

Tech: a RhoA GEF selectively expressed in hippocampal and cortical neurons

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Abstract

Recent studies implicating the Rho family of small G proteins in the regulation of neuronal morphology have focused attention on identifying key components of Rho signaling pathways in neurons. To this end, we have conducted studies aimed at defining the localization and function of Tech, a Rho guanine nucleotide exchange factor (GEF) family member that is highly enriched in brain. We have found that Tech is selectively expressed in cortical and hippocampal neurons with prominent Tech immunostaining apparent in the cell bodies and dendrites of these cells. *In vitro* studies with prototypical members of the major Rho subfamilies, RhoA, Rac1 and Cdc42, indicate that Tech binds selectively to and activates RhoA. To assess whether Tech may be involved in the regulation of neuronal morphology, we examined the

effects of Tech constructs on the morphology of cortical neurons grown in primary culture. We found that a constitutively active Tech construct, Tech 245 Δ C, decreases the number of dendritic processes present on these neurons. This reduction appears to be mediated by activation of RhoA as it is blocked by insertion of a point mutation into the DH domain of Tech which blocks its ability to activate RhoA or coexpression of a dominant negative RhoA construct. As Tech protein levels increase during post-natal development and remain at peak levels into adulthood, these results indicate that Tech regulates RhoA signaling pathways in developing and mature forebrain neurons.

Keywords: actin cytoskeleton, Dbl domain, dendritic morphology, PH domain, RhoA, stress fibers.

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Recent studies have identified members of the Rho family of small G proteins as key components of intracellular signaling pathways involved in controlling a broad range of cellular functions ranging from cell shape to gene transcription (Zheng 2001; Etienne-Manneville and Hall 2002). Although several Rho proteins are expressed widely, there is considerable heterogeneity in the expression patterns of proteins that control their activity, such as Rho GEFs and Rho GTPase activating proteins (GAPs), providing a mechanism for tailoring these signaling pathways to the diverse functional requirements of different cell types. In recent studies, we and others (De Toledo *et al.* 2001; Marx *et al.* 2002) have independently found a Rho GEF family member that is highly enriched in brain. As we identified it in northern blot studies on rat tissues as a transcript enriched in cortex and hippocampus, we called it Tech; the human orthologue has been designated KIAA0720.

In their initial characterization of the human orthologue of Tech, De Toledo *et al.* (2001) reported that this Rho GEF family member has several interesting properties. First, it is highly enriched in brain relative to a variety of peripheral

tissues. Second, its Rho GEF activity appears to be highly selective for RhoA. Third, consistent with its RhoA GEF activity, it blocks the ability of nerve growth factor (NGF) to induce neurite outgrowth in PC12 cells, a widely used model of neuronal differentiation. To help to define the role of Tech in the nervous system, we have pursued studies aimed at determining its localization within the brain. Furthermore, as these studies indicate that Tech is expressed selectively in neurons, we have also examined the effects of Tech on the morphology of brain neurons. Our results demonstrate that activated forms of Tech decrease the number of dendritic processes in cultured cortical neurons via stimulation of RhoA signaling pathways.

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Abbreviations used: GFP, green fluorescence protein; GST, glutathione S-transferase.

Materials and methods

Plasmids and antibodies

The full-length Tech cDNA sequence (submitted to GenBank) was determined from inserts isolated from a rat brain library generously provided by P. Worley and T. Lanahan (Johns Hopkins, Baltimore, MD, USA). Tech constructs were subcloned into the pRK5 expression vector with a C-terminal rhodopsin epitope tag (last 10 amino acids of the rhodopsin C-terminal tail) provided by J. Nathans (Johns Hopkins, Baltimore, MD, USA). Myc-tagged constitutively active RhoA (Q63L) and dominant negative RhoA (T19N) were obtained from A. Cerione (Cornell University, Ithaca, NY, USA) and G. Bokoch (Scripps Institute, La Jolla, CA, USA), respectively. Bacterial expression constructs of GST-tagged Rho proteins were obtained from A. Cerione. The following mouse monoclonal antibodies were used for immunostaining or immunoblotting: MAP-2 (1 : 2000; Sigma, St Louis, MO, USA), myc epitope tag (1 : 2000; Invitrogen, Carlsbad, CA, USA), actin C-2 (1 : 100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rhodopsin epitope tag (1 : 5000; provided by J. Nathans (Johns Hopkins, Baltimore, MD, USA)).

Northern blot analysis and *in situ* hybridization

To determine the tissue distribution of the Tech transcript, RNA samples were extracted from rat tissues using Triazole (Gibco-BRL Carlsbad, CA, USA), processed for northern blotting (10 µg/lane) and then hybridized with a probe prepared from a fragment of Tech spanning the DH and PH domains. For *in situ* hybridization studies, tissue sections were processed as described in Brakeman *et al.* (1999) with either an antisense or sense ³⁵S riboprobe prepared from the 3' portion of the Tech transcript.

Tech antibodies

The following peptide, NH₂-YPRVQPEPSPGISAQHRK-OH, derived from the C-terminal portion of Tech, was cross-linked to thyroglobulin and used to immunize rabbits. Crude antiserum was affinity purified on a column prepared by cross-linking the antigen peptide to Affigel 15 (Biorad, Hercules, CA, USA). For immunoblotting studies, human embryonic kidney 293 cells or brain regions were homogenized in a buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl and 1% Triton X-100, with the addition of one tablet of Complete EDTA free (Roche, Mannheim, Germany) protease inhibitor cocktail per 50 mL. The supernatant fluid was then boiled for 1 min in Laemmli buffer and processed for immunoblotting with the crude Tech antiserum (1 : 1000) or affinity-purified antiserum (1 : 100) after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (20 µg protein per lane). For immunohistochemical studies, rat brain sections were processed for immunostaining with affinity-purified Tech antiserum (1 : 100) using the procedure described in Brakeman *et al.* (1999).

Rho protein binding assay

The GST fusion proteins of Rho GTPases purified from *Escherichia coli* were depleted of bound nucleotide by incubation in 10 mM EDTA. Human embryonic kidney 293 cells transfected with rhodopsin-tagged Tech constructs were extracted in binding buffer (20 mM Tris-HCl, pH 7.5 and 0.5 M NaCl) containing 1% Triton X-100. For each binding reaction, 5 µg of GST-RhoGTPase bound

to 25 µL of glutathione-Sepharose beads was mixed with an aliquot of cell extract containing 1 mg of protein for 1 h at 4°C. The beads were washed three times with binding buffer and bound proteins eluted with Laemmli buffer were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting using a monoclonal antibody to the rhodopsin epitope tag (1 : 5000). Immunoblotting with a GST antibody (Amersham Pharmacia, Uppsala, Sweden; 1 : 1000) was used to confirm the presence of the GST-Rho fusion proteins in the binding reactions.

Rho activation assay

Activation of RhoA or Rac1 was measured with the RhoA or Rac1/Cdc42 activation assay kits (Upstate Biotechnology, Lake Placid, NY, USA), respectively. Briefly, human embryonic kidney 293 cells that had been transfected with a Tech construct (10 µg DNA/10-cm dish) 1 day earlier were serum starved overnight and cell lysates (~0.5 mg of protein) were incubated for 1 h at 4°C with 20 µg of GST-Rhotekin-PBD (RhoA activation assay) or 20 µg of GST-PAK1-PBD (Rac1 activation assay). Bound proteins were eluted and analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting with a RhoA polyclonal antibody (1 : 300) or a monoclonal Rac1 antibody (1 : 1000).

Cell culture

3T3 NIH fibroblasts were grown on Permanox chamber slides (Nalge Nunc International, Rochester, NY, USA) in Dulbecco's modified Eagle's medium (Gibco-BRL) containing 10% fetal bovine serum (Gibco-BRL), 10% Nu serum (BD Biosciences, Bedford, MA, USA), 2 mM glutamine and 1% penicillin/streptomycin (Gibco-BRL).

Dissociated cortical cultures were prepared from rat embryos at embryonic day 17. Cortical tissue was dissected quickly on ice in Hanks' balanced salt solution supplemented with 4.2 mM NaHCO₃, 10 mM HEPES, 33.3 mM glucose, 0.3% bovine serum albumin, 12 mM MgSO₄ and 5 µg/mL gentamycin adjusted to pH 7.3 (dissection medium). The dissected tissue was washed in Hanks' balanced salt solution and then incubated for 2 min in digestion medium (4.2 mM NaHCO₃, 7 mM Na₂HPO₄, 25 mM HEPES, pH 7.4, 137 mM NaCl, 5 mM KCl) containing trypsin-EDTA (2 mg/mL; Gibco) and DNase (0.6 mg/mL; Roche) at 37°C. After the digestion medium was removed, the tissue was placed back on ice and washed several times with dissection medium, followed by a 10-min incubation with dissection medium supplemented with trypsin inhibitor (2 mg/mL) on ice. After additional washes in dissection medium, the tissue was triturated in dissection medium supplemented with DNase (0.6 mg/mL). Any large tissue chunks remaining were allowed to settle and discarded. Cells in the supernatant fluid were pelleted and resuspended in neurobasal medium containing 1× B27 supplement (Gibco), 2 mg/mL gentamicin and 2 mM glutamine. Cells were plated at a density of approximately 0.5 × 10⁶ cells/well onto 18-mm circle coverslips coated with poly-D-lysine that had been placed in 12-well dishes.

Morphological analysis

To monitor the effect of Tech and Rho constructs on the morphology of 3T3 cells, these constructs (1 µg/slide) were transfected into cells using Lipofectamine Plus reagents according to the manufacturer's instructions (Invitrogen). After cells had been exposed to the

transfection reagent for 4 h, the transfection medium was replaced with serum-free Dulbecco's modified Eagle's medium. Cells were fixed 18 h later by placing them in phosphate-buffered saline containing 4% formaldehyde for 15 min. They were then permeabilized by placing them in phosphate-buffered saline containing 0.075% Triton X-100 and 2 mg/mL bovine serum albumin for 30 min. After the cells were placed in blocking solution (phosphate-buffered saline with bovine serum albumin; 2 mg/mL) for 60 min, they were incubated at 4°C overnight in the presence of blocking solution containing primary antibody. After the cells were washed in phosphate-buffered saline, they were incubated for 60 min at 25°C with a fluorophore-conjugated secondary antibody diluted in blocking solution. After another washing, coverslips were mounted onto the slides with Permafluor-DABCO (Beckman-Coulter, Marseille, France). Actin filaments were visualized with Texas red-phalloidin (1 : 200; Molecular Probes, Eugene, OR, USA). To examine the effects of the transfected constructs on cell morphology, fluorescence microscopy was used to collect images of cells that expressed the transfected constructs. For quantification studies, the slides were searched in a systematic fashion and images taken of each transfected cell identified. Visualization of phalloidin staining was used to score the cells as having stress fibers. Cells were scored as being round if they were round in shape and did not have any visible processes. In each experiment, at least 20 cells were scored for each condition tested. Each condition was scored in at least three independent experiments.

To assess the effects of Tech or RhoA constructs on dendritic morphology, dissociated cortical cultures were transfected with these constructs (3 µg/well) along with a GFP expression vector (3 µg/well) at 8 days *in vitro* (DIV) using Lipofectamine 2000 (Invitrogen). The next day, conditioned medium, that had been harvested just before switching to transfection medium, was added. Cells were kept in this medium until 12 DIV and then processed for microscopy as described above for 3T3 cells. To analyse the effects of Tech and RhoA constructs on dendritic morphology, slides were searched in a systematic fashion for GFP-positive cells. To ensure that each cell scored was also transfected with the expression construct of interest, only cells that also stained for the appropriate epitope tag were used for quantification. In preliminary studies, we processed cultures transfected with GFP for MAP-2 immunostaining and found that nearly all (> 95%) of the GFP-positive cells seen with this procedure were also MAP-2 positive. A circle with a radius of 40 µm was centered on the cell body of each GFP-positive neuron and the number of intersections of GFP-positive processes was counted by an investigator (J.M.B.) who was blind to the construct being evaluated. More than 20 neurons were evaluated for each construct in each of at least three independent experiments.

Results

Localization of Tech in brain

As an initial step in characterizing the expression pattern of Tech, we assessed its tissue distribution by northern blot analysis. These studies revealed that this transcript is highly enriched in cortex and hippocampus, hence the acronym Tech, with little or no detectable expression in other brain

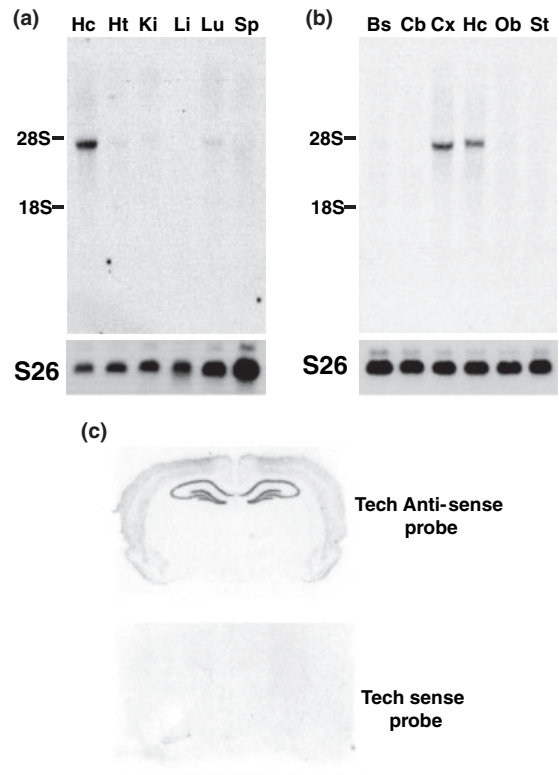


Fig. 1 Selective expression of Tech transcript in hippocampus (Hc) and cortex (Cx). (a) Top panel presents northern blot analysis of Tech mRNA levels in Hc and several peripheral tissues, heart (Ht), kidney (Ki), liver (Li), lung (Lu) and spleen (Sp). The blot was reprobbed for S26 ribosomal RNA (bottom panel) to check that strong Tech hybridization seen in the Hc does not reflect inadvertent overloading of hippocampal RNA sample relative to other lanes. (b) Northern blot showing distribution of Tech mRNA across several brain regions, brainstem (Bs), cerebellum (Cb), Cx, Hc, olfactory bulb (Ob) and striatum (St). Bottom panel shows similar levels of S26 ribosomal RNA in each lane as revealed by reprobbed the blot. (c) *In situ* hybridization autoradiograms show high levels of Tech transcript in Hc and Cx. The section shown in the top panel was hybridized with Tech antisense probe and that in the bottom panel with sense probe.

areas and a variety of peripheral tissues (Figs 1a and b). To help confirm these findings and obtain clues about which cell types express Tech, we conducted *in situ* hybridization studies on forebrain sections. Consistent with the northern blot results, we found strong hybridization in the hippocampus and cortex, with negligible staining in the thalamus. In addition, we noted prominent staining of the hippocampal pyramidal and dentate granule cell layers, indicating that Tech is selectively expressed in neurons (Fig. 1c).

As a first step in examining the expression of Tech protein, we conducted immunoblot studies with an antiserum generated against a C-terminal Tech peptide. In hippocampal extracts, we detected a major band that runs slightly higher than the predicted molecular weight of ~116 kDa and

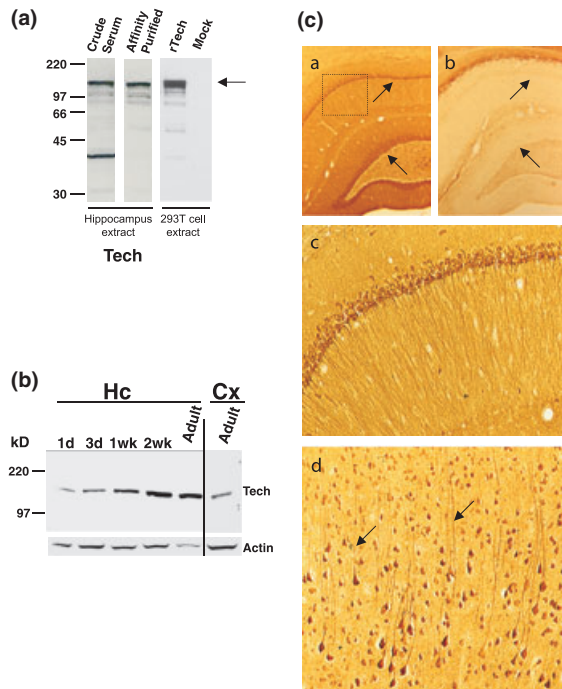


Fig. 2 Neuronal localization of Tech protein. (a) Immunoblot of hippocampal extract with crude Tech antiserum (left lane) reveals a major band that comigrates with recombinant rat Tech (indicated by arrow in right margin) expressed in 293T cells, as well as a faster migrating, non-specific band that is not detected with affinity-purified antiserum. To check that the prominent Tech band detected in 293T cells transfected with the rat Tech expression plasmid (rTech) does not reflect cross-reactivity of the serum, we confirmed that this band is absent in extracts from mock-transfected 293T cells (Mock). Pre-incubation of the purified antiserum with the antigen peptide (10 $\mu\text{g}/\text{mL}$) abolishes detection of Tech bands (not shown). (b) Analysis of Tech expression during post-natal development shows that Tech protein levels increase during the first 2 weeks and remain high in adulthood. The cortex (Cx) displays a similar development profile (not shown) but with lower levels of Tech expression seen at all of the ages assayed, as shown for the adult sample (right lane). Reprobing of this blot with actin antibodies confirms that the rise in Tech levels observed during post-natal development does not reflect inadvertent overloading of the samples harvested at older ages. (c) Immunohistochemical localization of Tech protein in rat brain. (i) Low power view of hippocampus (Hc) processed with affinity-purified Tech antiserum shows immunostaining in the hippocampal pyramidal cell layer (upper arrow) and the dentate granule cell layer (lower arrow). (ii) Adjacent section that was processed with affinity-purified Tech antiserum that was pre-incubated with the peptide antigen (10 $\mu\text{g}/\text{mL}$) confirms specificity of staining in those areas. In contrast, residual staining observed in the alveus, the white matter tract seen at the upper edge of the Hc, is not specific. This non-specific staining appears stronger than in (i) because the image shown in (ii) was overexposed to show the outlines of the anatomical structures. Arrows in (ii) point to the same position as those in (i). (iii) High power view of boxed area in (i) shows staining of pyramidal neuron cytoplasm that extends into their fine apical dendrites. (iv) High power view of Cx shows prominent staining of pyramidal neurons and their apical dendrites (arrows).

comigrates with recombinant Tech expressed in human embryonic kidney 293 cells (Fig. 2a). As expected, pre-incubation of the antiserum with the antigen peptide blocks detection of this band (data not shown). Consistent with the distribution of the Tech transcript, we found in immunoblotting studies that Tech protein levels are slightly higher in hippocampus than cortex (Fig. 2b) and barely detectable in cerebellum (data not shown). Examination of the developmental profile of Tech expression in the hippocampus revealed that it is present at low levels at birth and reaches peak levels by 2 weeks of age that are maintained in adulthood (Fig. 2b).

Immunohistochemical localization of Tech in rat brain sections matched that predicted by *in situ* hybridization studies with strong staining of cortical and hippocampal pyramidal neurons as well dentate granule cells. Tech staining was apparent in the cell body and extended into dendritic processes (Fig. 2c).

Tech binds selectively to RhoA

As these localization studies demonstrate that Tech is expressed in neurons, we wanted to test the possibility that it might be involved in regulating neuronal morphology via its ability to activate Rho signaling pathways (Threadgill *et al.* 1997; Li *et al.* 2000; Luo 2000, 2002). However, characterization of other Rho GEFs indicates that some contain autoinhibitory domains that render the full-length form inactive, while truncation yields constitutively active constructs (Schmidt and Hall 2002). Accordingly, before testing the effects of Tech on neuronal morphology, it was important to identify Tech constructs that display Rho GEF activity.

Previous studies using a yeast-based interaction assay indicated that KIAA0720, the human orthologue of Tech, displays a high degree of selectivity for RhoA (De Toledo *et al.* 2001). To check these results for Tech, we assessed its ability to bind *in vitro* to the prototypical members of the three major Rho subgroups: Rac1, RhoA and Cdc42. For this assay, we incubated epitope-tagged Tech constructs with GST fusion proteins for each of these three Rho GTPases. We found that full-length Tech and a truncated Tech construct, Tech 245 Δ C, which contains the DH and PH domains (Fig. 3c), bind selectively to RhoA (Fig. 3a), consistent with the results reported for KIAA0720. To check the assumption that the DH domain of Tech mediates its association with RhoA, we assessed the effect of mutating a conserved leucine residue (L530E), thought to be critical for interaction of the DH domain with Rho proteins, in the Tech 245 Δ C construct. As expected, we found that this mutant construct, Tech 245 Δ C-LE, does not display binding to RhoA. In contrast, a control mutant construct, Tech 245 Δ C-KA, containing a point mutation at a nearby residue (K526A), retains binding activity. In contrast, we found that a shorter construct, Tech 348 Δ C, generated by removing

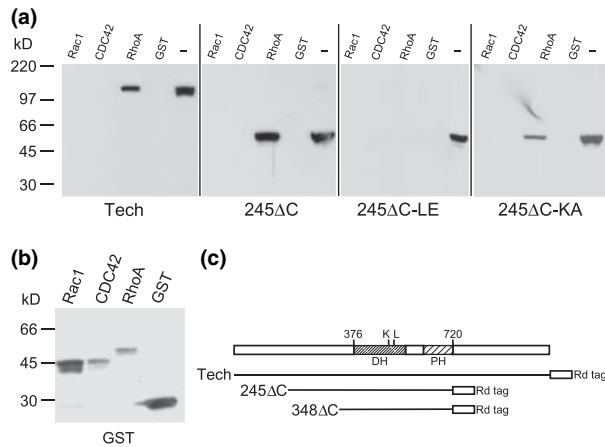


Fig. 3 Selective association of Tech with RhoA. (a) Recombinant Tech constructs were incubated with GST fusion proteins prepared from Rac1, Cdc42, RhoA or GST alone. The association of Tech proteins with these GST constructs was assayed by immunoblotting of proteins pulled down with glutathione-agarose beads. An aliquot of the Tech construct added to the incubation was loaded in the lanes labeled with a hyphen. As the Tech constructs used contained a rhodopsin-epitope tag, blots were probed with a monoclonal antibody to this tag. Full-length Tech and a truncated Tech construct, Tech 245 Δ C (diagrammed in c), bind selectively to RhoA. In contrast, the Tech 245 Δ C-LE construct, containing a point mutation (L530E) in the DH domain, does not associate with RhoA. However, mutation of a nearby residue (K526E) present in the Tech 245 Δ C-KA construct does not abolish RhoA binding activity. (b) An immunoblot obtained by reprobing the leftmost blot shown in (a) with a GST antibody, confirming that these GST constructs are present in the pellets, as expected. (c) Schematic diagram showing the position of the DH and PH domains in Tech and constructs used in these experiments. A small box labeled Rd tag indicates the position of the rhodopsin epitope tag.

more of the N-terminal flanking region, was inactive in this binding assay (data not shown).

Activation of RhoA

To check the prediction that Tech is able to activate RhoA, we assessed its ability to increase the association of RhoA with one of its downstream targets, rhotekin (Ren and Schwartz 2000). Furthermore, we also checked whether Tech was able to mimic morphological changes induced by RhoA activation in 3T3 NIH cells. In preliminary studies using full-length Tech in these assays, we observed weak and variable effects. Accordingly, we switched to testing the activity of the truncated construct, Tech 245 Δ C, which spans the DH and PH domains present in Tech and retains the ability to bind to RhoA.

In the rhotekin pull-down assay, we found that Tech 245 Δ C enhanced the association of RhoA with its binding domain in rhotekin (Fig. 4a), indicating that it is able to activate RhoA. To help to confirm that this effect is mediated by the interaction of Tech with RhoA, we checked that the mutant Tech construct, Tech 245 Δ C-LE, which does not bind

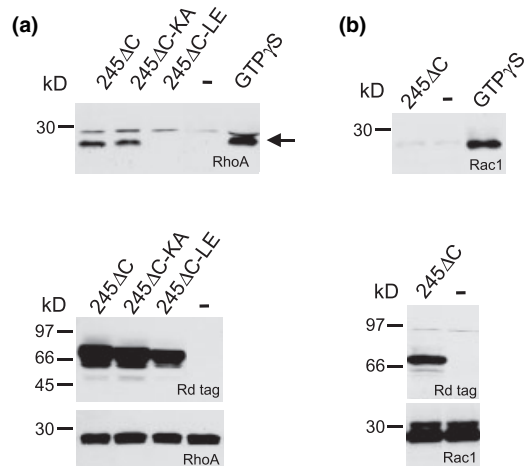


Fig. 4 Tech activation of RhoA. (a) Rhotekin pull-down assay. Extracts of human embryonic kidney (HEK)293 cells that had been transfected with the constructs listed at the top of the upper panel were incubated with beads coated with a fusion protein composed of GST linked to the RhoA-binding domain of rhotekin. The ability of Tech constructs to trigger the association of RhoA with this fusion protein was assayed by blotting the precipitates with antibody to RhoA (upper panel). The two right hand lanes represent control samples in which extracts from untransfected cells were incubated without (–) or with GTP γ S at a concentration of 1 mM. The arrow at the right of this blot points to the RhoA band. The presence of Tech constructs in the cell extracts used for binding was confirmed by immunoblot analysis with the rhodopsin epitope (Rd) tag contained in these constructs (middle panel). As shown in the bottom panel, these extracts contain similar levels of RhoA. (b) PAK pull-down assay. Extracts from HEK293 cells were incubated with beads coated with the Rac-binding domain of PAK and then blotted with antibody to Rac1 to check whether Tech expression enables endogenous Rac1 to associate with PAK (upper panel). In contrast to the effect of Tech on RhoA activation shown in (a), it is unable to elicit association of Rac1 with PAK. As mentioned above for the RhoA activation assay, we also confirmed that incubation of control extracts with GTP γ S does induce association of Rac1 with the PAK (right lane). Expression of Tech 245 Δ C constructs was confirmed by immunoblot analysis of these cell extracts with a rhodopsin epitope (Rd) tag antibody (middle panel). Furthermore, as shown in the bottom panel, blotting for Rac1 confirmed the presence of equivalent levels of this protein in these samples.

to RhoA, does not elicit this response. However, the KA mutant, which retains RhoA binding activity, also stimulates association of RhoA with rhotekin in this pull-down assay. To confirm the prediction that Tech 245 Δ C does not activate Rac1, we checked its ability to promote association of Rac1 with its binding partner Pak1. Consistent with the inability of Tech to bind to Rac1 *in vitro*, we did not detect activation of Rac1 in this pull-down assay (Fig. 4b).

To complement these biochemical studies, we also used 3T3 NIH cells to check whether, as found for RhoA, Tech elicits formation of stress fibers and increases the percentage of rounded cells (Fig. 5). We found that Tech 245 Δ C mimics

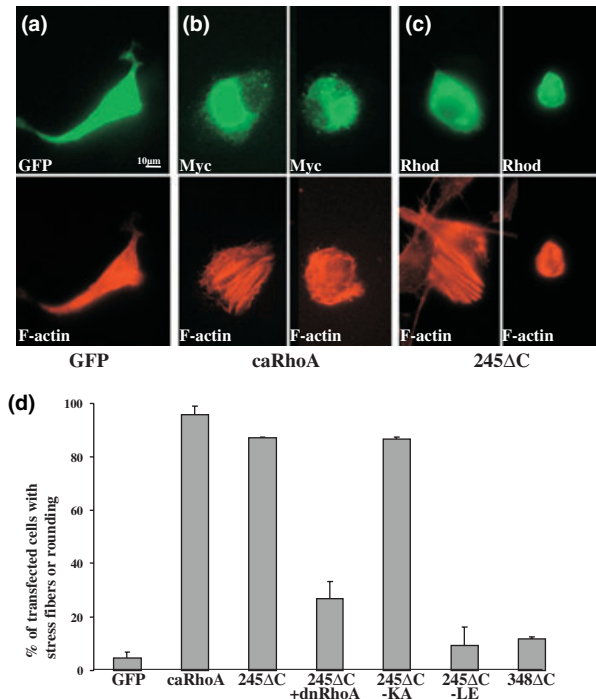


Fig. 5 Effects of Tech constructs on morphology of 3T3 NIH cells. (a–d) Images illustrate the effect of RhoA and Tech 245ΔC constructs on 3T3 cells. (a) Top panel labeled GFP shows green fluorescence displayed by a cell transfected with the GFP construct; bottom panel shows F-actin pattern as detected by labeling with Texas red-labeled phalloidin. (b) Cells were transfected with a constitutively active RhoA construct (caRhoA). Top panels show localization of the myc-tagged caRhoA construct; bottom panels show examples of cell rounding with (bottom left panel) or without (bottom right panel) stress fibers. (c) Cells were transfected with the Tech 245ΔC construct and illustrate the cell rounding and stress fiber formation produced by this construct. (d) Effect of Tech constructs on cell morphology. The bar graph shows the percentage of transfected cells scored as having stress fibers and/or a round shape. Tech 245ΔC mimics the ability of caRhoA to induce these morphological features (caRhoA vs. GFP, $p < 0.001$; Tech 245ΔC vs. GFP, $p < 0.001$). The effects of Tech 245ΔC are blocked by dominant negative RhoA (Tech 245ΔC vs. Tech 245ΔC + dnRhoA, $p < 0.005$) or a point mutation that blocks its ability to bind to RhoA (Tech 245ΔC-LE vs. GFP, $p > 0.2$) but not by a mutation that does not impair its ability to bind to RhoA (Tech 245ΔC-KA vs. GFP, $p < 0.001$). Furthermore, Tech 348ΔC, which is unable to bind to or activate RhoA, does not mimic these effects (Tech 348ΔC vs. GFP, $p > 0.2$). Data shown represent the average of three independent experiments. In each experiment, at least 20 cells were scored for each condition. Error bars, SEM.

these effects of RhoA. To check that these morphological effects of Tech 245ΔC are mediated by its activation of RhoA, we confirmed that its effects are blocked by coexpression with a dominant negative RhoA construct. Furthermore, we found that the Tech 245ΔC-LE construct is inactive in this assay, while the Tech 245ΔC-KA mutant

retains full activity. As expected, Tech 348ΔC, which does not bind to RhoA *in vitro*, is also inactive in this assay.

Effect of Tech on neuronal morphology

To check the effects of Tech on neuronal morphology, we cotransfected Tech and GFP expression constructs into cortical neurons grown in primary culture. As we had found that endogenous Tech expression rises during the first postnatal week, we chose to examine the effects of Tech constructs during the second week *in vitro*. To analyse the effects of Tech constructs on neuronal processes, we identified GFP-positive neurons that were cotransfected with a Tech construct and counted the number of times their GFP-positive processes crossed a circle drawn 40 μm from the center of the cell body. Using this method of quantification, we found that both Tech 245ΔC and constitutively active RhoA decreased the number of crossings (Fig. 6). In contrast, full-length Tech was ineffective in mimicking these morphological changes, consistent with its weak activity in 3T3 cells. To check that this effect of Tech 245ΔC is dependent on its ability to bind to and activate RhoA, we confirmed that the Tech 245ΔC-LE construct does not mimic this response and that it is blocked by cotransfection with RhoA T19N, a dominant negative RhoA construct. In contrast, the control mutant construct Tech 245ΔC-KA, which retains RhoA GEF activity, mimics the effect of Tech 245ΔC (data not shown).

Discussion

Our analysis of Tech expression and function in the brain has yielded several important findings. First, our results indicate that Tech is expressed selectively in neurons and displays a marked regional heterogeneity with prominent expression in the cortex and hippocampus. Second, we have found that Tech expression rises during early development and remains at peak levels in adulthood. Third, our results support the report by De Toledo *et al.* (2001) that Tech displays a high degree of selectivity for RhoA. Fourth, we have obtained evidence that a truncated, constitutively active form of Tech produces a marked reduction in the complexity of dendritic arbors via activation of RhoA. Taken together, these findings indicate that Tech regulates RhoA signaling pathways in developing and mature hippocampal and cortical neurons. In addition, they fit well with previous studies demonstrating that activation of RhoA signaling pathways elicits simplification of dendritic arbors (Luo 2000; Neumann *et al.* 2002; Ahnert-Hilger *et al.* 2004).

In addition to Tech, forebrain neurons contain multiple Rho GEF family members capable of activating RhoA, including Dbl (Hirsch *et al.* 2002), Ost (Horii *et al.* 1994), ephexin (Shamah *et al.* 2001), PDZ-Rho GEF, LARG (Kuner *et al.* 2002) and GEFT (Bryan *et al.* 2004). Some of these, such as Dbl, Ost and ephexin, have broader

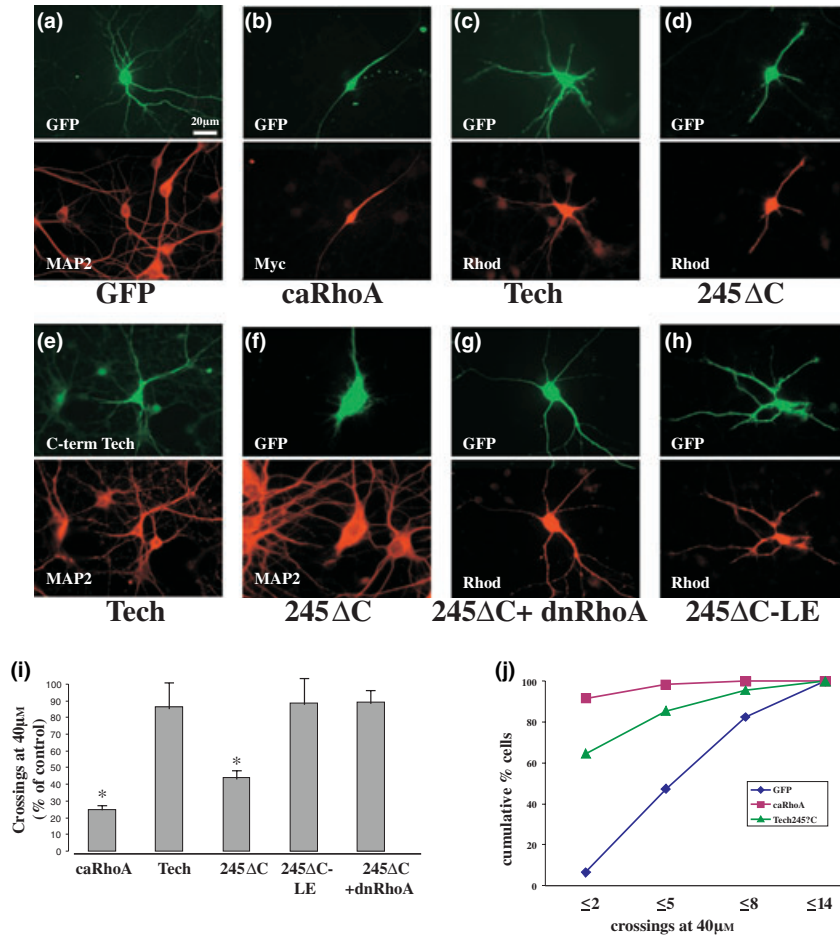


Fig. 6 Effect of Tech constructs on morphology of cortical neurons. (a–h) Immunofluorescence images illustrate the effect of Tech or RhoA constructs on neuronal morphology. (a) Morphology of a control cell transfected with GFP. Double staining with MAP-2 (shown in bottom part of this panel) confirms that this cell is a neuron. As shown in (b), cells transfected with constitutively active RhoA construct (caRhoA) display a dramatic reduction in the number of processes. Double staining for the myc or rhodopsin epitope tags was employed (bottom part of b–d, g and h) to confirm that GFP-positive cells were cotransfected with the construct being tested. (c and d) Examples of the effect of transfection with full-length Tech or Tech 245ΔC, respectively. (e and f) Additional confirmation that cells transfected with these constructs are MAP-2 positive. (g) Ability of dnRhoA to block the effect of Tech 245ΔC. Tech 245ΔC-LE, which contains a point mutation that blocks its RhoA GEF activity, does not mimic the

ability of Tech 245ΔC to reduce the number of processes (h). (i) Bar graph presents group data on the effects of these constructs on the number of GFP-positive processes that intersect a circle with a radius of 40 μm centered on the cell body. Although full-length Tech does not produce an effect on this measure, Tech 245ΔC decreases the average number of processes (GFP vs. Tech 245ΔC, $p < 0.001$) as found for caRhoA (GFP vs. caRhoA, $p < 0.001$). This effect of Tech 245ΔC is not mimicked by Tech 245ΔC-LE and is blocked by dnRhoA. Data are presented as a percentage of the number of crossings present in cells transfected with GFP alone. (j) Cumulative distribution graph. Plots of the cumulative percentage of cells scored as having a specific number of crossings or fewer illustrate that Tech 245ΔC produces a robust increase in the percentage of cells that have two processes or fewer compared with cells transfected with GFP alone. Few of the cells transfected with caRhoA have more than two processes.

specificity than Tech as they display GEF activity toward Rac and/or Cdc42 as well as RhoA (Horii *et al.* 1994; Olivo *et al.* 2000; Shamah *et al.* 2001). In contrast, PDZ-Rho GEF and LARG appear to be selective for RhoA (Fukuhara *et al.* 1999; Reuther *et al.* 2001), as found for Tech. Although little is known about the upstream signaling pathways that regulate Db1 and Ost, PDZ-Rho GEF, LARG and ephexin have been linked to cell surface receptors. PDZ-Rho GEF and LARG

mediate the activation of RhoA by plexins (Perrot *et al.* 2002; Swiercz *et al.* 2002) and G-protein-coupled receptors that stimulate $G\alpha_{12/13}$ and $G\alpha_{q/11}$ (Fukuhara *et al.* 1999; 2000; Booden *et al.* 2002; Vogt *et al.* 2003); ephexin binds to the intracellular tail of EphA receptors (Shamah *et al.* 2001). As neuronal morphology is extremely complex and dynamically regulated by multiple stimuli (Bonhoeffer and Yuste 2002), it is certainly plausible that all of these RhoA

GEFs are involved in regulating aspects of neuronal morphology. Conversely, it seems unlikely that each of these would be able, under physiological conditions, to elicit the full-blown effects induced by global activation of RhoA. Accordingly, it will probably be useful to examine how targeted deletion of individual or combinations of RhoA GEFs impacts neuronal morphology. In this vein, analysis of mice with targeted deletion of Dbl has revealed disruption of the dendritic morphology of cortical projection neurons (Hirsch *et al.* 2002), suggesting that further studies using this approach may help to clarify how multiple Rho GEFs act in concert to generate and regulate the complex morphological features of these neurons.

A major goal of current research on Rho signaling pathways is to identify the upstream signaling pathways that control the activity of Rho GEFs. In addition to plexins, ephrins and G-protein-coupled receptors linked to $G\alpha_{12/13}$ and $G\alpha_{q/11}$, several other extracellular signals have been shown to regulate RhoA in neurons, including tumor necrosis factor α (TNF α) (Neumann *et al.* 2002), p75 (Yamashita *et al.* 1999) and heparan sulfate (Calvet *et al.* 1998). As our results demonstrate that Tech is a RhoA GEF expressed in cortical and hippocampal neurons, studies aimed at examining whether Tech mediates the effects of these stimuli are warranted. As full-length Tech showed little or weak activity in several assays of RhoA GEF activity, it seems likely that Tech, as found for other Rho GEF family members, contains autoinhibitory domains that suppress its ability to activate RhoA under basal conditions. Although we have not detected any sequence similarity between Tech and autoinhibitory domains in other Rho GEF proteins, initial characterization of several Tech truncation mutants indicated that the C-terminal segment distal to the PH domain contains an autoinhibitory domain. We propose that this autoinhibitory influence can be relieved by, as yet unidentified, upstream signaling pathways which act to stimulate its RhoA GEF activity.

In characterizing the ability of Tech to bind to RhoA, we found, unexpectedly, that the Tech 348 Δ C construct does not bind to RhoA even though it contains the DH domain (Fig. 3c). In analysing a series of Rho GEF family members that display selectivity for RhoA, Alberts and Treisman (1998) pointed out that they share sequence similarity in a 25 amino acid segment that extends beyond the N-terminal border of the DH domain (Whitehead *et al.* 1997). Of note, Tech, which is selective for RhoA, appears to conform to this rule as it contains an N-terminal extension of the DH domain similar to that reported by Alberts and Treisman (1998). As the N-terminal edge of the Tech 348 Δ C construct is near the N-terminal border of the DH domain, its inability to bind to RhoA fits with the idea that this N-terminal extension of the DH domain plays a critical role in mediating binding of Tech and other RhoA-selective Rho GEF family members to RhoA.

It is now well established that the dendritic morphology of mature forebrain neurons can be regulated by endocrine (Woolley and McEwen 1993), behavioral (Kolb and Whishaw 1998) or pharmacological (Robinson and Kolb 1999) stimuli. However, we have only a rudimentary understanding of the molecular mechanisms underlying these changes. As our studies establish Tech as a RhoA GEF expressed in adult cortical and hippocampal neurons, it appears to be well suited to mediate remodeling of these neurons in the mature brain. Accordingly, it will be interesting, in future studies, to assess its role in morphological plasticity *in vivo*.

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