A kinase-deficient TrkC receptor isoform activates Arf6–Rac1 signaling through the scaffold protein tamalin

Pedro F. Esteban,[1] Hye-Young Yoon,[2] Jodi Becker,[1] Susan G. Dorsey,[1] Paola Caprari,[1] Mary Ellen Palko,[1] Vincenzo Coppola,[1] H. Uri Saragovi,[3] Paul A. Randazzo,[2] and Lino Tessarollo[1]

[1]Neural Development Group, Mouse Cancer Genetics Program, National Cancer Institute, Frederick, MD 21702
[2]Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, MD 20892
[3]Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada H3G 1Y6

Neurotrophins play an essential role in mammalian development. Most of their functions have been attributed to activation of the kinase-active Trk receptors and the p75 neurotrophin receptor. Truncated Trk receptor isoforms lacking the kinase domain are abundantly expressed during development and in the adult; however, their function and signaling capacity is largely unknown. We show that the neurotrophin-3 (NT3) TrkCT1-truncated receptor binds to the scaffold protein tamalin in a ligand-dependent manner. Moreover, NT3 initiation of this complex leads to activation of the Rac1 GTPase through adenosine diphosphate-ribosylation factor 6 (Arf6). At the cellular level, NT3 binding to TrkCT1–tamalin induces Arf6 translocation to the membrane, which in turn causes membrane ruffling and the formation of cellular protrusions. Thus, our data identify a new signaling pathway elicited by the kinase-deficient TrkCT1 receptor. Moreover, we establish NT3 as an upstream regulator of Arf6.

Introduction

The *trkB* and *trkC* loci encode a variety of receptor isoforms, in addition to the canonical full-length tyrosine kinase receptors (Tessarollo, 1998; Huang and Reichardt, 2001). Although several kinase-deficient Trk receptor isoforms have been identified over the years for both the *trkB* and the *trkC* genes, only TrkBT1 and the truncated TrkC isoform, which we call TrkCT1 in this study (also known as TrkCTK [Tsoulfas et al., 1993; Garner and Large, 1994; Palko et al., 1999], TrkCNC2 [Menn et al., 1998], and TrkCic158 [Valenzuela et al., 1993]), are believed to play important roles in vivo. The cytoplasmic tails of these truncated receptors are encoded by separate exons, which are evolutionarily conserved. Their protein products are present in both the embryo and in the adult animal, and their expression is dynamically regulated during development (Escandón et al., 1994; Menn et al., 1998). To date, the main function attributed to the kinase-deficient truncated Trk isoforms is inhibition of the kinase-active receptor isoforms, which is achieved by acting as a dominant-negative inhibitor of the full-length receptor or by a ligand-sequestering mechanism, which limits the neurotrophic factor available to bind the kinase-active receptor (Tessarollo, 1998; Huang and Reichardt, 2001). However, the high degree of sequence conservation of the intracellular domains of truncated receptors among species suggests the potential for other functions, such as interaction with cytoplasmic adaptor proteins and activation of signaling pathways (Baxter et al., 1997; Hapner et al., 1998). Indeed, it has recently been reported that brain-derived neurotrophic factor induces the production of calcium waves in astroglia through the truncated TrkBT1 receptor, and that TrkBT1 can alter astrocytic morphology via the regulation of Rho GTPase activity (Rose et al., 2003; Ohira et al., 2005). To date, no molecules have linked truncated TrkCT1 receptors to intracellular signaling pathways. Moreover, there are no data on direct biological functions, per se, although it has been reported that TrkCT1 with p75 can induce neural crest cell differentiation, and in animal models of glaucoma truncated TrkCT1 is overexpressed concomitantly with retinal ganglion cell death (Hapner et al., 1998; Rudzinski et al., 2004).

We present the identification of a new signaling pathway activated by the kinase-deficient TrkCT1 receptor that employs the scaffold protein tamalin (Nevrivy et al., 2000; Kitano et al., 2002), the cytohesin-2–Arf nucleotide-binding site opener (ARNO), the ADP-ribosylation factor 6 (Arf6), and the Rac1 GTPase. We show that neurotrophin-3 (NT3) activation of this

Correspondence to Lino Tessarollo: tessarol@ncifcrf.gov

Abbreviations used in this paper: Arf, ADP-ribosylation factor; ARNO, Arf nucleotide-binding site opener; coIP, coimmunoprecipitation; HEK, human embryonic kidney; PDZ, Psd-95/Dlg/ZO1.

The Journal of Cell Biology, Vol. 173, No. 2, April 24, 2006 291–299
http://www.jcb.org/cgi/doi/10.1083/jcb.200512013

signaling cascade by TrkCT1 causes Arf6 translocation to the membrane, followed by actin reorganization and membrane ruffling. Thus, we have identified a new pathway that provides a mechanism by which NT3 can control cell morphology, shedding light on the elusive role of abundantly expressed truncated Trk receptors in development. Moreover, it provides the only completely defined growth factor–activated pathway leading to Arf activation.

Results

The unique COOH terminus of TrkCT1 is encoded by two exons (13b and 14b in human; Fig. 1 A; Ichaso et al., 1998). Exon 14b is the most conserved among species such as mouse, human, rat, and chicken. Therefore, we used a yeast two-hybrid system to screen an adult mouse brain cDNA library with the 13-aa-long exon 14b (38 aa) as bait for interacting proteins (see Materials and methods). This approach yielded several candidate genes, including four independent clones for GRASP/tamalin (Nevrivy et al., 2000; Kitano et al., 2002). These clones initiated at proline 19, alanine 22, arginine 68, and arginine 80. Full-length cDNA for tamalin was not isolated.

Next, we analyzed the specificity of interaction between tamalin and TrkCT1 by assessing yeast two-hybrid β-galactosidase activity in liquid assays with a series of COOH- and NH_2-terminal deletions of tamalin and exon 13b and/or 14b of TrkCT1. All tamalin plasmids isolated from the brain cDNA library contained an intact Psd-95/Dlg/ZO1 (PDZ) domain, suggesting that this domain may be involved in the interaction with TrkCT1. Indeed, significant β-galactosidase activity can only be detected when the tamalin PDZ domain is intact (Fig. 1 B). Furthermore, the phylogenetically conserved exon 14b of TrkCT1 is both necessary and sufficient to promote association with the tamalin protein. Interestingly, full-length tamalin generates the lowest

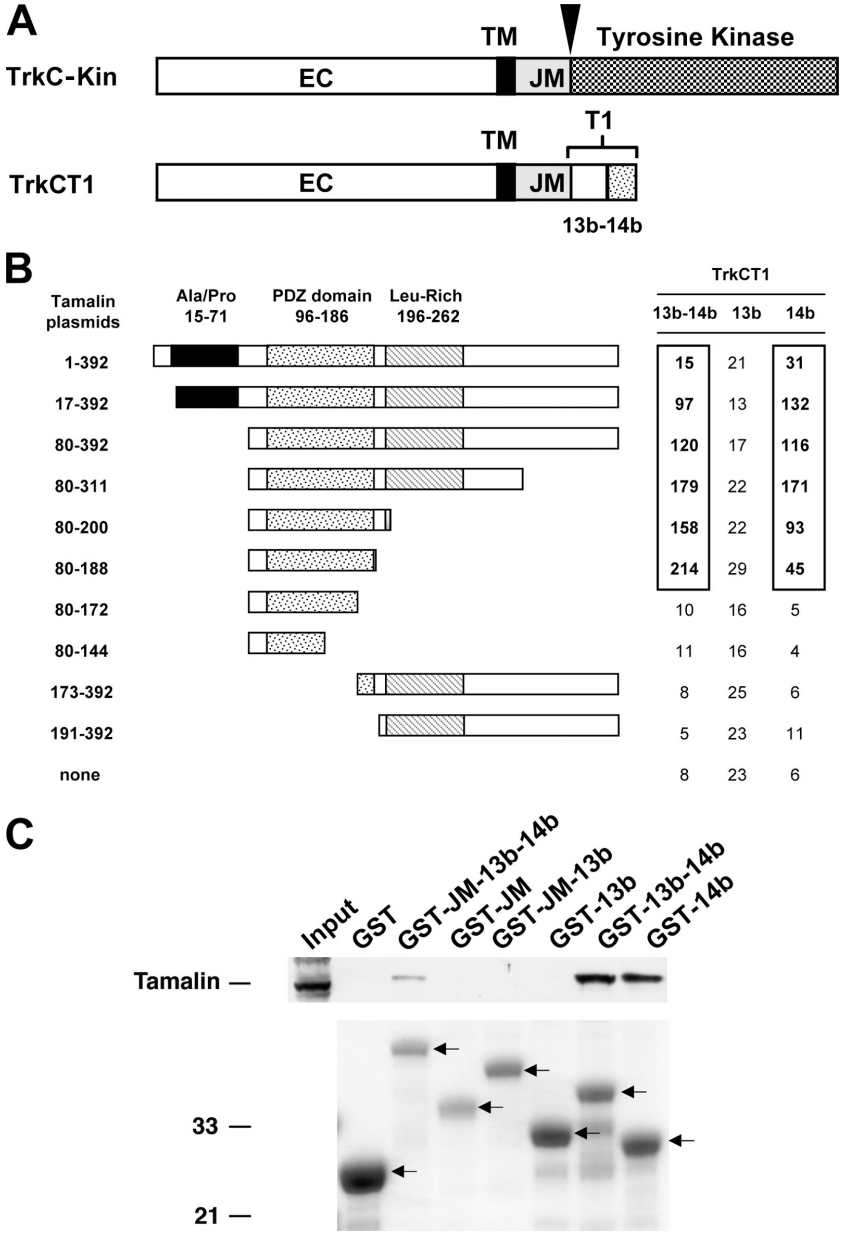

Figure 1. **The tamalin PDZ domain interacts with exon 14b of TrkCT1.** (A) Schematic representation of the full-length TrkC kinase (TrkC-Kin) and truncated TrkCT1 receptors. EC, extracellular domain; TM, transmembrane domain; JM, juxtamembrane domain. (B) Values of β-galactosidase activity obtained by combining different TrkCT1 bait and tamalin prey plasmids in liquid assay. Note the high β-galactosidase activity when both TrkCT1 exon 14b (aa 575–612) and an intact tamalin PDZ domain are used in the assay (box). Tamalin plasmid "none" indicates that an empty prey plasmid was used. (C) In vitro binding of tamalin to TrkCT1 protein fragments, which contain exon 14b. GST fusion proteins containing COOH-terminal portions of the TrkCT1 receptor were analyzed for their ability to interact in vitro with the full-length tamalin-HA protein by GST pull down-assay, as described in Materials and methods. The presence of tamalin was detected by immunoblotting with an anti-HA antibody (top). Input (bottom) represents 30% of the amount of GST–TrkCT1 fusion proteins (arrows) used in the assay and visualized by Coomassie staining.

β-galactosidase activity when compared with the other constructs that have an intact PDZ domain. Most likely, the full-length fusion protein does not localize to the nucleus to activate reporter expression. This could also explain why no full-length tamalin cDNA clones were isolated in the original screen.

To further confirm the interaction between tamalin and TrkCT1 observed in yeast, we performed in vitro GST pull-down assays. We tested whether a full-length, COOH-terminal, HA-tagged tamalin could bind different GST–TrkCT1 fusion proteins (Fig. 1 C). GST alone or GST–TrkCT1 fusion fragments containing the juxtamembrane domain or exon 13b failed to pull down tamalin. However, GST fusion proteins containing exon 14b were able to interact with tamalin. The deletion of even a single aa from the exon 14b COOH terminus was sufficient to abolish binding to tamalin, suggesting that the COOH-terminal tail is responsible for binding to the tamalin PDZ-binding domain (unpublished data). These data confirmed that the interaction between tamalin and TrkC requires an intact exon 14b, which is unique to the TrkCT1 isoform. Furthermore, the results suggest that the juxtamembrane region, which is common to both the TrkCT1 and the TrkC kinase–active (TrkC-kin) isoforms, is not involved in the interaction. Additional GST pull-down experiments performed with GST fusion proteins, including the kinase domain, also failed to pull down tamalin (unpublished data), confirming that the interaction between tamalin and TrkC receptors is specific to the truncated TrkCT1 isoform.

To verify that TrkCT1–tamalin interaction is physiologically possible in the animal, we analyzed their pattern of expression in the adult mouse brain. In situ hybridization experiments, using specific tamalin and TrkCT1 antisense riboprobes, showed that this scaffold protein gene and the truncated TrkC receptor have an overlapping pattern of expression in several areas of the mouse brain. For example, both genes are highly expressed in the hippocampus, cortex, striatum, and olfactory bulb (Fig. 2, A and B). Furthermore, immunofluorescence staining of human embryonic kidney 293 (HEK293) cells transfected with TrkCT1 and tamalin-HA shows that they colocalize at the plasma membrane, indicating that the subcellular localization of these proteins allows their association (Fig. 2, C–E).

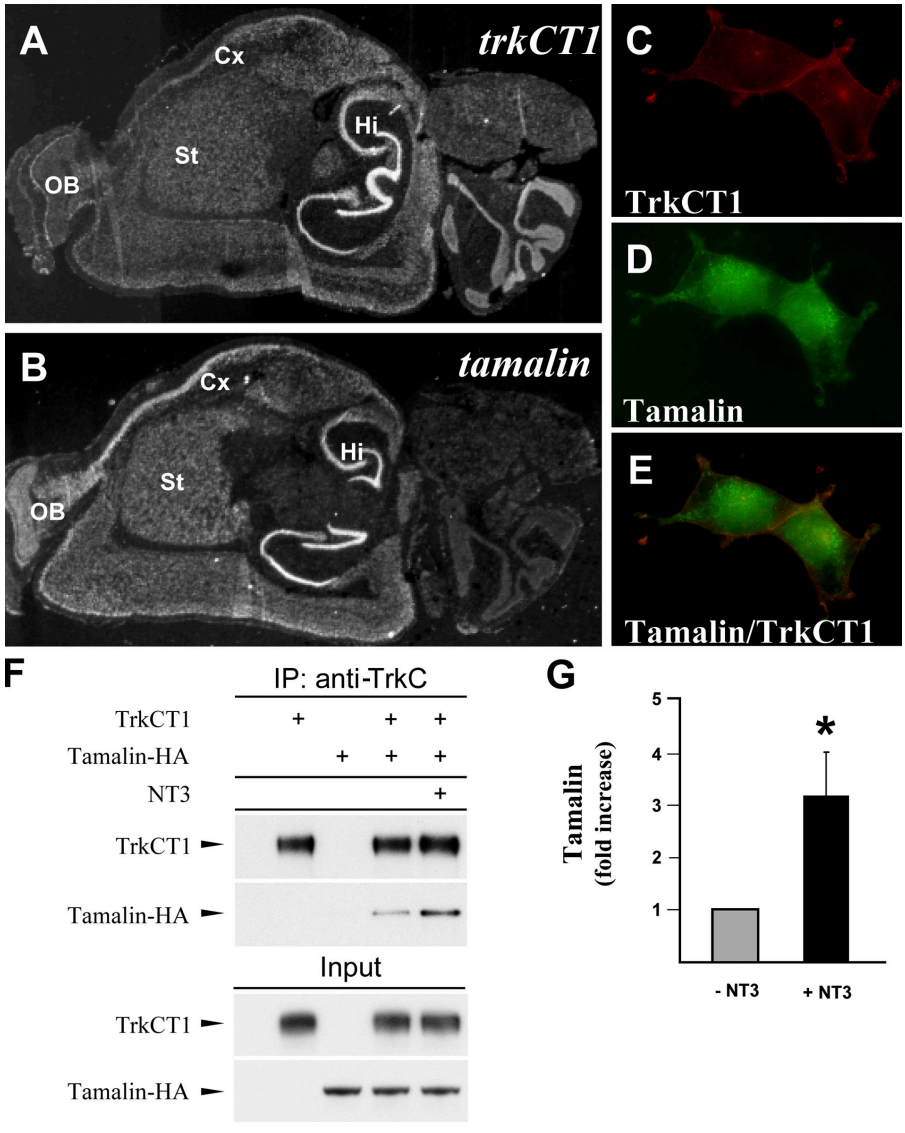

Figure 2. **TrkCT1 and tamalin physically interact and colocalize both anatomically and subcellularly.** (A and B) In situ hybridization analysis of sagital sections from an adult mouse brain with *trkCT1*- (A) and *tamalin*-specific (B) antisense riboprobes. Note the overlapping pattern of expression in most areas of the forebrain, with particularly high levels in the hippocampus. Hi, hippocampus; Cx, cortex; St, striatum; OB, olfactory bulb. (C–E) HEK293 cells transfected with TrkCT1 and tamalin-HA were stained with the rabbit anti-TrkC antiserum 656 and a mouse monoclonal anti-HA antibody. The secondary antibodies were anti–rabbit rhodamine-conjugated (C; TrkCT1) or anti–mouse fluorescein-conjugated antibodies (D; HA). Fluorescence imaging demonstrates the colocalization of both proteins (E) at the membrane of the cells, as indicated by the yellow color that results from the overlay of red and green fluorescence. (F) Tamalin and TrkCT1 coIP in mammalian cells. HEK293 cells transfected with the indicated plasmid and treated with NT3 were lysed, immunoprecipitated with a goat anti-TrkC antibody, and immunoblotted with anti-HA and anti-TrkC antibody (TrkC 656) as indicated. (top) Western blot of a representative experiment is shown. (bottom) The amount (input) of TrkCT1 and tamalin in 10% of the total cell lysates used for the coIP experiments. (G) Quantification of immunoprecipitated tamalin in double-transfected cells from three independent transfections, each immunoprecipitated and immunoblotted three times (fold difference relative to untreated cells). Error bars represent the mean ± SEM, relative to untreated cells. *, $P < 0.05$.

Next, we used coimmunoprecipitation (coIP) experiments to investigate if tamalin interacts with TrkCT1 in mammalian cells. Lysates obtained from HEK293 cells expressing tamalin-HA and the truncated TrkCT1 isoform were immunoprecipitated with an anti-TrkC serum and immunoblotted with an anti-HA antibody. As shown in Fig. 2 F, the anti-TrkC–specific antibody can coIP tamalin-HA only when both tamalin and TrkCT1 proteins are present. Therefore, TrkCT1 does interact with tamalin in mammalian cells.

To gain insight into the physiological relevance of TrkCT1–tamalin binding we next investigated whether the TrkC ligand NT3 would affect this interaction. TrkCT1 and tamalin were co-expressed in HEK293 cells. Before lysis, cells were serum starved for 4 h, followed by treatment with NT3 for 5 min (Fig. 2 F). Interestingly, the amount of tamalin-HA associated with TrkCT1 was significantly increased by NT3 (Fig. 2 F). Quantitation from multiple coIP experiments showed that the addition of NT3 led to a threefold increase in the amount of tamalin bound to TrkCT1 (Fig. 2 G). These data show that the ligand NT3 promotes interaction of TrkCT1 with tamalin and support the physiological relevance of the TrkCT1–tamalin association.

The finding that NT3 promotes the interaction of TrkCT1 with tamalin in vitro prompted us to expand this observation in a physiological context. Mouse hippocampal neurons, which in the adult express the highest levels of both tamalin and TrkCT1 (Fig. 2, A and B), were isolated from embryos, differentiated for 10–14 d in culture to allow neurons to mature and endogenously express truncated TrkCT1 and tamalin, and treated with NT3. Confocal microscopy analysis of control neurons stained with a specific antibody recognizing the tamalin COOH terminus showed that this protein has a diffuse, mainly cytoplasmatic pattern of expression (Fig. 3 A). Conversely, TrkC receptors visualized with a mouse monoclonal antibody recognizing the extracellular region of TrkC appear to be present uniformly along the plasma membrane, as previously described (Menn et al., 2000), with little or no colocalization with tamalin (Fig. 3 I). Interestingly, after NT3 treatment, tamalin distribution becomes more punctuate, especially along the neuronal axons, and

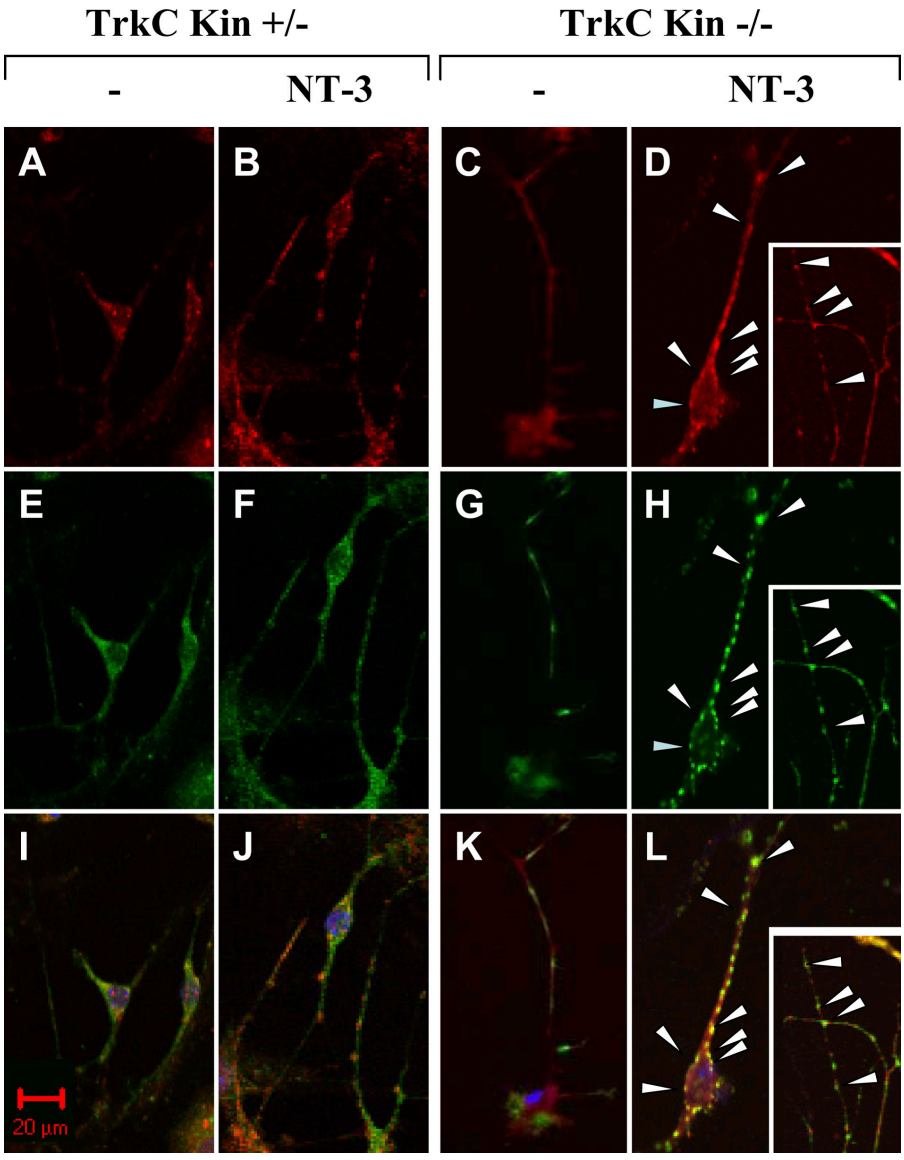

Figure 3. **Tamalin and TrkCT1 colocalize in hippocampal neurons in response to NT3.** Differentiated control (A, B, E, F, I, and J) and TrkC kinase-deficient (C, D, G, H, K, and L) mouse hippocampal neurons were stained with anti-tamalin– (A–D) and anti-TrkC–specific (E–H) antibodies and subjected to confocal microscopy to investigate tamalin (A–D) and TrkC cellular distribution in the presence (B, F, J, H, D, and L) or absence (A, E, I, C, G, and K) of NT3. Analysis of merged rhodamine and FITC signals reveals colocalization of tamalin and TrkC in response to NT3 treatment (I–L). TrkCT1 has a specific punctuated pattern of expression (G and H; Menn et al., 2000) compared with a more diffuse distribution of the kinase-specific TrkC receptor (E and F). Note the particularly strong punctuated overlapping distribution of tamalin with TrkC in the mutant neurons (D–L, insets) that have only the truncated TrkCT1 receptor (arrowheads).

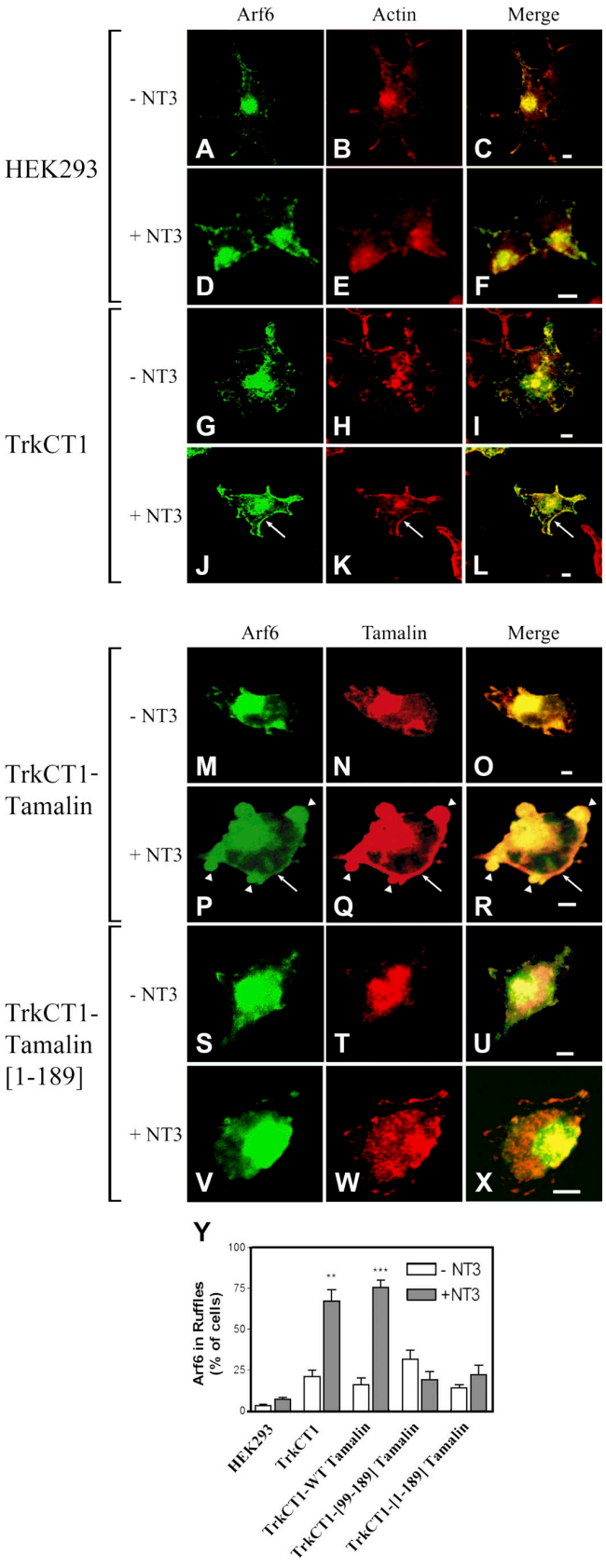

Figure 4. **NT3 binding to TrkCT1 induces Arf6 translocation to the cell membrane and ruffling.** (A–X) HEK293 (A–F) and HEK293 cells stably expressing TrkCT1 (G–X) were transfected with plasmids directing expression of FLAG-Arf6 (A–X), together with plasmids directing expression of wild-type (M–R) or inactive tamalin (S–X; aa 1–189), and treated with NT3 as indicated (D–F, J–L, P–R, and V–X). Cells were fixed, permeabilized, and stained for Arf6 (green; A, D, G, J, M, P, and S) and polymerized actin (red; B, E, H, and K) or tamalin (red; N, Q, T, and W). Arrows indicate colocalization of Arf6 and actin (J–L), and Arf6 and tamalin (P–R) at the cell membrane in response to NT3 treatment. Arrowheads in P–R indicate cell protrusions caused by NT3 when TrkCT1 and tamalin are coexpressed. (Y) Translocation of Arf6 to the membrane of TrkCT1-expressing cells in response to NT3 is inhibited by dominant-negative forms of tamalin. Percentage of cells with Arf6 at the cell edge in either protrusions or ruffles based on counts of at least 50 cells in each of two separate experiments. Two separate dominant-negative tamalin (aa 99–189 and aa 1–189) were used. Error bars represent the mean ± SEM, relative to untreated cells. $n \geq 100$. **, $P < 0.01$; ***, $P < 0.001$. ANOVA followed by a Tukey test were used for both. Bars, 5 μm.

appears to colocalize with the TrkC receptors. Because this experiment shows that tamalin redistributes in response to NT3 and colocalizes with TrkC receptors, we repeated this analysis in neurons from a TrkC mutant mouse that specifically lacks the TrkC kinase isoforms but retains the truncated ones (Klein et al., 1994; data not depicted). Immunostaining of neurons lacking the kinase TrkC isoform reveals a specific punctuated TrkC pattern of expression that is consistent with the previously reported pattern of expression of TrkCT1 (Menn et al., 2000). Remarkably, neuronal treatment with NT3 shows dotted neuronal redistribution of tamalin that matches the TrkCT1 cellular pattern of expression (Fig. 3, d, h, and l, and insets therein). Collectively, these data demonstrate that in neurons endogenous tamalin redistributes in response to NT3 and colocalize with TrkCT1.

Tamalin forms a protein complex with cytohesin-2–ARNO (Nevrivy et al., 2000; Kitano et al., 2002), which is a member of the guanine nucleotide exchange factors for the Arf1 and Arf6 GTP-binding proteins. ARNOs have been proposed to be part of signaling cascades based on the finding that growth factor or insulin treatment of cells results in the translocation of ARNOs to the cell edge (Langille et al., 1999; Venkateswarlu et al., 1999). These results motivated us to examine the effects of NT3 binding to the TrkCT1–tamalin complex on Arf subcellular localization and cell morphology. Because HEK293 cells express tamalin (although at a low level; unpublished data), cytohesin family exchange factors, and Arfs endogenously (Nevrivy et al., 2000; Kitano et al., 2002), expression of TrkCT1 should reconstitute the NT3-activated pathway. After the generation of a stable line expressing TrkCT1, we assessed the translocation of epitope-tagged Arf6 to actin-rich ruffles and protrusions as a measure of Arf6 activation in response to NT3 (Radhakrishna et al., 1996, 1999; Frank et al., 1998; Franco et al., 1999; Honda et al., 1999; Langille et al., 1999; Santy and Casanova, 2001). In both the parental HEK293 and TrkCT1 cells, Arf6 associated to a small extent with the edge of the cell, as well as with a tubular endosomal compartment (Fig. 4, A and G). Treatment of parental HEK293 cells with NT3 had no significant effect on the distribution of Arf6 (Fig. 4, D and Y); in contrast, treatment of the HEK293-TrkCT1 cells resulted in the redistribution of Arf6 from the tubular endosomal compartment to the cell edge in actin-rich ruffles (Fig. 4, J–L [arrows] and Y), whereas Arf1 had a perinuclear distribution that was not affected by NT3 (not depicted). This translocation from the tubular endosomal compartment to the cell edge is a well defined consequence of Arf6 activation (Radhakrishna et al., 1996; Donaldson, 2003).

Moreover, tamalin was necessary for Arf6 activation through TrkCT1 because transfection of two independent dominant-negative forms containing the PDZ domain of tamalin blocked these effects (Fig. 4, S–Y). In contrast, the response of TrkCT1 cells overexpressing tamalin to NT3 was more robust than that of the nontransfected cells, with Arf6 moving from a tubular endosomal compartment (Fig. 4 M) to the cell edge in ruffles (Fig. 4, P–R [arrows] and Y) and large protrusions (Fig. 4, P–R, arrowheads) colocalizing with tamalin.

Because we could not exclude that dominant-negative tamalin might block NT3 signaling by masking the TrkCT1 docking site encoded by exon 14b, we next investigated whether a catalytically inactive form of cythohesin-2–ARNO would affect NT3-mediated ruffling (Fig. 5). E156K-ARNO has a mutation in the Sec7 domain that blocks the ability of ARNO to promote GTP exchange at Arf6 but does not disrupt its ability to bind to other proteins (Frank et al., 1998). Transfection of E156K-ARNO into HEK293-TrkCT1 cells blocked actin polymerization (Fig. 5, J–L), whereas wild-type ARNO enhanced NT3-mediated actin polymerization at the cell edge (Fig. 5, D–F). Thus, these data strongly suggest that NT3 mediates ruffling through the TrkCT1–tamalin–cythohesin-2–ARNO pathway.

Arfs function as GDP/GTP-regulated switches in the pathways that stimulate actin reorganization and membrane ruffling (Randazzo et al., 2000). Part of this effect is mediated by Rac1, which is a well characterized Rho GTPase located

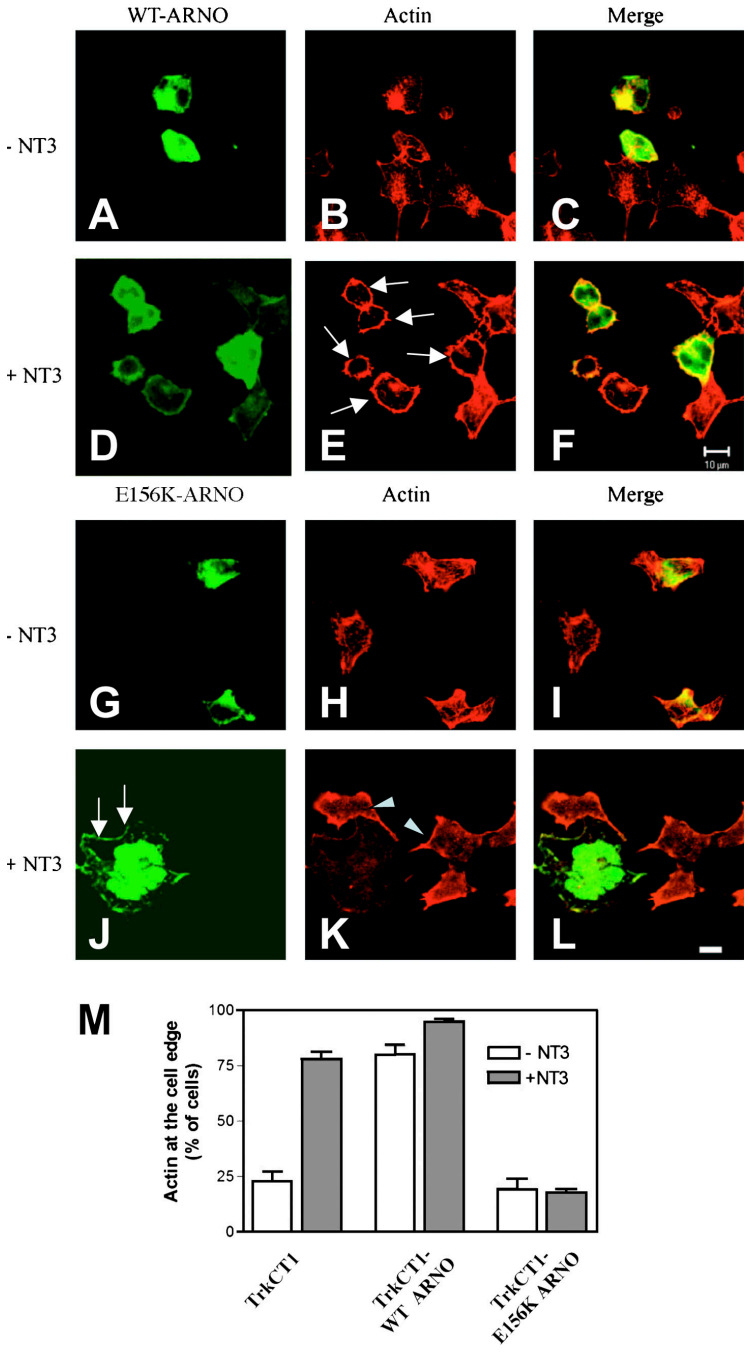

Figure 5. **Cytohesin-2–ARNO mediates NT3-induced membrane ruffling and actin protrusion formation.** HEK293 cells expressing TrkCT1 were transfected with a wild-type HA-tagged (WT-ARNO; A–F) or a catalytically inactive Myc-tagged ARNO (E156K-ARNO; G–L) and stained for polymerized actin. Ruffling, as indicated by the extensive presence of polymerized actin, is seen in cells expressing WT-ARNO in response to NT3 (D–F and arrows in E). On the contrary, the catalytically inactive E156-ARNO disrupts actin polymerization in response to NT3 (K), although it gets recruited to the cell edge because it can still bind tamalin (J, arrows). Arrowheads in K indicate ruffling in cells that do not express the inactive E156-ARNO, but are still able to use the endogenous ARNO for NT3 signaling. (M) Percentage of cells with actin at the cell edge in response to NT3 was determined from untransfected (TrkC.T1) or transfected (TrkC.T1-WT ARNO and TrkC.T1–E156K-ARNO) cells from two separate experiments. Error bars represent the mean ± SEM. Bars, 10 µm.

downstream of Arf6 (Radhakrishna et al., 1999; Santy and Casanova, 2001). Therefore, we investigated whether NT3 signaling trough TrkCT1–tamalin leads to Rac1 activation (Radhakrishna et al., 1996; Santy and Casanova, 2001). We found that NT3 treatment of HEK293 cells transfected with TrkCT1 and tamalin caused a 4.1 ± 0.47–fold increase in Rac1 activity (Fig. 6, A and B, compare lanes 5 and 6). Moreover, only expression of a dominant-negative form of Arf6 could block Rac1 activation, whereas expression of inactive Arf1 had no effect on Rac1 activation (Fig. 6, A and B, lanes 7 and 8). These data demonstrate that NT3 signaling through TrkCT1–tamalin can modulate Rac1 through Arf6 activation.

Discussion

In this study, we describe the identification of a new signaling pathway activated by an evolutionarily conserved truncated isoform of the TrkC receptor. This pathway links NT3 to downstream molecules affecting the regulation of actin cytoskeleton and membrane trafficking through TrkCT1 and the scaffold protein tamalin (Fig. 7).

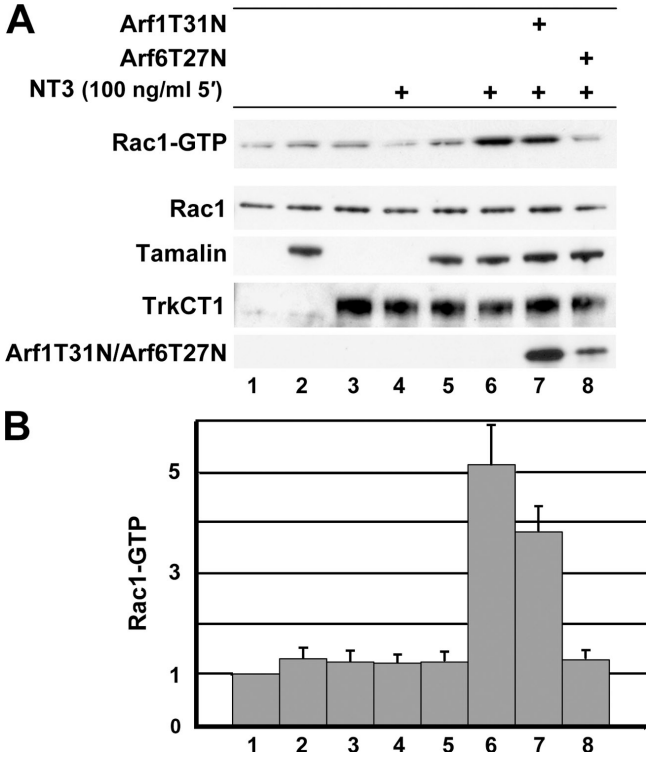

Figure 6. **NT3 induces Rac1 activation via the TrkCT1–tamalin–Arf6 pathway.** (A) HEK293 cells expressing TrkCT1 (lane 3), tamalin-HA (lane 2), and TrkCT1 and tamalin-HA (lanes 5–8) in the absence (lanes 5 and 6) or presence of inactive Arf1 (lane 7) or Arf6 (lane 8) were serum starved for 13 h and stimulated with 100 ng/ml of NT3 for 5 min, where indicated. Relative Rac1 activity to total Rac1 protein normalized to the basal activity present in untransfected HEK293 cells is shown. Input for Rac1, tamalin, TrkCT1, and Arf1T31N or Arf6T27N represents 1% of the total cell extract used to detect the active Rac1-GTP (top). (B) Quantification of Rac1 activation in response to NT3 treatment. The fold difference is mean ± SEM, relative to untreated cells (lane 1). Error bars represent the mean ± SEM from six independent experiments, except for the experiments performed with the Arf1 or Arf6 dominant-negative forms (three experiments).

The interaction of TrkCT1 with tamalin suggests a novel mechanism by which neurotrophins affect the development, maintenance, and function of the mammalian nervous system. Tamalin forms a protein complex with multiple postsynaptic and protein-trafficking scaffold proteins, suggesting that NT3 could be an extracellular trigger of these cellular processes by causing the recruitment of tamalin to TrkCT1 (Kitano et al., 2003). Among the several postsynaptic proteins with which tamalin interacts are the group 1 metabotropic glutamate receptors (mGluR1 and mGluR5) that affect ion channels and intracellular second messengers through G proteins (Schoepp et al., 1990; Aramori and Nakanishi, 1992). Glutamate receptors play a key role in neural development, especially in neuronal plasticity (Nakanishi and Masu, 1994; Nicoletti et al., 1999). It has been suggested that these mGluR1 functions may be mediated by tamalin, which provides a direct link to cytohesin-2–Arf GTP-binding proteins, which are involved in membrane trafficking, cytoskeletal reorganization, and phospholipase D activation (Nevrivy et al., 2000; Kitano et al., 2002). Thus, it is possible that the interaction of tamalin with TrkCT1 may provide a molecular pathway similar to the one activated by mGluR1 and by which neurotrophins can affect neuronal plasticity in mammals, a function that is still not well understood.

Several lines of evidence suggest that characteristic axonal and dendritic morphologies throughout the nervous system are determined, in part, by local patterns of expression of neurotrophins and neurotrophin receptors (Snider, 1988; Cohen-Cory and Fraser, 1995; McAllister et al., 1996, 1997; Lentz et al., 1999). For example, transfection of explant cultures of dorsal root ganglia neurons with the TrkC kinase or the TrkCT1 receptor revealed that specific ratios of receptor isoforms are associated with different axonal morphologies. Specifically, neurons overexpressing tyrosine kinase containing TrkC showed enhanced elaboration of major axonal processes, whereas the truncated isoform reduced elaboration of major processes and increased branching (Ichinose and Snider, 2000). Our data suggest that the effect on truncated TrkCT1 by NT3 may affect neuronal morphology, not by affecting the TrkC full-length receptor by a dominant-negative mechanism, but, rather, by activating Arfs and Rac1 GTPases that are known modulators of cell morphology.

In summary, this newly discovered neurotrophin-activated pathway may provide a means by which NT3 can exert some of its critical functions in development and might explain why mice lacking all TrkC receptor isoforms, including the TrkCT1, display a more severe phenotype than mice lacking the full-length TrkC tyrosine kinase receptors exclusively (Tessarollo et al., 1997). Importantly, these results identify a novel signaling pathway upstream of Arf6 in which an activated receptor binds directly to an ARNO-binding protein to stimulate ARNO and consequent nucleotide exchange on Arf6.

Materials and methods

Yeast two-hybrid screening

The PCR-amplified COOH-terminal region of the TrkCT1 isoform (corresponding to residues 575–612 of the rat TrkCT1 [Tsoulfas et al., 1993] or exon 14b of the human TrkC-truncated receptor [Ichaso et al., 1998]) was subcloned in frame to the GAL4-binding domain of the bait plasmid pGBD.C2.

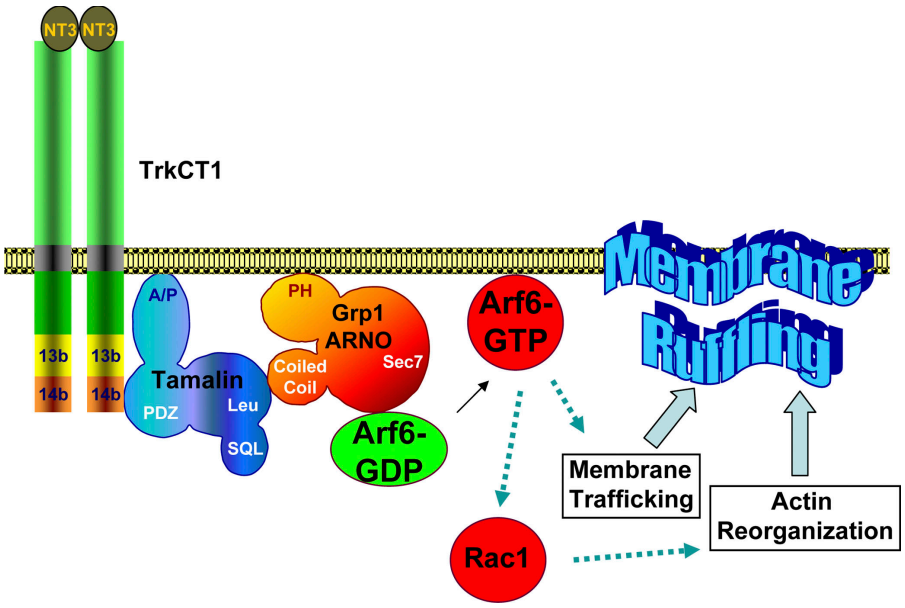

Figure 7. **Schematic representation of the newly identified NT3-activated signaling pathway.** NT3 binding to TrkCT1 causes the recruitment of tamalin to the TrkCT1 cytoplasmic domain (exon 14b). In turn, tamalin activates the guanine nucleotide exchange factors cytohesin-2–ARNO (Kitano et al., 2002), which causes the switch of Arf6 from its inactive to its active Arf6-GTP form. Arf6-GTP induces Rac1 activation and is translocated to the membrane, where ruffling and actin reorganization take place. Tamalin alanine/proline (A/P), PDZ, leucine rich (Leu), and PDZ-binding (SQL) domains are indicated, as well as ARNO's pleckstrin homology (PH), coiled coil, and Sec7 homology domains.

This fusion bait plasmid and an adult mouse brain cDNA library fused in frame to the GAL4 activation domain (Mouse Brain MATCHMAKER cDNA Library; CLONTECH Laboratories, Inc.) were sequentially transformed into the yeast strain PJ69-4A and subjected to colony selection and liquid β-galactosidase assays. Different tamalin deletion prey and TrkCT1 bait plasmids were generated by cloning PCR-generated cDNA fragments into pACT2 (CLONTECH Laboratories, Inc.) and pGBD.C2, respectively, and were used for subsequent two-hybrid analysis.

Cell culture

HEK293 cells were grown in DME (Invitrogen) supplemented with 10% FBS, 100 U/ml of penicillin/streptomycin at 37°C, and 5% CO_2. Cells were transfected using Fugene 6 (Roche) or Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Mouse embryonic hippocampus neurons were dissected from 18-d-old mouse embryos by the standard technique and seeded on coverslips pretreated with poly-D-lysine, collagen, and laminin. Cells were grown for 2 wk on DME supplemented with 2% B27, 5% fetal calf serum, 100 U/ml of penicillin/streptomycin, and 4 μM AraC; the media was changed every 3–4 d.

GST pull-down assay

GST–TrkC fusion proteins were generated by cloning the appropriate PCR products containing different rat TrkCT1 or TrkC full-length regions (Tsoulfas et al., 1993) in pGEX-4T-1 (GE Healthcare). The expression of the GST fusion proteins in *Escherichia Coli* strain BL21 was induced with IPTG, and soluble cell extracts were generated by sonication in lysis buffer containing PBS, pH 7.4, 1 mM EDTA, 1% Triton X-100 and complete mini EDTA-free protease inhibitor (Roche). The soluble proteins were immobilized on glutathione–Sepharose CL-4B beads (GE Healthcare) for 1 h at RT, washed five times with lysis buffer (PBS, pH 7.4, 1 mM EDTA, 1% NP-40, and complete mini EDTA-free protease inhibitor), and incubated with HEK293 cell extracts expressing the full-length COOH-terminal HA-tagged tamalin (tamalin-HA) protein in 500 μl of lysis buffer for 10–12 h at 4°C. The beads were washed five times with lysis buffer on a MicroSpin column (GE Healthcare). The bound proteins were eluted by centrifugation after heating at 95°C for 3 min in 2× SDS-PAGE loading buffer, separated by SDS-PAGE on 4–12% acrylamide gels (Invitrogen), and immunoblotted for detection with an anti-HA mouse monoclonal peroxidase-conjugated antibody (High Affinity 3F10; Roche).

coIP

A HEK293 line expressing TrkCT1 (HEK293-TrkCT1) was generated by transfection with a pcDNA3.1 puromycin-resistant plasmid containing the TrkCT1 cDNA. For protein expression experiments, a tamalin-HA plasmid was transiently transfected into naive HEK293 cells or the HEK293-TrkCT1 line, and lysates were prepared for protein analysis. A goat anti-TrkC (Upstate Biotechnology) antibody was used for coIP experiments. Immunoprecipitates were resolved by SDS-PAGE (4–12% gel) and immunodetected with the anti-HA antibody and the rabbit anti–TrkC 656 antiserum (raised against aa 484–501 of the juxtamembrane region; a gift from P. Tsoulfas, University of Miami, Miami, FL). Rac1 activation was measured using a nonradioactive Rac activation assay kit (CHEMICON International, Inc.) following the manufacturer's recommendations.

In situ hybridization and immunocytochemistry

In situ hybridization protocols using the antisense full-length tamalin (Kitano et al., 2002) or the TrkCT1-specific sequence (corresponding to exon 13b and 14b and the subsequent 286 bases of the 3′ un-translated region) riboprobes were performed as previously described (Tessarollo and Parada, 1995).

For immunocytochemical experiments, cells were transfected with the pcDNA3.1 (+) expression vector, containing the open reading frame for FLAG-tagged wild-type human Arf6 and ARNO (wild-type ARNO), Myc-tagged wild-type tamalin and mutant E156K-ARNO, and HA-tagged mutant (aa 1–189) tamalin. 10 μg of wild-type human Arf6 or 7 μg of wild-type ARNO and E156K-ARNO were used in single transfection; 5 μg of wild-type human Arf6 and 8 μg of wild-type or mutant tamalin were used in cotransfection experiments. Transfected cells were plated on 100-mm culture dishes precoated with poly-D-lysine (20 μg/ml for 10 min at 25°C). 24 h later, they were reseeded on fibronectin-coated coverslips in serum-free medium (OPTI-MEM; Invitrogen) and incubated for 12 h at 37°C. To determine their responses to NT3, cells or primary neurons were starved for 4 h and incubated with 100 ng/ml NT3 (Upstate Biotechnology) for 5 min at RT before fixation. HEK293 cells were fixed in 2% paraformaldehyde in PBS for 10 min at RT, washed three times with PBS and once in PBS containing 10% FBS and 0.04% sodium azide, and permeabilized with staining buffer (PBS containing 10% FBS, 0.2% saponin, and 0.04% sodium azide) for 10 min at RT. Mouse hippocampal neurons were fixed for 30 min in 4% paraformaldehyde, washed three times in PBS, and blocked overnight with mouse IgG blocking reagent (Vector Laboratories) in PBS. After two washes in PBS, they were incubated for 30 min in 0.1% Triton X-100, 5% mouse protein concentrate (Vector Laboratories), and 5% normal goat serum (S1000; Sigma-Aldrich) in PBS. After incubation with the primary antibodies in staining buffer for 1–3 h at RT, cells were washed three times for 10 min with staining buffer and incubated with FITC- or rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) for 1 h at RT. After three 10-min washes, glass coverslips were mounted on slides with fluorescent mounting medium (Dakocytomation). Cells were visualized at RT by confocal microscopy with a confocal system (LSM510 NLO) and with an inverted microscope (Axiovert 200M; both Carl Zeiss MicroImaging, Inc.) and operating with a 25-mW argon laser tuned to 488 nm and a 1-mW HeNe laser tuned to 543 nm. Cells were imaged with a 63×, 1.4 NA, oil immersion objective for HEK293 cells and Plan-Neofluar 40×, 1.3 NA, oil differential interference contrast objective for neuron analysis. Using AIM software (Carl Zeiss MicroImaging, Inc.), images were collected using a multitrack configuration where the FITC and rhodamine signals were sequentially collected with a bp 500–550-nm filter and a bp 565–615-nm filter after sequential excitation with the 488- and

543-nm laser lines, respectively. Antibodies were used at the following concentration in PBS: polyclonal anti-TrkC 656 (1:100), polyclonal anti-HA (1:5,000; Roche), monoclonal M5 anti-FLAG (1:1,000; Sigma-Aldrich), polyclonal anti-Myc (1:100; Santa Cruz Biotechnology, Inc.), polyclonal rabbit anti-tamalin (1:1,500, against the TEAREQALCGAGLRKTKYRSFR epitope of the mouse tamalin COOH terminus), and mouse monoclonal TrkC 2B7 (1:1,500, raised against epitope ESTDNFILFDEVSPTPPI of the human TrkC extracellular domain).

We thank Julie Donaldson, Robert Kalb, Ira Daar, Neal Copeland, and Nancy Jenkins for suggestions and discussions, Esta Sterneck for critical reading of the manuscript, and Alma Arnold and Steven Lockett for help with the confocal analysis.

This research was supported by the Intramural Research Program of the National Institutes of Health (NIH), the Center for Cancer Research, and the National Cancer Institute, and by grants from the Canadian Institutes of Health Research (MT-13265) and the NIH (CA82642) for H.U. Saragovi.

Submitted: 2 December 2005
Accepted: 20 March 2006

References

Aramori, I., and S. Nakanishi. 1992. Signal transduction and pharmacological characteristics of a metabotropic glutamate receptor, mGluR1, in transfected CHO cells. *Neuron*. 8:757–765.

Baxter, G.T., M.J. Radeke, R.C. Kuo, V. Makrides, B. Hinkle, R. Hoang, A. Medina-Selby, D. Coit, P. Valenzuela, and S.C. Feinstein. 1997. Signal transduction mediated by the truncated trkB receptor isoforms, trkB.T1 and trkB.T2. *J. Neurosci.* 17:2683–2690.

Cohen-Cory, S., and S.E. Fraser. 1995. Effects of brain-derived neurotrophic factor on optic axon branching and remodelling in vivo. *Nature*. 378:192–196.

Donaldson, J.G. 2003. Multiple roles for Arf6: sorting, structuring, and signaling at the plasma membrane. *J. Biol. Chem.* 278:41573–41576.

Escandón, E., D. Soppet, A. Rosenthal, J.-L. Mendoza-Ramírez, E. Szönyi, L.E. Burton, C.E. Henderson, L.F. Parada, and K. Nikolics. 1994. Regulation of neurotrophin receptor expression during embryonic and postnatal development. *J. Neurosci.* 14:2054–2068.

Franco, M., P.J. Peters, J. Boretto, E. van Donselaar, A. Neri, C. D'Souza-Schorey, and P. Chavrier. 1999. EFA6, a sec7 domain-containing exchange factor for ARF6, coordinates membrane recycling and actin cytoskeleton organization. *EMBO J.* 18:1480–1491.

Frank, S.R., J.C. Hatfield, and J.E. Casanova. 1998. Remodeling of the actin cytoskeleton is coordinately regulated by protein kinase C and the ADP-ribosylation factor nucleotide exchange factor ARNO. *Mol. Biol. Cell*. 9:3133–3146.

Garner, A.S., and T.H. Large. 1994. Isoforms of the avian TrkC receptor: A novel kinase insertion dissociates transformation and process outgrowth from survival. *Neuron*. 13:457–472.

Hapner, S.J., K. Boeshore, T.H. Large, and L. Lefcort. 1998. Neural differentiation promoted by truncated trkC receptors in collaboration with p75NTR. *Dev. Biol.* 201:90–100.

Honda, A., M. Nogami, T. Yokozeki, M. Yamazaki, H. Nakamura, H. Watanabe, K. Kawamoto, K. Nakayama, A.J. Morris, M.A. Frohman, and Y. Kanaho. 1999. Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell*. 99:521–532.

Huang, E.J., and L.F. Reichardt. 2001. Neurotrophins: roles in neuronal development and function. *Annu. Rev. Neurosci.* 24:677–736.

Ichaso, N., R.E. Rodriguez, D. Martin-Zanca, and R. Gonzalez-Sarmiento. 1998. Genomic characterization of the human trkC gene. *Oncogene*. 17:1871–1875.

Ichinose, T., and W.D. Snider. 2000. Differential effects of TrkC isoforms on sensory axon outgrowth. *J. Neurosci. Res.* 59:365–371.

Kitano, J., K. Kimura, Y. Yamazaki, T. Soda, R. Shigemoto, Y. Nakajima, and S. Nakanishi. 2002. Tamalin, a PDZ domain-containing protein, links a protein complex formation of group 1 metabotropic glutamate receptors and the guanine nucleotide exchange factor cytohesins. *J. Neurosci.* 22:1280–1289.

Kitano, J., Y. Yamazaki, K. Kimura, T. Masukado, Y. Nakajima, and S. Nakanishi. 2003. Tamalin is a Scaffold protein that interacts with multiple neuronal proteins in distinct modes of protein-protein association. *J. Biol. Chem.* 278:14762–14768.

Klein, R., I. Silos-Santiago, R.J. Smeyne, S.A. Lira, R. Brambilla, S. Bryant, L. Zhang, W.D. Snider, and M. Barbacid. 1994. Disruption of the neurotrophin-3 receptor gene trkC eliminates Ia muscle afferents and results in abnormal movements. *Nature*. 368:249–251.

Langille, S.E., V. Patki, J.K. Klarlund, J.M. Buxton, J.J. Holik, A. Chawla, S. Corvera, and M.P. Czech. 1999. ADP-ribosylation factor 6 as a target of guanine nucleotide exchange factor GRP1. *J. Biol. Chem.* 274:27099–27104.

Lentz, S.I., C.M. Knudson, S.J. Korsmeyer, and W.D. Snider. 1999. Neurotrophins support the development of diverse sensory axon morphologies. *J. Neurosci.* 19:1038–1048.

McAllister, A.K., L.C. Katz, and D.C. Lo. 1996. Neurotrophin regulation of cortical dendritic growth requires activity. *Neuron*. 17:1057–1064.

McAllister, A.K., L.C. Katz, and D.C. Lo. 1997. Opposing roles for endogenous BDNF and NT-3 in regulating cortical dendritic growth. *Neuron*. 18:767–778.

Menn, B., S. Timsit, G. Calothy, and F. Lamballe. 1998. Differential expression of TrkC catalytic and noncatalytic isoforms suggests that they act independently or in association. *J. Comp. Neurol.* 401:47–64.

Menn, B., S. Timsit, A. Represa, S. Mateos, G. Calothy, and F. Lamballe. 2000. Spatiotemporal expression of noncatalytic TrkC NC2 isoform during early and late CNS neurogenesis: a comparative study with TrkC catalytic and p75NTR receptors. *Eur. J. Neurosci.* 12:3211–3223.

Nakanishi, S., and M. Masu. 1994. Molecular diversity and functions of glutamate receptors. *Annu. Rev. Biophys. Biomol. Struct.* 23:319–348.

Nevrivy, D.J., V.J. Peterson, D. Avram, J.E. Ishmael, S.G. Hansen, P. Dowell, D.E. Hruby, M.I. Dawson, and M. Leid. 2000. Interaction of GRASP, a protein encoded by a novel retinoic acid-induced gene, with members of the cytohesin family of guanine nucleotide exchange factors. *J. Biol. Chem.* 275:16827–16836.

Nicoletti, F., V. Bruno, M.V. Catania, G. Battaglia, A. Copani, G. Barbagallo, V. Cena, J. Sanchez-Prieto, P.F. Spano, and M. Pizzi. 1999. Group-I metabotropic glutamate receptors: hypotheses to explain their dual role in neurotoxicity and neuroprotection. *Neuropharmacology*. 38:1477–1484.

Ohira, K., H. Kumanogoh, Y. Sahara, K.J. Homma, H. Hirai, S. Nakamura, and M. Hayashi. 2005. A truncated tropomyosin-related kinase B receptor, T1, regulates glial cell morphology via Rho GDP dissociation inhibitor 1. *J. Neurosci.* 25:1343–1353.

Palko, M.E., V. Coppola, and L. Tessarollo. 1999. Evidence for a role of truncated trkC receptor isoforms in mouse development. *J. Neurosci.* 19:775–782.

Radhakrishna, H., R.D. Klausner, and J.G. Donaldson. 1996. Aluminum fluoride stimulates surface protrusions in cells overexpressing the ARF6 GTPase. *J. Cell Biol.* 134:935–947.

Radhakrishna, H., O. Al-Awar, Z. Khachikian, and J.G. Donaldson. 1999. ARF6 requirement for Rac ruffling suggests a role for membrane trafficking in cortical actin rearrangements. *J. Cell Sci.* 112:855–866.

Randazzo, P.A., Z. Nie, K. Miura, and V.W. Hsu. 2000. Molecular aspects of the cellular activities of ADP-ribosylation factors. *Sci. STKE*. 2000:RE1.

Rose, C.R., R. Blum, B. Pichler, A. Lepier, K.W. Kafitz, and A. Konnerth. 2003. Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells. *Nature*. 426:74–78.

Rudzinski, M., T.P. Wong, and H.U. Saragovi. 2004. Changes in retinal expression of neurotrophins and neurotrophin receptors induced by ocular hypertension. *J. Neurobiol.* 58:341–354.

Santy, L.C., and J.E. Casanova. 2001. Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. *J. Cell Biol.* 154:599–610.

Schoepp, D., J. Bockaert, and F. Sladeczek. 1990. Pharmacological and functional characteristics of metabotropic excitatory amino acid receptors. *Trends Pharmacol. Sci.* 11:508–515.

Snider, W.D. 1988. Nerve growth factor enhances dendritic arborization of sympathetic ganglion cells in developing mammals. *J. Neurosci.* 8:2628–2634.

Tessarollo, L. 1998. Pleiotropic functions of neurotrophins in development. *Cytokine Growth Factor Rev.* 9:125–137.

Tessarollo, L., and L.F. Parada. 1995. In situ hybridization. *Methods Enzymol.* 254:419–430.

Tessarollo, L., P. Tsoulfas, M.J. Donovan, M.E. Palko, J. Blair-Flynn, B.L. Hempstead, and L.F. Parada. 1997. Targeted deletion of all isoforms of the trkC gene suggests the use of alternate receptors by its ligand neurotrophin-3 in neuronal development and implicates trkC in normal cardiogenesis. *Proc. Natl. Acad. Sci. USA*. 94:14776–14781.

Tsoulfas, P., D. Soppet, E. Escandon, L. Tessarollo, J.L. Mendoza-Ramirez, A. Rosenthal, K. Nikolics, and L.F. Parada. 1993. The rat trkC locus encodes multiple neurogenic receptors that exhibit differential response to neurotrophin-3 in PC12 cells. *Neuron*. 10:975–990.

Valenzuela, D.M., P.C. Maisonpierre, D.J. Glass, E. Rojas, L. Nuñez, Y. Kong, D.R. Gies, T.N. Stitt, N.Y. Ip, and G.D. Yancopoulos. 1993. Alternative forms of rat TrkC with different functional capabilities. *Neuron*. 10:963–974.

Venkateswarlu, K., F. Gunn-Moore, J.M. Tavare, and P.J. Cullen. 1999. EGF-and NGF-stimulated translocation of cytohesin-1 to the plasma membrane of PC12 cells requires PI 3-kinase activation and a functional cytohesin-1 PH domain. *J. Cell Sci.* 112:1957–1965.