Human Tumorous Imaginal Disc 1 (TID1) Associates with Trk Receptor Tyrosine Kinases and Regulates Neurite Outgrowth in nnr5-TrkA Cells*

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The human tumorous imaginal disc 1 (TID1) proteins including TID1L and TID1S, members of the DnaJ domain protein family, are involved in multiple intracellular signaling pathways such as apoptosis induction, cell proliferation, and survival. Here we report that TID1 associates with the Trk receptor tyrosine kinases and regulates nerve growth factor (NGF)-induced neurite outgrowth in PC12-derived nmr5 cells. Binding assays and transfection studies showed that the carboxy-terminal end of TID1 (residues 224–429) bound to Trk at the activation loop (Tyr(P)683-Tyr(P)684 in rat TrkA) and that TID1 was tyrosine phosphorylated by Trk both in yeast and in transfected cells. Moreover endogenous TID1 was also tyrosine phosphorylated by and co-immunoprecipitated with Trk in neurotrophin-stimulated primary rat hippocampal neurons. Overexpression studies showed that both TID1L and TID1S significantly facilitated NGF-induced neurite outgrowth in TrkA-expressing nmr5 cells possibly through a mechanism involving increased activation of mitogen-activated protein kinase. Consistently knockdown of endogenous TID1, mediated with specific short hairpin RNA, significantly reduced NGF-induced neurite growth in nmr5-TrkA cells. These data provide the first evidence that TID1 is a novel intracellular adaptor that interacts with the Trk receptor tyrosine kinases in an activity-dependent manner to facilitate Trk-dependent intracellular signaling.

Neurotrophins activate Trk receptor tyrosine kinases (RTKs)1 to stimulate a variety of cellular responses including survival, differentiation, proliferation, and apoptosis (1). The Trk RTKs comprise a family of three closely related proteins, TrkA, -B, and -C. TrkA is the preferred receptor for nerve growth factor (NGF), TrkB is the preferred receptor for brain-derived neurotrophic factor (BDNF) and NT-4/5, and TrkC preferentially binds NT-3 (2–4). Upon activation, Trk receptors are phosphorylated on five tyrosine residues in the intracellular domain (ICD), i.e. Tyr(P)794, Tyr(P)679, Tyr(P)683, Tyr(P)684, and Tyr(P)784 for rat TrkA receptor (5–8). Through these, the activated Trk receptors recruit, phosphorylate, and activate intracellular signaling proteins and maintain them in an active receptor complex (9). For example, fibroblast growth factor receptor sub- strate 2 and Shc directly bind to rat TrkA at Tyr(P)683 (10–12); Grb2, APS, and SH2B interact with the tyrosines within the activation loop of TrkA (Tyr(P)683-Tyr(P)684) (13, 14), whereas phospholipase Cγ-1 and CSK homologous kinase bind to Tyr(P)784 (1, 7, 15).

In the course of our studies to identify signaling proteins unique to specific Trk receptors, we identified tumorous imaginal disc 1 (TID1), the mammalian homologue of the Droso phila protein TID56, as an interacting protein by the yeast two-hybrid screen using the ICD of TrkCα as bait. Human TID1, a DnaJ domain-containing protein that is evolutionarily highly conserved, displays 54.9% amino acid identity with its Drosophila counterpart TID56 (16). Two isoforms of TID1 have been identified in human: TID1L (long form, 43 kDa) and TID1S (short form, 38 kDa) (17, 18). An additional isoform, designated mTID1S (mouse short form, 38 kDa), has also been described (19, 20). It is becoming increasingly evident that TID1 is involved in the regulation of multiple intracellular signaling pathways. For example, TID1 was first identified as an interacting protein with the E7 oncoprotein of human papilloma virus 16 (16). Consequently this group has shown that the TID1 isoforms act as both apoptotic and antiapoptotic regulators (17, 18). TID1 also binds to Tax, an oncogenic viral protein encoded by the human T cell leukemia virus type 1 (21), regulating NF-κB activity (22). Moreover TID1 interacts with p120 GTPase-activating protein, an activator of Ras, independently of TID1 tyrosine phosphorylation. In response to epidermal growth factor stimulation, TID1 and p120 GTPase-activating protein co-localize to distinct perinuclear subdomains resembling mitochondrial membranes (19). Most recently, it has been reported that TID1 interacts with the ErbB-2 receptor tyrosine kinase. TID1 overexpression down-regulates ErbB-2 expression in breast cancer cells and hence inhibits tumorigenesis induced by ErbB-2 activation in some tumor cell lines (23). Additionally TID1 interacts with Jak2 and the interferon-γ receptor subunit, interferon-γ R2, to regulate interferon-γ-mediated transcriptional activity (24). Finally TID1 expression is up-regulated by cellular senescence in rat and mouse embryo fibroblasts and also by premature senescence in rat embryo fibroblasts where TID1 acts as a repressor of NF-κB (25).

Most recently, it was reported that a complete loss of TID1...
results in embryonic death between embryonic days 4.5 and 7.5. Moreover TID1 removal in mouse embryo fibroblasts leads to massive cell death, which can be rescued by ectopic expression of wild-type TID1 (26). These observations suggest that TID1 is important for sustaining embryonic cell survival. This function of TID1 is possibly dependent on Hsp70 co-activation as cell death was not rescued by overexpression of the dominant negative TID1 mutant incapable of binding to Hsp70 (26).

In this study, we followed up on our initial yeast two-hybrid observation and investigated a role for TID1 in neurotrophin signaling via the Trk receptors. We demonstrated that TID1 bound to the Trk receptors in a phosphoryrosine-dependent manner at the tandem tyrosine residues Tyr683-Tyr684 of the activation loop. More importantly, TID1 was tyrosine phosphorylated by the activated Trk RTKs in response to neurotrophins in both transfected cells and primary hippocampal neurons. Functionally TID1 overexpression significantly enhanced NGF-induced neurite outgrowth as well as levels of activated MAP kinase in TrkA-expressing nrr5 cells. Finally we showed that TID1 knock-down, mediated by short hairpin RNA (shRNA), significantly reduced neurite outgrowth in PC12-derived neurotrophin-dependent cells. Collectively these data support a model that TID1 is a novel intracellular mediator of neurotrophin signaling and facilitates neurotrophin-induced neurite outgrowth through a mechanism that appears to involve increased levels of MAP kinase activation.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies—**Mouse NGF (2.5 S) was purchased from Harlan Bioproducts for Science, Recombinant BDNF and NT-3 were gifts from A. A. Welcher (Amgen Inc.). The Trk tyrosine kinase inhibitor K-252a (Calbiochem) was reconstituted in Me2SO and stored at −20 °C in the dark. The following antibodies were purchased from Santa Cruz Biotechnology: horseradish peroxidase-conjugated anti-Ga4 DBD (RK5C1) and anti-Ga4 TA (C-10) and horseradish peroxidase-conjugated anti-MYC (9E10), anti-TID1 (RS13), and anti-Trk (C-14 and MCTrks). The anti-HA (3F10) antibody was from Roche Diagnostics. The mouse anti-MYC monospecific antibody (9E10) and the rabbit anti-Trk (J203) polyclonal antibodies were prepared as reported using standard procedures (10, 14). The anti-TID1 monospecific antibody (RS13 clone) was the generous gift of Dr. K. Münger (Department of Pathology, Harvard Medical School, Boston, MA). We also generated a rabbit anti-TID1 antibody directed against a 15-amino acid epitope of TID1 (14)(CKYHPDTNKDDPKAKE133) (Genemed Synthesis, San Francisco, CA). The anti-TID1 antibody was affinity-purified by standard procedures (32) and was used at 10 μg/ml. GST (p190) and the wild type and mutant Trk receptors were expressed in eukaryotic cells co-transfected with the TrkC ICD as bait. The TID1 Phe217–Ser480 fragment was subsequently cut from pACT2-TID1 and then ligated into pEBG3 (10, 14) to make a bait with selective pressure for TID1 expression. For transfection, cells were grown to 50–70% confluency in 60-mm plates, and DNA transfections were performed by calcium phosphate precipitation for 293T cells or by Lipofectamine 2000 (Invitrogen) for other cell lines.

**Immunoprecipitation, GST Pull-down, and Western Blot—**Cells were lysed in Nonidet P-40 lysis buffer (1% Nonidet P-40, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 20 mM Tris (pH 7.5)) in the presence of 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 10 μg/ml aprotinin. For immunoprecipitations, cells were stimulated with NGF (BDNF, or NT-3 at 100 ng/ml for 5 min) and then solubilized in Laemmli sample buffer. Equivalent aliquots were analyzed by SDS-PAGE and detected by Western blots with the indicated antibodies. After washing, bound proteins were eluted in Laemmli sample buffer (100 mM dithiothreitol, 10% glycerol, 3% SDS) and analyzed by Western blotting.

**Hippocampal Neurons—**Hippocampi were dissected from postnatal day 3 Sprague-Dawley rat pups as described previously (30, 31). After careful removal of the meninges, the sliced hippocampal tissues were dissociated in papain solution (40 units/ml papain in Hank’s balanced salt solution supplemented with 15 mM HEPES, 0.02% DNase, 1 mM β-mercaptoethanol, 5 mM cysteine-HCl, and 1 mM EDTA) at 37 °C for 30 min followed by triturating with a P-1000 pipette tip. The hippocampal neurons, collected by centrifugation through a 30% fetal bovine serum cushion at 150 × g for 15 min, were resuspended in Neurobasal medium supplemented with 0.5 mM glutamine, 2% B27 (Invitrogen) and plated on a poly-L-lysine substrate at 375 neurons/mm². Cells were cultured in a humidified incubator at 37 °C in 5% CO₂/95% air.
Results
Identification of TID1 as an Interacting Protein with the Trk Receptors—As part of our ongoing work to understand the complexity of Trk-mediated signaling pathways, we carried out several yeast two-hybrid screens using the ICDs of the Trk\textsubscript{A}, -B, and -C receptors as baits as reported elsewhere (10, 11, 14, 32). During the course of this work, we isolated a clone corresponding to a carboxyl-terminal fragment (Phe\textsubscript{217}–Ser\textsubscript{480}) of human TID1\textsubscript{L} (16, 18) from a mammary gland cDNA library (Clontech) as an interacting partner of the TrkC ICD. As shown in Fig. 1, four clones with significant reduced TID1 expression were used in this study. As the control, four clones with pZeoH1 vector alone were also generated.

Viability and Neurite Outgrowth Assays—For neurite outgrowth assays, nmr5-TrkA cells were plated on poly-d-lysine-coated plates at low density (500 cells/mm\textsuperscript{2}). Cells were allowed to grow in media with or without the indicated concentration of NGF. The media with fresh NGF were changed every 24 h. The cells were observed twice a day on an inverted microscope (Olympus IX50), and images were captured with a video camera (Sony 3CCD, model DXC-950) using Image-Pro software. Neurites were measured with the MCID\textsuperscript{TM} analysis software (Amer sham Biosciences) in 10 independent fields (120–151 cells). The results are presented as cells bearing neurites and the length of neurites (\mu m).

Three independent experiments were performed. All data were analyzed using GraphPad Prism, version 4.0. A one-way analysis of variance with posthoc analysis by Student-Neuman-Keuls test was used to compare the differences between the groups. A \textit{p} value less than 0.05 was considered statistically significant.

TID1 Interacts with Trk Receptors—Sham Biosciences) in 10 independent fields (120–151 cells). The results after stimulation with neurotrophins, cells were lysed in Non-HALT media, whereas TID1 and TrkC co-transformed into yeast cells. After growing in \textit{LT} media (left panel), the cultures were then grown on \textit{HALT} plates (right panel). B, TID1-TrkA binding in yeast is phosphotyrosine-dependent. TID1 was co-expressed with wild type Trk or kinase-inactive TrkA mutant (K547A) in yeast and grown on \textit{LT} (left) or \textit{HALT} (right) plates. C, TID1 binds to Trk in a phosphotyrosine-dependent fashion in mammalian cells. GST-TID1 and HA-TrkA (wild type and kinase-inactive mutant) were co-transformed in COS cells. Lysates were incubated with glutathione-Sepharose, and the bound proteins were then detected with the anti-HA antibody (upper panel). Whole cell lysates were run separately, and Trk expression was detected using the anti-HA antibody (lower panel). A11, K547A in TrkA; B11, K571A in TrkB; C11, K572A in TrkC. Ppt, precipitate.

Seapaphore. Bound proteins were detected by Western blotting with the anti-HA antibody. As shown in Fig. 1C, all three Trk receptors were precipitated with GST-TID1 in a phosphorylation-dependent manner with no precipitation observed with kinase-inactive HA-Trks (A11, B11, and C11, respectively). The double Trk bands shown in the Trk expression panel (Fig. 1C, bottom panel) and in other figures thereafter indicate different glycosylation states of the receptors (10, 34, 35).

TID1 Is Tyrosine Phosphorylated by Trk Kinases—To evaluate whether TID1 is tyrosine phosphorylated by Trk in mammalian cells, MYC-TID1\textsubscript{L} and HA-Trks (wild type and kinase-inactive mutants) were co-expressed in COS cells. After stimulation with neurotrophins, cell lysates were precipitated with the anti-MYC antibody. The precipitated proteins were then detected by Western blotting with the anti-Tyr(P) antibody (Fig. 2A). No phosphorylated TID1\textsubscript{L} was detected in lysates expressing TID1\textsubscript{L} alone (Fig. 2A, lane 1). Consistent with the previous observation showing that GST-TID1 bound Trk receptors in a phosphotyrosine-dependent fashion, TID1\textsubscript{L} was tyrosine phosphorylated by wild type TrkA, TrkB, and TrkC but not by the kinase-inactive Trk mutants (Fig. 2A). Further

\textsuperscript{2} H.-Y. Liu and S. O. Meakin, unpublished data.
Fig. 2. **TID1 is tyrosine phosphorylated by Trk.** A, TID1 is tyrosine phosphorylated by Trk in COS cells. Cell lysates co-expressing MYC-TID1, and HA-Trks were immunoprecipitated with the anti-MYC antibody, and tyrosine phosphorylation of TID1 was determined using the anti-phosphotyrosine antibody (top panel). The expression of the Trk receptors was determined by Western blotting with the anti-HA antibody (bottom panel). B, TID1 is tyrosine phosphorylated after stimulation with NGF. MYC-TID1- and HA-Trks co-transfected COS cells were incubated with or without NGF (50 ng/ml, 5 min). Protein from whole cell lysates was analyzed by SDS-PAGE/Western blotting as described for A. C, K-252a inhibits the tyrosine phosphorylation of TID1. COS cells co-expressing MYC-TID1 and HA-TrkA were incubated with the indicated concentrations of K-252a for 30 min followed by NGF stimulation. The phosphorylation status of TID1 was assessed as described under “Experimental Procedures.” D and E, TID1 binds Trk and is tyrosine phosphorylated in primary hippocampal neurons. Lysates from hippocampal neurons stimulated with BDNF (100 ng/ml, 5 min) or not were immunoprecipitated with anti-TID1N (top panel in D), anti-Trk C-14 (E), or rabbit IgG. Bound proteins were detected by anti-phosphotyrosine (D, top panel, and E), anti-TID1 RS13 (D, middle panel), or anti-Trk (D, bottom panel) antibodies. IP, immunoprecipitate.

experiments demonstrated a level of constitutive TID1 phosphorylation (Fig. 2B, lane 2), however, NGF stimulation significantly enhanced the phosphorylation of MYC-TID1, (Fig. 2B, lane 3).

To address the specificity of TID1 tyrosine phosphorylation by Trk, we took advantage of the Trk tyrosine kinase inhibitor K-252a (36, 37). Consistently TID1 was tyrosine phosphorylated by activated TrkA in COS cells (Fig. 2C, lane 1). However, incubation of the cells with 3 nM K-252a significantly reduced TID1 tyrosine phosphorylation (Fig. 2C, compare lane 3 with lane 1), whereas at a higher concentration (30 nM), a complete blockage of TID1 phosphorylation was obtained (Fig. 2C, lanes 4 and 5). These observations suggest that TID1 is tyrosine phosphorylated by Trk in an NGF-dependent fashion in mammalian cells.

Although the data presented above suggest a TID1-Trk interaction, the results were observed in cell lines transfected to overexpress both TID1 and Trk proteins. Accordingly we wished to confirm the interaction in primary cells with endogenous proteins. For these studies we chose postnatal day 3 rat hippocampal neurons. After 4 days in culture, cells were stimulated with 100 ng/ml BDNF or medium alone for 5 min. Cell lysates were immunoprecipitated with anti-TID1N (Fig. 2D) or anti-Trk (Fig. 2E) antibodies or with rabbit IgG as a control (Fig. 2, D and E). As shown in Fig. 2D, TID1 was clearly tyrosine phosphorylated in response to BDNF (100 ng/ml, 5 min) (top panel, lane 4) in which lysate Trk activation was detected (Fig. 2E, top panel, lane 4). Importantly TID1 tyrosine phosphorylation was coincident with Trk activation (Fig. 2, compare D, lane 4, with E, lane 4), indicating that TID1 is a target of BDNF-dependent Trk activation in primary hippocampal neurons.

To identify the TID1-Trk interaction in primary hippocampal neurons, hippocampal lysates were immunoprecipitated either with the anti-TID1N antibody or with rabbit IgG. The precipitated proteins were then detected by Western blotting with an anti-Trk (MCTrk) antibody. No Trk was observed when immunoprecipitations were performed with control rabbit IgG (Fig. 2D, bottom panel, lanes 1 and 2). Co-immunoprecipitated Trk, however, was detected from BDNF-stimulated lysates with an anti-TID1N antibody (Fig. 2D, bottom panel, compare lane 4 with lane 3), suggesting that TID1 interacts with Trk in primary hippocampal neurons in a BDNF-dependent manner.

The Carboxyl-terminal Portion of TID1 Mediates Binding to Trk—To further identify the sites and requirements of tyrosine phosphorylation by Trk, we generated a series of TID1 truncation mutants in which amino acids were progressively deleted from the carboxyl terminus (Fig. 3A). All mutants were MYC-tagged and co-expressed with HA-TrkA in 293T cells. Lysates were immunoprecipitated with an anti-MYC antibody, and the precipitated proteins were detected by Western blotting with an anti-Tyr(P) antibody, RC20H. As shown in Fig. 3B, both TID1NE429 (residues 1–429) and TID1NV304 (residues 1–304) are tyrosine phosphorylated (Fig. 3B, lanes 3 and 4, respec-
Actively), but TID1NG223 (residues 1–223) and TID1NJ (residues 1–159) are not (Fig. 3B, lanes 5 and 6, respectively) as indicated by the arrows in Fig. 3B. These observations suggest that the carboxyl-terminal portion, including both cysteine-rich and carboxyl-terminal domains, is required for TID1 tyrosine phosphorylation. We then further tested the Trk binding ability of MYC-tagged TID1 fragments using a co-immunoprecipitation assay. As expected, both MYC-TID1NE429 (residues 1–429) and MYC-TID1NV304 (residues 1–304) immunoprecipitated HA-TrkA (Fig. 3C, top panel, lanes 1 and 2, respectively), whereas the shorter forms MYC-TID1NG223 (residues 1–223) and TID1NJ (residues 1–159) did not bind HA-TrkA (Fig. 3C, top panel, lane 3, and data not shown) and were consequently not tyrosine phosphorylated (Fig. 3B, lanes 5 and 6, the positions as indicated by the arrows). These data further confirm that the carboxyl-terminal portion of TID1 mediates binding to Trk.

**TID1 Interacts with Trk Receptors**

TID1 Binds to Rat TrkA at Tyr(P)683-Tyr(P)684—As stated above, phosphotyrosines Tyr499, Tyr679, Tyr683, Tyr684, and Tyr794 in rat TrkA (Fig. 4A) serve as docking sites for various signaling proteins (10, 11, 14). To identify the site(s) for TID1 binding on Trk, a series of Trk mutants (Fig. 4A), including point mutants defective in binding specific signaling proteins and two deletion mutants, were tested. Neither of the Trk deletion mutants (A3 and A17) affected Trk-dependent TID1 tyrosine phosphorylation (Fig. 4B, lanes 2 and 3). With respect to the activated tyrosines, we found that the A8/A9 double mutant (Y499F/Y794P) did not affect TID1 phosphorylation (Fig. 4C, lane 3). These data suggest that neither Tyr499 nor Tyr794 are required for TrkA-TID1 interactions. We next explored a series of TrkA mutants involving the activation loop. TID1 phosphorylation was observed with the A26 (Y679A) mutant (Fig. 4C, lane 1), indicating that TID1-TrkA binding is independent of Tyr679. However, the A13 (Y683E/Y684E) mutant significantly reduced TID1 phosphorylation (Fig. 4C, lane 2), indicating that TID1 may interact with TrkA at one or both of the activation loop tyrosines.

To further determine whether the TID1-TrkA interaction site(s) involves the activation loop, single point mutants A13e (Y683E) and A13g (Y684E) were used. ShcB, which binds TrkA at Tyr499 (10), was used as a control. Wild type TrkA showed the highest degree of tyrosine phosphorylation in response to NGF stimulation, whereas all other double or single point mutations showed a reduced level of TrkA tyrosine phosphorylation (Fig. 4D, top panel). ShcB was phospho-

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**Fig. 3.** The carboxyl region of TID1 mediates Trk binding. A, schematic diagrams representing TID1L, TID1S, and the carboxyl-terminal deletions. The mitochondrial targeting sequence and processing site (MAARCS and LRPGGV, respectively) are labeled. DnaJ, cysteine-rich (CXXCXXGXX), and C terminus domains are shown. The underlined letters indicate retention signals for endoplasmic reticulum membrane. The last amino acid of each truncated mutant is indicated from the amino terminus (denoted as "N"). B, wild type TID1, and the truncation mutants were co-expressed with TrkA in 293T cells. NGF-stimulated cells were lysed and immunoprecipitated with the anti-MYC antibody. The phosphorylation status of TID1 was determined by Western blotting with the anti-Tyr(P) antibody (top panel), and expression was determined by probing with the anti-MYC antibody (bottom panel). The arrows indicate the electrophoretic positions of the mutant TID1 proteins. C, TID1 mutants differentially co-immunoprecipitates Trk. TID1 mutants were co-expressed with Trk in 293T cells as described under “Experimental Procedures.” After NGF stimulation, lysates were immunoprecipitated with the anti-MYC (top panel) or anti-Trk J203 (second panel) antibodies, and immunocomplexed proteins were detected by the anti-Trk antibody. TID1 mutant protein expression was determined by Western blotting with the anti-MYC antibody (bottom panel). IP, immunoprecipitate; aa, amino acids.
Tyrosine phosphorylation was observed with the double A13 mutant (Fig. 4D, third panel, lane 4). Interestingly both the A13e and A13g single point mutations (Y683E and Y684E, respectively) also resulted in a significant reduction in TID1 phosphorylation (Fig. 4D, third panel, lanes 5 and 6), indicating that both Tyr683 and Tyr684 are required for TID1 binding. Similar expression levels of TrkA mutants (Fig. 4D, second panel) and TID1 (Fig. 4D, fourth panel) were detected. Taken together, these data strongly suggest that TID1 binds to TrkA at Tyr683-Tyr684 in a phosphotyrosine-dependent manner.

We further confirmed the binding of TID1 at Tyr683-Tyr684 of rat TrkA by performing a yeast two-hybrid experiment. As expected, cultures co-expressing ShcB and the A8 mutant failed to grow in −HALT media. All other co-transformants with ShcB showed robust growth in −HALT media (Fig. 5A, lower plate). In contrast, cells co-transformed with TID1 and A8 grew well in −HALT media. Most importantly, cultures co-expressing TID1 and A13, A13e, or A13g mutants failed to grow in −HALT media strengthening the argument that Tyr683-Tyr684 are the sites for TID1 binding to TrkA (Fig. 5A). Stable expression of the Trk, TID1, and ShcB Gal4 fusion proteins were confirmed by Western blots with the anti-Gal4 DBD (Fig. 5B) or the anti-Gal4 TA antibody (Fig. 5C), respectively.

**TID1 Regulates NSF-dependent Neurite Outgrowth**—nnr5 cells are derived from PC12 cells and do not express Trk receptors consequently making them non-responsive to neurotrophins (38). However, this response is reconstituted upon transfection with exogenous Trk (39). To determine whether TID1 overexpression enhances NSF-dependent differentiation in nnr5-TrkA cells, we generated several cell lines stably overexpressing either MYC-tagged TID1L or TID1S (four lines each). By Western blotting with the anti-MYC and anti-TID1 monoclonal antibodies, the expression of exogenous TID1 was evident in all TID1L- and TID1S-overexpressing lines (Fig. 6A). We next tested whether TID1 overexpression affects neurite outgrowth. Neurite response assays using a titration of NSF on

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**Fig. 4. Identification of TID1 binding sites on TrkA.** A, schematic of the ICD of rat TrkA mutants including A3 (Δ497IMENP497), A17 (Δ500KFG502), A8 (Y499F), A9 (Y794F), A26 (Y679A), A13 (Y683E/Y684E), A13e (Y683E), A13g (Y684E), and A11 (K547A). Shc and fibroblast growth factor receptor substrate 2 (FRS-2) bind Tyr(P)499, and phospholipase C-1 (PLC-1) and CSK homologous kinase (CHK) bind Tyr(P)794. The ATP binding site in rat TrkA is Lys547. TM, transmembrane domain. B–D, tyrosine phosphorylation assays. MYC-TID1L or MYC-ShcB and HA-TrkA (wild type and mutant) were co-expressed in COS7 cells. After stimulation with NSF, cell lysates were immunoprecipitated and immunoblotted as described under “Experimental Procedures” with the indicated antibodies. Whole cell lysates were used to evaluate TID1L and Trk expression. IP, immunoprecipitate; wt, wild type.
a nnr5-TrkA-reconstituted cell line (B5 clone) (39) revealed that these cells respond well to high doses of NGF (data not shown) but remain poorly responsive to low NGF doses (i.e., 2–5 ng/ml for 2 days) (Fig. 6, panels b and c). Both TID1L and TID1S overexpression significantly enhanced neurite outgrowth of such B5 cells in response to NGF (one line of each shown in Fig. 6B, panels d–f). Even at a very low dose of NGF (2 ng/ml), to which parental nnr5-TrkA cells are only weakly responsive (Fig. 6B, panel b), TID1L- and TID1S-overexpressing nnr5-TrkA cells underwent extensive neurite outgrowth (Fig. 6B, panels c and f). At a higher dosage of NGF (5 ng/ml), both TID1L- and TID1S-overexpressing nnr5-TrkA cells displayed more extensive neurite development than parental nnr5-TrkA cells (Fig. 6B, compare panels c, f, and i).

To further demonstrate that TID1 overexpression facilitates NGF-dependent differentiation in nnr5-TrkA cells, neurite outgrowth assays were performed using four TID1L clones and four TID1S clones stimulated by NGF at 5 ng/ml for 2 days. The percentage of cells bearing neurites was scored and found to be 80.33 ± 11.97% in TID1S cells and 86.43 ± 9.85% in TID1L cells as compared with 21.65 ± 2.51% in the parental cells (Fig. 6C).

Moreover neurites in TID1S cells (323 ± 11 μm) and in TID1L cells (234 ± 26 μm) were significantly longer than those in parental nnr5-TrkA cells (66 ± 15 μm) (Fig. 6D). These data agree with the observations of the highest TID1 expression levels in TID1S-overexpressing cell lines (Fig. 6A). The results show that TID1L and TID1S overexpression enhances NGF-induced neurite outgrowth in nnr5-TrkA cells.

It is known that signaling proteins such as fibroblast growth factor receptor substrate 2/Shc are implicated in the neurotrophin-dependent activation of AKT and ERK/MAP kinase and that the activation of these pathways is important for NGF-induced neurite outgrowth (1). Thus, we assayed changes in NGF-dependent ERK/MAP kinase and AKT activation in nnr5-TrkA cells overexpressing TID1. Cells were stimulated with increasing concentrations of NGF for 2 min to activate TrkA. Changes in activation of ERK/MAP kinase were detected by Western blot with the anti-phospho-ERK antibody. The activated ERK/MAP kinase activation was apparent in even nnr5-TrkA cells cultured in media without NGF addition (Fig. 6E, lanes 1, 5, and 9); however, ERK/MAP kinase was activated in these cells with NGF stimulation (5–5 ng/ml for 5 min). Importantly overexpression of both TID1L and TID1S clearly enhanced the levels of NGF-dependent ERK/MAP kinase activation in nnr5-TrkA cells (Fig. 6F). The densitometric analysis showed that ERK/MAP kinase was significantly enhanced by overexpression of both TID1L and TID1S in TID1-expressing nnr5-TrkA cells (Fig. 6F).

To further elucidate the effect of TID1 on the ERK/MAP kinase activation in TID1-overexpressing nnr5-TrkA cells, we treated the cells with 5 ng/ml NGF for up to 60 min. The activation of ERK1/2 was analyzed by Western blotting with the anti-phospho-ERK antibody. As expected, weak activation of ERK1/2 was observed after 5 min of NGF treatment (Fig. 6G, lane 2) and decreased thereafter up to 1 h (Fig. 6G, lane 3–5). In contrast, ERK1/2 activation in the TID1L- and TID1S-overexpressing cells was quite robust after 5 min of stimulation and continued in this vein up to 1 h (Fig. 6G, lanes 7–15). The effect of TID1L and TID1S overexpression on ERK/MAP kinase activation is summarized in Fig. 6H. Conversely, AKT phosphorylation at Ser473 was increased by high doses of NGF stimulation (50 ng/ml), but no enhancement was apparent by overexpression of TID1 (data not shown). These data indicate that TID1 overexpression amplifies NGF-dependent MAP kinase activation and facilitates neurite outgrowth in nnr5-TrkA cells.

As a corollary to the overexpression studies, we used small interfering RNA (siRNA) technology to reduce the endogenous expression of TID1. Using the pZeoH1 mammalian expression vector, four nnr5-TrkA cell lines stably expressing a shRNA directed against TID1 and the control with empty vector only were generated. Western blot analysis shows that the expression levels of both TID1L and TID1S were significantly reduced in interfering RNA-expressing (RNAi) cell lines but not in control lines (Fig. 7A). When assayed for neurite outgrowth in response to NGF, the cells in which TID1 expression was reduced (lane 5) were incapable of extending neurites, whereas the control cells were indistinguishable from non-transfected nnr5-TrkA cells (Fig. 7B, panels a and b). A similar result was obtained with the other four cell lines. Quantitative analysis showed that both the number and length of neurites were significantly reduced in the TID1 knock-down cell lines (Fig. 7C).

We next determined whether TID1 knock-down affects the NGF-dependent activation of ERK/MAP kinase in nnr5-TrkA cells. Both the shRNA-expressing and control nnr5-TrkA cells

![Image](http://www.jbc.org/)
were treated with the indicated concentrations of NGF for 10 min. A total of 30 μg of lysate protein from each treatment was separated by SDS-PAGE and analyzed by Western blotting with the anti-phospho-ERK (p42/44) antibody. The results indicated that the NGF-dependent activation of ERK/MAPK was reduced in the TID1 knock-down cells relative to controls (Fig. 7D).

Indeed concentrations of NGF that induce ERK/MAPK activation in control cells had no effect on activation in TID1 knock-down cells (Fig. 7D, compare lanes 2 and 3 with lanes 6 and 7). Moreover these data support our observations that low concentrations of NGF are incapable of supporting neurite outgrowth in TID1 knock-down cell lines (data not shown).
DISCUSSION

The Trk family of receptor tyrosine kinases plays a central role in many cellular processes inherent in the mammalian nervous system. For example, TrkA functions in the differentiation and survival of nociceptive pain and temperature-sensitive neurons as well as sympathetic neurons (40–42). TrkB plays important roles in the differentiation and survival of motor neurons and sensory neurons responsive to tactile stimuli (43–45). TrkC is a key factor for the development of sensory proprioceptive neurons (46). In addition, the Trk receptors may also function in a mitogenic or apoptotic capacity (47), depending upon the cellular context, and constitutive activation of these receptors leads to cellular transformation with a resultant loss of proliferative control (48). Central to our understanding of how the Trk proteins regulate such diverse functions is to understand the signaling pathways inherent in these receptors. To date a number of proteins, including Shc (10, 12), Grb2 (14), fibroblast growth factor receptor substrate 2 (11), SH2B (13), APS (13), CSK homologous kinase (49), phospholipase Cγ-1 (12, 14, 33), the guanine nucleotide exchange proteins Ras-guanine-releasing factor (50) and GRIT (51), and scaffolding proteins such as the atypical protein kinase C-interacting protein p62 (52), have been shown to interact directly with TrkA. Many of these proteins also interact directly with TrkB and TrkC (1). Despite this, our understanding of the molecular switches operating through the Trk receptors in the regulation of growth versus differentiation and potential cross-talk between signaling pathways remains poorly understood.

To gain a better understanding of the physiological aspects of Trk-mediated signaling we used the yeast two-hybrid system to screen a human mammary gland library using the intracellular domain of TrkC as bait. To this end we identified the mammaalian homologue of the Drosophila tumor suppressor TID56 protein, lethal(2)tumorous imaginal discs (l(2)TID) protein (16, 53), as an interacting protein with TrkC. Structurally TID1 contains an amino-terminal DnaJ domain and as such belongs to the DnaJ family of molecular chaperone proteins. In addition to the DnaJ domain, TID1 also has a centrally located cysteine-
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TID1 protein is characterized by a cysteine-rich domain (CXXC(XG)_{1-2} repeats) and a distinct carboxyl-terminal domain (16). Two isoforms of TID1 have been identified, TID1S and TID1L, and both proteins are expressed in a variety of tissues (16, 19). Interestingly, alternate splicing of TID1S and TID1L introduces two different types of PDZ domain binding sites (25), although at date no functional significance has been ascribed to these putative domains. With respect to function, the TID1 proteins appear to play a fundamental role in both cell death and growth. For example, recessive mutations in TID56 bring about neoplastic transformation of imaginal disc cells resulting in larval death (53, 54), and it has been shown that the TID1 isoforms function in both a pro-and antiapoptotic capacity (17, 18).

The TID1 fragment identified in our yeast two-hybrid screen comprises the carboxyl-terminal portion of the protein (Phe^{217-Ser^{480}}) including the cysteine-rich and carboxyl-terminal domains but not the DnaJ domain. Moreover, although the two-hybrid screen identified interacting proteins with TrkC as bait, we found that TID1 interacts with all three Trk receptors. Mapping experiments indicate that TID1 interacts via the carboxyl-terminal domain with tyrosines Tyr^{683}/Tyr^{684} of rat TrkA in a phosphorylation-dependent fashion. Tyrosines 683 and 684 are found in the activation loop of rat TrkA and, when phosphorylated, stabilize the receptor in an activated state (27, 55, 56). Initially it was believed that these tyrosines do not participate as docking sites for signaling adapter proteins, but recently a number of proteins (APS, SH2B, and Grb2) have been reported to bind at these sites (13, 14, 57). It is curious that TID1 interacts with TrkA at these phosphorylated tyrosines residues as TID1 does not contain a classical phosphotyrosine binding domain such as a PTB or Src homology 2 domain. However, the interaction may not occur at these phosphorylated tyrosine residues per se but rather may be a consequence of the conformational change the receptor undergoes upon activation. Having said this, however, the TrkA13 mutants are constitutively active; albeit this activity is reduced relative to the wild type (27, 55). The reduction in the level of activity observed in these TrkA13 mutants (27, 55) suggests that, although these receptor mutants may assume an active conformation, it is probably less stable than that in the wild type receptor.

The carboxyl-terminal domain of TID1 has been observed to interact with other tyrosine kinases such as Jak2 and the R2 subunit of the human interferon-γ receptor (24), the intracellular domain of the ErbB-2 receptor tyrosine kinase (23), and the TPR/MET receptor tyrosine kinase (58). Although the sites of interaction within these individual kinases for TID1 were not identified, the interaction with Jak2 appeared to be phosphorylation-dependent (24). Moreover the interaction with ErbB-2 occurred with a constitutively active receptor (23), suggesting that receptor phosphorylation is an important factor for this interaction.

It is interesting that overexpression of TID1 in nnr5-TrkA cells potentiated the effects of NGF through TrkA. Accordingly we observed an enhancement in both neurite outgrowth and ERK/MAP kinase activity. Similarly knock-down of TID1 resulted in a decreased cellular sensitivity toward NGF reflected in a decreased level of NGF-dependent ERK/MAP kinase phosphorylation. Thus, with respect to TrkA, TID1 acts as a positive modulator of NGF. Conversely the action of TID1 with the ErbB-2 receptor is negative in that overexpression of TID1 exerts a suppressive effect on ErbB-2 by enhancing the degradation of the receptor (23). Thus, increased expression of TID1 in tumor cell lines overexpressing ErbB-2 leads to growth arrest and cell death (23). Similarly TID1 suppresses interferon-γ-mediated gene expression through the receptor and Jak2 (24). Exactly how TID1 potentiates Trk activity through activation of ERK/MAP kinase is not presently known. We have observed, however, that TID1 constitutively binds Grb2 (data not shown), and it is possible that the enhanced recruitment of Grb2 through TID1 overexpression results in a higher level of ERK/MAP kinase activity by amplifying signaling through Ras or Rap1. Alternatively TID1 may function as a molecular chaperone of activated Trk receptors, helping to maintain the protein in an active form upon NGF binding and internalization. It has been reported that TID1 interacts with Ras-GTPase-activating protein (19). In this sense NGF activation of Trk may enhance the ability of TID1, perhaps through tyrosine phosphorylation, to bind inhibitory proteins such as Ras-GTPase-activating protein thus prolonging the ability of Ras, or possibly other small GTPases, to promote neurite outgrowth. The effect of TID1 overexpression on small GTPase activity is currently being explored.

It has recently been reported that endogenous TID1 expression is up-regulated during the onset of senescence where cells undergo a process of growth arrest in vitro (25). Moreover overexpression of TID1S in rat embryonic fibroblasts resulted in growth suppression and enhanced pathways involved in senescence (25). Because TID1 is a tumor suppressor it is possible that its ability to promote senescence in rat embryonic fibroblasts and enhance neurotrophin-dependent growth arrest and differentiation are related. Evidence suggests that TID1 promotes senescence by repressing NF-κB in rat embryonic fibroblasts (25). Others have also shown that TID1 exerts effects on NF-κB. Cheng et al. (21, 22) showed that the viral oncoprotein Tax forms a complex with TID1/Hsp70 the effect of which is to antagonize the IκBα kinase-mediated phosphorylation of IκBα (21, 22) and prevent the nuclear translocation of NF-κB. Although NF-κB has been implicated in neurotrophin-dependent pathways, this has been observed with signaling through p75<sup>NT</sup>B, a low affinity neurotrophin receptor (59). Although ectopic expression of TID1 in nnr5-TrkA cells does not promote growth arrest and neurite outgrowth per se, we cannot rule out that the NF-κB pathway is not a target of TID1 in conjunction with neurotrophin stimulation. Future work may reveal a novel role for NF-κB in Trk signaling.

TID1 localizes to a number of cellular compartments, most strikingly the mitochondrial matrix but also the cytosol (18, 19). In addition, using the PSORT program, TID1 has two endoplasmic reticulum membrane retention signals, one amino-terminal, AARC<sub>5</sub>, and one carboxyl-terminal, KMFT<sub>4</sub>. This suggests a role for TID1 in the endoplasmic reticulum to assist in protein processing, and juxtaposed against the report where TID1 overexpression enhances ErbB-2 degradation (23), overexpression of TID1 in nnr5-TrkA cells may facilitate the expression of developmentally regulated proteins. Indeed one of those proteins may be Trk itself. Thus, nnr5-TrkA cells overexpressing TID1 may be expressing more receptors than control cells, and the enhancement of neurite outgrowth observed in the overexpressers may be a dosage effect. Further analysis will improve our understanding of the physiological role(s) that TID1 serves in the mechanisms regulating neuronal development and survival.

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