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Activation of RhoA and SAPK/JNK signalling pathways by the RhoA-specific exchange factor mNET1

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We have characterized the DH domain protein mNET1, a Rho-family guanine nucleotide exchange factor (GEF). N-terminal truncation of mNET1 generates an activated transforming form of the protein, mNET1ΔN, which acts as a GEF for RhoA but not Cdc42 or Rac1. In NIH 3T3 cells, activated mNET1 induces formation of actin stress fibres and potentiates activity of the transcription factor serum response factor. Inhibitor studies show that these processes are dependent on RhoA and independent of Cdc42 or Rac1. In contrast to the GTPase-deficient RhoA.V14 mutant, however, expression of activated mNET1 also activates the SAPK/JNK pathway. This requires mNET1 GEF activity, since it is blocked by point mutations in the mNET1 DH domain and its C-terminal pleckstrin homology (PH) domain, and by the dominant-interfering RhoA mutant RhoA.N19. Although mNET1ΔN-induced SAPK/JNK activation requires a C3 transferase-sensitive GTPase, it occurs independently of the generation of titratable GTP-bound RhoA. Thus, mNET1 can activate signalling pathways in addition to those directly controlled by activated RhoA.

Keywords: Cdc42/mNET1/Rac1/RhoA/SRF

Introduction

The Rho family of GTPases are Ras-like proteins that regulate diverse processes including cytoskeletal rearrangements, gene transcription, cell-cycle progression and cytokinesis (for reviews see Lim *et al*., 1996; Van Aelst and D'Souza-Schorey, 1997). Activated forms of RhoA, Rac1 and Cdc42 can induce DNA synthesis in quiescent cells (Olson *et al*., 1995), and activation of Rho-family signalling pathways is both capable of inducing transformation of cells in culture and required for Ras-induced transformation (Perona *et al*., 1993; Khosravi-Far *et al*., 1995; Prendergast *et al*., 1995; Qiu *et al*., 1995a,b, 1997; van Leeuwen *et al*., 1995; Lebowitz *et al*., 1997; Lin *et al*., 1997). The activity of Rho-family GTPases is in part controlled by the Dbl homology (DH) family of guanine nucleotide exchange factors (GEFs), which catalyse their conversion to the active GTP-bound state (for reviews see Cerione and Zheng, 1996; Whitehead *et al*., 1997). In addition to the DH domain, which is required for GEF activity (Hart *et al*., 1994), the biological activity of each of these GEFs requires a conserved neighbouring pleckstrin homology (PH) domain, which apparently mediates subcellular localization (Whitehead *et al*., 1995b; Zheng *et al*., 1995c). Several Rho-family GEFs have been implicated directly in tumorigenesis *in vivo*, but activated forms of Rho-family GTPases have not been recovered in screens for transforming oncogenes (e.g. Moscow *et al*., 1994; Toksoz and Williams, 1994). One potential explanation for this is that the GEFs activate multiple signalling pathways, and indeed several transforming GEFs can activate more than one Rho-family protein (Cerione and Zheng, 1996; Whitehead *et al*., 1997).

Recent studies have provided direct evidence that Rho-family GTPases can signal to the cell nucleus to potentiate the activity of transcription factors, including serum response factor (SRF) and NF-κB, and targets of the stress-activated SAPK/JNK and p38 MAPK signalling pathways such as Jun and ATF-2 (Coso *et al*., 1995; Hill *et al*., 1995; Minden *et al*., 1995; Zhang *et al*., 1995; Perona *et al*., 1997). In transfection assays, activated RhoA potentiates SRF activity, and functional cellular RhoA is required for the activation of SRF that occurs when cells are stimulated with agents such as serum or LPA that act through heterotrimeric G protein-coupled receptors (Hill *et al*., 1995). Activated Cdc42 and Rac1 can also potentiate SRF activity in transfection assays independently of RhoA, although the extracellular signals which activate SRF through Rac1 or Cdc42 have yet to be identified (Hill *et al*., 1995). The nature of the SRF-linked signal pathway, which is distinct from the ERK, SAPK/JNK and p38 pathways, remains to be elucidated. In addition, although activated RhoA efficiently activates SRF-controlled reporter genes microinjected into cells, it is incapable of activating stably integrated SRF reporter genes or the chromosomal c-*fos* gene unless stress-activated MAPK pathways are activated simultaneously. In contrast, activated Cdc42, which can activate the SAPK/JNK pathway, can potentiate the activity of both chromosomal and microinjected templates (Alberts *et al*., 1998a).

The mouse homologue of the human NET1 DH domain protein (Chan *et al*., 1996), mNET1, interacts with both GDP- and GTP-bound RhoA in biochemical and two-hybrid assays, suggesting that it may be a RhoA-specific GEF (Alberts *et al*., 1998b). Here we show that mNET1 indeed possesses RhoA-specific GEF activity *in vitro*, and that it activates RhoA to induce stress fibre formation and potentiate SRF activity *in vivo*. However, unlike RhoA itself, activated mNET1 also efficiently induces the SAPK/JNK pathway in microinjection assays, enabling it to activate chromosomal SRF-controlled reporter genes. SAPK/JNK activation requires GEF activity and a C3-sensitive GTPase, and does not involve Cdc42 or Rac1. The activation of both RhoA and SAPK/JNK signalling pathways by activated mNET1 may contribute to the potent transforming activity of this GEF.

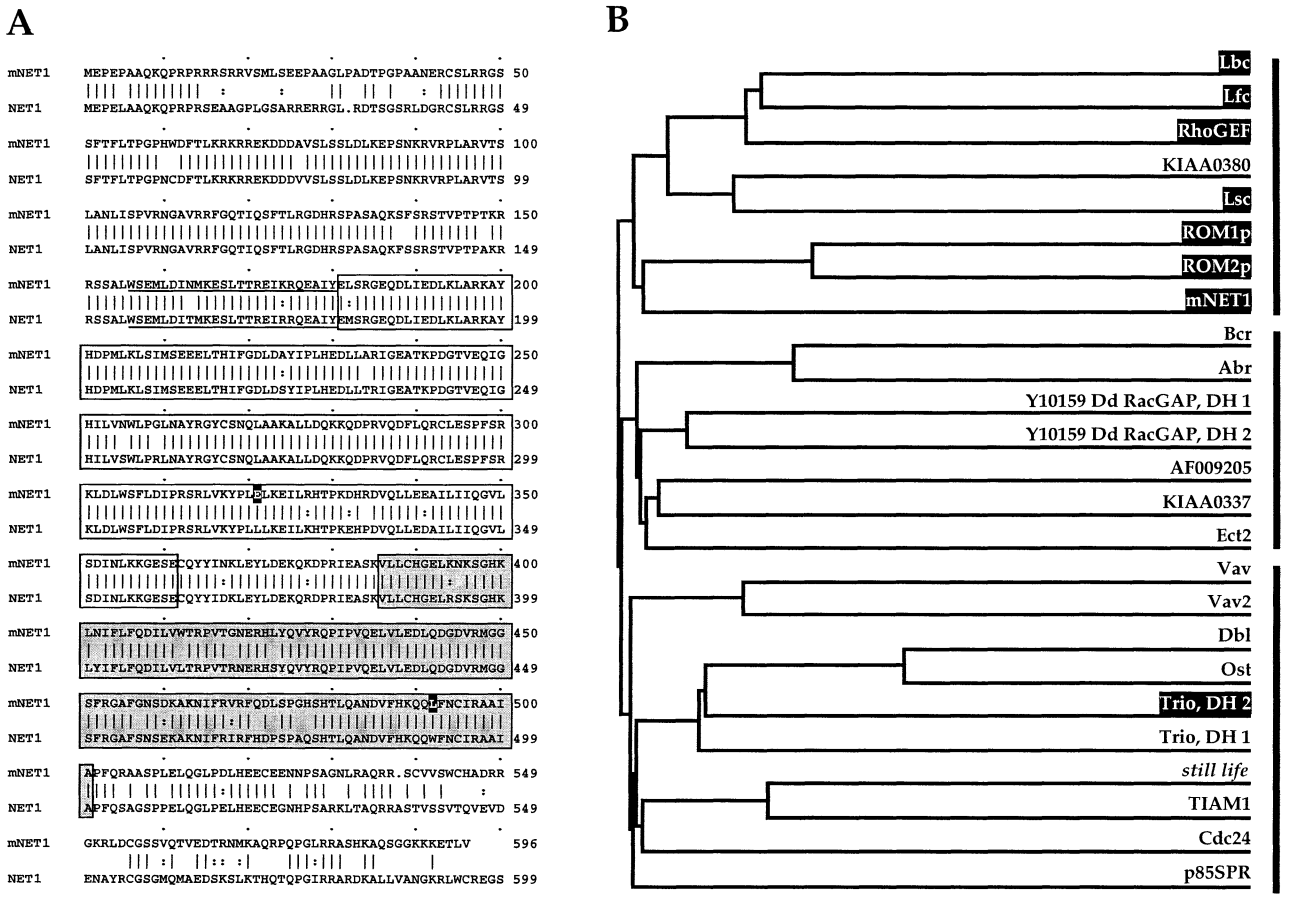

Fig. 1. mNET1 is related to RhoA-specific GEFs. (**A**) Sequence of mNET1. The sequence of mNET1 is shown compared with the human NET1 sequence (Chan *et al.*, 1996). Identical residues are shown as vertical lines and conserved residues by colons. The DH domain is boxed, and its N-terminal extension is underlined; the PH domain is indicated by the shaded box. The point mutations L321E and W492L discussed in the text are shown in reverse shading. Note that the N-terminal sequence of NET1 shows greater conservation between residues 14–30 if read in frame –1. (**B**) Sequence relationship between DH domains. Sequences corresponding to the conventional DH domain plus the N-terminal extension shown in (A) were compared using the GCG Pileup and Growtree programs; gene names and sequence identifiers are indicated on the figure. Reverse shading indicates known RhoA-specific GEFs. The highly divergent DH domains of Tim, Dbs and SOS were omitted for clarity.

Results

Sequence of mNET1

We identified mNET1 in a two-hybrid screen for proteins that interact with activated RhoA. Biochemical studies indicated that mNET interacts with both GTP- and GDP-bound wild-type RhoA, but only with the GTP-bound form of Cdc42 (Alberts *et al.*, 1998b). Prior to further studies of mNET1, we sequenced the coding region in its entirety (Figure 1A). The DH and PH domain sequences are, respectively, 95 and 89% identical to human NET1, and the N-terminal domain is substantially conserved (87%; the human sequence shown contains a frameshift between codons 15 and 30). During analysis of the sequence, we noted that the homology between the mNET1 sequence and a subset of other DH proteins extends some 25 residues N-terminal to the conventional DH domain. A comparison of this extended DH domain with the corresponding sequences from other DH domain proteins is shown in Figure 1B. This indicates that mNET1 belongs to a distinct subgroup of nine DH proteins, of which all six that have been investigated encode RhoA-specific GEFs (see Discussion); its closest relatives are the *Saccharomyces cerevisiae* ROM1 and ROM2 GEFs.

Transformation and exchange factor activity

For functional studies of mNET1, we constructed expression plasmids encoding the intact protein and an N-terminally truncated derivative, mNET1ΔN, analogous to the transforming human NET1 cDNA (Chan *et al.*, 1996), each N-terminally tagged with the haemagglutinin (HA) epitope. In addition, we constructed two mNET1ΔN point mutants, L321E and W492L, which contain point mutations predicted to inactivate the DH and PH domains, respectively (Hart *et al.*, 1994; Whitehead *et al.*, 1995a,b, 1996). We also used an 9E10-tagged derivative of the Dbl oncogene, which has GEF activity on RhoA, Cdc42 and Rac1 (Olson *et al.*, 1996), as a control to visualize the effects of activation of multiple Rho-family GTPases.

In transformation experiments, to be described in more detail below, we established lines of NIH 3T3 cells transformed by mNET1ΔN and Dbl. Immunoblotting experiments confirmed that the cells expressed proteins of the expected sizes (data not shown). To investigate the biochemical properties of mNET1, extracts were prepared from the cells, immunoprecipitated with antibodies specific for each GEF, and the immunoprecipitates tested for their ability to promote release of radiolabelled GDP from or

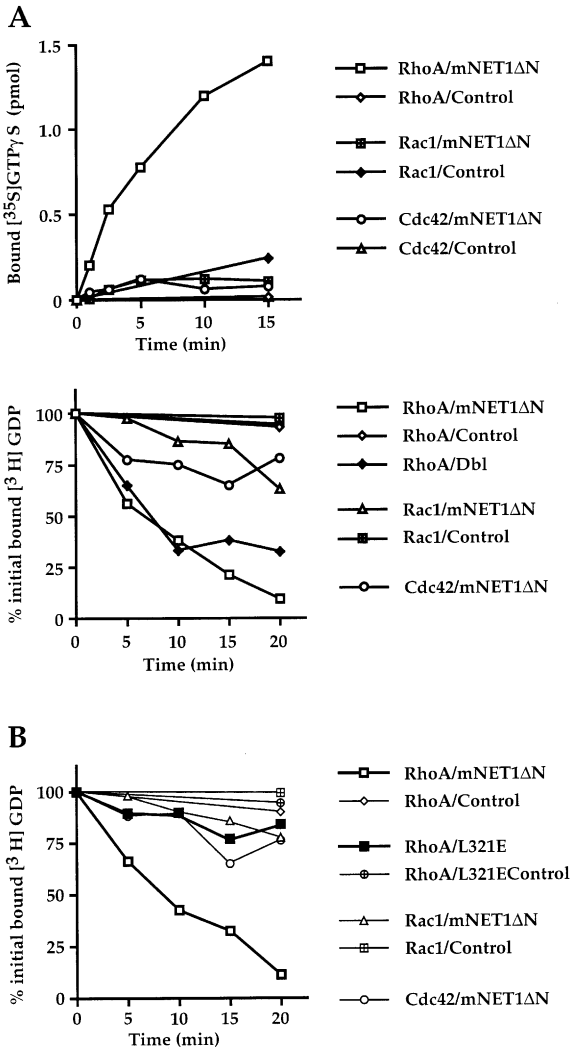

Fig. 2. Exchange factor assays. (**A**) mNET1 encodes a RhoA-specific GEF. Extracts were prepared from cells transformed with HA-mNET1ΔN (EF-HAmNET1ΔN; clone D) and 9E10-tagged Dbl (RK5-myc-Dbl; clone E) and immunoprecipitated using HA or 9E10 antibodies. The precipitates were then tested for GEF activity by measurement of released [^{35}S]GTPγS (upper panel) or bound [^{3}H]GDP (lower panel) as described in Materials and methods. Controls were 9E10 immunoprecipitates from HA-mNET1ΔN-transformed cells and HA immunoprecipitates from 9E10-Dbl-transformed cells. (**B**) The mNET1 DH domain is required for GEF activity. Extracts were prepared from cells transiently transfected with expression plasmids encoding either HA-mNET1ΔN or HA-mNET1ΔN(L321E) and immunoprecipitated using HA or 9E10 (control) antibodies. The precipitates were then tested for GEF activity by measurement of released [^{3}H]GDP as described in Materials and methods.

loading of radiolabelled GTPγS onto different Rho-family GTPases. mNET1ΔN promoted GTPγS loading onto RhoA, but showed no activity against Rac1 or Cdc42, while control immunoprecipitates showed no activity with any of the GTPases (Figure 2A, upper panel). In the GDP release assay, mNET1ΔN immunoprecipitates displayed activity against RhoA but not Rac1 or Cdc42 (Figure 2A, lower panel).

As an alternative approach to investigate mNET1 GEF specificity, we transiently transfected NIH 3T3 cells with expression plasmids encoding either mNET1ΔN or the DH domain mutant mNET1ΔN(L321E) and analysed GEF activity in cell extracts using the GDP release assay. Again, immunoprecipitates from cells expressing mNET1ΔN displayed specificity for RhoA rather than Rac1 or Cdc42; moreover, the L321E mutation reduced GEF activity to background levels (Figure 2B). Taken together with the previous results, these data strongly suggest that mNET1 encodes a RhoA-specific GEF, and are consistent with our previous biochemical studies which demonstrated that mNET1 interacts with both GDP- and GTP-bound RhoA (Alberts *et al*., 1998b).

mNET1ΔN activates stress fibre formation in NIH 3T3 cells

To investigate signalling pathways activated by mNET1 *in vivo*, we microinjected serum-deprived NIH 3T3 cells with expression plasmids encoding mNET1, mNET1ΔN or Dbl, and after 3 h stained the cells with fluorescently labelled phalloidin to visualize filamentous actin. Expression of mNET1ΔN induced a polar morphology and formation of actin stress fibres, while Dbl expression induced membrane ruffling, stress fibre formation and increased peripheral filamentous actin, as previously observed (Figure 3A, panels 1–3; Olson *et al*., 1996). Cells expressing intact mNET1 were indistinguishable from mock-injected cells (data not shown; Figure 4A, panel 2). In contrast, cells stably transformed by mNET1ΔN and Dbl exhibited substantially similar morphologies, possessing pronounced lamellipodial extensions visible by phase-contrast microscopy (Figure 3B). This similarity was dependent on serum factors since both cell types appeared similar to their microinjected counterparts upon maintenance in 0.5% serum for 48 h (Figure 3B).

We next investigated the downstream signalling pathways involved in mNET1ΔN-induced cytoskeletal rearrangements. Results are shown in Figure 4 and summarized in Table I. The DH domain mutation L321E, which abolishes mNET1ΔN GEF activity, also abolished its ability to induce stress fibres, as did the PH domain mutation W492L (Figure 4A, panels 3 and 4; data not shown). In addition, expression of the dominant-interfering RhoA mutant RhoA.N19, which should exhibit high affinity for GDP (Feig and Cooper, 1988), also abolished mNET1ΔN-induced stress fibre formation (Figure 4A, panel 5); in a control experiment, RhoA.N19 expression did not affect the activity of the Rac-specific GEF Tiam1 (see below).

We next investigated the role of different Rho-family GTPases in mNET1ΔN-induced cytoskeletal changes. Overexpression of the N-terminal domains of the p38-activated kinase (PAK) kinases can block signalling by the activated forms of both Cdc42 and Rac1 in both microinjection experiments and transfection assays, presumably by competition with effector molecules for the GTPase (Minden *et al*., 1995; Zhang *et al*., 1995; Alberts *et al.*, 1998a). However, expression of the N-terminal 252 residues of PAK (PAK.N) did not block the effect of mNET1ΔN expression on actin organization (Figure 4A, panel 6). Next, we used two methods to inhibit cellular RhoA function: we expressed either C3 transferase, which inactivates RhoA by ADP ribosylation of residue N41, or the N-terminal RhoA-binding domain of the kinase PKN, which binds RhoA.GTP. Both treatments abolished mNET1ΔN-induced actin stress fibre formation (Figure 4A, panel 7; data not shown). Expression of C3 together

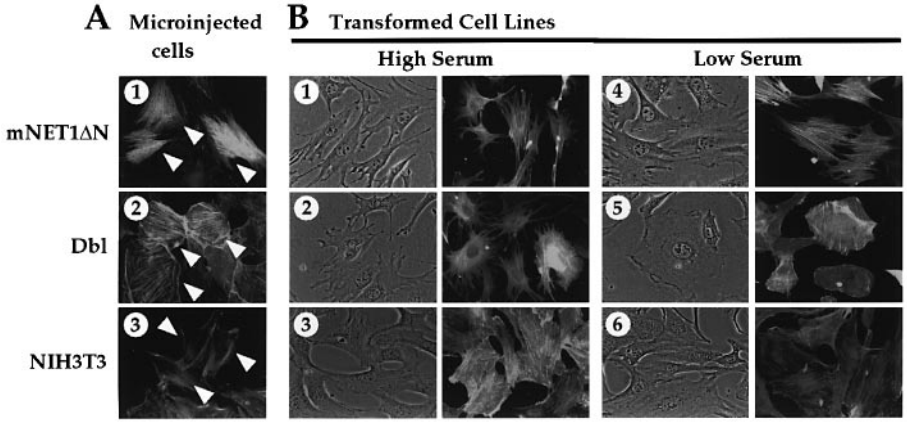

Fig. 3. Morphology of cells expressing mNET1ΔN or Dbl. (**A**) Microinjected cells. Serum-starved NIH 3T3 cells were injected (arrowheads) with expression plasmids encoding mNET1ΔN (panel 1), Dbl (panel 2) or GFP (panel 3) and the filamentous actin visualized by TRITC–phalloidin staining 3 h later. (**B**) Transformed cell lines. mNET1ΔN-transformed clone D (panels 1 and 4), Dbl-transformed clone E (panels 2 and 5) or untransformed NIH 3T3 cells (panels 3 and 6) examined following growth for 48 h in 10% or 0.5% calf serum. Cells were examined either by phase contrast microscopy (left panels) or by TRITC–phalloidin staining (right panels).

with the C3-resistant RhoA mutant RhoA.I41 restored mNET1ΔN-induced stress fibre formation (Figure 4A, panels 7 and 8).

To demonstrate the specificity of the inhibitors, we examined cells expressing Dbl which exhibit both ruffling and stress fibres (Figure 4B). Expression of PAK.N blocked the ability of Dbl to induce cell ruffling and peripheral actin, but not stress fibres (Figure 4B, panels 1 and 2). Co-expression with C3 or PKN.N abolished stress fibre formation but did not prevent ruffling (Figure 4B, panels 3 and 4). These observations are consistent with previous studies which showed that ruffling and stress fibres are dependent on Cdc42/Rac1 and RhoA, respectively (Ridley *et al*., 1992; Olson *et al*., 1996). Moreover, in biochemical and functional studies, each inhibitor exhibited interactions specific for its cognate GTPase (data not shown; Hill *et al*., 1995; Alberts *et al.*, 1998a,b). The results in this section thus demonstrate that mNET1ΔN induces cytoskeletal reorganization by a mechanism requiring GEF activity on RhoA, but not Cdc42/Rac1, consistent with the biochemical data.

mNET1ΔN activates SRF

Previous studies have shown that activated forms of RhoA, Cdc42 and Rac1 can potentiate the activity of SRF (Hill *et al*., 1995; Alberts *et al.*, 1998a). We therefore investigated the ability of mNET1ΔN to activate SRF-linked signal pathways. Serum-starved NIH 3T3 cells were injected with expression plasmids encoding mNET1ΔN, its point mutants or Dbl, together with the SRF-controlled reporter gene 3D.AFos-HA, in which transcription of c-*fos* mRNA sequences is regulated by a synthetic promoter containing three SRF-binding sites (Mohun *et al*., 1987; Hill *et al*., 1995; Alberts *et al.*, 1998a). Three hours after injection, cells were stained for reporter expression using anti-HA antibody (Figure 5A; results summarized in Table I). Both mNET1ΔN and Dbl could activate the reporter gene (Figure 5A, panels 1 and 6); SRF activation by mNET1ΔN was dependent on intact DH and PH domains, and was blocked by RhoA.N19 expression, indicating that it requires mNET1 GEF activity (Table I). To examine the role of Rho-family GTPases in mNET1ΔN- and Dbl-induced SRF activation, we again tested the effect of overexpression of the PAK and PKN GTPase-binding domains or C3 transferase. SRF activation by mNET1ΔN was prevented by expression of PKN.N but not by PAK.N (Figure 5A, panels 2 and 3). In contrast, activation of the SRF reporter gene by Dbl, which activates Cdc42 and Rac1 in addition to RhoA, could be blocked by neither PAK.N nor PKN.N alone, but effectively by a combination of both these GTPase-binding domains (Figure 5A, panels 7–9). Similar results were obtained when C3 transferase expression was used to inactivate RhoA, and when the PAK.N and PKN.N were introduced into cells as recombinant proteins (Table I). Activation of the reporter by either GEF was unaffected by co-expression of SEK-AL, an inhibitor of the SAPK/JNK pathway (Sanchez *et al*., 1994; Figure 5A, panels 5 and 10). Thus, consistent with the biochemical data, mNET1ΔN activates SRF via RhoA, and not via Rac1 or Cdc42.

We next tested whether mNET1ΔN expression could activate a chromosomally located SRF-controlled reporter. Our previous studies have shown that although activated Cdc42 can activate such templates, activated RhoA cannot unless a cooperating signal is supplied, and that the SAPK/JNK pathway can supply this signal (Alberts *et al.*, 1998a). To investigate activation of a chromosomal reporter, we microinjected mNET1ΔN and Dbl expression plasmids into SRE-Fos.HA cells, which contain an integrated 3D.AFosHA reporter gene. Both GEFs induced reporter gene expression (Figure 5B, panels 1 and 4), and both were also able to activate expression of the immediate-early gene *Egr-1* (data not shown). Similar results were obtained using a different cell line, SRE-lacZ, which contains a distinct SRF-controlled reporter gene (data not shown). Thus, in contrast to activated RhoA, mNET1ΔN can activate chromosomally located templates.

We next examined the signal pathways required for activation of the chromosomal reporters by mNET1ΔN. In contrast to the results obtained with the microinjected reporter, mNET1ΔN-induced expression of the chromosomal reporter was abolished by expression of SEK-AL, suggesting that its activation involves the SAPK/JNK pathway (Figure 5B, panel 2; Table I). As observed with

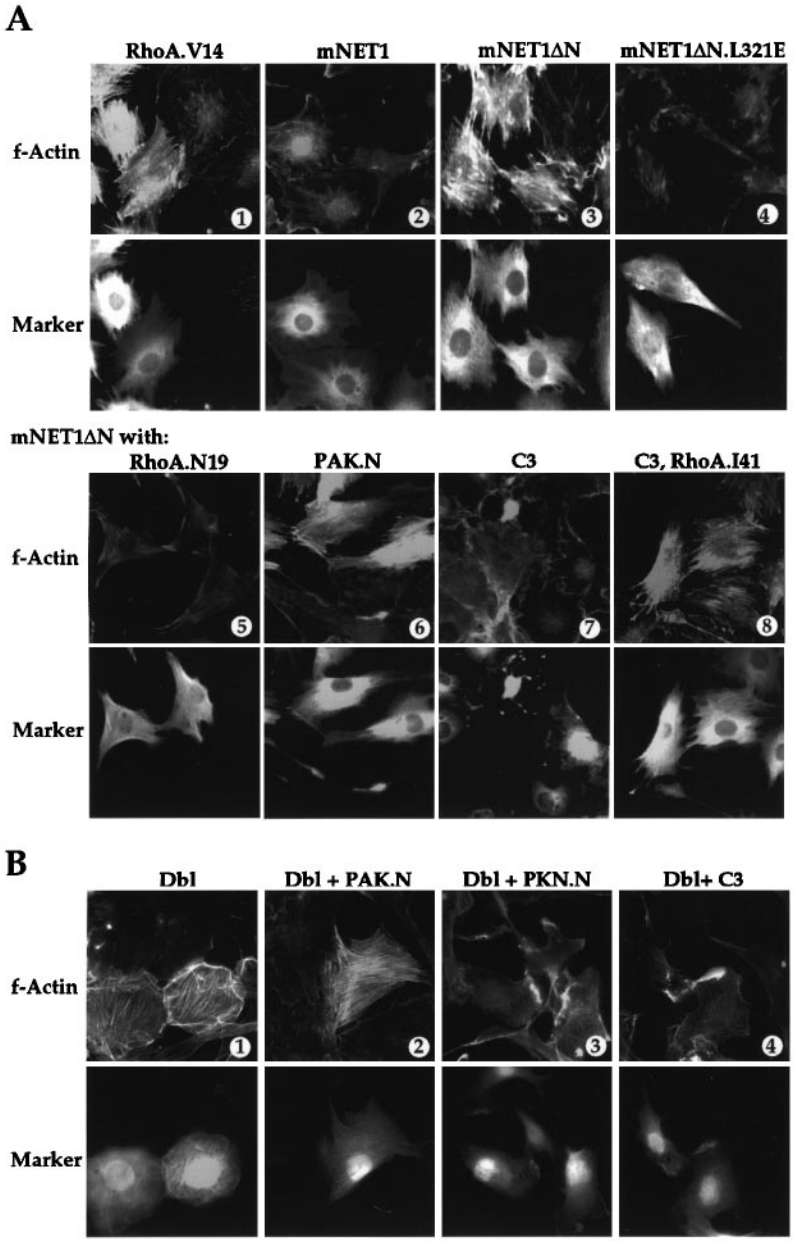

Fig. 4. Actin reorganization by mNET1ΔN and Dbl. (**A**) RhoA mediates mNET1ΔN-induced actin stress fibre formation. Cells were microinjected with expression plasmids as indicated on the figure; for RhoA.N19 expression, recombinant protein was injected at 3 mg/ml. Cells were fixed and stained 3 h later with TRITC–phalloidin (top panels). To visualize injected cells, expressed proteins were stained with either 9E10 (panel 1) or anti-HA (panels 2–8) antibodies which detect RhoA.V14 and mNET1 proteins respectively. (**B**) Dbl induces both ruffling and actin stress fibres. Cells were microinjected with a Dbl expression plasmid together with the following plasmids or proteins and a GFP expression plasmid as marker: panel 1, Dbl only; panel 2, Dbl and recombinant PAK.N; panel 3, Dbl and recombinant PKN.N; panel 4, Dbl and C3 plasmid. Cells were fixed and stained 3 h later with TRITC–phalloidin (top panels); injected cells were visualized via GFP fluorescence.

the injected reporter genes, inactivation of cellular RhoA, by expression of PKN.N or C3 transferase, effectively abolished mNET1ΔN-induced expression, while inactivation of Rac1 and Cdc42 by PAK.N expression had no effect, suggesting that only RhoA is involved in activation of the chromosomal SRE (Figure 5B, panel 3; Table I). We also examined activation of the chromosomal SRF-controlled reporter by Dbl, which activates the SAPK/JNK pathway via Cdc42 and/or Rac1 in transfection assays (Coso *et al*., 1995; Minden *et al*., 1995). Dbl-induced activation of the chromosomal reporter was again inhibited by SEK-AL (Figure 5B, panels 4 and 5); however, unlike the injected reporter, it was also blocked by expression of PAK.N alone, suggesting a specific role for Cdc42 and/or Rac1 in Dbl-induced activation of the chromosomal reporter (Table I).

Activation of the SAPK/JNK pathway by mNET1ΔN

The results presented above suggest that in contrast to Dbl, mNET1ΔN can induce expression of a chromosomal reporter gene by activating the SAPK/JNK pathway independently of Cdc42 or Rac1. To investigate SAPK/JNK activation by the GEFs more directly, we used an immunofluorescence assay. The Jun N-terminal sequences were overexpressed as a LexA fusion protein (NLex.JunN;

Table I. Functional studies of mNET1 and Dbl

Microinjected plasmid	Cytoskeleton[a]	Co-injected SRE[b]	Chromosomal SRE[c]	Focus formation[d]
mNET1	NE	4 ± 4 (2)	2 ± 2 (2)	2, 3
mNET1ΔN alone	stress fibres	92 ± 6 (5)	77 ± 18 (3)	109, 131
mNET1ΔN + C3	no stress fibres	10 ± 9 (2)	4 ± 2 (2)	nt
mNET1ΔN + PKN.N	no stress fibres	2 ± 2 (4)	6 ± 3 (2)	15, 20
mNET1ΔN + PAK.N	stress fibres	93 ± 6 (3)*	88 ± 1 (3)*	3, 31
mNET1ΔN + PAK.N + PKN.N	no stress fibres	12 ± 2 (3)*	nt	nt
mNET1ΔN + SEK-AL	stress fibres	86 ± 4 (2)	3 ± 4 (2)	3, 43
mNET1ΔN(L321E)	NE	2 ± 1 (4)	nt	1
mNET1ΔN(W492L)	NE	1 ± 1 (4)	nt	0
mNET1ΔN + RhoA.N19	NE*	10 ± 5 (3)*	1 ± 2 (3)*	nt
Dbl alone	stress fibres, ruffles	85 ± 4 (3)	62 ± 11 (2)	89, 66
Dbl + C3	ruffles, no stress fibres	93 ± 1 (2)	76 ± 5 (2)	nt
Dbl + PKN.N	ruffles, no stress fibres*	84 ± 2 (3)	66 ± 6 (2)	32, 17
Dbl + PAK.N	stress fibres, no ruffles*	98 ± 4 (3)*	14 ± 7 (3)*	5, 6
Dbl + PAK.N + PKN.N	NE*	6 ± 4 (3)*	nt	nt
Dbl + SEK-AL	stress fibres, ruffles	89 ± 5 (2)	4 ± 3 (2)	23, 3

Cells were injected with the indicated expression plasmids or proteins as described in Materials and methods. C3 expression plasmid was injected at 10 μg/ml. Asterisks indicate use of recombinant RhoA.N19, PAK.N and PKN.N proteins at 3.0 mg /ml. NE, no effect; nt, not tested.
[a]Cytoskeletal rearrangements. Stress fibres, scored as unbranched parallel fibres of filamentous actin running the length of the cell, and peripheral filamentous actin were visualized by TRITC–phalloidin staining. Ruffles were scored visually.
[b]Activation of co-injected SRF reporter 3D.AFosHA. Numbers indicate the percentage of injected cells staining positive for the HA-tagged Fos reporter protein, ± SEM or half range, from the number of independent experiments indicated in parentheses. Similar results were obtained when GEF expression plasmid DNA was injected at 1 μg/ml (data not shown).
[c]Activation of a chromosomal SRF reporter gene in the cell line SRE-FosHA. Numbers indicate the percentage of injected cells staining positive for the HA-tagged Fos reporter protein, ± SEM or half range, from the number of independent experiments indicated in parentheses. Similar results were obtained when GEF expression plasmid DNA was injected at 1 μg/ml (data not shown).
[d]Focus formation assays in NIH 3T3 cells. The number of foci obtained in two independent experiments is shown. Transformation by mNET1ΔN is 4- to 7-fold more efficient than RhoA alone in these assays (E.Sahai, personal communication).

Price *et al*., 1995) and phosphorylation of Jun serine 63 monitored using a phospho-specific antibody. In this assay, Jun S63 phosphorylation could be induced by expression of activated Cdc42, but not by activated RhoA (Figure 6A, panels 1 and 2), in agreement with previous findings that activated Cdc42 can activate the SAPK/JNK pathway (Coso *et al*., 1995; Minden *et al*., 1995). Cdc42-induced SAPK/JNK activation was blocked by expression of SEK-AL and, as expected, was inhibited by PAK.N but not by PKN.N (Figure 6A, panels 3–5).

We used the immunofluorescence assay to monitor SAPK/JNK activation by mNET1ΔN (Figure 6B and C; data summarized in Figure 7A). Expression of mNET1ΔN induced phosphorylation of Jun S63, even at concentrations of injected plasmid DNA insufficient to induce stress fibres (Figure 6B, panel 1; data not shown). Phosphorylation of Jun S63 was blocked effectively by co-expression of SEK-AL, indicating that it reflected activation of the SAPK/JNK pathway (Figure 6B, panel 2). Surprisingly, however, mNET1ΔN-induced SAPK/JNK activation was unaffected by injection of the recombinant PAK or PKN GTPase-binding domains, either singly or in combination (Figure 6B, panels 3–5). In control experiments, activation of the SAPK/JNK pathway by Dbl, which is mediated by Cdc42 and/or Rac1 (Coso *et al*., 1995; Minden *et al*., 1995), was blocked effectively by co-injection of PAK.N, indicating that the recombinant GTPase-binding domain could titrate Cdc42 and/or Rac1 effectively under our experimental conditions (Figures 6C, panels 1, 2 and 4, and 7A). Dbl-induced SAPK/JNK activation was unaffected by PKN.N expression (Figures 6C, panels 3 and 4, and 7A), under conditions sufficient to block Dbl-induced stress fibre formation (Figure 4B, panels 1 and 3). Taken together, these results strongly suggest that although mNET1ΔN can activate the SAPK/JNK pathway, this does not involve titratable GTP-bound forms of Cdc42, Rac or RhoA (see Discussion).

We next investigated the role of mNET1 GEF activity in SAPK/JNK activation. Both the L321E and W492L mutations blocked SAPK/JNK activation (Figure 7A, left panel); moreover, SAPK/JNK activation by mNET1ΔN was sensitive to increasing concentrations of RhoA.N19, while activation by the Rac-specific GEF Tiam1 remained unaffected (Figure 7B). These results show that mNET1ΔN GEF activity is required for SAPK/JNK activation, so we used expression of C3 transferase to test whether RhoA itself is required. In control experiments, C3 expression alone was sufficient to induce SAPK/JNK activation (Figure 7C, left). We therefore tested whether expression of C3 at low levels would affect the ability of mNET1ΔN to activate SAPK/JNK. Under these conditions, mNET1ΔN-induced SAPK/JNK activity was reduced to background level, indicating that a C3-sensitive GTPase is required for mNET1ΔN to induce SAPK/JNK. As with other RhoA-dependent processes, SAPK/JNK activation was restored by expression of the C3-resistant RhoA.I41 mutant (Figure 7C, right). Taken together, these results indicate that activation of the SAPK/JNK pathway by mNET1ΔN requires both functional GEF activity and a C3-sensitive GTPase, probably RhoA (see Discussion).

Transformation by mNET1ΔN requires both RhoA- and Cdc42/Rac1-linked signal pathways

Finally, we investigated the role of different signal pathways in transformation by mNET1. Intact mNET1 did not generate transformed foci upon transfection into NIH

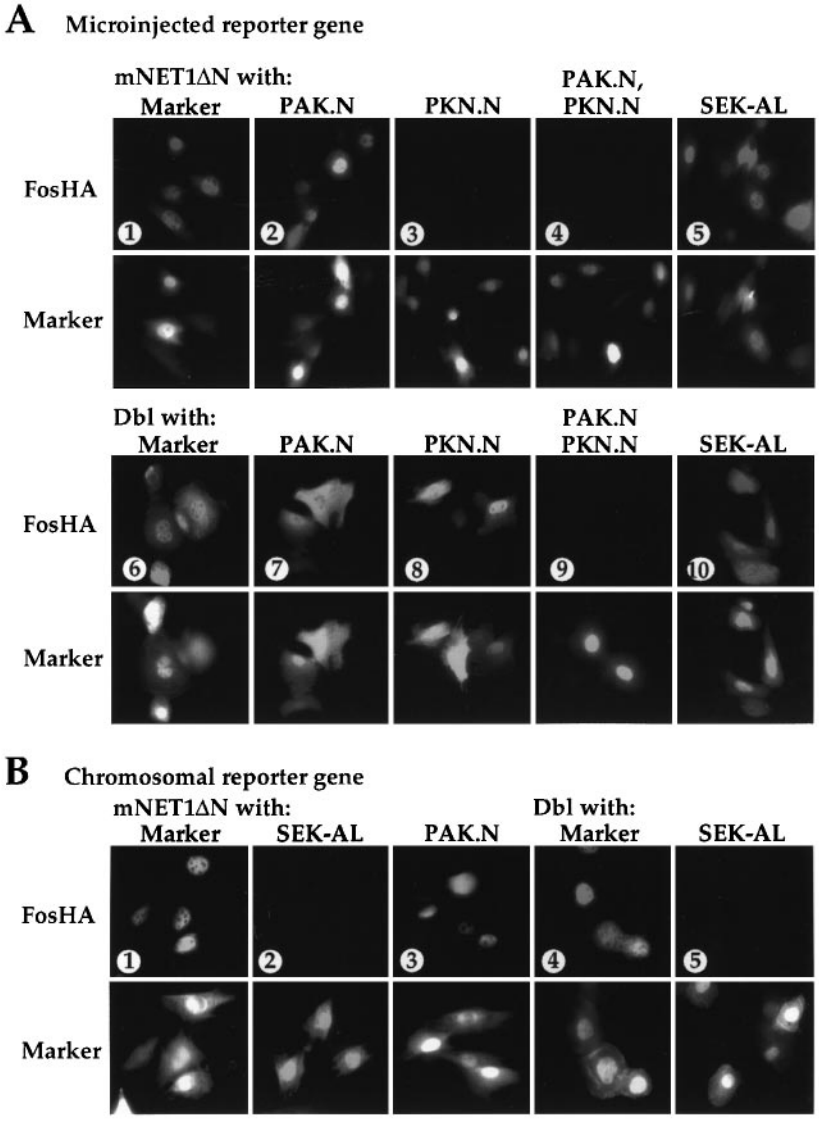

Fig. 5. SRE activation by mNET1ΔN and Dbl. (**A**) mNET1ΔN-induced SRF activity is RhoA-dependent. Serum-deprived NIH 3T3 cells were microinjected with the SRF-controlled reporter gene 3D.AFosHA (Hill *et al*., 1995; Alberts *et al*., 1998a) and expression plasmids encoding either mNET1ΔN (panels 1–5) or Dbl (panels 6–10), together with expression plasmids encoding the indicated proteins and a plasmid encoding GFP as injection marker. After 3 h, the cells were fixed and stained for FosHA protein and GFP. Similar results were obtained when recombinant PAK.N or PKN.N proteins were injected (Table I). (**B**) Activation of a chromosomal SRF-controlled reporter by mNET1ΔN requires SAPK/JNK activity. Serum-deprived SRE-FosHA cells were microinjected with expression plasmids encoding either mNET1ΔN (panels 1–3) or Dbl (panels 4 and 5) together with either a SEK-AL expression plasmid or PAK.N protein (at 3 mg/ml in the needle) and a plasmid encoding GFP as injection marker. After 3 h, the cells were fixed and stained for FosHA protein and GFP.

3T3 cells, whereas both mNET1ΔN and Dbl efficiently generated transformed foci (Table I; Chan *et al*., 1996). mNET1ΔN formed foci 4- to 5-fold more efficiently than did RhoA.V14 (E.Sahai, personal communication). Transformation was dependent on the integrity of the DH and PH domains, since it was abolished by the L321E and W492L mutations (Table I). Since the morphology of mNET1ΔN-transformed cells suggests that signalling pathways other than those controlled directly by RhoA contribute to transformation, we carried out transformation assays in which with mNET1ΔN was co-transfected with plasmids encoding PKN.N, PAK.N or SEK-AL, which inhibit signalling via RhoA-, Cdc42/Rac1- and SAPK/JNK-controlled signal pathways, respectively. Focus formation by both GEFs was significantly inhibited in each case, showing that transformation requires the activity of multiple signalling pathways (Table I). Thus, although mNET1ΔN acts as a RhoA-specific GEF in biochemical assays *in vitro* and functional assays *in vivo*, transformation by this GEF requires the activity of multiple signalling pathways.

Discussion

We have characterized a member of the DH family of GEFs, mNET1. Several lines of evidence show that mNET1 acts as a RhoA-specific GEF. Immunoprecipitates from cells transformed by an activated derivative of mNET1, mNET1ΔN, exhibit RhoA-specific GDP release and GTP loading activities *in vitro*. Microinjection studies *in vivo* show that activated mNET1 induces actin stress fibre formation and potentiates transcriptional activation

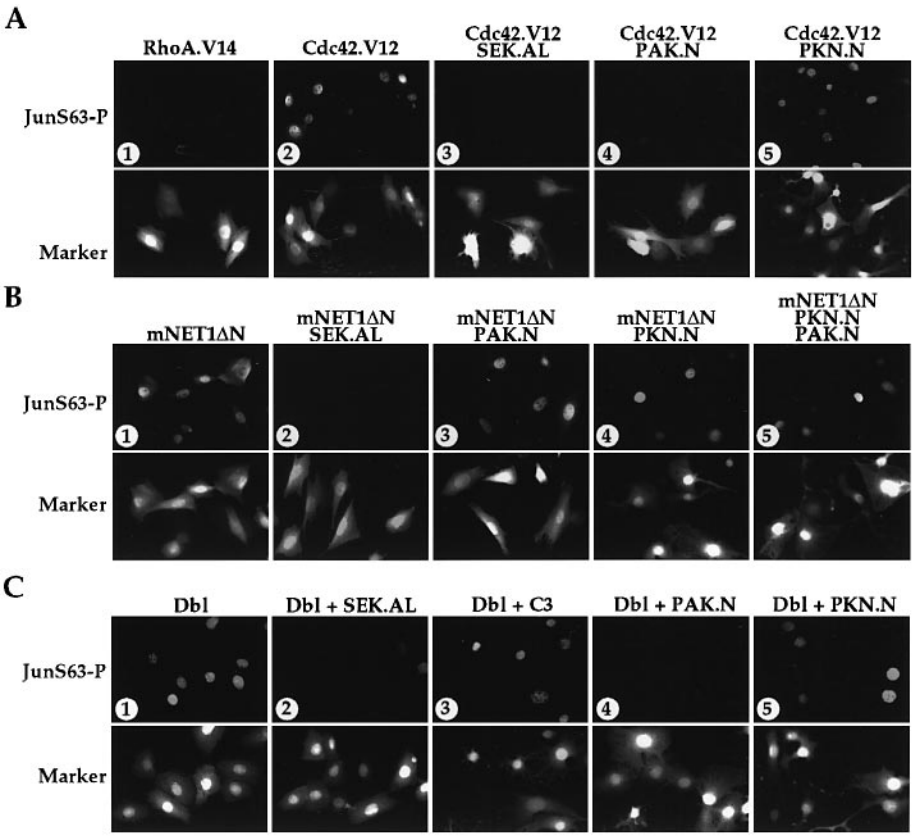

Fig. 6. Activation of the SAPK/JNK pathway by GEFs and Rho-family proteins. Cells were injected with various expression plasmids and proteins, including expression plasmids encoding NLex.JunN and marker GFP. PAK.N and PKN.N were injected as recombinant proteins at 3 mg/ml. After 3 h, cells were stained for JunS63 phosphorylation using a phosphospecific antibody. (**A**) Cdc42.V12 but not RhoA.V14 induces SAPK/JNK activity. In three independent experiments, SAPK/JNK activation occurred as follows: RhoA.V14 alone, 2 ± 1%; Cdc42.V12 alone, 95 ± 6%; Cdc42.V12 plus SEK-AL, 7 ± 7%; Cdc42.V12 with PAK.N, 10 ± 1%; Cdc42.V12 with PKN.N, 94 ± 8%. (**B**) mNET1ΔN activates the SAPK/JNK pathway independently of GTP-bound RhoA or Cdc42/Rac1 proteins. The results are summarized in Figure 7A. (**C**) Dbl-induced SAPK/JNK activation is dependent on GTP-bound Cdc42 or Rac1. Cells were injected with the indicated expression plasmids (C3 plasmid at 10 μg/ml) or proteins and analysed for JunS63 phosphorylation. The results are summarized in Figure 7A.

by SRF: both of these functions are dependent on RhoA, and are unaffected by blockade of Cdc42 or Rac1 signalling pathways. Complementation experiments in cells in which endogenous RhoA is inactivated demonstrated that RhoA itself can mediate mNET1ΔN-induced stress fibre formation. These results are consistent with our previous biochemical and two-hybrid studies, which show that mNET1 interacts with both GTP- and GDP-bound forms of RhoA (Alberts *et al*., 1998b). These studies also showed that mNET1 can interact specifically with GTP-bound Cdc42. Several other Rho-family GEFs, including lfc, ost and yeast Cdc24, have been found to bind GTP-bound GTPases distinct from those for which they act as GEFs (Horii *et al*., 1994; Zheng *et al*., 1995a; Glaven *et al*., 1996), and the interaction of the Cdc24 GEF with the Rsr1 GTPase has been shown to be functionally significant *in vivo* (Zheng *et al*., 1995a). It is therefore possible that mNET1 may somehow be regulated by Cdc42.

The DH domain conventionally would be regarded as spanning mNET1 residues 178–354 (Whitehead *et al*., 1997) or 181–361 (ISREC ProfileScan server; http://ulrec3.unil.ch/software/profilescan.html). However, comparison of the mNET1 sequence with other Rho-family GEFs revealed additional limited sequence conservation between mNET1 and sequences N-terminal to the conventional DH domain. This N-terminal homology, which has the signature W-x(3)-[V,I,L]-x(12)-[V,M,I]-x-[K,R]-Q-[D,E]-I-E, is present in only a subgroup of DH domain proteins (Figure 1B). The nucleotide exchange specificity of seven of these nine proteins, including mNET1, has been investigated, and all have been found to be RhoA specific. In contrast, only one of the 11 other DH domain proteins analysed, Trio DH2 (Debant *et al*., 1996), encodes a RhoA-specific GEF, the others being active on multiple GTPases (Whitehead *et al*., 1997). We suggest that the GEFs with this N-terminally extended DH domain encode RhoA-specific exchange factors.

Truncation of mNET1 at its N-terminus appears to be required for mNET1 to manifest transforming activity in focus formation assays in NIH 3T3 cells, as previously found in the case of several Rho-family GEFs including human NET1 (for review see Whitehead *et al*., 1997). We found that N-terminal truncation was also necessary for mNET1 to induce actin stress fibre formation and SRF activation, and that these activities are also dependent on the integrity of the DH and PH domains. These results are consistent with the idea that N-terminal truncation relieves an inhibitory constraint on mNET1 GEF activity, which is in turn responsible for both short- and long-term effects on cell behaviour.

Although both biochemical and functional assays demonstrate that activated mNET1 functions as a RhoA-

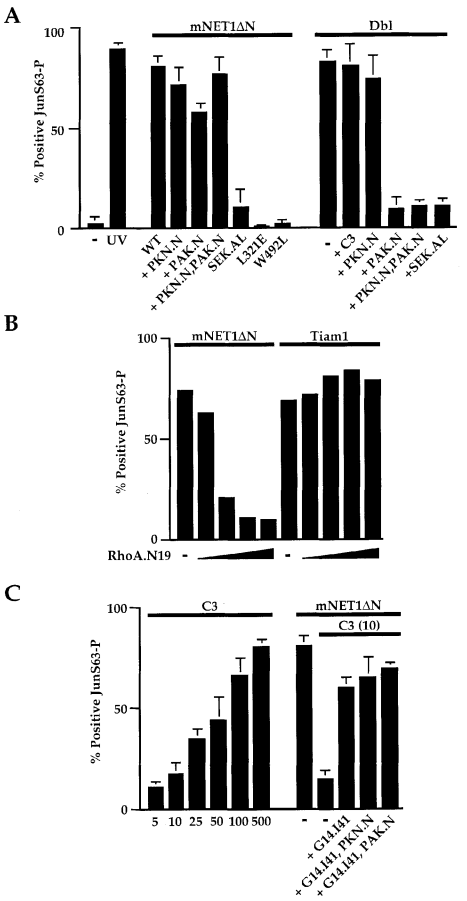

Fig. 7. mNET1ΔN-induced SAPK/JNK activation requires GEF activity and a C3-sensitive GTPase. Cells were injected with various expression plasmids including expression plasmids encoding NLex.JunN and marker GFP. Ater 3 h, cells were stained for JunS63 phosphorylation. Bars indicate the percentage of S63-P-positive injected cells, error bars indicate SEMs. (**A**) mNET1ΔN-induced SAPK/JNK induction is dependent on SEK, sensitive to DH and PH mutations, and independent of GTP-bound RhoA or Cdc42/Rac1. Left, control induction by UV irradiation; centre, SAPK/JNK activation by mNET1ΔN; right, SAPK/JNK activation by Dbl. Results are from 3–5 independent experiments. (**B**) mNET1ΔN-induced SAPK/JNK induction is inhibited by expression of RhoA.N19. Cells were injected with expression plasmids encoding either mNET1ΔN or the Rac-specific GEF Tiam1, together with increasing amounts of recombinant RhoA.N19 protein (0.01, 0.3, 1, 3 mg/ml). In three independent experiments, injection of RhoA.N19 at 3 mg/ml reduced SAPK/JNK activation by mNET1ΔN from 78 ± 5% to 7 ± 9%. (**C**) mNET1ΔN-induced SAPK/JNK induction requires a C3-sensitive GTPase. Left, induction of SAPK/JNK by microinjection of increasing concentrations (μg/ml) of C3 transferase expression plasmid; right, mNET1ΔN was expressed either alone or together with C3 transferase (10 μg/ml plasmid) in the presence or absence of RhoA.G14I41. PAK.N and PKN.N proteins were injected at 3 mg/ml.

specific GEF, we were surprised to discover that in microinjection assays it can activate the SAPK/JNK pathway efficiently. This allows it to activate both injected and chromosomal SRF-controlled reporter genes, in contrast to activated RhoA, which can only activate injected reporters (Alberts *et al.*, 1998a). Both SRF and SAPK/JNK activation by mNET1ΔN were not inhibited by RasN17, indicating that they occur independently of Ras (A.S. Alberts, unpublished observations). SAPK/JNK activation requires mNET1 GEF activity, since it is abolished by mutations in the mNET1 DH or PH domains and is sensitive to titration of mNET1 by overexpression of RhoA.N19; it also involves a C3-sensitive GTPase. Nevertheless, activated RhoA.V14, which is constitutively GTP-bound, does not induce SAPK/JNK in our cells even when expressed at very high levels (although it has been reported to do so in 293T cells; Teramoto *et al.*, 1996). It is unlikely that SAPK/JNK activation merely reflects extremely high levels of RhoA.GTP generated by constitutive GEF activity, because it occurs at mNET1ΔN expression levels insufficient to induce cytoskeletal rearrangements.

Two kinds of model can reconcile these apparently contradictory observations. SAPK/JNK activation either might occur as a result of the nucleotide exchange process itself, or require the hydrolysis of GTP on RhoA. While the latter model is attractive, it is not supported by our observation that RhoA does not contribute to Dbl-induced SAPK/JNK activation; moreover, mNET1ΔN-induced SAPK/JNK activation is insensitive to titration of RhoA.GTP by PKN.N, the RhoA-binding domain of PKN. We therefore favour the view that SAPK/JNK activation is somehow brought about by the active GEF complex. It remains possible, however, that it involves a Rho-family GTPase distinct from those we have tested, that it can occur at levels of active Cdc42 or Rac1 undetectable in our assays, or that it is induced by RhoA.GTP even in the presence of excess PKN.N. It will be interesting to examine signalling by activated RhoA mutants that exhibit high spontaneous nucleotide exchange rates but remain able to hydrolyse GTP analogous to the Cdc42 mutant, F28L (Lin *et al.*, 1997).

In contrast to microinjected cells expressing mNET1ΔN or Dbl, cells transformed by these GEFs appear morphologically similar, with pronounced lamellipodial extensions, suggesting that both maintain active Rac signalling pathways. This similarity is dependent on serum factors since, upon serum starvation withdrawal, both mNET1ΔN- and Dbl- transformed cells adopt morphologies similar to those of the microinjected cells. Although mNET1 appears specific for RhoA in biochemical and short-term assays, focus formation by mNET1ΔN was inhibited by blockade of both RhoA- and Cdc42/Rac1-linked signalling pathways. Similar results were previously obtained with lsc, another RhoA-specific GEF (Whitehead *et al.*, 1996). Transformation by mNET1ΔN and lsc might therefore either involve indirect activation of Rac1- and/or Cdc42-linked signalling pathways, or require basal activity of these pathways. We found that inhibition of SAPK/JNK signalling also reduces the efficiency with which mNET1ΔN acts in focus formation assays. This apparent requirement for SAPK/JNK signalling is not restricted to DH domain oncogenes, however, since it is also observed with the Ras and Met oncogenes, and a transforming epidermal growth factor receptor mutant (Clark *et al.*, 1997; Rodrigues *et al.*, 1997; Antonyak *et al.*, 1998).

Although mutated Rho-family GTPases have not been detected in gene transfer screens for transforming oncogenes, several Rho-family GEFs have been recovered in such assays (for review see Cerione and Zheng, 1996; Van Aelst and D'Souza-Schorey, 1997; Whitehead *et al.*, 1997). A potential explanation for this is that the GEFs activate multiple signal pathways, and indeed several DH proteins can activate more than one Rho-family GTPase. However, at least one RhoA-specific GEF, lbc, has been

recovered as an oncogene in gene transfer experiments (Toksoz and Williams, 1994). It is not known whether lbc activates signalling pathways in addition to RhoA: it does not activate Cdc42, Rac1, TC10 or RhoG (Zheng *et al*., 1995c), although it remains possible that it activates unidentified additional Rho-family GTPases. We speculate that the ability of mNET1ΔN, and perhaps other RhoA-specific GEFs, to activate the SAPK/JNK pathway may in part underlie their apparent potency as transforming oncogenes.

Materials and methods

Plasmids and cells

Plasmids were constructed by standard techniques. The following have been described previously: Rho-family and C3 transferase expression plasmids (Hill *et al*., 1995); SRE-FosHA reporter, EF-PAK.N, EF-PKN.N, GST–PKN.N, GST–PAK.N (Alberts *et al.*, 1998a); RK5-myc-Dbl (Olson *et al*., 1996); MLVNLex.JunN (Price *et al*., 1995), pcDNA3-SEK, SEK-AL (Sanchez *et al*., 1994). EF-9E10-mNET1ΔN encodes 9E10-MAGS-(mNET1 codons 122–595). EF.GFP encodes green fluorescent protein (GFP) and EF-9E10-mNET encodes 9E10-MAGS-(mNET1 codons 1–595). EF-HA-mNET1ΔN is as EF-mNET1ΔN except with an N-terminal HA epitope tag. Mutant HA-mNET1ΔN derivatives L321E and W492L were constructed by standard techniques.

GEF assays

Clones of EF-HAmNET1ΔN- and RK5-myc-Dbl transformed cells were expanded (clones HAmNET1ΔN-D and 9E10Dbl-E, respectively; ~5×10^6 cells per assay) and lysed in 300 µl of 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.2% Triton X-100, 20 mM Tris–HCl pH 8.0, containing protease inhibitors. Alternatively, extracts were prepared similarly from single 9 cm plates of cells transiently transfected with 3 µg of expression plasmid. Clarified extracts were pre-cleared with protein G–Sepharose (Pharmacia/LKB) bound to non-specific mouse immunoglobulins then incubated with protein G–Sepharose immune complexes [100 µl 50% (v/v) in lysis buffer] bearing either 12CA5 or 9E10 monoclonal antibodies. GDP-dissociation and GTPγS-binding assays were performed exactly as described (Zheng *et al*., 1995b) using 20 µl of slurry per assay. Control immune complexes were prepared from HAmNET1ΔN-D and 9E10Dbl-E using 9E10 and HA antibodies, respectively.

Microinjection and immunofluorescence techniques

Cells were grown on glass coverslips and injected using a Zeiss 5171 semi-automated machine using pulled glass capillaries. For reporter gene assays, 50–100 cells per experiment were injected for each treatment (Alberts *et al*., 1993, 1998a). In general, reporter DNAs were injected at 10 µg/ml, and expression plasmid DNAs at 50 µg/ml unless otherwise stated. As a microinjection control, EF.GFP was injected at 10 µg/ml. Recombinant GTPase-binding domains PAK.N and PKN.N, and RhoA.N19 were injected at 3.0 mg/ml. Reporter gene expression analysis by indirect immunofluorescence was with 9E10 and 12CA5 monoclonal antibodies, respectively (ICRF; Alberts *et al.*, 1998a). Phospho-specific JunS63 antibody (NEB) was used at 1:50 dilution.

Other methods

DNA sequences were determined using an ABI automated sequencer using appropriate primers and assembled using Sequence Navigator software. Sequence comparisons and tree assembly used GCG PileUp and Growtree programs (GCG; Feng and Doolittle, 1987). Immunoblotting and immunoprecipitations were by standard methods. Focus formation assays in NIH 3T3 cells were as described (Sahai *et al*., 1998). Preparation of recombinant GTPase-binding domains and RhoA.N19 was by thrombin cleavage from recombinant GST–PKN.N, GST–PAK.N and GST–RhoA.N19 proteins (Alberts *et al.*, 1998a).

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