuttling of GEFs might therefore be a common mechanism for regulating their activity.

Is Net1 exported from the nucleus? Our results suggest that Net1 is exported and that this is mediated by its PH domain. Thus, Net1ΔN lacking the PH domain accumulates in the nucleus, whereas the Net1 PH domain fused to the export-deficient nuclear protein Rev is sufficient to induce export of Rev into the cytoplasm. Analysis of the nuclear-cytoplasmic shuttling of Bruton’s tyrosine kinase (Btk) has shown that a PH domain deletion mutant also accumulates in the nucleus, suggesting that some PH domains might act as mediators of nuclear export (33).

Interestingly, we find that the W492L PH domain point mutation does not prevent nuclear export of Net1ΔN, although it abolishes its cytoplasmic GEF activity. This suggests that the PH domain of Net1 has two functions: 1) mediating export of Net1 from the nucleus, presumably by interaction with another, NES-containing protein via a “piggy-back” mechanism; and 2) activation of Rho in the cytoplasm, possibly via an interaction with PIPs or other proteins. This interaction might be required for targeting Net1 to the plasma membrane or might directly influence the activity of the DH domain (Fig. 6).

In the case of Btk or the β-adrenergic receptor kinase, it has been shown that mutation of the conserved tryptophan disrupts the interaction of the PH domain with both phosphorylated phosphoinositides as well as Gephy subunits (34, 35). It remains to be seen whether nuclear export of Btk, like export of Net1, is also independent of this tryptophan residue. Identifying the binding partners of the Net1 PH domain will be important to further understand the regulation of Net1.

What triggers export of Net1 from the nucleus? A large variety of stimuli are known that activate Rho and induce stress fiber formation. These include activated Cdc42, Rac, Ras, Go, and Go13, growth factors such as lysophosphatidic acid, sphingosine 1-phosphate, platelet-derived growth factor, insulin, thrombin, bombesin, transforming growth factor-β, stress conditions (hypotonic shock, oxidative stress, CO2-depletion, heat shock), treatment of cells with sodium vanadate or nocodazole, adhesion to extracellular matrix, integral ligation during CR3-mediated phagocytosis, or cadherin-mediated cell-cell adhesion (26, 36–47). However, we have been unable to stimulate release of Net1 from the nucleus using any of these conditions. Recently, the Rho-specific GEF Ect2, which is involved in cytokinesis, has been shown to localize to the nucleus (48, 49). Nuclear envelope breakdown during nuclear division leads to cytoplasmic localization of Ect2, thus enabling it to activate Rho during cytokinesis. It is therefore possible that Net1, like Ect2, is released from the nucleus only during nuclear division and plays a role in cytokinesis. However, we think this is unlikely, as our results indicate that Net1 has an active export signal that allows the protein to shuttle between the nucleus and the cytoplasm. Furthermore, we have been unable to inhibit cytokinesis using a dominant-negative Net1 construct. This suggests that there might be a physiological condition that leads to relocation of Net1 to the cytoplasm and to activation of Rho.

Finding a stimulus for Net1 export will be of great importance to understand the biological implications of Net1 nuclear localization and will help to unravel the signaling pathway that controls Net1 regulation. Finally, it will be interesting to determine whether Net1 is localized in the cytoplasm in any human cancer cells and thereby contributes to tumor formation and progression.

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