Transfection of TRK-A into Human Neuroblastoma Cells Restores Their Ability to Differentiate in Response to Nerve Growth Factor

Erik Lavenius, Carolina Gestblom, Irja Johansson, Ewa Nänberg, and Sven Pählin

Department of Pathology, University of Uppsala, University Hospital, S-75 18 Uppsala, Sweden

Abstract

Human neuroblastoma cell lines frequently express the TRK-A proto-oncogene and bind nerve growth factor (NGF) but do not differentiate when exposed to NGF. Transient transfection of an exogenous TRK-A gene into SH-SY5Y and LA-N-5 neuroblastoma cells restored the ability of these tumor cells to differentiate with NGF. Stable TRK-A-transfected SH-SY5Y cell clones were isolated, and they responded to NGF by autophosphorylation of p140TRK-A, c-fos induction, morphological differentiation, and increased expression of two neuronal marker genes, neuropeptide tyrosine and GAP-43. In phorbol ester-induced differentiated wild-type cells, TRK-A expression was increased with no change in NGF responsiveness. Thus, the restoration of the NGF-induced differentiation pathway by exogenous TRK-A presents a system of NGF-responsive human cultured cells and focuses attention on the TRK-A protein and its function or malfunction in neuroblastoma.

Introduction

Neuroblastomas are considered to be derived from sympathetic neuroblasts of the peripheral nervous system. The tumor cells are arrested at various stages of neuronal differentiation, and there is a positive correlation between favourable outcome and high degree of biochemical maturation (1). In rare cases, a malignant tumor can mature spontaneously into a benign form (2). A number of neuroblastomas have been established as in vitro cultured cell lines. Most of them are derived from highly malignant tumors with an amplified N-myc gene, which only represents approximately 20% of all neuroblastomas (3). None of the established cell lines respond by differentiation to NGF stimulation, although many of them bind NGF with high and low affinity (4). Examples of NGF responsiveness in neuroblastoma cells, after treatment with antimotic drugs such as aphidicolin, have been reported (5, 6). Furthermore, the high affinity NGF receptor trk-A is frequently

expressed in neuroblastoma tumors, with the highest levels found in low malignant, highly differentiated, tumors (7–10), and in a few tested cases, these tumor cells require NGF for survival in vitro (7). Transfection and overexpression of the TRK-A gene in the HTLA230 neuroblastoma cell line resulted in cells that differentiate in response to NGF stimulation, suggesting that one major defect in neuroblastoma is a too low NGF receptor level (11).

The human SH-SY5Y neuroblastoma cells differentiate into a functional neuronal sympathetic phenotype via a PKC-dependent mechanism when treated with the phorbol ester TPA in serum-containing medium or in combination with one of several different growth factors, such as IGF-I; IGF-II; bFGF; PDGFs AA, AB, and BB; or EGF (12–16). However, NGF did not induce differentiation in these cells under the culture conditions investigated, nor did NGF support survival of the SH-SY5Y cells in serum-free medium as opposed to the other tested, non-neurotrophin growth factors (16). Thus, with respect to the lack of NGF responsiveness, our previously reported data are in agreement with those reported by Azar et al. (4).

The TRK-A proto-oncogene product is the high affinity receptor for NGF, belonging to the gene family coding for protein tyrosine kinase receptors (17–21). SH-SY5Y and LA-N-5 cells express the trk-A receptor (p140TRK-A) and the low affinity p75 NGF receptor (4, 17). Furthermore, stimulation of these cells with NGF can result in activation of the p140TRK-A as shown by the increased autophosphorylation (17). To investigate whether the lack of measurable secondary NGF responses in SH-SY5Y and LA-N-5 cells were due to low TRK-A expression or whether the trk-A protein in some way is defective and is not transducing NGF-evoked signal(s) and induction of genes necessary for a differentiation response, an exogenous TRK-A gene was expressed transiently or stably in these cells.

Results

NGF Does Not Induce Differentiation or c-fos Expression in Neuroblastoma Cells. As reported previously, many neuroblastoma cell lines show high affinity NGF binding (4) and present data confirmed at the mRNA level that six different neuroblastoma cell lines express varying levels of the TRK-A proto-oncogene (Fig. 1A). The amounts of TRK-A transcripts were similar to that in rat PC-12 cells (Fig. 1B), although a direct quantitative interspecies comparison cannot be done. NGF has no apparent effects on the SH-SY5Y cells in terms of induced survival, proliferation, or differentiation when they are cultured in either serum-free or FCS-containing medium (12, 16). None of the other neuroblastoma cell lines differentiated with NGF, including the LA-N-2 cells, which had the highest endogenous TRK-A expression (Fig. 1A). To further test whether NGF did induce a response in neuroblastoma cells, SH-SY5Y and LA-N-5 cells were stimulated with either 100 ng/ml of NGF or 5 nm of IGF-I for 30 min, followed by analysis of the

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: NGF, nerve growth factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; IGF, insulin-like growth factor; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
expression of the immediate early gene, c-fos. IGF-I but not NGF induced c-fos expression in both neuroblastoma cell lines. In contrast, PC-12 cells treated in parallel showed a NGF-induced increase in c-fos expression, thus demonstrating that the ligand was active (Fig. 1C).

**Transient Transfection of TRK-A into Neuroblastoma Cells.** Previous data (17) and those presented in Fig. 1 suggest that the SH-SYSY and LA-N-5 cells have low or modest levels of TRK-A expression. Thus, the lack of response to NGF treatment, as exemplified by SH-SYSY cells (Fig. 2), might be explained by a defective trk-A protein. We decided to test whether introduction of the TRK-A proto-oncogene into these cells could restore their ability to differentiate in response to NGF. Using lipofectin and plasmid pDM-69 DNA containing the human TRK-A cDNA (22), SH-SYSY and LA-N-5 cells were transiently transfected. Two days after transfection, cells were stimulated with 100 ng/ml of NGF, and 3 days thereafter, morphological differentiation was apparent (Fig. 2). Thus, introduction of an exogenous TRK-A gene restored the ability of these tumor cells to morphologically differentiate in response to NGF.

**Generation of SH-SYSY Cell Clones Stably Expressing an Exogenous TRK-A Gene.** To generate SH-SYSY cell clones that stably express an exogenous TRK-A gene, the cells were transfected with calcium-precipitated plasmid pDM-69 DNA containing the TRK-A cDNA and a neomycin resistance gene (22). After G-418 selection, several clones were isolated and characterized with respect to growth properties and morphology in FCS-containing medium (Fig. 3 and data not shown). Most of the clones showed a morphology and had growth properties similar to the wild-type SH-SYSY cells (Fig. 3). A slightly increased spontaneous neurite formation was noted in some TRK-A-transfected clones (e.g., clones 1:3 and 6:2; Fig. 3), which could suggest that the FCS contain small amounts of NGF-like activity or that the SH-SYSY cells themselves produce low levels of endogenous NGF. A few transfected clones with SH-EP morphology (an SK-N-SH subclone with epitheloid morphology and a phenotype lacking neuronal characteristics; Ref. 23) were isolated, but they did not respond to NGF and were not
Further analyzed (data not shown). In addition, SH-SY5Y cells were transfected with an expression plasmid containing the bacterial neo gene, and such transfected clones were also isolated (Fig. 3).

As exemplified in Fig. 3, several TRK-A-transfected clones responded to NGF by neurite growth, whereas neo-transfected clones showed no response to NGF treatment (Fig. 3). The TRK-A-transfected clones could be grouped into high (e.g., clones 1:3 and 6:2) and modest (e.g., clone 2:1) responders to NGF. Northern blot analysis of the exogenous TRK-A mRNA levels, distinguished from the endogenous mRNA by a slightly higher molecular weight (3.5 versus 3.2 kb), revealed that all responding clones that have been analyzed, but not wild-type and neo-transfected cells, expressed the transfected gene with the highest mRNA levels in clones 1:3 and 6:2 (Fig. 4, A and B). NGF stimulation induced c-fos expression in the TRK-A-transfected cell clones (1:3 and 6:2), whereas wild-type cells and the neo-transfected (9:1) clone did not respond. The 2:1 TRK-A clone also responded with c-fos expression, although the signal was very weak (Fig. 4C).

**TRK-A Receptor Activation in Transfected SH-SY5Y Cells.** Although there was a clear difference in TRK-A mRNA levels and NGF responsiveness between wild-type and TRK-A transfected (Figs. 3 and 4), the differences in p140TRK-A levels were not that apparent, although the highest level was found in the 1:3 clone (Fig. 5B). Therefore, we investigated whether there was a difference in NGF-induced p140TRK-A autophosphorylation between these cells, using TRK-A-transfected NIH 3T3 cells as a positive control. The cells were stimulated with NGF in the presence of orthovanadate and trk (Fig. 5, A and B), or tyrosine-phosphorylated proteins (Fig. 5C) were immunoprecipitated. Whereas autophosphorylated p140TRK-A easily could be detected in the NGF-treated 1:3 and 6:2 clones and in NIH 3T3-TRK-A cells, no apparent trk-A receptor autophosphorylation was found in either the wild-type cells or in the neo clone (Fig. 5, A and C). To estimate the trk-A protein levels in these clones, the filter in Fig. 5A was reanalyzed with an anti-pan-trk antibody. This revealed that all SH-SY5Y-derived clones, including the wild-type cells, had low trk protein levels, as compared to the NIH 3T3-TRK-A positive control (Fig. 5B). Although the p140TRK-A expression levels in the analyzed SH-SY5Y cell clones are at the limit of what can be detected, the 1:3 and 6:2 clones appear to express slightly more p140TRK-A than did the wild-type cells and the 9:1 neo clone.

The apparent lack of NGF-induced p140TRK-A autophosphorylation in wild-type SH-SY5Y cells might reflect suboptimal experimental conditions, preventing the detection of the autophosphorylated protein in these cells.
of an autophosphorylation signal in these cells. However, to
detect autophosphorylation in wild-type cells, we have un-
successfully tried a number of different pretreatment and
NGF incubation protocols, including 5-min NGF incubations
at varying temperatures (data not shown), and we are
inclined to believe that either the receptor number in SH-
SY5Y is too low to allow detection of a phosphorylated
receptor, or the receptor does not become autophos-
phorylated. A more sensitive measure of protein tyrosine kinase
receptor activation is to assay for phosphorylation of spec-
cific receptor substrates, and the overall NGF-induced pro-
tein tyrosine phosphorylation, therefore, was studied. Im-
munoprecipitation by anti-phosphotyrosine antibodies
followed by anti-phosphotyrosine Western blotting re-
vealed a slight increase in tyrosine-phosphorylated proteins
in NGF-stimulated SH-SY5Y wild-type cells. However, p140
^-A^- could not be detected, and compared to the
PDGF-evoked tyrosine phosphorylation, NGF induced a
minimal response (Fig. 6A). The experiment was repeated
with the 1:3 and 6:2 TRK-A-transfected clones, which
showed a substantial increase in protein tyrosine phosphor-
ylation after NGF stimulation (Fig. 6B). Finally, the tyrosine
phosphorylation of a specific substrate, Shc (24–26), was
not increased in NGF-treated SH-SY5Y wild-type cells,
whereas this was the case in the NGF-stimulated 6:2 TRK-A
clone (Fig. 6C). In these cells, NGF induced the highest
tyrosine phosphorylation of the M, 66,000 and M, 53,000
forms of Shc, whereas the M, 48,000 form only became
modestly phosphorylated.

The 1:3 and 6:2 Clones Differentiate Biochemically in
Response to NGF. The 1:3 and 6:2 clones responded well
to NGF both, in the presence of FCS (Fig. 3) and in serum-
free SHTE medium (Fig. 7, B-D, and data not shown).
The treated cells showed the morphological characteristics of in
vitro differentiated neuronal cells, i.e., long growth cone-
terminated processes with frequent varicosities, and
rounded cell bodies (Fig. 7, B-D). Treatment of clone 1:3
with NGF for more than a week revealed that the morpho-
dological differentiation continued to develop, although
the cells obviously did not stop proliferating (Fig. 7, C and D).
To verify this observation, wild-type cells and neo-
and TRK-A-transfected SH-SY5Y cells were stimulated
with NGF and pulsed with [3H]thymidine, after 2 days of serum
starvation in the presence of a low NGF (3 ng/ml) con-
tentration to avoid cell death in the TRK-A clones. Counting
of labeled nuclei revealed a comparatively high basal thymi-
dine incorporation, ranging from 21 to 27% labeled cells in
the four untreated clones (Table 1). These numbers are in
agreement with previous findings (12), showing that the
wild-type tumor cells cannot be completely growth arrested
by serum or growth factor starvation. NGF stimulation gave
an almost 2-fold increase in labeled nuclei in the TRK-A-
transfected cells, whereas the wild-type and the neo-trans-
fected cells did not respond to NGF by increased uptake of
thymidine (Table 1).

SH-SY5Y wild-type cells differentiate into a functional
sympathetic phenotype when treated with 16 nM TPA in
serum or growth factor-containing medium (12–14, 16, 27).
In such cells, transcription of the NPY gene, coding for a
peptide neurotransmitter, and the GAP-43 gene, coding for
an axonal growth cone protein, are induced. To test that
the basal properties of the SH-SY5Y cells with respect to pro-
hormon expression were unchanged after transfection and
subcloning, the 1:3 clone was stimulated with 16 nM
TPA in the presence of FCS. The 1:3 clone still responded
to TPA with differentiation, as judged by changes in morphol-
ygy (data not shown), and an increase in NPY and GAP-43
mRNA levels (Fig. 8). Treatment with NGF in FCS contain-
ing or in SHTE medium for 4 days also induced NPY and
GAP-43 expression in the 1:3 and 6:2 clone (Fig. 8 and data
not shown), which was not the case in NGF-treated wild-
type cells (16). The increase in GAP-43 mRNA levels was
of the same magnitude or higher in NGF as compared with
TPA/FCS-treated cells. Interestingly, TPA and NGF induced
similar levels of NPY expression in FCS containing medium,
whereas NGF was more potent under serum-free conditions
(Fig. 8).

A combined treatment of the SH-SY5Y wild-type cells
with IGF-I and bFGF induces the cells to mature morpho-
logically and biochemically, with no additional effect if
NGF is included (16). However, the 1:3 clone, treated with
a combination of these three factors, differentiated morpho-
logically (data not shown), and biochemically (induced expres-
sion of GAP-43 and NPY; Fig. 8). Furthermore, this
protocol induced the highest mRNA levels of the two neu-
rotransmitter marker genes of the tested differentiation protocols
(Fig. 8), suggesting that the expression of exogenous TRK-A
as such was not sufficient for a maximally differentiated
phenotype.
TRK-A Expression Increases in Differentiated Wild-Type SH-SYSY Cells. Since neuroblastomas are considered to be developmentally arrested embryonal tumors, low TRK-A expression in neuroblastoma cell lines could reflect an early stage of neuronal differentiation. TRK-A expression was, therefore, monitored in TPA/FCS-treated, sympathetically differentiating, SH-SYSY wild-type cells, which showed that the TRK-A mRNA levels increased with time, as did the expression of the neuronal marker gene GAP-43 (Fig. 9A). In cells treated for 24 h, there was a 8-fold increase in the TRK-A mRNA level, using GAPDH expression as reference. This should be compared to the TRK-A mRNA levels in the 1:3 and 6:2 TRK-A transfected cell lines, which by the same type of analysis of the data presented in Fig. 4B showed to be 20 and 10 times higher than in the wild-type SH-SYSY cells. With this limited difference in TRK-A expression, it appeared relevant to test whether the TPA/FCS-differentiated SH-SYSY cells could be affected by NGF stimulation. As reported earlier (12), NGF had no drastic effects on the differentiated SH-SYSY phenotype. However, there was a slight increase (2-fold) in c-fos expression after 30 min of NGF stimulation of these cells, whereas no induction was found in NGF-stimulated, non-treated wild-type SH-SYSY cells (Fig. 9B). This should be compared with the more than 20-fold induction of c-fos expression in the NGF-stimulated 1:3 clone shown in Fig. 4C. The slightly higher c-fos expression in the differentiated cells in comparison with non-treated cells was in accordance with previous findings (28, 29). The NGF-induced response in TPA-treated cells and in the 6:2 clone was further compared by analyzing the p140-Trk-A autophosphorylation status. Pan-trk immunoprecipitation, followed by Western blotting with anti-phosphotyrosine antisera, demonstrated receptor autophosphorylation in the 6:2 clone, whereas only a faint receptor signal was seen in the NGF-stimulated, TPA-treated SH-SYSY cells (Fig. 9C). The same filter was probed with an anti-pan-trk (Fig. 9D), followed by an anti-trk-A antisera (Fig. 9E), which showed small differences in p140-Trk-A levels between the TPA-treated cells and the 6:2 clone, although the p140-Trk-A signal was very weak (Fig. 9E). Thus, the p140-Trk-A expression levels appear to be similar in the 6:2 TRK-A-transfected and in the TPA/FCS-differentiated wild-type cells. The identity of the major, nonphosphorylated protein reproducibly recognized by the anti-trk-A and trk-A antisera (Figs. 5B, 9D, and 9E) has thus far not been revealed.

Discussion

Many neuroblastoma cell lines express the low affinity NGF receptor (4, 30), and here we show that they also express the TRK-A gene, coding for the high affinity NGF receptor. Consequently, cultured neuroblastoma cells often bind NGF both by high and low affinity, as exemplified by the SH-SYSY cells (4). However, these and other cultured neuroblastoma cells do not respond to NGF as evident by growth, survival, or differentiation (4, 12, 16). Introduction of an exogenous TRK-A gene into SH-SYSY and LA-N-5 neuroblastoma cells resulted in cells that were inducible to differentiation with NGF, a finding that could be explained either by a quantitative effect due to increased TRK-A expression and/or a qualitative effect due to introduction of a normal TRK-A gene coding for a functional protein. Furthermore, it appears unlikely that this NGF responsiveness could be explained by drastically increased p75 low affinity receptor expression as a result of the transfection and selection procedures, especially since also transiently transfected cells responded to NGF. Thus, the restoration of p140-Trk-A-coupled intracellular signal transduction pathway(s) by expression of an exogenous TRK-A gene offers a human NGF-responsive in vitro system and focuses atten-
mature phenotype and that the capacity for NGF-induced signaling is intact in most low-stage neuroblastomas. In contrast, high-stage tumors either lack or have low expression levels of these two receptor proteins, possibly due to an early differentiation arrest. As neuroblastoma cell lines are established from high-stage tumors, the increased TRK-A expression in differentiating SH-SYSY cells lends support to this conclusion.

SH-SYSY cells treated with nanomolar TPA concentrations in FCS containing medium is an established cell system to study neuronal sympathetic differentiation in vitro.
The hallmarks of the differentiated phenotype are morphological differentiation and formation of neurites terminated by growth cones; increased neurotransmitter synthesis, i.e., noradrenalin and NPY; high expression of a number of neuronal genes such as GAP-43, tyrosine hydroxylase, synaptophysin, synaptic vesicle protein-2, and secretogranin II; and down-regulation of genes like chromogranin A, c-myc, and N-myc (12, 13, 27, 31–34). Functionally, the cells express acetylcholine receptors and acquire an increased resting membrane potential, which is depolarized by aceetylcholine followed by release of stored transmitters (35). Taken together, the differentiated SH-SYSY phenotype is similar to that of noradrenergic sympathetic neurons. Although this maturation is a PKC-dependent process (15), TPA alone cannot induce SH-SYSY cells to differentiate in serum- or growth factor-free medium (12). However, several tested growth factors can replace FCS and induce a differentiated phenotype together with TPA (13, 14, 16). None of these growth factors alone can differentiate SH-SYSY cells, whereas a combination of bFGF and IGF-I will (16). This is of relevance for the analysis of the sympathetic phenotype of differentiated SH-SYSY cells, since IGFs have been implicated, and bFGF has been shown to be involved in sympathetic neuronal differentiation by up-regulating NGF binding and responsiveness in v-myc-immortalized sympathetic adrenal stem cells (36–39). The increase in expression of the TRK-A gene during conditions in which the SH-SYSY cells differentiate neuronomally, as shown here, is consistent with the comparatively late expression of TRK-A during development of human fetal sympathetic ganglia4 and suggests that the regulation of this gene is normal in SH-SYSY cells and is related to the stage of differentiation of these cells. It is thus conceivable that induced expression of TRK-A is an important part of the differentiation program in these cells but is not necessarily required during the onset of differentiation.

The failure of the wild-type SH-SYSY cells to respond to NGF treatment might be a consequence of low NGF receptor expression. The most simple interpretation of the NGF-

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Fig. 9. Induction of TRK-A expression and NGF responsiveness in differentiating SH-SYSY wild-type cells. A, Northern blot analysis of TRK-A, GAP-43, and GAPDH transcripts. Cells were treated with 16 nM TPA in FCS-containing medium and were harvested at the indicated time points. Total RNA (15 μg/lane) was analyzed. B, c-fos and GAPDH transcript levels in control (FCS, 4 days) and TPA-differentiated (TPA, 4 days) SH-SYSY cells with or without 30-min treatment with 100 ng/ml NGF. The relative c-fos transcript levels were quantitated with GAPDH expression as reference, using phosphorimaging technique. C, D, and E, expression of p140TRK A protein and NGF-dependent receptor autophosphorylation in differentiating wild-type and TRK-A-transfected (6:2) SH-SYSY cells. SH-SYSY wild-type cells were cultured for 48 h in medium containing FCS (C) or FCS + 16 nM TPA (TPA), and the TRK-A-transfected clones 6:2 were kept in FCS-containing medium. The cultures were then incubated with (+) or without (−) 100 ng/ml NGF for 5 min at 25°C in the presence of 0.1 mM orthovanadate. Proteins were immunoprecipitated (IP) with anti-pan-trk (Trk 203) antiserum, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. C, immunoprecipitated tyrosine-phosphorylated proteins were detected by immunoblotting (IB) using the 4G10 anti-phosphotyrosine mAb (α-PY). The membrane was subsequently stripped and probed with an anti-pan-trk (Trk 203) antiserum (D) and after a second stripping procedure with the anti-trk-A (763) antiserum (E). Immunoreactivity was visualized using ECL technique in C and D and alkaline phosphatase-coupled antibodies in E.

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induced differentiation of TRK-A-transfected neuroblastoma cells reported here would be that these cells have acquired sufficiently high p410ktrkA levels to be able to transduce a sustained or stronger differentiation signal into the cell and thereby overcome the differentiation arrest. Many of our data support this interpretation; the transfected SH-SYSY clones had a higher TRK-A expression in comparison to wild-type cells, which was reflected in readily detectable p140trkA autophosphorylation, induced protein tyrosine phosphorylation in general, and phosphorylation of Shc in particular, and induced c-fos expression after NGF treatment of the TRK-A transfectants.

However, the lack of detectable p140trkA autophosphorylation activity and c-fos induction in wild-type SH-SYSY cells stimulated with NGF may be difficult to explain solely by low TRK-A expression, since the difference in expression between wild-type and transfected cells at the protein level was not striking. Thus, we could not repeat the previously published result, where NGF induces p140trkA autophosphorylation in SH-SYSY cells (17), although a weak NGF-induced receptor kinase activity was detected when total protein tyrosine phosphorylation was analyzed (Fig. 6, A and B). There are several conceivable explanations for this discrepancy, one being that the two laboratories are working with different SH-SYSY subclones. The TRK-A clones analyzed here showed a good correlation between the amount of exogenous TRK-A transcript and c-fos induction. A gradual morphological response was also seen, and the low level-expressing clone 2:1 showed a morphological response clearly distinguishable from NGF-treated wild-type cells. Furthermore, TPA/FCS-differentiated wild-type SH-SYSY cells had TRK-A mRNA levels only slightly lower (2-fold) than that of the transfected 1:3 clone and comparable with that of the 6:2 clone. Still, the differentiated cells remained virtually unresponsive to NGF, as judged by the lack of effect on the differentiated phenotype (morphology and marker gene expression; data not shown; Refs. 12 and 16), the c-fos gene expression, and most importantly, the minute NGF-induced induction of the p140trkA autophosphorylation. The possibility for a potentiated differentiation response is indicated in Fig. 8, where a combination of growth factors gave a stronger induction of neuronal marker genes as compared with TPA/FCS-treated cells. The apparently higher efficiency of exogenous TRK-A to mediate an NGF signal than the endogenous TRK-A (e.g., compare c-fos expression in NGF-induced wild-type and clone 6:2 cells in Fig. 4C) suggests a qualitative difference between the protein products. Thus, an alternative interpretation of the presented results, and of previously reported data, would be that many neuroblastoma cell lines have a defect or truncated NGF-induced signaling rather than, or in addition to, a low expression of a functional NGF receptor complex. However, as the c-fos expression was slightly induced in the differentiated wild-type cells and a weak receptor autophosphorylation could be induced, we conclude that the trkA receptor complex in these cells has some signaling capacity. Present data do not discriminate between our two suggested mechanisms for the restored NGF responsiveness of TRK-A-transfected SH-SYSY cells, but cloning and sequence characterization work in progress of SH-SYSY wild-type TRK-A cDNA might give conclusive information.

With the NGF-responsive TRK-A-transfectant clones presented here, a cell line that allows the study of NGF-induced signal transduction mechanisms in a human cell system has been established. This appears to be important in understanding the molecular defects responsible for the childhood neuroblastoma and might be relevant also for other neurological diseases. In the case of SH-SYSY cells, lack of trk-A signaling is probably not the only defect, since the NGF-treated TRK-A-transfected cells still proliferate. Similarly, bFGF- and IGF-I-differentiated wild-type SH-SYSY cells do also continue to proliferate (16), whereas phorbol ester treatment in combination with growth factors induce differentiation and growth arrest (12–14). However, the TRK-A-transfected HTLA230 neuroblastoma cell lines do differentiate and stop dividing when treated with NGF (11). Although the direct comparison has not been made, the TRK-A expression levels might be higher in these transfectants than in the SH-SYSY clones reported here, which could explain the different results. It is not obvious which NGF receptor level represents the physiological situation, but vast overexpression of the EGF receptor and the insulin receptor, respectively, in PC-12 cells results in transfected cells that differentiate with either EGF or insulin (40, 41). This indicates that overexpression of any protein tyrosine kinase receptor might result in cellular responses that are not physiological. With this in mind, we suggest that the blocked differentiation of SH-SYSY is related to an impairment of the p140trkA signaling, which can be restored by introduction of a normal TRK-A gene expressed at modest levels, whereas the ligand-induced growth arrest might be a quantitative event requiring high expression of the TRK-A gene. However, during sympathetic neuronal development in vitro, TRK-A expression is preceded by growth arrest, suggesting that differentiation and growth arrest are two distinctly regulated phenomena in nontransformed neuroblasts (37, 42).

Materials and Methods

Cell Cultures. SH-SYSY (Ref. 43; kindly provided by Dr. June Biedler, Sloan Kettering Institute, New York, NY), IMR-32, U-2674, LA-N-1, LA-N-2, and LA-N-5 (44) neuroblastoma cells were grown as described (45). NIH 3T3 cells, transfected with TRK-A (NIH 3T3-TRK-A; Ref. 17; kindly provided by Dr. David R. Kaplan, Frederick Cancer Research and Development Center, Frederick, MD) and PC-12 cells were cultured according to Kaplan et al. (17). For serum-free conditions, cells were cultured in SHTE medium (RPMI 1640 containing 30 nm selenium, 10 nm hydrocortisone, 30 mg/ml transferrin, and 10 nm β-estradiol). In these experiments, cells were plated for 1 day in serum-containing medium. They were then washed with RPMI 1640 before changing to SHTE medium, with or without further addition of growth factors and/or 16 nm TPA. Except when explicitly indicated, the cells were cultured for 4 days before harvest. The following growth factors were used: human recombinant IGF-1 (Pharmacia, Stockholm, Sweden), human recombinant bFGF, and mouse NGF 2.55 (Promega, Madison, WI).

Thymidine Incorporation. Cells (5 × 10⁴) of SH-SYSY wild-type neocline 9:1 and the TRK-A clones 1:3 and 6:2 were plated on 3.5-cm dishes and cultured 4 days in Eagle’s medium, rinsed twice in PBS, and starved for 2 days in RPMI 1640 with 3 mg/ml of NGF. Fresh RPMI 1640 was then added, and NGF (100 ng/ml) stimulation was performed for 24 h with 10 μCi/ml of [3H]thymidine included during the final 1 h. The cells were fixed in methanol-acetic acid (3:1), and the plates were covered with Kodak NTB2 emulsion
diluted 1:1 in water and exposed for 7 days at 4℃. After development, the cells were counterstained.

**Transient and Stable Transfection of Neuroblastoma Cells with the TRK-A Proto-Oncogene.** The plasmid pDM69, containing the proto-TRK cDNA inserted into pMEXneo (22), is driven by the Moloney murine sarcoma virus long-terminal repeat, was kindly provided by Dr. Mariano Barbacid (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). For transient transfections, 2 × 10⁵ SH-SY5Y and LA-N-5 cells were plated in 10% FCS-containing Eagle’s and RPMI 1640, respectively, 24 h prior to the transfection. The cells were transfected with 10 μg of the TRK-A plasmid and lipofectin (GIBCO) in serum-containing medium. Four h after transfection, the cultures were changed to fresh serum-containing medium. Forty-eight h after transfection, the cells were stimulated with 100 ng/ml of NGF and cultured for 3 days before they were photographed.

For stable transfection of SH-SY5Y cells, 2 × 10⁵ cells were plated in FCS-containing Eagle’s medium for 24 h, and 3 h prior to transfection, the cultures received fresh medium. Plasmid pDM-69 DNA (14.5 μg), diluted in 1 ml Tris-HCl and 0.1 mM EDTA buffer (pH 8.0) to a volume of 440 μl, was mixed with 62 μl 2 mM CaCl₂, and DNA was precipitated by dropwise addition into 500 μl of 280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄, and 12 mM dextrose in 50 mM HEPES for 30 s during constant mixing. The DNA solutions were incubated for 30 min at room temperature, followed by vortexing before dropwise addition to the cultures. As control, SH-SY5Y-cells were also transfected with RSV neo in the same way as described for the TRK-A transfection. Sixteen h after transfection, the cultures were washed with cold PBS, and fresh serum-containing Eagle’s medium was added. Forty-eight h after transfection, the medium was changed again with the addition of 600 μg/ml of G-418 sulfate, Geneticin (GIBCO). The cultures received fresh serum-containing medium complemented with G-418 twice a week.

**Northern Blot Analysis.** Total RNA was prepared from frozen cell pellets using guanidine-isothio-cyanate and phenol according to Chomczynski and Sacchi (46). RNA (15 μg) was separated on formaldehyde agarose gels according to Sambrook et al. (47). The RNA was blotted onto Hybond-C extra filters (Amersham International, Amersham, Bucks, United Kingdom), which were hybridized with the following cDNA probes: NPY (Ref. 48; kindly provided by Dr C. Minth), GAPDH (Ref. 49; kindly provided by Dr. R. Wu), GAP-43 (34), TRK-A (Ref. 22; kindly provided by Dr. M. Barbacid), and human c-fos (kindly provided by Dr. T. Curran). The probes were labeled using Amersham’s megaprime system according to the manufacturer’s protocol. Hybridizing RNA was visualized by autoradiography using Kodak X-Omat film, and RNA levels were quantified and related to the corresponding GAPDH mRNA level by phosphorimage analysis using Fuji BAS 2000 equipment (Fuji, Inc., Tokyo, Japan).

**Immunoprecipitations and Western Blot Analysis.** For immunoprecipitation of trk-A protein, cells were lysed for 20 min in 1% NP40, 10% glycerol, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1 mM Na₂VO₄, 100 units/ml transylol (Sigma Chemical Co.), 10 μg/ml leupeptin (Sigma), and 1 mM phenylmethylsulfonyl fluoride (Sigma) and precipitated with the Trk 203 anti-pan-trk rabbit antiserum (kindly provided by Dr. D. Kaplan). The amount of protein in the supernatant was determined according to a modified Lowry procedure (50), and aliquots of 1 μg of protein were resolved under reducing conditions with 7.5% SDS-PAGE (51). The proteins were electrophoretically blotted onto a nitrocellulose membrane. For detection of tyrosine kinase activity, cells were serum-starved overnight and pretreated with 100 μM Na₂VO₄ for 30 min and ligands as described in the figure legends. Cells were harvested and lysed in 1% Triton X-100, 10 mM Tris-HCl (pH 7.6), 50 mM NaCl, 30 mM sodium PPi, 50 mM NaF, 5 mM EDTA, 1 mM Na₂VO₄, and protease inhibitors, as above, for data in Fig. 5C, and in 0.5% Triton X-100, 0.5% sodium deoxycholate, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1 mM Na₂VO₄, and protease inhibitors for data in Fig. 6. Tyrosine-phosphorylated proteins were immunoprecipitated using PY20-agarose (Transduction Laboratories, Lexington, KY; Fig. 6) or PY 20 and a rabbit antimouse IgG, together with fixed Staphylococcus aureus (Fig. 5C). Immunoprecipitated proteins were separated by reducing SDS-PAGE and transferred to nitrocellulose. The membranes were blocked either in 5% nonfat dry milk (for shc proteins) or in 2% BSA (for trk-A protein and phosphotyrosine). The nitrocellulose membranes were probed with the following antibodies and sera: anti-pan-trk (C-14) antiserum (Santa Cruz Biotechnol. Inc., Santa Cruz, CA), anti-trk-A (763) antiserum (Santa Cruz), the PY 20 mouse monoclonal antiphosphotyrosine antibody, the 4G10 mouse monoclonal antiphosphotyrosine antibody (kindly provided by Dr. D. Kaplan), and a rabbit anti-shc antiserum (Transduction Laboratories). Proteins were visualized by either alkaline phosphatase (Bio-Rad Laboratory, Inc., Hercules, CA) or ECL technique (Amersham) as described in the figure legends. Where indicated, the bound antibodies were stripped of the membrane by incubation in 2% SDS, 100 mM β-mercaptoethanol, and 62.5 mM Tris-HCl (pH 6.7) at 70℃ for 1 h.

**References**