

Nesca, a novel adapter, translocates to the nuclear envelope and regulates neurotrophin-induced neurite outgrowth

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We provide the first characterization of a novel signaling adapter, Nesca, in neurotrophic signal transduction. Nesca contains a RUN domain, a WW domain, a leucine zipper, a carboxyl-terminal SH3 domain, and several proline-rich regions. Nesca is highly expressed in the brain, is serine phosphorylated, and mobilizes from the cytoplasm to the nuclear membrane in response to neurotrophin, but not epidermal growth factor, stimulation in a MEK-dependent process. Overexpression studies in PC12 cells indicate that Nesca facilitates neurotrophin-dependent neurite outgrowth at nonsaturating doses

of nerve growth factor (NGF). Similarly, short interfering RNA studies significantly reduce NGF-dependent neuriteogenesis in PC12 cells. Mutational analyses demonstrate that the RUN domain is an important structural determinant for the nuclear translocation of Nesca and that the nuclear redistribution of Nesca is essential to its neurite outgrowth-promoting properties. Collectively, these works provide the first functional characterization of Nesca in the context of neurotrophin signaling and suggest that Nesca serves a novel, nuclear-dependent role in neurotrophin-dependent neurite outgrowth.

Introduction

Ligand activation of the NGF receptor tyrosine kinase, *trkA*, results in the downstream activation of several signaling proteins including Ras, MAPK, phosphatidylinositol-3 kinase (PI-3 kinase), Raf-1, FRS2, Shc, and AKT (Meakin, 2000; Huang and Reichardt, 2003). As a consequence of the activation of such a varied array of signaling intermediates, *trkA* plays a vital role in a variety of cellular processes such as the survival and development of sympathetic neurons as well as the pain and temperature sensitive sensory neurons of the peripheral nervous system (Meakin, 2000; Huang and Reichardt, 2003). Given the diverse roles that *trkA* plays in regulating neuronal development and function, an understanding of the complex intracellular signal transduction pathways stimulated after *trkA* activation is highly relevant.

Incumbent upon our understanding of how *trkA* functions is an understanding of how the kinase and downstream targets are regulated and how this translates into changes in gene expression and cell function. Several signaling proteins bind

directly to activated *trkA*, including Shc, phospholipase C γ -1 (PLC γ -1), rAPS, SH2B, FRS2, Grb2 (MacDonald et al., 2000; Meakin, 2000; Qian and Ginty, 2001; Huang and Reichardt, 2003), the Csk homologous kinase (CHK; Yamashita et al., 1999), and the atypical PKC-interacting protein p62 (Wooten et al., 2001a). Many of these proteins are recruited into signaling complexes and activate downstream targets such as MAPK, whereas others act as scaffold proteins to stabilize active *trkA* complexes. Activated *trkA* also binds to the intermediate filament proteins peripherin and α -internexin (MacDonald et al., 1999), which may also have scaffolding implications, by coupling *trkA* to the cytoskeleton.

In neuroendocrine rat pheochromocytoma (PC12) cells, NGF induces a differentiative response, whereas in non-neuronal cells, the response is proliferative (Meakin, 2000). Although the regulatory elements governing these diverse responses are not completely understood, several pathways have been implicated. Included among these pathways are the tyrosine phosphorylation and recruitment of FRS2 (Meakin et al., 1999; Ong et al., 2000; Zeng and Meakin,

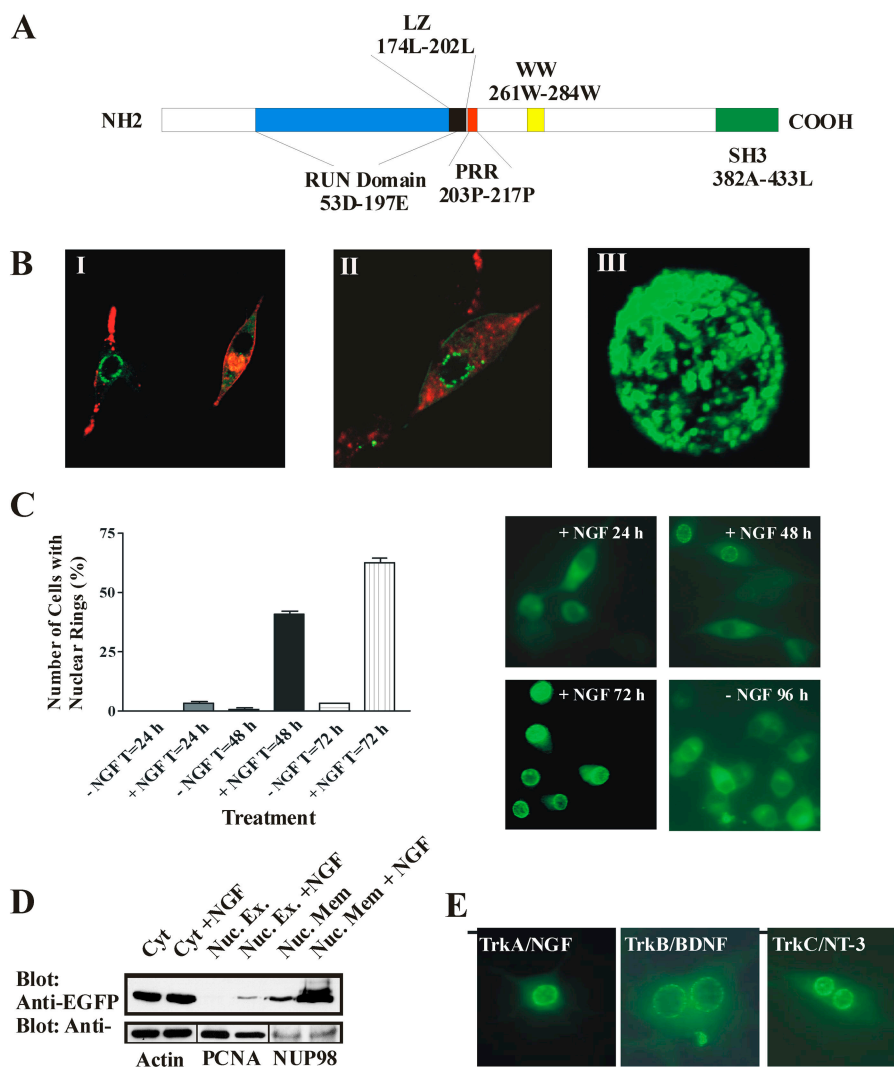
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Abbreviations used in this paper: BDNF, brain-derived neurotrophic factor; CHK, Csk homologous kinase; EGFR, EGF receptor; GAPDH, glycerol-3-phosphate dehydrogenase; NT-3, neurotrophin-3; RFP, red fluorescent protein; siRNA, short interfering RNA.

Figure 1. Nesca localizes with the nuclear envelope in NGF-treated cells.

(A) Schematic representation of Nesca. The RUN domain lies between 53D and 197E. The leucine zipper (174L–202L), proline-rich region (203P–217P), putative WW domain (261W–284W), and the SH3 domain (382A–433L) are indicated. (B) Confocal analysis of EGFPNesca and RFP-trkA in *nnr5* cells treated with 100 ng/ml NGF for 72 h. Panels I and II show the intracellular distribution of RFP-trkA and EGFPNesca. Panel III reveals the association of NescaEGFP with the nuclear membrane. (C) The nuclear trafficking of Nesca was assessed over 72 h of NGF treatment (100 ng/ml) in *trkA*-expressing B5 cells by fluorescence microscopy. The ratio of cells with nuclear rings relative to transfected cells was determined at 24, 48, and 72 h of NGF stimulation. (D) Subcellular fractions of B5 cells expressing EGFPNesca (–/+ NGF) were prepared as described, and equal protein amounts were assayed by SDS-PAGE. Immunoblots were probed with an anti-EGFP antibody to localize EGFPNesca and with antibodies against actin, PCNA, and NUP98 as markers for cytoplasmic extracts, nuclear extracts, and the nuclear envelope, respectively. (E) Nesca mobilizes to the nuclear membrane after BDNF and NT-3 stimulation (100 ng/ml, 4 d) of *trkB*- and *trkC*-expressing *nnr5* cells.



2002), prolonged activation of MAPK (York et al., 1998; Kao et al., 2001; Wu et al., 2001; Yasui et al., 2001), activation of p38 MAPK (Morooka and Nishida, 1998; Iwasaki et al., 1999), and atypical PKC-mediated activation of NF- κ B (Wooten et al., 2001a,b).

One recently described putative signaling adapter protein is Nesca (new molecule containing an SH3 domain at the carboxyl terminus; Matsuda et al., 2000). Although the signaling capacity of Nesca has yet to be shown, its molecular architecture is highly reminiscent of a signaling adapter protein. In addition to an SH3 domain, Nesca contains a putative WW domain, a leucine zipper, and several distinct proline-rich regions that may serve to recruit other SH3 domain-containing proteins (Fig. 1 A; Kay et al., 2000). Nesca also contains a newly described RUN domain spanning 144 amino acids from 53D–197E (Callebaut et al., 2001). Although the precise role of the RUN domain remains to be determined, many RUN domain-containing proteins are involved in Ras-like GTPase signaling (Callebaut et al., 2001; Mari et al., 2001; Yang et al., 2002).

Herein, we provide the first characterization of a role for Nesca in cell signaling. Specifically, we demonstrate that

Nesca mobilizes from the cytoplasm to the nuclear envelope in response to neurotrophin stimulation. The intracellular redistribution of Nesca takes place gradually, increasing between 24–72 h of growth factor stimulation, and is coincident with increased serine phosphorylation. From overexpression and short interfering RNA (siRNA) studies in PC12 cells, we also propose a possible function for Nesca in regulating NGF-dependent neurite outgrowth. Collectively, these data suggest that Nesca plays a role in neuronal differentiation, specifically in signaling pathways that are regulated by the prolonged activation of MAPK.

Results

Nesca translocates to the nucleus in response to NGF in neurotrophin-responsive cells

In our ongoing efforts to delineate the signal transduction pathways mediated through *trkA*, we identified a poorly characterized protein Nesca (Fig. 1 A; Matsuda et al., 2000) in a yeast two-hybrid screen using the intracellular domain of *trkA* as bait. As shown in Fig. 1 A, the molecular architecture of Nesca resembles a signaling adapter, suggesting a potential role in signal transduction. Initially, we

examined the subcellular localization of Nesca in NGF-stimulated cells using confocal microscopy to determine if EGFP-tagged Nesca colocalized with red fluorescent protein (RFP)-tagged *trkA* in the *nnr5* cell line. *Nnr5* cells do not express *trkA* or respond to NGF, but transfection with *trkA* reconstitutes their NGF-dependent neurite outgrowth. However, instead of a colocalization between Nesca and *trkA*, we observed that Nesca localized primarily to the nuclear envelope (Fig. 1 B) with a small amount at the plasma membrane, whereas *trkA* was predominantly located in the neurites (Fig. 1 B). Serial confocal images suggest an association between Nesca and the nuclear pores, the major proteinaceous component of the nuclear envelope (Fig. 1 B, III; Mattaj and Englmeier, 1998). The lack of colocalization, in conjunction with our inability to coprecipitate *trkA* and Nesca (unpublished data), suggests a lack of direct interaction between these proteins in NGF-stimulated cells.

Despite the lack of interaction between *trkA* and Nesca, the nuclear localization of Nesca in NGF-treated cells was striking, and we examined this process in further detail. Our results revealed the nuclear localization of Nesca to be a dynamic process in that NGF stimulates a time-dependent translocation of Nesca from the cytosol to the nuclear envelope. The translocation process becomes readily apparent only after 48 h (Fig. 1 C), and by 72 h, greater than two-thirds of transfected cells displayed prominent nuclear rings (Fig. 1 C). In contrast, we observed no Nesca nuclear translocation in the absence of NGF, even after 96 h (Fig. 1 C). Cellular fractionation analysis confirmed the microscopic data in that the bulk of Nesca localizes to the cytosol in unstimulated cells, whereas NGF stimulates an increase of Nesca in the nuclear membrane (Fig. 1 D). A small amount of Nesca was also observed in the NGF-treated nuclear extract (Fig. 1 D), suggesting the possibility that Nesca may also be present in the soluble nucleoplasm. We assessed the equivalence of protein loading by stripping and reprobing with antibodies specific to proteins expressed in each fraction (Fig. 1 D). In contrast to *trkA*-expressing *nnr5* cells (B5), we observed no nuclear localization of Nesca in nonneuronal HEK293 cells, even when coexpressing *trkA* (unpublished data). The nuclear translocation of Nesca was unaffected by treatment with the microtubule-disrupting agents nocodazole or colchicine (Wang et al., 1998; Nusser et al., 2002), despite a complete collapse of the neuritic network (unpublished data). These data suggest that the nuclear translocation of Nesca is not a passive process and is not dependent on the microtubule network.

NGF is a member of the neurotrophin family of growth factors that signal through a family of related *trk* receptors. Thus, we determined if other neurotrophins, specifically brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) also induced the nuclear translocation of Nesca. Stable *nnr5* lines expressing *trkB* and *trkC* were stimulated with BDNF and NT-3, respectively, and assessed for neurite outgrowth and the nuclear translocation of Nesca. In both cases, both BDNF/*trkB* and NT-3/*trkC* stimulated the nuclear redistribution of Nesca (Fig. 1 E) comparably to NGF/*trkA*.

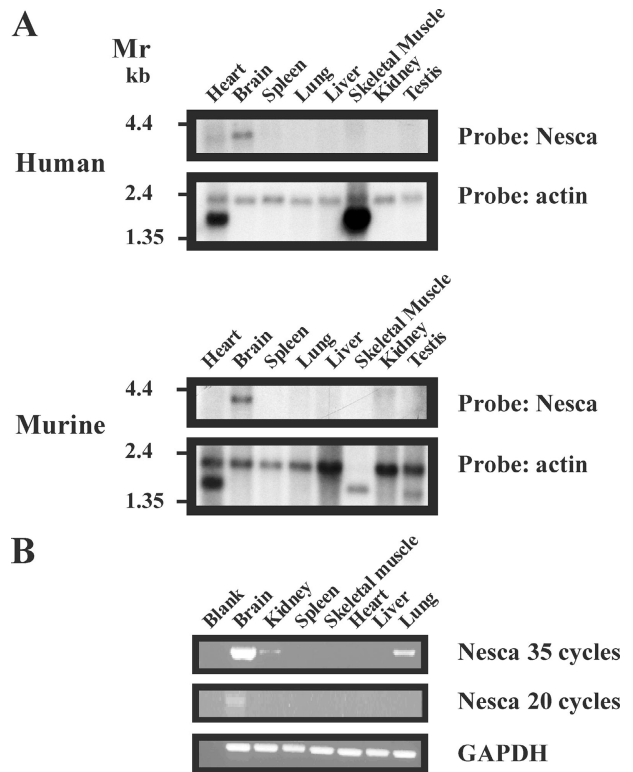


Figure 2. **Nesca is expressed primarily in the brain.** (A) Multiple tissue Northern blots were probed with random primed Nesca-specific probes. (B) Total RNA was isolated from various murine tissues, and RT-PCR was performed as described in Materials and methods. GAPDH served as a control.

Nesca expression is highly enriched in the brain

Because we observed the neurotrophin-dependent nuclear translocation of Nesca only in neuronal-like cells, we addressed the tissue distribution of Nesca expression. Previously, it had been reported that Nesca is expressed at equal levels across a variety of human tissues (Matsuda et al., 2000). However, we obtained a predominantly neuronal expression pattern upon probing human and murine multiple tissue Northern blots (Fig. 2 A). Therefore, we performed nonquantitative RT-PCR on a series of murine tissues using primers directed against the mouse Nesca (655C-1552T) sequence obtained from the NCBI EST database (GenBank/EMBL/DDBJ accession no. AK039664). Included in the predicted 895 bp murine Nesca fragment is 175 bp of 3'UTR. Our results partially agree with previous results (Matsuda et al., 2000) in that Nesca is expressed in some nonneuronal tissues; however, we find a considerable enrichment in brain (Fig. 2 B). At 20 cycles, Nesca was apparent only in brain. In each case, the putative Nesca band was confirmed by sequence analysis. Glycerol-3-phosphate dehydrogenase (GAPDH) served as a control (Fig. 2 B).

The nuclear translocation of Nesca is mediated through sequences contained in the amino terminus and occurs independently of an NLS

Many proteins that mobilize to the nucleus do so by means of an NLS. Classically, two types of NLS have been described: (1) a single stretch of 5–6 basic amino acids, and (2) a bipar-

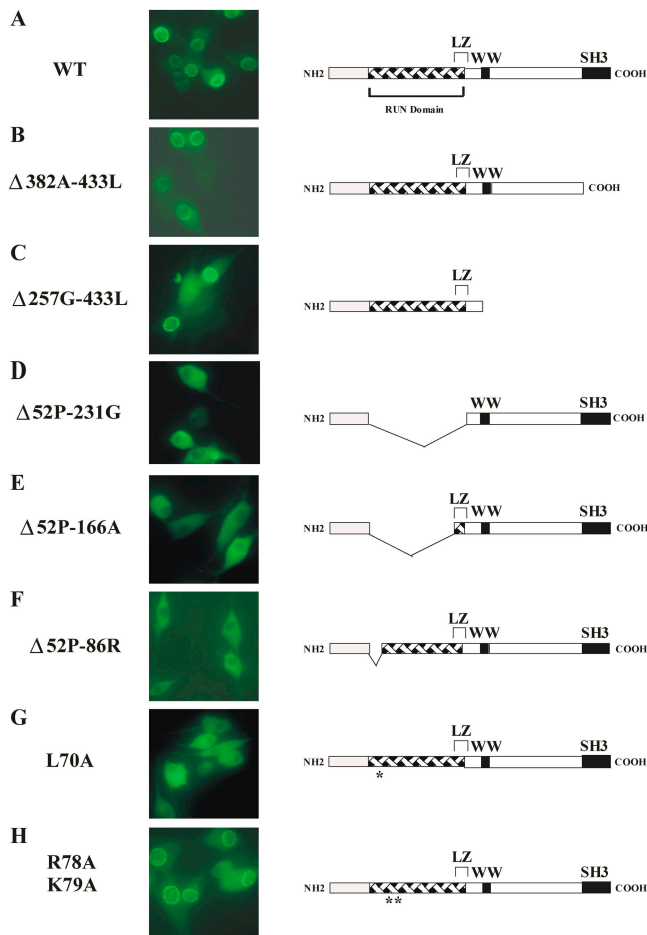


Figure 3. The RUN domain is essential for nuclear translocation. Nesca deletion and point mutants (EGFP tagged; A–H as indicated) were assessed for NGF-dependent (100 ng/ml NGF, 5 d) nuclear translocation in *trkA*-expressing B5 cells.

tite NLS consisting of 2 basic amino acids followed by a cluster of 5 basic amino acids that are separated by a spacer region of ~ 10 amino acids (Mattaj and Englmeier, 1998). Sequence analysis of Nesca using PSORT (<http://psort.nibb.ac.jp>) failed to reveal the presence of an NLS, although several basic amino acid stretches are present ($^{10}\text{QLQEQQK}^{16}$, $^{335}\text{KSRR}^{338}$, and $^{75}\text{KPRKDLITGQRR}^{87}$). None of these sequences supported the nuclear localization of a β -Gal/EGFP fusion protein (Sorg and Stamminger, 1999), comparable to the NLS of p53, or enhanced the nuclear localization of EGFP alone, indicating that they do not function as an NLS (unpublished data).

Consequently, we generated a series of carboxyl- and amino-terminal deletion mutants to identify sequences important to the nuclear translocation. The ΔSH3 mutant deletes amino acids 382A–433L and the $\Delta 257\text{G}$ –433L mutant deletes the entire carboxyl terminus up to the WW domain at 261W. This latter mutant retains the proline-rich region between 203P and 217P and the leucine zipper between 174L and 202L. Both of these deletion mutants retained the ability to translocate to the nucleus upon NGF stimulation (compare Fig. 3, B and C, with Fig. 3 A). The amino-terminal deletion mutants include $\Delta 52\text{P}$ –231G, deleting the entire RUN domain and the leucine zipper, $\Delta 52\text{P}$ –166A deletes the

entire RUN domain and $\Delta 52\text{P}$ –86R deletes the first conserved block of the RUN domain. In contrast to deletions at the carboxyl terminus, deletions within the RUN domain completely abrogated the translocation of Nesca, even after 5 d (compare Fig. 3, D–F, with Fig. 3 A). Point mutations within the RUN domain had variable effects on the translocation of Nesca. Alanine substitution of 78R and 79K had no effect on the nuclear translocation of Nesca, whereas alanine substitution of 70L had a deleterious effect (Fig. 3, G and H). Leucine 70 constitutes an important conserved residue within the first block comprising the structure of the RUN domain, whereas 78R and 79K are not believed to play specific roles as structural determinants (Callebaut et al., 2001). No mutant underwent nuclear translocation in the absence of NGF. These data indicate that the RUN domain serves an essential role in the nuclear redistribution of Nesca.

Nesca is phosphorylated in response to NGF

The intracellular localization of many proteins is regulated by their phosphorylation state; thus, we investigated if NGF induces changes in the phosphorylation of Nesca. Previous results indicated that Nesca is not tyrosine phosphorylated (unpublished data), however, this did not exclude the possibility that Nesca may be a target of a serine/threonine kinase(s) activated in response to *trkA*. To address this issue, *trkA*-expressing B5 cells were transfected with EGFP, EGFP-Nesca, or HA-Nesca and incubated with NGF for 72 h, the last 12 h of which included [^{32}P]orthophosphate in phosphate/serum-free media. We selected a 72-h time point because the translocation of Nesca occurs maximally between 48 and 72 h (Fig. 1). Labeled cells were lysed and EGFP-Nesca/HA-Nesca were immunoprecipitated and analyzed for ^{32}P incorporation and expression by Western blotting. As shown in Fig. 4, a substantial increase in ^{32}P incorporation in both

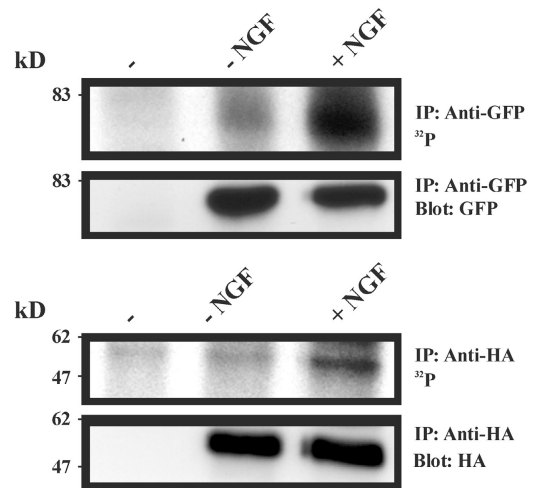


Figure 4. Nesca is phosphorylated in response to NGF. B5 cells were transfected with EGFPNesca or HA-Nesca (–/+ 100 ng/ml NGF, 72 h) or untransfected (–). Cells were loaded with [^{32}P]orthophosphate, washed, lysed, and immunoprecipitated with anti-EGFP or anti-HA antibodies. Immune complexes were separated by SDS-PAGE and analyzed for ^{32}P incorporation and immunoblot analysis using 3F10 (anti-HA) or anti-EGFP antibodies.

EGFPNesca and HA-Nesca was observed in the presence of NGF (Fig. 4, $-/+$ NGF). We obtained identical results with a myc-tagged Nesca construct (unpublished data). Densitometry of ^{32}P -labeled Nesca in Fig. 4, normalized to the amount of EGFPNesca or HA-Nesca in each lane, revealed an approximate sixfold increase in the phosphorylation state of EGFPNesca in response to NGF and a fourfold increase in the phosphorylation state of HA-Nesca. We detected no ^{32}P incorporation in EGFP alone (unpublished data). Phosphoamino acid analysis of phospho-Nesca revealed only the presence of phosphoserine (unpublished data).

The aforementioned data confirms that the nuclear translocation and phosphorylation of Nesca occur coincidentally. However, we wanted to determine the earliest point at which Nesca is phosphorylated. Accordingly, B5 cells expressing HA-tagged Nesca were labeled with [^{32}P]orthophosphate and stimulated with NGF for various times up to 24 h. No Nesca phosphorylation was detected up to 24 h of NGF stimulation (unpublished data), suggesting a correlation between Nesca's phosphorylation and its nuclear translocation.

Translocation of Nesca to the nucleus is growth factor specific and is dependent on prolonged MAPK activation

Because the neurotrophins comprise a closely related family of growth factors eliciting similar cellular effects, we examined the effect of an unrelated growth factor, namely, EGF. Although many of the same signaling proteins are activated upon stimulation with EGF and NGF (Wells, 1999; Meakin, 2000), EGF does not induce cell cycle arrest or neurite outgrowth in PC12 cells but rather elicits a proliferative response (Wells, 1999; Kao et al., 2001). A major difference between NGF and EGF signaling involves the activation of MAPK. In NGF-treated cells, the activation of MAPK is prolonged, in contrast to the transient activation in response to EGF (York et al., 1998; Kao et al., 2001). To address this difference, B5 cells were cotransfected with EGFPNesca and the EGF receptor (EGFR) and assayed for both EGF-dependent nuclear translocation of Nesca and/or the ability of Nesca overexpression to possibly facilitate the ability of EGF to stimulate neurite outgrowth. As shown in Fig. 5, EGF treatment greatly enhanced the EGFR phosphorylation but resulted in neither the induction of neurites nor a change in the intracellular localization of Nesca (Fig. 5, A and B).

In a similar experiment, we analyzed the ability of *trkA* mutants, unable to support NGF-dependent differentiation, for their ability to promote the translocation of Nesca. *TrkA* mutants designated *trkAS3* ($\Delta^{493}\text{IMENP}^{497}$) and *trkAS8* (Y^{499}F) are impaired in their ability to bind Shc and FRS-2 (Stephens et al., 1994; Meakin and MacDonald, 1998; Meakin et al., 1999). Despite being fully kinase active, neither mutant supports NGF-dependent neurite outgrowth (Meakin and MacDonald, 1998; Meakin et al., 1999) and both are impaired in their ability to activate MAPK when assayed at comparable levels of receptor expression relative to wild-type *trkA* (Meakin and MacDonald, 1998; Meakin, 2000). Not surprisingly, these mutants are incapable of supporting the NGF-dependent nuclear translocation of Nesca

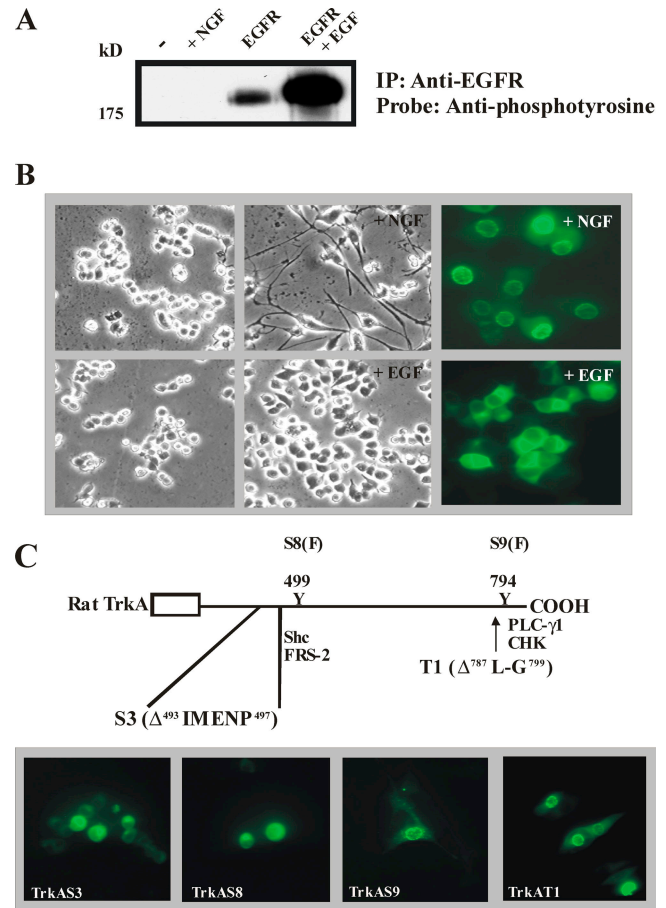
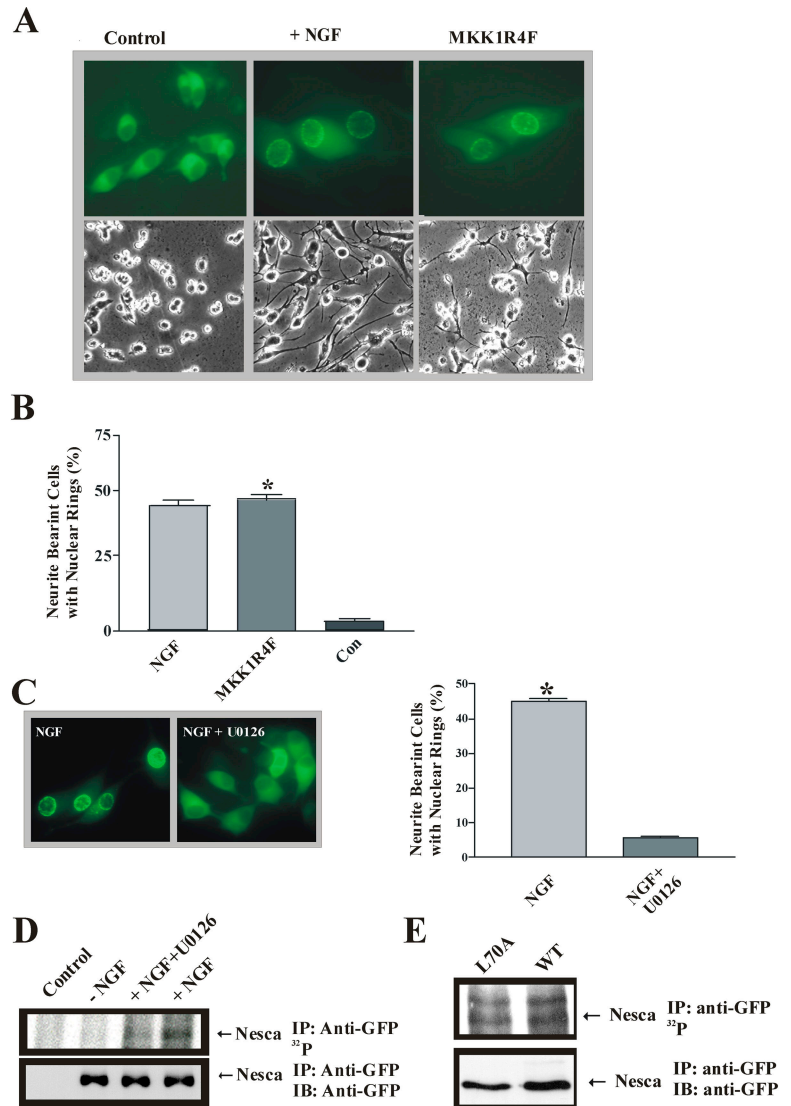


Figure 5. Nesca does not translocate to the nucleus in response to EGF or after activation of *trkA* mutants incapable of supporting neurite outgrowth. B5 cells were cotransfected with EGFPNesca and the EGFR and stimulated with 100 ng/ml EGF for 72 h to assess EGFR activation (A) and cell phenotype/nuclear rings (B). (C) Nnr5 cells expressing the *trkA* mutants *trkAS3* ($\Delta^{493}\text{IMENP}^{497}$), *trkAS8* (Y^{499}F), *trkAS9* (Y^{794}F), and *trkA-T1* ($\Delta^{787}\text{L-G}^{799}$) were transfected with EGFPNesca and stimulated with 100 ng/ml NGF for 5 d.

(Fig. 5 C). Two other *trkA* mutants, *trkAS9* (Y^{794}F) and *trkAT1* ($\Delta^{787}\text{L-G}^{799}$), were assessed for their ability to support the nuclear translocation of Nesca. Neither mutant binds PLC γ -1 or CHK (Stephens et al., 1994; Yamashita et al., 1999); however, both support NGF-dependent neurite outgrowth and the nuclear translocation of Nesca. These data indicate that the mobilization of Nesca is independent of PLC γ -1 or CHK (Fig. 5 B).

The aforementioned data indicates a link between Nesca translocation and the prolonged activation of MAPK. Pursuant to this, we mimicked the effects of NGF by activating MAPK with a constitutively active MKK1 construct (R4F; Emrick et al., 2001). Thus, differentiation in the absence of *trk* activation was obtained. As expected, B5 cells transfected with MKK1R4F extended neurites (Fig. 6, A and B). Moreover, when coexpressing EGFPNesca, neurite extension was coincident with a translocation of Nesca to the nucleus (Fig. 6 A). In a similar experiment, we inhibited MAPK with the selective MKK1/MKK2 inhibitor UO126 (Favata et al., 1998). Neurotrophin-responsive B5

Figure 6. The nuclear translocation of Nesca is stimulated by constitutively active MKK1 and is dependent on prolonged MAPK activation. (A) The nuclear translocation of Nesca was assessed in B5 cells coexpressing EGFPNesca and a constitutively active MKK1 (R4F; Emrick et al., 2001). (B) The nuclear translocation of Nesca was determined as the ratio of total transfected cells containing nuclear rings after 48 h. The error bars represent the SD from the mean. Statistical significance (*, $P < 0.001$) of neurite induction by MKK1 R4F relative to the control was determined using *t* test. (C) B5 cells expressing EGFPNesca were stimulated with NGF for 48 h in the presence and absence of the MKK1 inhibitor U0126 (10 μ M). Quantitation (right) was performed as described in B. The error bars represent the SD from the mean. (D) Cells were labeled with [32 P]orthophosphate and analyzed for [32 P]phosphate incorporation. The control represents untransfected cells. The bottom panel shows the expression levels of EGFPNesca. (E) B5 cells expressing the L70A Nesca mutant and/or wild-type Nesca were loaded with [32 P]orthophosphate and analyzed for [32 P]phosphate incorporation.



cells expressing EGFPNesca were incubated with 10 μ g/ml U0126 before stimulation with 100 ng/ml NGF. Cells treated with U0126 and NGF displayed considerably smaller, less developed neurites relative to controls (unpublished data). Coincident with the lack of neurite development, we observed a significant decrease in the nuclear translocation of Nesca in the U0126-treated cells (Fig. 6 C). Moreover, we found that treatment of B5 cells with U0126 also led to an inhibition of NGF-dependent phosphorylation of Nesca (Fig. 6 D). Although Nesca contains several potential MAPK phosphorylation sites, we have not been able to show Nesca to be a substrate of MAPK *in vitro*. Alternatively, Nesca may be phosphorylated by a kinase activated downstream of MAPK in the neurotrophin signaling pathway.

Although the nuclear translocation of Nesca is dependent on the prolonged activation of MAPK, we determined if Nesca is phosphorylated in the absence of translocation. Accordingly, we compared the incorporation of [32 P]phosphate in EGFPNesca and the EGFPNesca L70A mutant incapable of undergoing NGF-dependent nuclear translocation (Fig. 3 G). Surprisingly, we found that the wild type and L70A mu-

tant were phosphorylated to similar levels (Fig. 6 E). Thus, although prolonged activation of MAPK is essential to the nuclear translocation of Nesca, phosphorylation appears to precede nuclear translocation.

Nesca potentiates neurite outgrowth in PC12 cells

It has been suggested that RUN domains function in Ras-like GTPase signaling (Callebaut et al., 2001). Because Nesca contains a RUN domain and may play a role in small GTPase signaling, we determined the effects of Nesca overexpression on NGF-dependent PC12 cell differentiation. Stable PC12 cell lines overexpressing EGFPNesca and EGFP alone were generated and, as overexpression of Nesca alone induced no discernible phenotype, we assayed for phenotypic changes in response to NGF. As shown in Fig. 7 A, neurite outgrowth in PC12 cells expressing EGFPNesca after a 48-h incubation with 20 ng/ml NGF was consistently more robust than that observed in the EGFP-expressing controls. The cells were divided into three categories and the degree of neurite extension was quantified, the results of which are shown in Fig. 7 B. After 48 h with NGF, all Nesca-overexpressing clones dis-

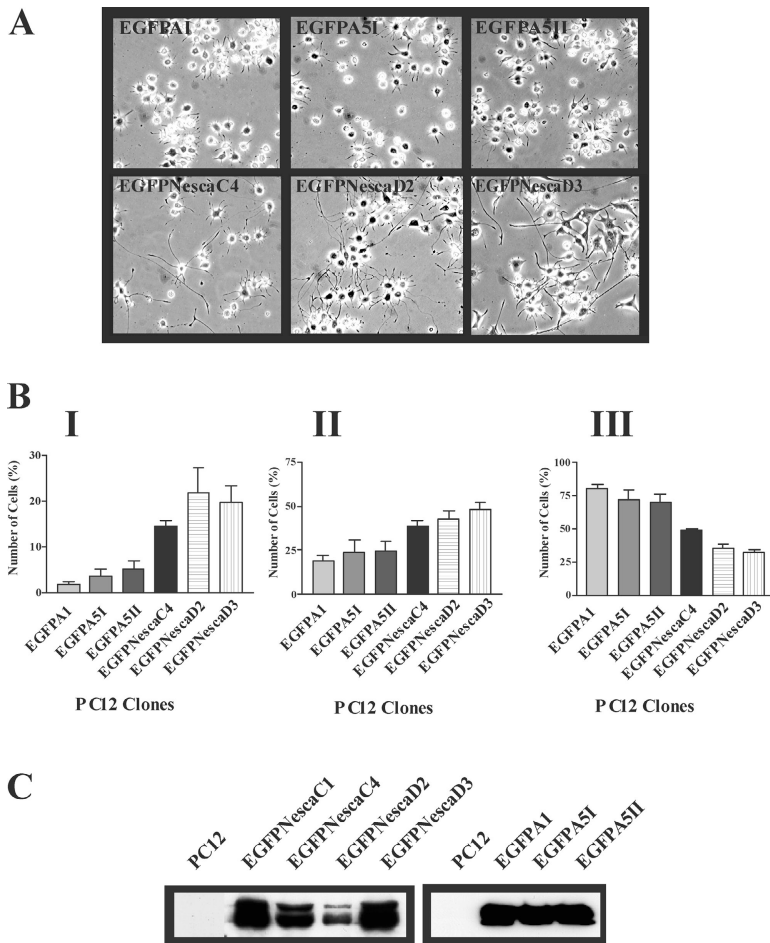


Figure 7. Nesca potentiates neurite outgrowth in NGF-treated PC12 cells. (A) EGFP-expressing PC12 cells (A1, A5I, and A5II) and EGFPNesca-expressing lines (C1, C4, D2, and D3) were stimulated with 20 ng/ml NGF for 48 h and assessed for neurite extension. (B) Quantification of neurite outgrowth: cells with a neurite to cell body length ratio of >2 (I); cells with a neurite to cell body length ratio of 1–2 (II); and cells with a neurite to cell body length ratio of <1 (III). 10 random fields were counted for each cell line in each experiment ($n = 3$). The error bars represent the SD from the mean. (C) Expression of EGFP and EGFPNesca was assessed by immunoblotting using 10 and 50 μ g of total lysate from each cell line.

played significantly more neurites with a neurite/cell body length ratio of >2 (Fig. 7 B, I). In addition, the Nesca-overexpressing lines displayed more neurites with a neurite/cell body ratio of 1–2 relative to controls (Fig. 7 B, II). Conversely, the number of cells with a neurite/cell body length ratio of <1 was consistently higher in the EGFP-expressing clones (Fig. 7 B, III). Levels of EGFP and EGFPNesca expression in each of the clones was relatively comparable, except in NescaD2, as shown in Fig. 7 C.

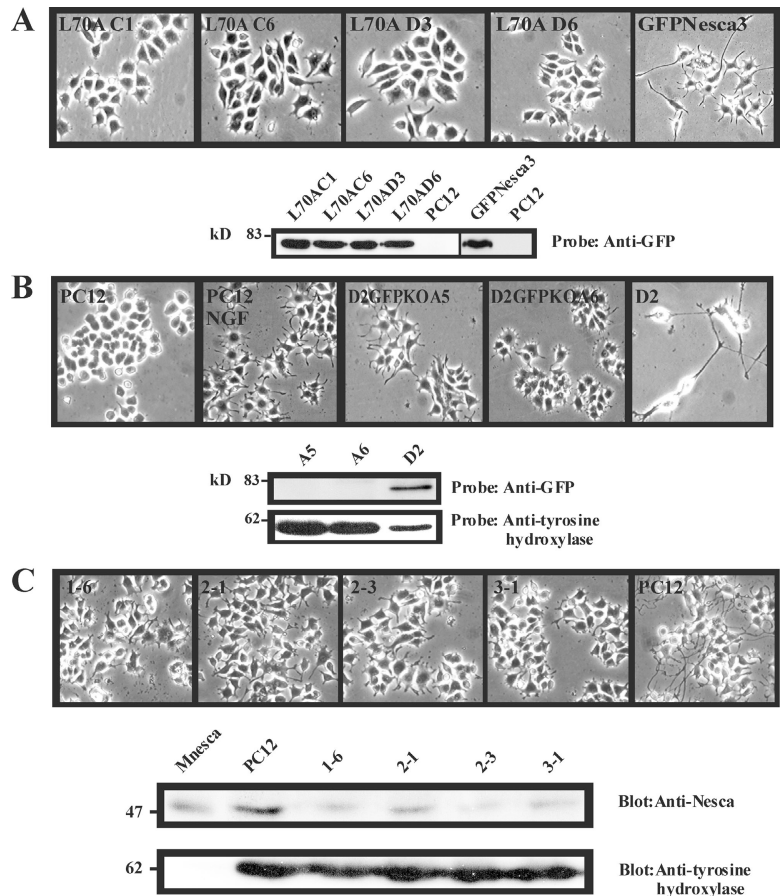
Although compelling, the data outlined here do not firmly establish a role for Nesca in neurotrophin-dependent neurite outgrowth. Therefore, we used several additional strategies. First, we determined whether nuclear translocation of Nesca is essential to its neurite outgrowth-promoting properties by taking advantage of the L70A Nesca mutant, incapable of nuclear translocation. We generated PC12 cell clones overexpressing L70A and assayed NGF-dependent neurite outgrowth relative to EGFP- and EGFPNesca (wild type)-expressing cell lines. Four EGFP-L70A cell lines and a single additional wild-type-overexpressing cell line were analyzed under conditions identical to those described for Fig. 7. In each case, NGF-dependent neurite outgrowth was less robust in the L70A lines compared with wild-type Nesca (Fig. 8 A). Approximately 20% of the wild-type Nesca-overexpressing cells displayed neurites of 2 soma diameters, whereas none of the L70A Nesca-overexpressing cells had neurites >1 soma diameter

after 48 h of NGF (unpublished data). All cell lines displayed similar levels of Nesca expression (Fig. 8 A).

In a corollary experiment, we used an EGFP siRNA oligonucleotide (Hannon, 2002) to silence EGFPNesca in the EGFPNesca-overexpressing D2 cell line (Fig. 7) and compared neurite outgrowth relative to the parental D2 line and PC12 cells. After 48-h incubation with 20 ng/ml NGF, neurite outgrowth in the D2 knockdown cell lines (A5 and A6) was significantly reduced relative to the parental D2 line and was comparable to PC12 cells (Fig. 8 B). Western blot analysis for EGFP clearly shows a reduction of EGFPNesca expression in the A5 and A6 cell lines (Fig. 8 B). Subsequent analysis of two other D2 knockdown cell lines revealed identical results to those described in Fig. 8 B (unpublished data).

Finally, we reduced the expression of endogenous Nesca in PC12 cells using specific siRNA oligonucleotides homologous to rat Nesca. Stable clones expressing the siRNAs were analyzed for Nesca expression by Western blotting as well as for their effects on NGF-induced neurite outgrowth (10 ng/ml NGF, 5 d). Although we observed no neurites >2 cell bodies in the knockdown cell lines comparable to parental PC12 cells, neurite outgrowth was not completely abrogated (Fig. 8 C). This residual neurite outgrowth is probably a result of our inability to completely eliminate endogenous Nesca expression (Fig. 8 C). Collectively, these data strongly suggest a role for Nesca in the regulation of neurite outgrowth in NGF-treated PC12 cells.

Figure 8. Overexpression of the L70A Nesca mutant and/or loss of endogenous Nesca expression negatively affects NGF-dependent neurite outgrowth. (A) PC12 cell lines stably expressing the nuclear translocation defective L70A Nesca mutant were assayed for neurite outgrowth (20 ng/ml NGF; 48 h). (B) EGFPNesca expression was silenced in PC12 EGFPNesca D2 cells with the pSilencer vector expressing a siRNA targeted against EGFP. Clones were analyzed for NGF-dependent neurite outgrowth and Nesca expression by Western blotting. Antityrosine hydroxylase antibodies served as a control for protein loading. (C) Endogenous Nesca expression was silenced in stable PC12 cells as described in Materials and methods. Cells were treated for up to five days with 10 ng/ml NGF. Control PC12 cells expressed a scrambled siRNA with no homology to rat mouse or human genomic sequences. Changes in endogenous Nesca expression were determined with an affinity-purified mouse anti-Nesca antibody. Left lane represents murine Nesca expressed in HEK293 cells.



Overexpression of Nesca prolongs the activation state of MAPK

As stated earlier, the prolonged activation of MAPK is a strong correlate of differentiative signaling, as opposed to the transient kinetics of activation during proliferation. Therefore, it was of interest to determine if Nesca overexpression had any effect on the kinetics of NGF-induced phosphoMAPK. Cell lines overexpressing wild-type EGFP-Nesca were stimulated with NGF for varying lengths of time, under serum starvation, and analyzed for active ERK1/ERK2 by Western blotting. As shown in Fig. 9 A, PC12 cell lines overexpressing Nesca (C1, C4, D2, and D3) maintain levels of phosphoERK1/ERK2 at higher absolute levels at 24 and 48 h relative to lines expressing EGFP alone ($n = 4$).

We also analyzed the D2 EGFPNesca knockdown cell lines for MAPK activation after 72 h stimulation with NGF. In each of the four D2 knockdown lines examined, the level of phospho-MAPK after 72 h approximated that observed for PC12 cells and was considerably lower than that observed for the parental D2 Nesca-overexpressing cell line (Fig. 9 B). Stripping and reprobing the blot with an anti-MAPK antibody revealed equal levels of kinase (Fig. 9 B).

Discussion

In addition to signaling proteins recruited directly to trkA (Meakin, 2000; Huang and Reichardt, 2003), many molecules function downstream in response to NGF stimulation. Included amongst these downstream targets are MAPK,

Rac1 (Yasui et al., 2001), cdc42 (Chen et al., 1999), and the myotonic dystrophy kinase-related cdc42 binding kinase MRCK α and β (Chen et al., 1999), which are involved in regulating cytoskeletal changes necessary for neurite outgrowth.

To reconstitute the pathways involved in trkA-mediated differentiation, it is necessary to identify the proteins that act downstream of an active receptor complex. Here, we describe the translocation of Nesca, a novel signaling adapter, from the cytosol to the nuclear envelope in response to neurotrophin stimulation. Several lines of evidence indicate that the nuclear translocation of Nesca is dependent on the prolonged activation of MAPK, a hallmark of the differentiative response (Marshall, 1995; York et al., 1998). First, Nesca does not translocate in response to EGF or in cells expressing trkA receptor mutants (trkAS3 and trkAS8) incapable of supporting NGF-dependent cell cycle arrest or neurite outgrowth. Activation of the EGFR results in only transient activation of MAPK (Kao et al., 2001), whereas the trkAS3 and trkAS8 mutants impair Shc and FRS2 recruitment and can activate MAPK only fractionally relative to wild-type trkA (Meakin and MacDonald, 1998; Meakin, 2000). In combination, the current data points to the importance of either the FRS2 or Shc adapters in the trk-dependent mechanisms regulating the nuclear translocation of Nesca. Second, Nesca translocates to the nucleus in the absence of NGF when MAPK is constitutively activated; and third, the nuclear translocation of Nesca is abrogated in the presence of NGF when the MAPK pathway is inhibited with U0126.

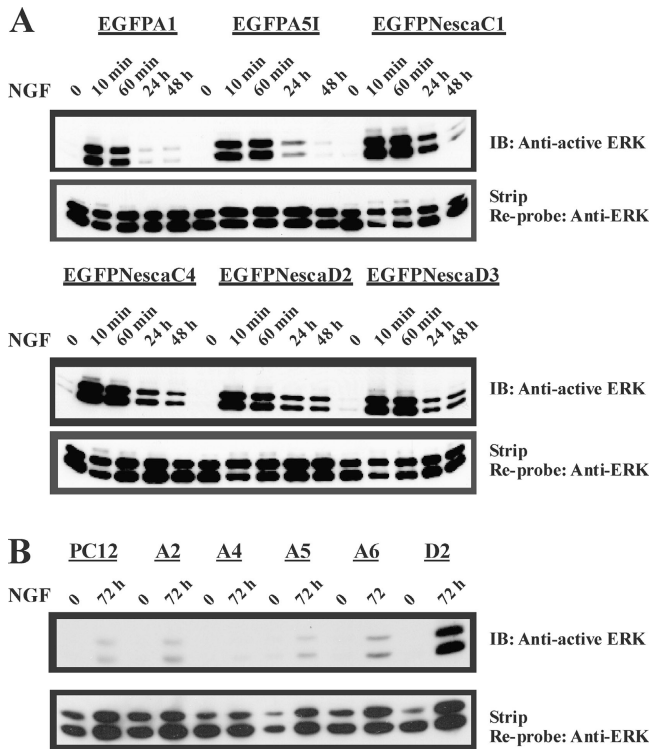


Figure 9. Nesca overexpression in NGF-treated PC12 cells results in enhanced levels of activated MAPK. (A) PC12 cell lines stably expressing EGFP and EGFPNesca and (B) EGFP knockdown cell lines (A2, A4, A5, and A6) derived from the EGFPNesca D2 line were serum starved for 24 h followed by the addition of 20 ng/ml NGF for varying lengths of time or for 72 h (EGFPNesca D2 knockdowns). Lysates were analyzed for phosphoMAPK, and the blots were stripped and reprobed with a MAPK antibody to assess protein loading.

Although we do not yet fully understand the mechanism underlying the neurotrophin-dependent nuclear redistribution of Nesca, it is clear that the process is dependent on the structural integrity of the RUN domain. Specifically, a single point mutation, L70A, predicted to be integral to the structure of the RUN domain (Fig. 3 G), abrogates the nuclear localization of Nesca. Although the function of RUN domains is not clear, many RUN domain-containing proteins are involved in signaling by members of the Ras superfamily of small GTPases, including members of the Rap and Rab families (Janoueix-Lerosey et al., 1998; Callebaut et al., 2001; Mari et al., 2001; Yang et al., 2002). Whether or not Nesca interacts with and/or regulates cytoplasmic/nuclear transport mediated through the Ran GTPase is the subject of ongoing investigations.

Because Nesca does not contain a nuclear translocation sequence, the question is raised as to the underlying mechanism by which Nesca translocates to the nucleus. An obvious starting point involves the kinetics of NGF-dependent phosphorylation of Nesca that correlates with the kinetics of translocation (>24 h of NGF treatment). Thus, it may be postulated that phosphorylation-dependent changes in protein-protein interactions govern the intracellular localization of Nesca. Interestingly, the intracellular localization of the MAPKs is also determined by changes in protein-protein interactions that are ultimately determined by changes

in protein phosphorylation. In their inactive form, the MAPKs are cytoplasmic and are tethered to the MEKs and MAPK phosphatase-3 (Cyert, 2001; Robinson et al., 2002). Upon activation, the MAPKs are phosphorylated and released from their cytoplasmic tethers, and, after dimerization, they translocate to the nucleus (Cyert, 2001; Whitehurst et al., 2002). However, having made this analogy, the L70A Nesca mutant still undergoes NGF-dependent phosphorylation, despite not undergoing nuclear translocation, raising the possibility that the translocation of Nesca involves a binding partner. Essentially, phosphorylation appears to precede translocation, and sequences within the RUN domain are involved in either the translocation process itself or in retention at the nuclear envelope. In addition, the temporal regulation of phosphorylation and nuclear translocation of Nesca raises the possibility that the process may depend on the synthesis of a chaperone protein and/or kinase. However, attempts to address this possibility by inhibiting protein synthesis with cycloheximide have been difficult to interpret due to the long incubation times required to observe Nesca's redistribution coupled with the cytotoxicity of the drug.

Currently, we have no information regarding the nature of the kinase(s) responsible for Nesca phosphorylation, and we have not been able to demonstrate direct *in vitro* phosphorylation of Nesca by MAPK itself. The rapid activation of MAPK, coupled with the fact that Nesca is not phosphorylated until after 24-h NGF stimulation, provides an argument against Nesca being a direct target of MAPK. Rather, the data suggest that Nesca may be phosphorylated by an unidentified kinase activated downstream of, or transcriptionally regulated by, MAPK itself. Consensus phosphorylation sites for cAMP-dependent kinase, PKA, calmodulin-dependent protein kinase II, and PKC exist in Nesca. Whether or not any of these kinases contribute to the phosphorylation of NGF-stimulated Nesca is the subject of ongoing investigations. Obviously, the identification of the site(s) of Nesca phosphorylation would benefit our understanding of the mechanism of nuclear translocation and aid in the overall understanding of trk-mediated signaling.

The enhancement of neurite outgrowth in PC12 cells overexpressing Nesca, and the reduction in process formation in siRNA knockdown cells, suggests a functional role for Nesca in NGF signaling, although the nature of this role is not yet clear. Cytoskeletal rearrangements, necessary for neurite outgrowth, are regulated through members of the Rho subfamily of the Ras superfamily of small GTPases. In PC12 cells, Rac1 regulates neurite outgrowth (Chen et al., 1999; Yasui et al., 2001; Nusser et al., 2002) through a mechanism that involves Ras and PI-3 kinase (Yasui et al., 2001). Moreover, RhoA, which negatively affects neurite outgrowth, is inactivated in a process that involves a change in its intracellular localization (Nusser et al., 2002). Although the kinetics of RhoA inactivation make it unlikely that Nesca plays a role in this process, Nesca may enhance the activity of Rac and/or cdc42.

Alternatively, Nesca may indirectly influence neurite outgrowth by amplifying trk-mediated signaling pathways, perhaps by prolonging activation in NGF-induced signaling endosomes (Wu et al., 2001). RUN domain-contain-

ing proteins have been shown to promote endosomal fusion (Mari et al., 2001). However, having said this, the appearance of NGF-induced signaling endosomes is rapid (Wu et al., 2001), whereas the appearance of Nesca at the nucleus is a comparatively slow process. Thus, if Nesca does have a role in endosomal fusion, it would be confined to late signaling endosomes.

It is also possible that Nesca could differentially regulate nuclear trafficking and, in so doing, regulate mechanisms of transcription and/or translation in response to neurotrophin stimulation. It is also possible that Nesca plays multiple roles in trk-mediated signaling. The existence of a small subsidiary pool of Nesca at the plasma membrane in NGF-treated *nnr5*-B5 cells is alluded to by confocal microscopy (Fig. 1 B, I and II) and biochemical analyses. This secondary pool may function in a stimulatory role for plasma membrane-bound small GTPases involved in neurite outgrowth as discussed earlier in this section. Insofar as neurite outgrowth represents a phenotypic measure of neurotrophin-dependent differentiation, we can only conclude at this point that the evidence indicates that Nesca regulates some aspect of development, the final measure of which is process formation.

At present, we cannot rule out an intranuclear role for Nesca. Clearly, the confocal images reveal little Nesca within the nucleus. However, as stated earlier, cellular subfractionation studies show a small amount of Nesca within the nuclear extracts of NGF-treated *nnr5*-B5 cells. If the primary site of Nesca activity is within the nucleus, then the mass of Nesca found on the nuclear periphery may simply represent protein congregating at the nuclear envelope before transport. Conversely, it is possible that this intranuclear Nesca simply represents a contaminant in the nuclear extract fraction originating from the nuclear envelope. We are directing future efforts at distinguishing between these models by identifying proteins and/or RNAs that may bind to Nesca in order to understand more fully the role of Nesca in neurotrophin-dependent signaling.

Materials and methods

Materials

All primary antibodies, except the anti-myc antibody 9E10 and the pan-trk antibody 203, were obtained from Roche Molecular Biochemicals (anti-HA: 12CA5 or 3F10). Other primary antibodies used were EGFR and GFP (Santa Cruz Biotechnology, Inc.), phospho-p44/42 MapK (New England Biolabs, Inc.), Erk2 (BD Biosciences), and RC20 (Transduction Laboratories). The secondary antibodies, HRP-goat anti-mouse and HRP-goat anti-rabbit, were obtained from Jackson ImmunoResearch Laboratories. β -NGF and EGF were obtained from Harlan Products for Bioscience. BDNF and NT-3 were provided by A.A. Welcher (Amgen Inc., Thousand Oaks, CA). A polyclonal anti-Nesca antibody was generated against the peptide ³²³CPSETESGAFKSRRP³³⁷ of mouse Nesca by Gen-Med Synthesis. The antibody was affinity purified by passage over a peptide affinity column generated by coupling the Nesca peptide to NHS-activated Sepharose (Amersham Biosciences). The constitutively active MKK1 mutant (R4F; Emrick et al., 2001) was the gift of N. Ahn (University of Colorado, Boulder, CO). The siRNA plasmid pSilencer 3.1H-1 Hygro was obtained from Ambion. The vector pSuper was a gift of R. Agami (The Netherlands Cancer Institute, Amsterdam, Netherlands; Brummelkamp et al., 2002).

Plasmids and constructs

The amino terminal HA-epitope (YPYDVPDYASL) tagged Nesca was cloned into the mammalian expression vector pcDNA3.1 (Invitrogen), whereas a carboxyl-terminal myc-epitope tagged Nesca was prepared in pcDNAMycHis (Invitrogen). The EGFP-Nesca fusion protein and all subse-

quent Nesca mutants were constructed in pEGFPN3 (CLONTECH Laboratories, Inc.). The RFP-trkA fusion protein was constructed in pDsRedN1 (CLONTECH Laboratories, Inc.). A cDNA encoding the human EGFR in the expression vector pRK5 was obtained from M. Moran (Samuel Lunenfeld Research Institute, Toronto, Canada). The Nesca deletion mutants Δ 52P-231G, Δ 52P-166A, and Δ 52P-86R were generated by PCR-mediated introduction of an XbaI site at the indicated amino acid junctions (e.g., 52P and 231G) in EGFPNesca, followed by digestion with XbaI and re-ligation. The carboxyl-terminal deletions (Δ 382A-433L and Δ 257A-433L) and single site Nesca mutants (L70A and R78A:K79A) were generated by PCR using standard procedures. All PCR reactions used to generate constructs employed Herculase-enhanced DNA polymerase (Stratagene), and all products were sequenced completely to ensure fidelity. The siRNA sequence GGTATGTACAGGAACGCCA directed against EGFP (Ambion) was cloned into the vector pSilencer (Ambion). For Nesca silencing, two 19-mer oligonucleotides, ¹¹²GCTTGGTTCAGAAAGCTCAA¹³¹ and ²⁹⁵ACCAGGTTCTGCACACATT³¹⁴, homologous to rat Nesca, were selected. The former oligonucleotide was cloned into the pSuper plasmid (Brummelkamp et al., 2002) that was modified by the addition of a zeocin resistance marker (pSuperZeo). The latter oligonucleotide was cloned into the pSilencer vector (hygromycin resistant). Cells were transfected using Lipofectamine 2000 (Invitrogen) at a 2:1 ratio of LF2000/DNA and selected with zeo and hygromycin.

Cell culture

PC12 cells expressing EGFP (clones A1, A5I, and A5II), EGFPNesca (clones C1, C4, D2, and D3), and EGFPNescaL70A (clones C1, C6, D3, and D6) and *nnr5* cells expressing HA-tagged trk receptors (trkA[B5], trkB[B5], trkC[C3], trkAS8 [Y499F], trkAS3 [Δ ⁴⁹³IMENP⁴⁹⁷], trkAS9 [Y794F], and trkAT1 [Δ ⁷⁸⁷L-G⁷⁹⁹]) were generated as described previously (Meakin and MacDonald, 1998; Meakin, 2000). HEK293 cells were maintained in DME supplemented with 10% FBS (Hyclone). EGFPNesca knockdowns (clones A5 and A6) were generated by transfecting PC12 EGFPNesca expressing D2 cells with pSilencer expressing a siRNA against EGFP, selected with 500 μ g/ml hygromycin and maintained in 50 μ g/ml hygromycin. We generated PC12 lines with loss of endogenous Nesca (clones 1-6, 2-1, 2-3, and 3-1) by cotransfecting cells with pSuperNesca112 and pSilencer-Nesca295 and selecting with 400 μ g/ml zeocin and 500 μ g/ml hygromycin. Cells were maintained in 100 μ g/ml zeocin and 50 μ g/ml hygromycin. Negative control PC12 cells were generated by transfecting cells with pSilencer containing a scrambled siRNA oligonucleotide with no homology to mouse, human, or rat sequences (Ambion).

Microscopy and image processing

For confocal microscopy, *nnr5* cells were transfected with 2.5 μ g each of pEGFPN3Nesca and pDsRedN1HA-trkA as described in the previous paragraph, grown overnight, and seeded onto poly-D-lysine-coated coverslips in 35-mm culture dishes and stimulated with 100 ng/ml NGF (72 h). Cells were washed with PBS, fixed in 4% PFA (15 min, RT), washed and mounted on glass slides under PBS, and visualized with a confocal microscope (model LSM 510; Carl Zeiss MicroImaging, Inc.). For epifluorescence microscopy, cells were visualized for EGFP and RFP using appropriate filter sets (B-3A [excitation of 420nm, barrier of 520nm] and G-2A [excitation of 510 nm, barrier of 590 nm]) on inverted microscopes (1 \times 70 [Olympus]; Diaphot 300 [Nikon]) at a magnification of 200–600. Phase-contrast images were visualized with a magnification of 200 using an inverted microscope (model Diaphot 300; Nikon). All images were captured with digital cameras (model Pro-series 3CCD) using Image-Pro software. Images were processed in Adobe Photoshop 7.0 and imported into CorelDraw (version 11) for figure presentation.

RT-PCR and Northern analysis

RNA was isolated using Trizol (Invitrogen) and cDNA was transcribed using Superscript (Invitrogen). RT-PCR was performed with primers specific to mouse Nesca (5'-CCTGGACCTGCTCTTTGAGCAC-3' and 5'-CAG-ATTTACACCTCACAGGTGGG-3') and GAPDH (5'-CTCACTACATGTGTACATGTTCC-3' and 5'-ATGGACTGTGGTCATGAGYCCTTCC-3'). Multiple tissue Northern blots (CLONTECH Laboratories, Inc.) were probed with ³²P-dCTP-labeled probes corresponding to a carboxyl-terminal sequence (822C-1424G) of mouse Nesca or nucleotides 1736T–1885T of the 3'-UTR of human Nesca (Matsuda et al., 2000).

Immunoprecipitations and immunoblotting

Immunoprecipitations were performed as described previously (Meakin and MacDonald, 1998). In brief, cells were washed with ice-cold PBS containing 10 mM NaF and 1 mM vanadate before being lysed in NP-40 lysis

buffer (Meakin and MacDonald, 1998) containing 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM vanadate, 10 mM NaF, and 1 mM PMSF. Immune complexes were precipitated by the addition of Gamma Bind Plus (Amersham Biosciences) and Pansorbin (rabbit specific; Calbiochem) or Tachisorb (mouse specific; Calbiochem). The immune complexes were analyzed by SDS-PAGE and Western blots as described previously (Meakin and MacDonald, 1998). Blots were stripped for reprobing in 62.5 mM Tris-Cl, pH 6.8, 1% SDS, and 100 mM β-mercaptoethanol at 50°C for 15 min. Antibodies for Western blotting were used at the following dilutions: phospho-Mapk, 1:2500; Erk2, 1:2000; GFP, 1:1000; RC20, 1:2500.

Subcellular fractionation

Nnr5-B5 cells were transfected with EGFPNesca and stimulated with NGF for 72 h. The cells were washed with ice-cold PBS and lysed in 10 mM Tris-HCl, 3 mM MgCl₂, 10 mM NaCl, 5 mM EGTA, 0.05% NP-40, 5 mM NaF, 1 mM vanadate, and protease inhibitors, pH 7.5. After centrifugation (400 g), the nuclear pellet was washed twice with lysis buffer and twice with nuclear wash buffer (10 mM Hepes, 300 mM sucrose, 3 mM MgCl₂, 25 mM NaCl, 1 mM EGTA, 5 mM NaF, 1 mM vanadate, and protease inhibitors, pH 6.8). Supernates were pooled and centrifuged at 100,000 g to obtain the cytosolic fraction. The pellet was resuspended in nuclear wash buffer containing 0.5% Triton X-100, incubated on ice for 5 min, and centrifuged at 700 g to separate nucleoplasm from nuclear membranes. The nuclear membrane was resuspended in nuclear wash buffer.

In vivo phosphorylation study

The phosphorylation state of Nesca was analyzed by in vivo labeling with [³²P]orthophosphate of nnr5-trkA B5 cells transfected with EGFPNesca or HA-Nesca and stimulated with NGF. In brief, cells were plated in a 6-well plate, transfected with EGFPNesca, HA-Nesca, or EGFP, and then left untreated or stimulated with 100 ng/ml NGF (72 h) to generate nuclear rings in ~75% of cells. The media were replaced with serum/phosphate-free DME and NGF, where applicable, and the cells further incubated for 2 h followed by the addition of 0.1 mCi/ml [³²P]orthophosphate for 12 h. The cells were washed and lysed, and EGFPNesca or EGFP were immunoprecipitated with a monoclonal anti-GFP antibody (1–2 µg antibody/IP) or HA-Nesca with 12CA5. Immune complexes were collected, analyzed by SDS-PAGE, and exposed to X-ray film for visualization. To localize EGFPNesca or HA-Nesca, the blots were probed with the anti-EGFP antibody or 3F10. Radiolabeled EGFPNesca was excised and Cherenkov radioactivity determined. Phosphoamino acid analysis was performed as described previously (Kamps, 1991), and phosphoamino acids were separated by thin-layer electrophoresis on cellulose plates (Boyle et al., 1991).

Identification of Nesca

Nesca was isolated in a yeast two-hybrid screen using the intracellular domain of rat trkA as bait. The yeast two-hybrid screen was performed using the yeast strain PJ694A as described previously (MacDonald et al., 1999) using commercially available human fetal brain and mammary libraries (CLONTECH Laboratories, Inc.).

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