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Human Melanoma TrkC: Its Association With a Purine-Analog-Sensitive Kinase Activity

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Abstract The various members of the Trk tyrosine kinase family and p75 neurotrophin receptor ($p75^{NTR}$) have been identified as signaling receptors for the structurally related members of the neurotrophins (NT) family. We have previously reported that NT treatment of murine and human brain-metastatic melanoma cells affects their invasive capacities and increases the production of extracellular-matrix degradative enzymes. These cells express aberrant levels of functional $p75^{NTR}$ and TrkC, the putative high-affinity receptor for the neurotrophin NT-3. Here we demonstrate that, by using sensitive immune-complex kinase assays in human brain-metastatic (70W) melanoma cells, TrkC receptors associate with a kinase activity exhibiting a dose-dependent susceptibility to inhibition by the purine-analogs 6-thioguanine and 2-aminopurine. The activity of this purine-analog-sensitive kinase (PASK) was induced by NT-3 in a time-dependent fashion, phosphorylating exogenous myelin basic protein (MBP) but not denatured enolase. It is similar to the one reported to relate with $p75^{NTR}$ and TrkA receptors and stimulated by the prototypic NT, nerve growth factor. Thus, PASKs may represent unique signaling components common to NT receptors that could engage joint downstream signaling effectors in brain-metastatic melanoma. J. Cell. Biochem. 88: 865–872, 2003. © 2003 Wiley-Liss, Inc.

Key words: neurotrophins; neurotrophin receptors; TrkC; purine-analog-sensitive-kinase (PASK); 6-thioguanine (6-TG); 2-aminopurine (2-AP); brain-metastatic melanoma

Melanoma frequency is growing at a faster rate than any other human malignancy. The brain is often a primary target of melanoma metastasis, brain-metastatic melanoma being of biological and clinical relevance. However, mechanisms responsible for malignant melanoma progression to highly aggressive brain-metastatic disease are not completely understood [Herlyn et al., 1990; Marchetti, 2002a]. Of the phenotypic changes that occur during melanoma development, differences in the production of various autocrine growth factors and in the expression of receptors for paracrine growth factors are important [Albino et al., 1991]. Brain-metastatic murine and human melanoma cells respond to select members of a family of neurotrophic factors called the neurotrophins (NT) [Marchetti et al., 1993, 1996; Marchetti, 2002a]. Mammalian NT include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) [Chao and Bothwell, 2002]. NT can bind not only to the low-affinity p75 neurotrophin receptor ($p75^{NTR}$) [Benedetti et al., 1993; Chao and Bothwell, 2002] but also to the high-affinity NTR represented by the Trk family of tyrosine kinase receptors: mainly, NGF binds TrkA, BDNF interacts with TrkB while TrkC is the putative receptor for NT-3 [Barbacid, 1991; Kaplan and Miller, 2000; Chao and Bothwell, 2002]. Like other tyrosine kinase receptors, NTR are involved in a sequence of events that includes ligand binding leading to receptor dimer formation, transactivation resulting in tyrosine phosphorylation, and activation of serine/threonine phosphorylation cascades [Berg et al., 1991; Batistatou et al., 1992; Bibel and Barde, 2000; Kaplan and Miller, 2000].

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Malignant melanoma cells express $p75^{NTR}$ in direct relation to their malignancy and ability to metastasize to the brain [Herrmann et al., 1993; Marchetti et al., 1993, 1995; Marchetti, 2002a] within regions that synthesize and respond to NT [Marchetti et al., 1995]. We have found that NT affect invasive processes in brain-metastatic (murine B16B15b and human 70W variants) melanoma cells and the production of extracellular matrix-degrading enzymes, notably heparanase [Marchetti et al., 1993, 1996; Marchetti, 2002a,b]. Of relevance, human brain-metastatic 70W cells display a highly aggressive pattern of in vivo experimental metastases: a unique model, it is the first example of human melanoma capable of brain metastasis formation when injected intravenously into nude mice [Ishikawa et al., 1988]. As such, target organ site colonization by 70W cells is similar to the clinical presentation of human melanoma metastasis in advanced disease stages.

We have shown that $p75^{NTR}$ is overexpressed in 70W cells, resulting in NGF binding to these cells and NGF-stimulated extracellular matrix invasion and heparanase production [Herrmann et al., 1993; Marchetti et al., 1993]. Importantly, these effects have been found to be mediated by functionally active $p75^{NTR}$ in absence of high-affinity TrkA NGF receptors [Herrmann et al., 1993; Marchetti et al., 1993]. Furthermore, TrkC was expressed in 70W cells, and capable of binding NT-3: treatment of these cells with NGF and NT-3 augmented in vitro invasion and heparanase activity, whereas exposure with NT other than NGF or NT-3 did not [Herrmann et al., 1993; Marchetti et al., 1996]. These findings support the notion that $p75^{NTR}$ and TrkC are capable of transducing NT signals, which are important determinants in the pathogenic properties of melanoma in its progression to the brain-metastatic phenotype.

Functions of TrkC receptors in neuronal tissues have been investigated [Lamballe et al., 1991; Kaplan and Miller, 2000]; however, their roles in nonneuronal cells is less certain. Of note, Yaar et al. [1994] have demonstrated presence and functionality of $p75^{NTR}$/TrkC in melanocytes and mediating NT effects in these cells. The potential $p75^{NTR}$- trkC complex formation and their cross-talk [Bibel et al., 1999] have yet to be explored in malignant melanoma.

In the present work, we have investigated whether there is an association between TrkC receptors and a PASK in 70W cells. Secondly, we have determined presence of such activity and its specific regulation by NT-3.

MATERIALS AND METHODS

Materials and Reagents

Superscript AMV reverse transcriptase, 123-bp ladder fragment size and [^{14}C]-protein standards were purchased from GIBCO/BRL (Gaithersburg, MD). Protein A/G agarose was procured from Pierce (Rockford, IL). Leupeptin and phenylmethylsulfonylfluoride (PMSF) were obtained from Boehringer Mannheim (Indianapolis, IN). 3-[3-chloroamidopropyl)-dimethylammonio]- 1-propane sulfonate (CHAPS), sodium orthovanadate, 2-aminopurine (2-AP), 6-thioguanine (6-TG), acid-denatured enolase and myelin basic protein (MBP) were purchased from Sigma (St. Louis, MO). Rabbit anti-mouse IgG was procured from Zymed (San Francisco, CA). Affinity-purified anti-trkC(798) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). It is specific for TrkC and it does not cross-react with TrkB or TrkA. Anti-trk(790) antibody, generated against residues 777 to 790 of TrkA and cross-reactive with Trk receptors, was obtained from Oncogene Research Products (La Jolla, CA). Antiserum 443 (pan-trk 443), generated against residues 807 to 821 of gp145 TrkB and cross-reactive with TrkC receptors [Soppet et al., 1991] was a gift of Dr. Louis F. Parada (The University of Texas Southwestern Medical Center, Dallas, TX). Human NT-3 was purchased from Promega (Madison, WI). Alkaline-phosphatase-conjugated anti-phosphotyrosine antibody RC20 was purchased from Signal Transduction Laboratories (Lexington, KY). Nitrocellulose transfer membranes (0.1-μm size) were obtained from Schleicher and Schuell (Keene, NH).

Cell Culturing

The human brain-metastatic melanoma cell line 70W has been previously described [Ishikawa et al., 1988; Marchetti et al., 1993, 1996]. The cells were cultured in 1:1 (v/v) Dulbecco-modified Eagle's medium and F12 medium (DMEM/F12) supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO_2-95% air atmosphere. Cells were subcultured using ethylenediaminetetra-acetic acid (2 mM in phosphate buffer saline) treatment and were grown without the addition of antibiotics. 70W cells were regularly screened for

possible mycoplasma contamination using a GEN-PROBE[3] (San Diego, CA) [^{3}H]-single-stranded DNA detection procedure and were found to be mycoplasma-free. In all experiments, 70W cells were extensively washed in serum-free DMEM/F12 medium (four washes), then cultured for 16 h in serum-free medium, washed once, cultured an additional 2 h, washed once more and finally processed.

RT-PCR Analysis

Total RNA obtained from asynchronous early-passage 70W cells was used as template for first-strand cDNA synthesis using Superscript™ AMV reverse transcriptase. Oligonucleotide primers for NT-3 were based on the cDNA sequence for NT-3 [Maisonpierre et al., 1990] and were selected for their predicted annealing specificity using GCG sequence analysis software (Genetics Computer Group, Madison, WI). Oligonucleotide primer design was further enhanced with respect to thermodynamic considerations and target sequence specificity using the OLIGO-4 Primer/Probe Analysis Software Package (National Biosciences, Plymouth, MN). Primer, template, salt, and divalent cation (Mg^{2+}, Mn^{2+}) concentration, as well as primer annealing temperature, were provided by the OLIGO-4 program. The NT-3 primers were: forward (amino acid position—106) 5′-AAGTT-GATCCAGGCGGATATCTT-3′ and reverse (amino acid position—48), 5′-ACAGGAGAGT-TGCCGGTTTGAT-3′. Amplification was performed for 30 cycles, and PCR products were analyzed on a 1% agarose/TBE gel using a 123-bp ladder fragment size standard.

Immunoprecipitation and Immune-Complex Kinase Assays

Cells were adjusted to reflect equivalent cell numbers (1×10^6) and lysed for 5 min on ice in 100 μl of ice-cold CHAPS lysis buffer (10 mM CHAPS, 50 mM NaCl, 25 mM HEPES, pH 7.5, 10 μM leupeptin, 200 μM PMSF, 1 mM sodium orthovanadate). Lysates were cleared of insoluble material by centrifugation at 14,000*g* for 3 min. The soluble supernatant was transferred to a fresh tube and precleared of nonspecific Fc-binding proteins by incubating with nonrelevant polyclonal rabbit anti-mouse IgG for 60 min at 4°C, followed by protein A/G agarose beads (prewashed twice with CHAPS lysis buffer) for 60 min at 4°C. Nonspecific complexes were collected by centrifugation as described above. The supernatant was transferred to a fresh tube and used for subsequent antibody reactions [either with 2 μg of anti-trkC (798), or 2 μg of anti-trk(790), or 5 μl of antiserum 443]. The supernatant was incubated for 60 min at 4°C with occasional mixing on a Vortex Genie at a setting of 2. After the incubation, the prewashed protein A/G agarose bead mixture (25 μl) was combined with the specific antibody-CHAPS-cell lysate and incubated for an additional 12 h at 4°C with gentle agitation. The agarose beads were then washed three times with CHAPS lysis buffer, transferred to a fresh tube, and washed a fourth time with solubilization buffer. Afterwards, the pellet was resuspended in reaction buffer (25 mM HEPES, pH 7.5, 4 mM $MgCl_2$, 5 mM $MnCl_2$ 20 μM [γ-^{32}P]ATP (5 μCi, 300 Ci/mmol) with or without 6-TG or 2-AP. Some kinase assays included acid-denatured enolase or bovine brain MBP as exogenous substrates (400 ng). The reactions were performed at 25°C for 15 min and terminated by the addition of sample loading buffer (2% SDS, 30% glycerol, 0.1% bromophenol blue, 15 mM Tris, pH 8.3). The reaction mixtures were then frozen at −70°C and later resolved after heating to 95°C for 4 min using 6–16% moving boundary velocity gradient-SDS-polyacrylamide gel electrophoresis (MBVG-SDS-PAGE). Gels were fixed, vacuum dried, and exposed using Kodak XAR-5 autoradiography film. For the filter retention assays, the samples were treated as described above with sample loading buffer and heated. Next the samples were absorbed under vacuum onto a nitrocellulose membrane using a slot-blot apparatus as described [Volonté et al., 1992] with the exception that the nitrocellulose membrane was preblocked for nonspecific ATP-binding by incubation with 1 mM cold (4°C) ATP in 100 mM HEPES buffer, pH 7.4, for 20 min.

Western Blot Analysis

Pan-trk and anti-trkC immunoprecipitates were resolved by high resolution MBVG-SDS-PAGE. MBVG gels were designed to improve stacking and resolution of SDS-treated immunoprecipitated samples and consisted of continuous, linear pH, and buffer strength gradients without SDS. The gradients were made up of a stacking leading phase buffer (0.37 M Tris, 0.17 M HCl, $pH_{25^\circ} = 8.3$) and a resolving leading phase buffer (0.82 M Tris, 0.068 M HCl, $pH_{25^\circ} = 9.2$). The anolyte buffer consisted of

20 mM Tris and 10 mM HCl, $pH_{25^\circ} = 8.1$, and the catholyte buffer consisted of 20 mM glycine, 10 mM Tris, 0.03% SDS, $pH_{25^\circ} = 9.2$. The Mr standards consisted of 50 ng each of [^{14}C]-proteins. Electrophoresis was conducted at 50 V until the dye-fronts entered the continous gels and then at 30 mA/gel until the dye-fronts were 5 mm from the bottom of the gel. Protein transfers were performed in a 10 mM Tris, 10 mM Tricine low-ionic strength buffer. Protein transfers were performed using Immobilon PVDF membranes (Millipore, Bedford, MA). The transfer was performed at 4 V constant voltage on ice for 18 h, with a transfer efficiency of >95%. The transfer membrane was blocked using Tween-20 detergent and probed using a 1:2,000 dilution of alkaline-phosphatase-conjugated RC20, in a recombinant version of anti-phosphotyrosine monoclonal antibody PY20.

RESULTS

Detection of a 6-Thioguanine Sensitive Kinase Activity in TrkC Melanoma Receptors Immunoprecipitates

We sought to determine whether 70W TrkC receptors might be associated with a constitutive kinase activity. Human brain-metastatic 70W cells were washed extensively in serum-free medium prior to lysis and TrkC receptors immunoprecipitation. Lysis of the cells and immunoprecipitations were performed using CHAPS detergent in order to preserve native structures important in mediating protein–protein interactions. Immunoprecipitations and immune-complex kinase assays (ICKAs) were carried out similar to those described for detection of a $p75^{NTR}$- and TrkA receptors-associated NGF-stimulated kinase activity [Volonté et al., 1993a,b].

Using these methods, we found that both anti-trkC(798) and anti-trk(790) were capable of co-precipitating a PASK activity that was inhibited by 6-TG (Fig. 1). Possible substrates for this kinase activity included proteins of approximately 200, 150, 125, 90, and 55 kDa in size (Fig. 1). The 150 kDa species may represent TrkC receptors. Control experiments were performed to assess the signal specificity of anti-trk(790), one of the TrkC-recognizing antibodies used in these assays. The peptide (ALANAPPVYLDVLG) corresponding to 14-amino acid residues (777–790) within the C-terminal domain of c-Trk used for immunization

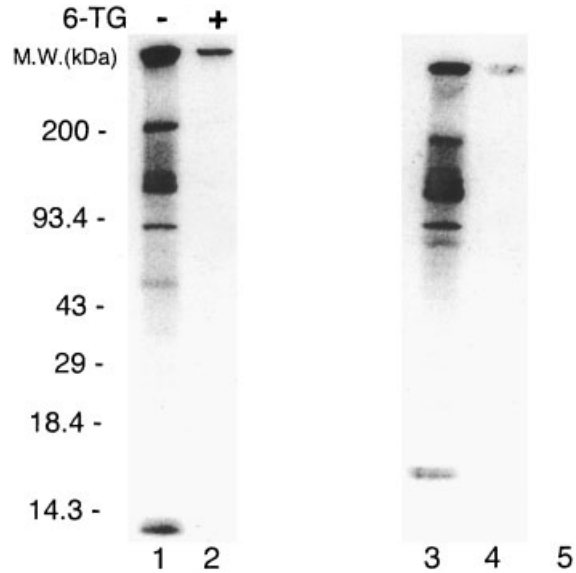

Fig. 1. Detection of a 6-TG sensitive kinase activity associated with melanoma TrkC receptors. MBVG-SDS-PAGE autoradiography of immunoprecipitates performed with TrkC-recognizing antibodies and resuspended in a buffer optimized for the immune-complex kinase assays (ICKA). ICKA using anti-trkC(798) without (**lane 1**) or with 500 μM 6-TG (**lane 2**). **Lane 3**: ICKA performed using anti-trk(790). **Lane 4**: ICKA performed following neutralization of anti-trk(790) with a 10-fold mass (w/w) excess of its target peptide (ALANAPPVYLDVLG) prior to immunoprecipitation. **Lane 5**: ICKA performed using immunoprecipitate obtained from control nonrelevant polyclonal rabbit anti-mouse $IgG_{(H+L)}$ antibody plus recombinant protein A/G agarose.

was incubated as a competitor in a neutralization reaction [10:1 peptide:antibody (w/w) for 2 h at 25°C] prior to the immunoprecipitation. The immunizing peptide essentially abolished coimmunoprecipitation of the PASK activity, indicating the immunospecificity of our ICKAs using this antibody (Fig. 1). As a control for Fc-binding cell surface receptors, the ICKA was performed using a nonrelevant rabbit anti-mouse IgG polyclonal antibody. Such control experiments also indicated the specificity of this assay. Similar results were obtained using pan-trk 443 antiserum (data not shown).

Purine-Analogs Inhibit TrkC Melanoma Receptors-Associated Kinase in a Dose-Dependent Manner

We investigated the selective phosphorylation of exogenous substrates by this PASK activity. 70W melanoma cells cultured in serum-free medium were lysed and the precleared lysates were immunoprecipitated with affinity-purified anti-trkC antibodies. The washed immunocomplex was then assayed for kinase

activity in presence of either acid-denatured enolase or MBP. Exogenous acid-denatured enolase did not serve as effective in vitro substrate for the melanoma TrkC receptors-associated PASK; however, MBP was found to be a good exogenous substrate for this activity (Fig. 2A).

To further characterize the purine-analog sensitivity of the kinase activity associated with TrkC, we performed ICKAs with anti-trkC(798) immunoprecipitates using various doses of either 6-TG or 2-AP (Fig. 2B) [Volonté et al., 1993a,b]. The results of these experiments indicated a dose-dependent attenuation of TrkC receptor-associated kinase activity with these two purine-analogs (Fig. 2B). Although low concentrations of 6-TG (20 μM) were only slightly effective at diminishing TrkC receptor-associated kinase activity, higher concentrations (100 or 500 μM) effectively inhibited this activity. As a control for anti-trkC(798) immunospecificity, the antibody was incubated with a 10-fold mass (w/w) excess of its target peptide (QQRLNIKEIYKILHA) prior to immunoprecipitation. We found that 2-h incubation with the peptide was sufficient to block greater than 90% of the kinase activity coimmunoprecipitated by anti-trkC(798) (Fig. 2B). Additionally, we found that 2-AP inhibited the human 70W malignant melanoma TrkC receptor-associated kinase activity in a dose-dependent manner although at higher concentration values than 6-TG (Fig. 2B) [Volonté et al., 1993a,b]. Comparable results were obtained with pan-trk 443 antiserum (data not shown).

70W Melanoma TrkC Activation Is Not Due to Autocrine NT-3 Production

Since malignant melanoma cells can produce autocrine growth factors, we examined whether 70W cells synthesize the TrkC ligand NT-3. RT-PCR using NT-3-specific oligonucleotide primers were performed on cDNA generated from 70W cells cultured using the conditions that result in TrkC phosphorylation [Herrmann et al., 1993]. Using various primer concentrations, an NT-3 amplification product was not found (Fig. 3). Additionally, performing RT-PCR under various pH conditions and Mg^{2+} concentrations did not result in the generation of a specific NT-3 amplification product (data not shown). However, this primer pair performed

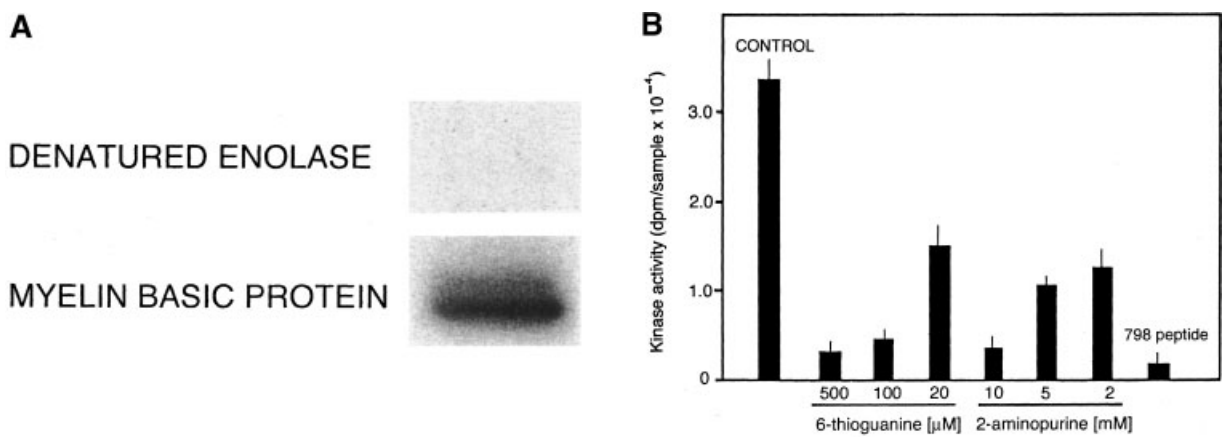

Fig. 2. **A**: Selective phosphorylation of exogenous substrates (acid-denatured enolase or myelin basic protein) by purine-analog-sensitive kinase (PASK) activity. 70W melanoma cells cultured in serum-free medium were lysed and precleared lysates were immunoprecipitated with affinity-purified anti-trkC(798) antibody. The washed immunocomplex was assayed for kinase activity (see Materials and Methods) with 400 ng of either acid-denatured enolase or MBP. After washing, phosphorylated proteins were resolved on a 6–16% MBVG-SDS-PAGE and autoradiographed, as shown. The positions of exogenous substrates were identified by their molecular weights and by comparison to lanes without added exogenous substrate. **B**: Purine-analogs inhibit TrkC receptors-associated kinase in a dose-dependent manner. Anti-trkC(798) immunoprecipitates were subjected to ICKA analysis and phosphorylated proteins were slot-blotted onto nitrocellulose membranes. **Slot 1**: Analysis in absence of purine-analogs (positive control); **slots 2**, **3**, and **4**, analysis in presence of 6-TG (500, 100, 20 μM respectively); **slots 5**, **6**, and **7**, analysis in presence of 2-AP (10, 5, 2 mM, respectively); **slot 8**, analysis in presence of peptide (798) (negative control). Free ATP-binding sites on the nitrocellulose were blocked by prior incubation with 1 mM cold (4°C) ATP. Following filtration and absorption, samples were washed five times. Slots were excised and bound radioactivity determined by liquid scintillation counting. For slot 8, the anti-trkC(798) antibody was incubated with a 10-fold (w/w) excess of its target peptide (QQRLNIKEIYKILHA) prior to immunoprecipitation. Assays were performed in triplicate. Means ± SEM of three independent experiments are shown.

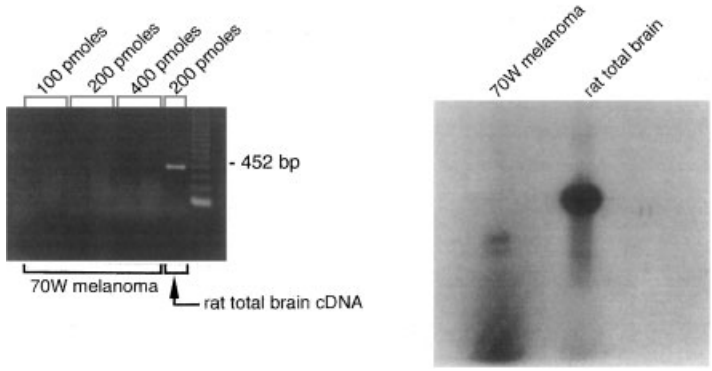

Fig. 3. Tyrosine phosphorylation of 70W malignant melanoma TrkC receptors is not due to catalytic activation via an autocrine NT-3 production. 70W cells were cultured and analyzed by RT-PCR for NT-3 mRNA production. **Left panel** shows results using various amounts of input oligonucleotide primer (100–400 pmoles/100 μl reaction). Rat brain total RNA-generated cDNA served as a positive control for NT-3 mRNA production. The **right panel** shows overexposure of [^{32}P]-labeled amplified products corresponding to the 70W melanoma and rat brain cDNA amplified with 200 pmoles of primer. Various pH modifications and adjustments to the Mg^{2+} concentration were made without affecting the results shown.

well using rat total brain cDNA as a template and positive control (Fig. 3).

Time Course of NT-3-Dependent Induction of TrkC PASK

70W cells cultured for different times with or without NT-3 were solubilized and subjected to immunoprecipitation with anti-trkC antibodies. The immunoprecipitates were extensively washed and then tested for protein kinase activity using MBP as an exogenous substrate. After treatment of 70W cells with human NT-3 (100 ng/ml) for 5–10 min, a five- to six-fold increase in TrkC-associated MBP phosphorylation activity was obtained (Fig. 4). The extent of induction significantly decreased after the cells were cultured for 1 h with NT-3 and returned to basal levels following a 24 h exposure. Basal as well as NT-3-induced PASK activities were inhibited between 75% and 90% in the presence of 500 μM 6-TG, indicating presence and functional association of PASK with TrkC receptors.

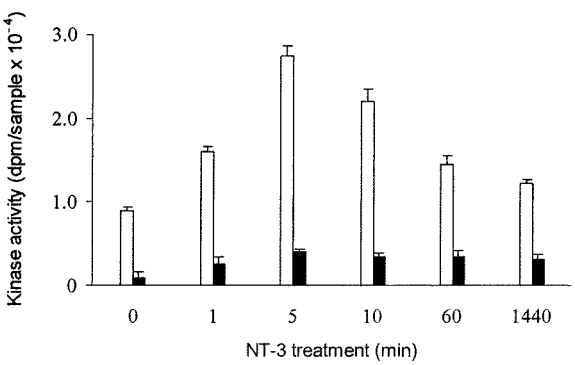

Fig. 4. Time-course of NT-3-dependent induction of TrkC-associated purine-analog-sensitive kinase (PASK). 70W cells were treated for different times (0–1,440 min) in the absence or presence of NT-3 (100 ng/ml). Extracts were then subjected to immunoprecipitation using anti-trkC(798) antibody, and the immunoprecipitates were incubated with myelin basic protein (MBP) under phosphorylating conditions for 15 min at 37°C, with or without 500 μM 6-TG (see Materials and Methods). Samples/treatment were then assessed by liquid scintillation counting. Results are means of triplicate determinations ± SEM (n = 3).

DISCUSSION

We have presented evidence that brain-metastatic 70W melanoma cells (1) express a PASK activity, which is found in anti-trkC immunoprecipitates, and (2) this PASK activity is stimulated by exogenous NT-3 and not by an autocrine NT-3 production in these cells.

We found that the TrkC receptors are associated with a kinase activity that is inhibited by purine-analogs, such as 6-thioguanine (6-TG) and 2-aminopurine (2-AP). These compounds have previously been shown to inhibit some of the biological activities of NGF on NGF-responsive cells [Volonté et al., 1993a,b] and are thought to function by inhibiting the activity of a kinase that associates with TrkA as well as $p75^{NTR}$ [Herrmann et al., 1993; Volonté et al., 1993a,b]. Our results indicate that PASK in 70W cells (1) associates with TrkC, (2) is induced by exogenously added NT-3, (3) is inhibited greater than 90% by 6-TG, and (4) phosphorylates in vitro MBP but not acid-denatured enolase, thus demonstrating its selectivity. These results suggest that TrkC receptors in human melanoma cells may be capable of NT-3-dependent signaling via a TrkC receptor-PASK

complex. One key observation demonstrated that the co-immunoprecipitation of PASK activity with 70W TrkC is specific: prior incubation of anti-trkC(798) or anti-trk(790) antibodies with their respective competing peptides neutralized co-immunoprecipitation of the PASK activity.

The functional role of TrkC receptors in the biology of human malignant melanoma is currently under investigation. We propose that the PASK activity described in anti-trkC immune complexes can be responsible for catalytic activation of TrkC receptors via transphosphorylation. This activity, together with a similar activity known to associate with melanoma $p75^{NTR}$ [Herrmann et al., 1993] can represent a signaling component(s) common to NTR, playing roles in melanoma cell pathogenesis by generating constitutive downstream signaling. Previous studies indicated that 6-TG inhibits a soluble (cytosolic) kinase, named protein kinase N (PKN), with an apparent K_i of 5–10 μM, while the $p75^{NTR}$-PASK activity had a K_i on the order of 20–35 μM [Volonté et al., 1993a]. Volonté et al. [1993b] also found that higher concentrations of 6-TG in the kinase reaction were needed to effectively inhibit a similar kinase activity associated with the TrkA receptors. Thus, it is quite possible that the soluble, $p75^{NTR}$ and Trk receptor-PASK activities may be the isoforms of the same enzyme. As a precedent for this, although protein kinases ERK1 and ERK2 are very closely related, only the former appears to associate with TrkA [Loeb et al., 1992]. Alternatively, these apparent differences in PASK-like activities can be due to alterations in structure or to accessibility of Trk receptor-associated molecules. Finally, we cannot rule out the possibility that they are unrelated.

Irrespective of whether TrkC-associated PASK activity is related to cytosolic PKN or to the $p75^{NTR}$- associated enzyme, these findings raise the issue of the significance of this interaction. One possibility is that TrkC itself is the target of PASK by transphosphorylation mechanisms. Alternatively, other molecules associated with TrkC are substrates for PASK activity and transphosphorylation may regulate their functional properties. Interaction/phosphorylation of receptor or receptors-associated enzymes by a serine/threonine kinase have been documented [Northwood et al., 1991; Ohmichi et al., 1991]. A third possibility is that PASK maybe a substrate of another TrkC-associated activity.

Studies on the Trk family of receptors have focused on downstream signaling events and alterations in cellular behavior evoked by catalytic activation of these receptors [Kaplan and Miller, 2000]. Introduction of TrkA receptors into mutant pheochromocytoma (PC12) cells unresponsive to NGF and ectopic expression of TrkB or TrkC in these cells can result in neuronal-like differentiation following treatment with NGF, BDNF, or NT-3 [Loeb et al., 1991; Hanzopoulos et al., 1994]. Coexpression of $p75^{NTR}$ can accelerate some of these events by cooperating with each of the Trks [Hempstead et al., 1991; Chao and Bothwell, 2002]. It has been previously shown that application of purine-analogs to cells suppresses a subset of early as well as late responses to NGF [Volonté et al., 1989; Volonté and Greene, 1990; Batistatou et al., 1992]. For example, NGF-enhanced neuronal survival is not affected by either 2-aminopurine or 6-thioguanine, whereas both these molecules diminish NGF-stimulated neurite outgrowth and the induction of ornithine decarboxylase [Volonté et al., 1989; Volonté and Greene, 1990]. Interestingly, 2-AP but not 6-TG can block NGF-induction of early response genes such as c-*fos*, c-*jun*, and *TIS 11* [Batistatou et al., 1992]. Such observations indicate that purine-analogs specifically target metabolic pathways and that these compounds are useful in dissecting certain NT pathways.

In light of the present and recent data, both $p75^{NTR}$- and TrkC-associated PASK activities must be considered as targets for purine-analogs and potential elements in the NT mechanism of action in malignant melanoma. NTR signaling in melanoma can thus proceed through common signal transduction pathways requiring a common effector(s). We speculate that PASK may be involved in such pathways.

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REFERENCES

Albino AP, Davis BM, Nanus DM. 1991. Induction of growth factor RNA expression in human malignant melanoma: Markers of transformation. Cancer Res 51:4815–4820.

Barbacid M. 1991. The trk family of tyrosine kinase receptors. Biochem Biophys Acta 1072:115–127.

Batistatou A, Volonté C, Greene LA. 1992. Nerve growth factor employs multiple pathways to induce primary response genes in PC12 cells. Mol Biol Cell 3:363–371.

Benedetti M, Levi A, Chao MV. 1993. Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. Proc Natl Acad Sci USA 90:7859–7863.

Berg MM, Sternberg DW, Hempstead BL, Chao MV. 1991. The low-affinity p75 nerve growth factor (NGF) receptor mediates NGF-induced tyrosine phosphorylation. Proc Natl Acad Sci USA 88:7106–7110.

Bibel M, Barde YA. 2000. Neurotrophins: Key regulators of cell fate and cell shape in the vertebrate nervous system. Genes Dev 14:2919–2937.

Bibel M, Hoppe E, Barde Y. 1999. Biochemical and functional interactions between the neurotrophin Trk and $p75^{NTR}$. EMBO J 18:616–622.

Chao MV, Bothwell M. 2002. Neurotrophins: To cleave or not to cleave. Neuron 33:9–12.

Hanzopoulos PA, Chitra S, Glass DJ, Goldfarb MP, Yancopoulos GD. 1994. The low affinity NGF receptor, p75, can collaborate with each of the trks to potentiate functional responses to the neurotrophins. Neuron 13: 187–201.

Hempstead BL, Martin ZD, Kaplan DR, Parada LF, Chao MV. 1991. High-affinity NGF binding requires coexpression of the *trk* proto-oncogene and the low-affinity NGF receptor. Nature 350:678–683.

Herlyn M, Kath R, Williams N, Valyi-Nagy I, Rodeck V. 1990. Growth regulatory factors for normal, premalignant and malignant human cells. Adv Cancer Res 54:213–234.

Herrmann JL, Menter DG, Hamada J, Marchetti D, Nakajima M, Nicolson GL. 1993. Mediation of NGF-stimulated extracellular matrix invasion by the human melanoma low-affinity p75 neurotrophin receptor: Melanoma p75 functions independently of *trkA*. Mol Biol Cell 4:1205–1216.

Ishikawa M, Fernandez B, Kerbel RS. 1988. Highly pigmented human melanoma variant which metastasize widely in nude mice, including to skin and brain. Cancer Res 48:4897–4903.

Kaplan DR, Miller FD. 2000. Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 10:381–391.

Lamballe F, Klein R, Barbacid M. 1991. TrkC, a new member of the TRK family of tyrosine protein kinases, is a receptor for neurotrophin-3. Cell 66:967–979.

Loeb DM, Maragos J, Martin-Zanca D, Chao MV, Parada LF, Greene LA. 1991. The *trk* proto-oncogene rescues NGF responsiveness in mutant NGF-nonresponsive PC12 cell lines. Cell 66:961–966.

Loeb DM, Tsao H, Cobb MH, Greene LA. 1992. NGF and other growth factors induce an association between ERK1 and the NGF receptor, $gp140^{prototrk}$. Neuron 9:1053–1065.

Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Furth ME, Lindsay RM, Yancopoulos GD. 1990. Neurotrophin-3: A neurotrophic factor related to NGF and BDNF. Science 23:1446–1451.

Marchetti D. 2002a. Brain-metastasis associated genes. In: Welch DR, editor. Cancer-metastasis-related genes. The Netherlands: Kluwer Academic Press. p. 89–108.

Marchetti D. 2002b. Heparanase: A target for therapy of brain invasive tumors? Expert Rev Neurotherapeutics 2(4):89–93.

Marchetti D, Menter DG, Jin L, Nakajima M, Nicolson GL. 1993. Nerve growth factor effects on human and mouse melanoma cell invasion and heparanase production. Int J Cancer 55:692–699.

Marchetti D, McCutcheon I, Ross M, Nicolson GL. 1995. Inverse expression of neurotrophins and neurotrophin receptors at the invasion front of human melanoma brain metastases. Int J Oncol 7:87–94.

Marchetti D, McQuillan DJ, Spohn WC, Carson DD, Nicolson GL. 1996. Neurotrophin stimulation of human melanoma cell invasion: Selected enhancement of heparanase activity and heparanase degradation of specific heparan sulfate subpopulations. Cancer Res 56:2856–2863.

Northwood IC, Gonzalez GA, Wartman M, Raden DL, Davis RJ. 1991. Isolation and characterization of two growth factor-stimulated protein kinases that phosphorylate the epidermal growth factor receptor at threonine 669. J Biol Chem 277:15266–15276.

Ohmichi M, Decker SJ, Saltiel AR. 1991. Nerve growth factor induces the association of a 130-kDa phosphoprotein with its receptor in PC12 pheochromocytoma cells. Cell Regul 2:691–697.

Soppet D, Escandon E, Maragos J, Middlemas DS, Reid SW, Blair J, Burton LE, Stanton BR, Kaplan DR, Hunter T, Nikolics K, Parada LF. 1991. The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the *trk*B tyrosine kinase receptor. Cell 65:895–903.

Volonté C, Greene LA. 1990. Induction of ornithine decarboxylase by nerve growth factor in PC12 cells: Dissection by purine analogues. J Biol Chem 265:11050–11055.

Volonté C, Rukenstein A, Loeb DM, Greene LA. 1989. Differential inhibition of nerve growth factor responses by purine analogues: Correlation with inhibition of a nerve growth factor-activated protein kinase. J Cell Biol 109:2395–2403.

Volonté C, Nichols RA, Greene LA. 1992. Rapid measurement of protein kinase and phophatase activities by slot filtration. BioTechniques 12:854–863.

Volonté C, Ross AH, Greene LA. 1993a. Association of a purine-analogue-sensitive kinase activity with p75 nerve growth factor receptors. Mol Biol Cell 4:71–78.

Volonté C, Loeb DM, Greene LA. 1993b. A purine-analogue-sensitive protein kinase actively associates with Trk nerve growth factor receptors. J Neurochem 61:664–672.

Yaar M, Eller MS, DiBenedetto P, Reenstra WR, Zhai S, McQuaid T, Archambault M, Gilchrest BA. 1994. The trk family of receptors mediates nerve growth factor and neurotrophin-3 effects in melanocytes. J Clin Invest 94(4):1550–1562.