Critical Role for TrkB Kinase Function in Anoikis Suppression, Tumorigenesis, and Metastasis

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Abstract

Anoikis, or cell death induced by cell detachment, provides protection against the metastatic spread of tumor cells. We have previously shown that the neurotrophic receptor tyrosine kinase TrkB suppresses anoikis in rat intestinal epithelial cells and renders them highly tumorigenic and metastatic. Because TrkB is overexpressed in several aggressive human cancers, first attempts are being made to target TrkB in cancer therapy. However, the mechanisms underlying TrkB-mediated anoikis suppression, tumorigenesis, and metastasis still remain largely elusive. Although, to date, most attempts to neutralize TrkB in tumors aim to inactivate its kinase activity, it is unclear whether TrkB kinase activity is required for its oncogenic functions. Indeed, it has been suggested that also other properties of the receptor contribute to functions that are relevant to tumor cell survival. Specifically, several adhesion motifs reside within the extracellular domains of TrkB. In line with this, TrkB-expressing epithelial cells form large cellular aggregates in suspension cultures, possibly facilitating tumor cell survival. Therefore, we set out to study the relative contributions of TrkB’s kinase activity and its adhesion domains to anoikis suppression and oncogenicity. On the basis of a structure-function analysis, we report that TrkB kinase activity is required and, unexpectedly, also sufficient for anoikis suppression, tumor formation, and experimental metastasis. Thus, TrkB can act tumorigenically independent of its adhesion motifs. These results suggest that targeting the enzymatic activity of TrkB might be beneficial in cancer therapy. [Cancer Res 2007;67(13):6221–9]

Introduction

Cells disseminating from a primary tumor to metastasize to different sites in the body represent the main problem in cancer treatment and often are responsible for the death of cancer patients. A better understanding of the underlying mechanisms of this process could reveal new targets for cancer treatment. Metastasis involves several discrete steps, each serving to overcome a physiologic barrier against the spread of tumor cells (1). One such barrier, which is intrinsic to epithelial cells particularly, is anoikis, or apoptosis induced by unfamiliar (or loss of) cell adhesion signals (2, 3). Tumor cells can encounter foreign environments as soon as they start invading neighboring tissue, but also during and after the process of intravasation (tumor cell invasion into lymphatic or vascular vessels) and extravasation, when tumor cells seed in distant tissues. In the case of normal cells, these new environments would trigger an anoikis response, resulting in the elimination of cells upon the activation of an apoptotic program. By contrast, many tumor cells are anoikis resistant. Therefore, suppression of anoikis by disseminating tumor cells is predicted to prolong their survival in unfamiliar environments, thereby facilitating metastasis (2–4).

In a recent functional genomic screen for genes that suppress anoikis, we identified the neurotrophic receptor TrkB as an oncoprotein associated with metastatic capacity (5). Overexpression of TrkB rendered nonmalignant epithelial cells anoikis resistant and highly tumorigenic. Consistent with the model that anoikis suppression facilitates metastasis, TrkB-expressing cells formed highly invasive and metastatic tumors in nude mice, with very short latencies (5).

As to its physiologic role, the TrkB receptor tyrosine kinase, together with its ligand brain-derived neurotrophic factor (BDNF), is essential for the development and function of the nervous system, at least in part by providing an important survival signal (6–8). On the other hand, high TrkB expression levels have been implicated in several human malignancies. A prime example is neuroblastoma, in particular the subgroup associated with \( MYCN \) amplification and bad prognosis (9, 10). Likewise, TrkB overexpression has been found in pancreatic and prostate carcinomas and some other solid and lymphoid cancers (11–17). It has therefore been suggested that TrkB provides a potential target for therapeutic intervention (18–20). The fact that TrkB is a receptor tyrosine kinase in principle allows various ways of targeted interference. Indeed, attempts are being made already to develop inhibitors of Trk kinase signaling. Preclinical xenograft studies suggest that such inhibitors can delay tumor growth (11, 16, 18, 20–22). However, in spite of these efforts, the mechanism of TrkB-mediated anoikis resistance and oncogenicity is largely unknown. To date, most attempts to interfere with Trk function have focused on inhibiting its kinase activity. Somewhat surprisingly, however, to our knowledge, it has not yet been shown that TrkB enzymatic activity is required to suppress anoikis or more importantly, to execute its oncogenic functions. Although it seems likely that (sustained) TrkB kinase activity and downstream signaling contribute to, or are responsible for, anoikis suppression and tumorigenicity, alternative or additional mechanisms might be involved as well.

For example, we have observed that TrkB-expressing cells form large aggregates in suspension as well as during malignant invasion in vivo (5). It is a longstanding hypothesis that aggregate formation of tumor cells in vivo plays an essential role in tumor cell survival and metastasis (23–25). Consistent with this, TrkB harbors several adhesion motifs in its extracellular domain and has been proposed to act as an adhesion molecule (26, 27). This raises the question as to whether TrkB directly (through its extracellular domain) mediates intercellular adhesion and, if so, whether this is required for anoikis suppression and tumorigenesis. In such a scenario, the...
adhesion domains could represent another target for TrkB inhibition in cancer, independent of its kinase domain. Therefore, our aim in this study was to determine the contribution of the conserved domains of TrkB to its cancer-relevant functions. To this end, we undertook a structure-function analysis and generated a set of TrkB mutants carrying deletions or point mutations in the different functional domains of the receptor. These mutants were subsequently tested for their potential to suppress anoisik in vitro and to form tumors in nude mice.

Materials and Methods

Vector constructs. Human TrkB receptor (also called NTRK2, Genbank accession number NM_006180) was cloned by reverse transcription-PCR (RT-PCR) from cDNA derived from SY5Y cells. Human BDNF (NM_170735) was cloned from CDNA derived from IMR32 neuroblastoma cells. TrkB and BDNF were subcloned into the retroviral pBabe-puro (pBP) and pBabe-hygro (pBH) vectors, respectively (28). The COOH-terminally truncated rat Trk-T1 receptor (accession number M55292) was a kind gift from T. Hunter (Molecular and Cell Biology Laboratory, Salk Institute, La Jolla, CA; ref. 29). The KSS8M mutant, harboring a point mutation in the kinase domain, was generated by site-directed mutagenesis (Stratagene) according to the manufacturer's instructions and using a 5′-CTTGGTGGCA-GTGATGACCTCTGAGGCTGG-3′ and complementary primer pair (the bold letter indicates the base substitution A to T). The Δlug1 mutant was generated by a partial restriction digest of pCR2.1-TrkB with SpI, a full digest with HinCII and subsequent re-ligation, leading to an in-frame deletion of base pairs 550 to 837 (corresponding to amino acid residues 184 to 279, deleting the entire Ig-1 domain, including part of the second cystein-rich stretch). We constructed the ΔLRM, Δlug2 and Δlug1-2 mutants by overlap extension PCR (30). First, fragments 5′ and 3′ of the desired breakpoints and with overlapping extensions were PCR amplified. Subsequently, we did a PCR reaction with the two fragments at equimolar ratios to create a template, which was amplified with TrkB-forward and TrkB-reverse primers, thereby fusing the 5′ and 3′ input fragments. The TrkB-forward primer used was 5′-CGCGGATCCATCCATGCTTCTGGATAAGG-3′, TrkB-reverse primer was 5′-CGCGGTGCACCTGACAGTGAATGCACA-3′. The breakpoint forward primers used were ΔLRM: 5′-CTAAGCATGTAAGCTGAG* CCATTACATGGCTCCTGGATC-3′; Δlug2: 5′-GAGAAGATCAAGATT- CTTGT* CACTTCTAGGCGGCTGGTGTG-3′; Δlug1-2: 5′-GTTGTGTTCA- TCTGCAAA* CACTTCTAGGCGGCTGGTGTG-3′ (* indicating the breakpoint), resulting in TrkB-interdomain fusions linking amino acid residues E66 to P149, generating by a partial restriction digest of pCR2.1-TrkB with SpI, a full digest with HinCII and subsequent re-ligation, leading to an in-frame deletion of base pairs 550 to 837 (corresponding to amino acid residues 184 to 279, deleting the entire Ig-1 domain, including part of the second cystein-rich stretch). We constructed the ΔLRM, Δlug2 and Δlug1-2 mutants by overlap extension PCR (30). First, fragments 5′ and 3′ of the desired breakpoints and with overlapping extensions were PCR amplified. Subsequently, we did a PCR reaction with the two fragments at equimolar ratios to create a template, which was amplified with TrkB-forward and TrkB-reverse primers, thereby fusing the 5′ and 3′ input fragments. The TrkB-forward primer used was 5′-CGCGGATCCATCCATGCTTCTGGATAAGG-3′, TrkB-reverse primer was 5′-CGCGGTGCACCTGACAGTGAATGCACA-3′. The breakpoint forward primers used were ΔLRM: 5′-CTAAGCATGTAAGCTGAG* CCATTACATGGCTCCTGGATC-3′; Δlug2: 5′-GAGAAGATCAAGATT- CTTGT* CACTTCTAGGCGGCTGGTGTG-3′; Δlug1-2: 5′-GTTGTGTTCA- TCTGCAAA* CACTTCTAGGCGGCTGGTGTG-3′ (* indicating the breakpoint), resulting in TrkB-interdomain fusions linking amino acid residues E66 to P149.

Phosphotyrosine (4G10, Upstate), phospho-Akt (Ser 473, Cell Signaling), Akt1/2 (H-136, Santa Cruz Biotechnologies), and α-tubulin (DM 1A, Sigma). All antibodies were used in a 1:1,000 dilution, except α-tubulin, which was diluted to 1:3,000. Phosphorytrosine and phospho-Akt antibodies were diluted in 4% bovine serum albumin and all other antibodies were diluted in 4% skimmed milk. For immunoprecipitation, equal amounts of lysates were incubated with 0.5 μg pan-Trk [or TrkB [TK-]] (C-13, Santa Cruz Biotechnologies) for the T1 splice variant antibody for 2 h at 4°C. Proteins were immobilized with Protein A sepharose beads, washed four times with lysis buffer and immunoblotted for analysis.

Cell culture, retroviral transduction, and anoisik assays. RIE-1 cells (a kind gift from R.D. Beauchamp, Surgical Oncology Research Laboratories, Vanderbilt University Medical Center, Nashville, TN, and K.D. Brown, Signalling Programme, The Babraham Institute, Cambridge, United Kingdom) were cultured in DMEM (Life Technologies) supplemented with 9% FCS (Greiner bio-one) and penicillin + streptomycin (Life Technologies). Ecotropic retrovirus was produced in Phoenix packaging cells. We transduced RIE-1 cells with viral supernatant for pBH-hBDNF or empty vector in the presence of 3.5 μg/mL polybrene (Sigma). Cells were subsequently selected with 110 μg/mL hygromycin B (Calbiochem). pBH-hBDNF- (or pBH for Tpr and Tpr-TrkB) transduced RIE-1 cell pools were superinfected with retrovirus for the various TrkB mutants and selected in medium containing 1.5 μg/mL puromycin. To induce anoisik, we seeded freshly trypsinized cells (1 × 106) into ultra-low cluster (ULC) six-well culture dishes (Costar). Photographs were taken with a Sony DSC-75 digital camera, and ULC plates were scanned on an Eppon expression 1680 Pro scanner. Total protein amounts were measured as described above. Apoptosis was measured as described before (5). In brief, cells were seeded into ULC plates and after 3 days incubated for 10 min at 37°C in PBS with DIO6C (a fluorescing dye that accumulates in mitochondria as a function of membrane potential; Molecular Probes). Subsequently, cells were analyzed by fluorescence-activated cell sorting (FACS).

Stimulation with BDNF. Cells were serum starved overnight. The following day, we stimulated the cells for 5 min with serum-free DMEM supplemented with 100 ng/mL recombinant human BDNF (Peprotech). To inhibit phosphoinositide-3-kinase (PI3K) activity, we preincubated serum-starved cells with 20 μmol/L L3294002 (Calbiochem) for 30 min and subsequently treated them with serum-free medium containing 100 ng/mL recombinant human BDNF plus 20 μmol/L L3294002 inhibitor.

Immunofluorescence. Cells were grown on glass coverslips, fixed in 4% formaldehyde in PBS (or methanol/ethanol, 1:1, for staining with C-13 antibody), permeabilized with 0.2% Triton-X and treated with PBS + 0.2% Tween + 5% normal goat serum for 30 min. Coverslips were then incubated with either pan-Trk antibody (C-14), diluted 1:200 in blocking solution or with TrkB [TK-] antibody (C-13), diluted 1:100 for 1 h at room temperature. After washing with PBS + 0.2% Tween, we incubated the cells with Alexa Fluor 488 goat–anti-rabbit secondary antibody (1:1,000 for 1 h) and with TO-PRO (1:500 for 15 min; both from Molecular Probes). Coverslips were mounted with Vectashield mounting solution (Vector Laboratories) and analyzed by confocal microscopy on a Leica TCS NT (Leica Microsystems) confocal system, equipped with an Ar/Kr laser. Images were taken using a 63× numerical aperture 1.32 objective with standard filter combination(s) and Kalman averaging.

In vivo assays. Female BALB/c nude mice, 9 to 10 weeks of age, were s.c. injected with 1 × 106 cells into both flanks. Mice were inspected daily and euthanized by CO2 when the total tumor burden reached 2 cm3. Tumor size was measured with a caliper and tumor volume calculated by the formula (a × b × c)/2, with a being the longest diameter and b the shortest diameter of the tumor. For experimental metastasis, 1 × 106 cells were i.v. injected into the tail veins of female BALB/c nude mice of 11 weeks of age. Mice were inspected daily and euthanized by CO2 13 to 15 days later, when clinical symptoms became apparent.

1 http://www.stanford.edu/group/nolan/retroviral_systems/phx.html
Results

Design of TrkB mutants. TrkB has a characteristic domain architecture that is shared by its two relatives, TrkA and TrkC. At its NH2-terminal end that is exposed to the extracellular environment, three leucine-rich motifs (LRMs) are flanked by two cysteine-rich stretches and followed by two immunoglobulin-like domains (Ig-1 and Ig-2; Fig. 1A). The LRMs as well as the Ig-domains all contain adhesion motifs (26). At the COOH-terminal cytoplasmic end of Trk receptors, a tyrosine kinase domain mediates downstream signaling.

To assess the functional contribution of each individual domain, we introduced the following mutations into a full-length, wild-type, human TrkB cDNA (Fig. 1A). To inactivate the kinase domain, the Lys588 residue was replaced by methionine. The Lys588 residue is required for ATP-binding and abolishes kinase function if mutated (30, 33). In addition, we used a truncated splice variant, TrkB-T1, which lacks the entire kinase domain (29). Complementary to these kinase-inactive mutants, we generated a constitutively active Tpr-TrkB fusion protein that contains only the kinase domain but lacks kinase-inactive TrkB protein levels by immunoblotting (IB). The COOH-terminally truncated splice variant T1 (left) was detected with an antibody recognizing the Ig domains of TrkB (H-181). All other mutants (right) were detected with a COOH-terminal pan-Trk antibody (C-14). α-Tubulin serves as a loading control.

Relative expression of TrkB mutants. The functional consequences of these mutations were tested in the immortal rat intestinal epithelial cell line RIE-1, which is highly sensitive to anoikis and nonmalignant upon injection into nude mice (5, 35, 36). To assess the contribution of the various conserved domains of TrkB, we transduced RIE-1 cells first with recombinant retrovirus encoding BDNF and subsequently with retrovirus driving the expression of one of the different TrkB mutants. As Tpr-TrkB was expected to signal in a constitutive and ligand-independent manner, Tpr-TrkB and the Tpr control were expressed in the absence of BDNF. Following the selection for successful proviral integration, Western blot analysis of the cell line pools revealed that all the mutants were expressed to similar levels, except for Tpr-TrkB, which was expressed at relatively low levels (Fig. 1B). All but the Tpr-TrkB and ΔLRM mutants were detected as multiple species, most likely resulting from glycosylation (37).

Ligand activation and downstream signaling of TrkB mutants. Two of the adhesion domains within TrkB (i.e., LRM and Ig-2) are also involved in ligand binding and kinase activation (38–40). To assess whether the deletion of the adhesion domains would interfere with kinase signaling (thereby complicating a distinction between the functional contribution of the enzymatic and adhesion functions of TrkB), we first measured kinase activation of the mutants following stimulation with ligand. Serum-starved cells expressing the TrkB mutants in the absence of coexpressed ligand (RIE-1 cells have undetectable levels of BDNF) were briefly stimulated with recombinant human BDNF. Subsequently, TrkB was analyzed for the presence of phosphorylated tyrosine residues as a consequence of ligand-induced receptor cross-phosphorylation.

Only wild-type TrkB receptor and the ΔIg-1 mutant showed strong phosphorylation of tyrosine residues upon stimulation with BDNF (Fig. 2A). In contrast, the kinase-inactive K588M mutant and T1 splice variant, as well as the ΔLRM, ΔIg-2, and ΔIg-1+2 mutants.
failed to show receptor phosphorylation upon ligand stimulation. As expected, the constitutively active Tpr-TrkB mutant contained phosphotyrosine irrespective of the presence of ligand (Fig. 2A).

We have shown previously that TrkB signaling involves the activation of protein kinase B (PKB/AKT) in a PI3K-dependent manner, and that this represents a critical property of TrkB in mediating anoikis suppression (5). Therefore, we determined the ability of the TrkB mutants to activate PKB in response to stimulation with recombinant BDNF. Consistent with our previous data (5), wild-type TrkB stimulated the activation of PKB, as judged by the phosphorylation of the latter on residue Ser473 (Fig. 2B). Similarly, Δlg-1 stimulated activation of PKB. By contrast, all other mutants failed to activate PKB upon ligand stimulation. Of note, we were unable to detect significant activation of PKB by Tpr-TrkB. However, in contrast to the short-term stimulation with exogenous ligand, Tpr-TrkB signals constitutively. It might therefore be subject to regulatory (negative) feedback signaling loops, resulting in a low but sustained level of PKB activation. In

Figure 2. Ligand activation and downstream signaling of TrkB mutants. RIE-1 cell pools stably expressing TrkB mutants but not BDNF were serum starved overnight and subsequently stimulated with 100 ng/mL recombinant human BDNF for 5 min. A, cell lysates were prepared and used for immunoprecipitation (IP) with pan-Trk antibody and analyzed by immunoblotting (IB) with phospho-tyrosine antibody (pY, top), pan-Trk antibody recognizing the COOH terminus (middle), or NH2-terminal TrkB antibody (bottom). Arrows, positions of the respective (phosphorylated ‘p’) TrkB mutants. (Phosphorylated) Tpr-TrkB co-migrates with the immunoglobulin heavy chain (hc). Samples were derived from the same experiment but analyzed on parallel gels. B, cell lysates were prepared and analyzed by immunoblotting with PKB-pSer473 (pPKB, top) or total PKB (bottom) antibody. As a control for identifying pPKB (arrow), cells were treated with the PI3K inhibitor LY294002 (20 μmol/L) for 30 min before and during BDNF stimulation. *, aspecific bands; samples were analyzed on parallel gels. C, intracellular localization of TrkB mutants by immunofluorescence and confocal microscopy. Cells expressing the indicated TrkB mutants were stained with Trk antibody [TrkB [TK-] (C-13) for T1, pan-Trk (C-14) for wild-type and all other mutants] and analyzed by confocal microscopy on a 63x numerical aperture 1.32 objective. Cell nuclei were labeled with TO-PRO. Cells expressing only the vector served as a control to visualize unspecific signal (bottom).
summary, these results indicate that the deletion of the extracellular LRM and Ig-2, but not Ig-1, domains interferes with BDNF-mediated stimulation of TrkB as well as activation by TrkB of one of its primary downstream effectors, PKB. This implies that for these mutants, we are unable to distinguish any direct roles of the particular domains in mediating cell adhesion from mediating ligand-induced kinase activity. Although we did include the full set of mutants in all of our subsequent in vitro and in vivo functional assays, we therefore focused our attention on the role of kinase activity of TrkB.

To ensure that the kinase-defective TrkB mutants properly localized at the plasma membrane, we assessed their intracellular distribution by immunofluorescence and confocal microscopy analysis. Although specific signal was detected only in a small proportion of the cells, there were no significant differences between wild-type TrkB, T1, and K588M, which all showed enriched staining at the plasma membrane (Fig. 2C). As expected, Tpr-TrkB, which contains no signal peptide and no transmembrane domain, was evenly distributed throughout the cytoplasm. A second, independent approach using cell surface protein biotinylation (41) led to the same findings: membrane localization of wild-type TrkB, K588M, and T1 and intracellular localization of Tpr-TrkB (data not shown). These results allowed us to address the contribution of the kinase domain versus the adhesion domains to TrkB’s functions in vitro and in vivo.

TrkB kinase activity is required and sufficient for morphologic transformation and anoikis suppression. Consistent with our previous results (5), expression of wild-type TrkB in conjunction with BDNF led to a dramatic morphologic transformation of RIE-1 cells (Fig. 3). Specifically, TrkB-expressing cells largely lost their cell-cell contacts and acquired a spindle-shaped morphology. In contrast, both TrkB kinase-defective mutants, T1 and K588M, failed to induce morphologic transformation. The constitutively active Tpr-TrkB mutant did transform cells, although it was expressed to low levels and in the absence of coexpressed ligand. The ΔIg-1 mutant showed the same functional phenotype as wild-type TrkB, whereas ΔLRM, ΔIg-2, or ΔIg-1+2 failed to induce a morphologic change (Fig. 3). These results indicate that TrkB kinase activity is both required and sufficient to induce morphologic transformation of epithelial cells.

The mechanism by which TrkB-expressing cells form viable aggregates in suspension is unknown (see Introduction). To address the relative roles of TrkB’s kinase function and of its extracellular adhesion motifs in this process, we determined the ability of RIE-1 cells expressing our set of TrkB mutants to survive in suspension. Parental RIE-1 cells failed to survive upon seeding into ULC plates and massively underwent apoptosis, as judged by visual inspection (Fig. 4A), total protein content (Fig. 4B), and apoptosis analysis by FACS (Fig. 4C). Consistent with our previous results (5), epithelial cells expressing ligand-activated wild-type TrkB survived in suspension. Furthermore, these cells continued to proliferate, forming large cell clusters and markedly changing the color of the culture medium. Similarly, Tpr-TrkB and ligand-activated ΔIg-1 mutant rendered RIE-1 cells anoikis resistant, with continued proliferation and formation of cellular aggregates in suspension. By contrast, all other mutants analyzed were unable to suppress anoikis and the cells underwent massive apoptosis in suspension, comparable to cells transduced with empty vector. These results show that, just as we observed for morphologic transformation, anoikis resistance requires the TrkB kinase domain to be intact and active, whereas unexpectedly, its extracellular domains are completely dispensable. Of note, in all cases where anoikis was suppressed, (mutant) TrkB-expressing cells grew in the form of large cellular aggregates.

TrkB kinase activity is required and sufficient for oncogenicity and experimental metastasis. Although our previous observations suggested that the abilities of TrkB to suppress anoikis and to induce tumors were functionally linked (5), our set of mutants offered a powerful tool to study this in more detail.
Therefore, we assessed the capability of the various TrkB mutants to induce tumors in vivo. Because TrkB-expressing cells are highly tumorigenic (5), we used a relatively small amount of cells to increase the likelihood of detecting any difference in oncogenic potential of the mutants. To this end, $1 \times 10^4$ cells coexpressing (mutant) TrkB and BDNF were injected s.c. into both flanks of BALB/c nude mice, which were subsequently monitored for the appearance of tumors.

Mice that had received cells expressing wild-type TrkB, Tpr-TrkB, or ΔIg-1 developed rapidly growing tumors, reaching a size of about 1 cm$^3$ within 23 to 34 days (Fig. 5A). Given the small amount of cells we used, as well as the fact that the empty vector- or Tpr-expressing cells were devoid of any tumorigenic activity, this confirmed the marked oncogenic potential of TrkB. Although we observed small differences in the tumorigenicity of Tpr-TrkB and ΔIg-1 relative to wild-type TrkB, only these three forms of TrkB

![Figure 4](image_url)

**Figure 4.** TrkB kinase activity is required and sufficient for anoikis suppression A, a total of $1 \times 10^6$ cells of cell pools stably coexpressing BDNF (except for Tpr and Tpr-TrkB) and TrkB mutants as indicated were seeded into ULC cell culture plates and scanned at 10x magnification or photographed at 50x magnification 7 d later. B, quantification of the results shown in (A) by measuring the total cellular protein amounts. Columns, average values of three independent experiments; bars, SD. C, apoptosis was analyzed by DiOC6 staining (a measure of mitochondrial membrane potential) and FACS analysis. Left- and right-hand peaks, apoptotic and living cells, respectively.
These results indicate, in line with our experiments, that TrkB's adhesion motifs are not strictly required for its oncogenic function, but that its kinase domain is indispensable.

Several studies suggest that TrkB may drive tumorigenicity and metastasis in a number of different human malignancies (9–17). As a consequence, TrkB has been proposed to represent a target for anticancer therapy (18–20). Indeed, two compounds, CEP-751 and CEP-701/lestaurtinib (derivatives of the alkaloid K252a), have been developed as inhibitors of pan-Trk kinase activity (22, 44). Xenograft experiments in mice treated with CEP-751 and CEP-701 showed impaired tumor growth of cell lines from neuroblastoma and medulloblastoma (21) and from prostate (11, 44) and pancreatic tumors (22). However, unmasking the relevant target involved in these settings has not always been straightforward mainly because of the lack of specificity of the inhibitor. K252a, CEP-751, and CEP-701 block not only TrkA, TrkB, and TrkC, but also other kinases, including FLT3, MET, and RET, which can all contribute to tumorigenesis (45–47). The antitumorigenic effect of the inhibitors could therefore be caused by inhibition of any or several of these kinases. Recently, lestaurtinib (CEP-701) has been used in phase 2 clinical trials for acute myelogenous leukemia because of its inhibitory effect on FLT3 (48). Phase 2 clinical trials based on Trk inhibition have not been reported thus far. Attempts are now being made to develop new TrkB inhibitors. We propose that anoikis resistance could thereby represent a useful readout for screening inhibitors of TrkB function. The strict correlation we observed between anoikis suppression, tumor formation, and experimental metastasis (Table 1) predicts that compounds effective against anoikis suppression could also impair TrkB-driven oncogenicity.

Our finding that TrkB kinase activity is sufficient for tumorigenesis and experimental metastasis suggests that kinase-activating TrkB mutations could endow TrkB with oncogenic properties. Recent large-scale mutational analysis studies have revealed point mutations in TrkB in colon carcinoma (49) and lung adenocarcinoma (50), although their oncogenic significance is as yet unclear.

TrkB causes cells to form large aggregates when seeded as suspension cultures (ref. 5 and this paper). It has been suggested that its extracellular domains, in particular the LRM and Ig-like domains, mediate adhesion functions (26, 27). As such, these domains could contribute to or even drive cellular aggregate formation. Consistent with this prediction, we observed that the LRM, Ig-1, and Ig-1+2 extracellular domain mutants failed to suppress anoikis. However, these mutants also failed to activate TrkB kinase signaling in response to BDNF. Therefore, the analysis were highly oncogenic, in contrast to all the other TrkB mutants. Of note, when we inoculated 10 times more cells, K588M, ΔLRM–, ΔIg-2–, and ΔIg-1+2 (but not T1)–expressing cells did produce tumors, but with much longer latencies (between 48 and 71 days, compared with 18–21 days for wild-type, ΔIg-1–, and Tpr-TrkB–expressing cells) and with lower penetrance (data not shown).

These results indicate, in line with our in vitro observations described above, that TrkB kinase activity is both required and sufficient for tumorigenicity. Similar to our findings, the TrkA oncogene requires kinase activity for oncogenic transformation of NIH 3T3 fibroblasts (42, 43).

Finally, we wished to assess whether the extracellular adhesion domains of TrkB are required for its metastatic properties. To this end, we did an experimental metastasis assay, in which several steps of metastasis (i.e., extravasation, seeding of micrometastases, and outgrowth of macrometastases) can be mimicked by injecting tumor cells i.v. We inoculated 1 × 10⁶ RIE cells expressing wild-type TrkB, Tpr-TrkB, or ΔIg-1 via the tail vein of nude mice and inspected the lungs for the presence of tumor lesions. Multiple foci were apparent on the lungs of all animals 13 to 15 days after injection (Fig. 5B), indicating that TrkB kinase activity is not only sufficient for bringing about anoikis resistance and tumor formation, but also for experimental metastasis.

Discussion

On the basis of the structure-function analysis presented here, we conclude that kinase activity is required for TrkB to induce anoikis suppression in vitro and to produce tumors with metastatic capacity in vivo. Unexpectedly, we found that an artificially activated TrkB mutant completely lacking its extracellular adhesion motifs did as well as ligand-activated wild-type TrkB in all our in vitro and in vivo assays. Together, these results reveal that TrkB's adhesion motifs are not strictly required for its oncogenic function, but that its kinase domain is indispensable.

Figure 5. TrkB kinase activity is required and sufficient for oncogenicity and experimental metastasis. A, 1 × 10⁶ RIE-1 cells expressing TrkB mutants and BDNF (except for Tpr and Tpr-TrkB) were s.c. injected into both flanks of BALB/c nude mice (n = 6 for wild-type, Tpr-TrkB, and ΔIg-1; n = 3 for all other mutants), which were subsequently monitored for tumor formation. Kaplan-Meier plots are shown. Numbers in parentheses, number of growing tumors out of the total number of injection sites per cell line. B, 1 × 10⁶ RIE-1 cells expressing the indicated TrkB mutants and BDNF (except for Tpr-TrkB) were i.v. injected via tail veins of BALB/c nude mice (n = 5 for each cell line). Photographs show dissected lungs 13 to 15 d after inoculation. Numbers, numbers of mice with macroscopic lesions on their lungs out of the total numbers of injected mice. Top, a picture of a normal lung is shown for comparison.
of those mutants did not allow us to discriminate between the contribution of kinase activity and direct cell adhesion function in mediating anoikis resistance.

This issue was solved when we studied the role of TrkB kinase activity. The Tpr-TrkB mutant (completely lacking all extracellular domains) seemed as capable as BDNF-activated wild-type TrkB in mediating anoikis resistance and stimulating the formation of large cellular aggregates when maintained as suspension cultures. Conversely, the two TrkB mutants lacking ligand-induced kinase activity (K588M and T1) failed to bring about anoikis resistance, although their extracellular domains were intact. Thus, in the context of an activated TrkB receptor, there is no critical requirement for the extracellular domains in anoikis suppression, whereas kinase signaling is sufficient to mediate both anoikis suppression and aggregate formation. Of course, our observations do not rule out the possibility that in the context of an intact TrkB receptor, the extracellular domains contribute not just to ligand binding but also to other functions, thereby cooperating with kinase-dependent functions. We conclude that aggregate formation is an indirect TrkB-induced effect, mediated by cell-intrinsic TrkB kinase signaling. It will be important to analyze how this is mediated, e.g., by the activation and/or induction of specific adhesion molecules.

It is remarkable that in all cases in which cells were able to survive in suspension, they formed large aggregates. This observation suggests an important role for cellular adhesion in anoikis resistance. It is in agreement with previous suggestions that cell-cell interactions are crucial for the survival of cells in foreign environments (2–4, 23–25). It also raises the possibility that this particular function represents a target for therapeutic approaches aimed at reducing tumor cell survival.

In conclusion, we show that TrkB-induced anoikis resistance and tumorigenesis are consequences of TrkB kinase signaling activity. Because TrkB is overexpressed in a variety of human malignancies, our results imply that the development of inhibitors of TrkB kinase signaling is justified. Furthermore, given the strict correlation between the ability of TrkB to induce cellular aggregation and its tumorigenic potential, our results indicate that it will be interesting to determine the role and mechanism of TrkB-induced cell-cell adhesion in these settings. Any causal role for TrkB-activated regulators of cell adhesion in tumorigenesis and metastasis might present us with additional targets for therapeutic intervention.

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References


Table 1. Summary of the phenotypes of all TrkB mutants analyzed in vitro and in vivo

<table>
<thead>
<tr>
<th>Kinase activity</th>
<th>Activation of PKB</th>
<th>Morphologic transformation</th>
<th>Anoikis suppression</th>
<th>Aggregation in suspension</th>
<th>Tumor formation</th>
<th>Experimental metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>wt</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>K588M</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tpr</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tpr-TrkB</td>
<td>+</td>
<td>(–)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ΔIg1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>n.d.</td>
</tr>
<tr>
<td>ΔIg2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ΔIg1+2</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tr>
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Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O2 consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if \[ M = +0.27 \text{ and } L = -0.16 \] and the normal differential is 65 per cent M and 35 per cent L, then

\[
0.65 (+0.27) + 0.35 (-0.16) = +0.12
\]

a figure identical to the observed +0.12 for normal leukocytes.
Critical Role for TrkB Kinase Function in Anoikis Suppression, Tumorigenesis, and Metastasis

Thomas R. Geiger and Daniel S. Peeper


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