Autophosphorylation of Activation Loop Tyrosines Regulates Signaling by the TRK Nerve Growth Factor Receptor*

Matthew E. Cunningham‡, Robert M. Stephens‡, David R. Kaplan‡, and Lloyd A. Greene‡

From the ¤Department of Pathology and Center of Neurobiology and Behavior, College of Physicians and Surgeons, Columbia University, New York, New York 10032, the §§ABL-Base Research Program, Frederick, Maryland 21702, and the ¶Montreal Neurological Institute, McGill University, Montreal, Quebec H3A 2B4, Canada.

Many receptor tyrosine kinases possess an “activation loop” containing three similarly placed tyrosine autophosphorylation sites. To examine their roles in the TRK NGF receptor, these residues (Tyr-670, Tyr-674, and Tyr-675) were mutated singly and in all combinations to phenylalanine and stably expressed in Trk-deficient PC12mn5 cells. All mutant receptors showed significantly diminished nerve growth factor (NGF)-stimulated autophosphorylation, indicating impaired catalytic activity. NGF-induced neurite outgrowth exhibited dose-responsive behavior when transfectants were compared by relative receptor expression and exhibited a functional hierarchy: wild type > Y670F Y674F >> Y675F > YY670/74FF >> YY745FF > YY670/674/675FF. NGF-induced tyrosine phosphorylation of Shc and PLC-1, Src homology and collagen; PLC, phospholipase C γ-1; Shc, Src homology and collagen; PLC, phospholipase C γ-1; SNT (9). Activation of these signal transduction molecules culminates in downstream signaling events, including the induction of immediate early and late genes (10, 11).

As with other ligands and their cognate RTKs (1, 2), NGF appears to initiate signaling through TrkA by the sequential steps of dimerization, trans-autophosphorylation on tyrosine residues and kinase activation, followed by binding and tyrosine phosphorylation of substrates (9, 12, 13). In vitro studies indicate that TRKA autophosphorylates at tyrosines 490, 670, 674, 675, and 785 (14, 15). To analyze the significance of these phosphorylation events in NGF signaling, we have introduced TRK A receptors modified at these residues into the mutant PC12 cell line, PC12mn5, that expresses no detectable endogenous TrkA (16, 17). This analysis reveals that phosphoryrosines 490 and 785 are required for binding and activation of Shc and PLC γ-1, respectively (14, 15, 18). The three remaining tyrosine autophosphorylation sites, Tyr-670, Tyr-674, and Tyr-675, are contained within the kinase domain of the receptor and are within a sequence motif that is not typical for an SH2 protein binding site (19) or for other protein-protein interactions (20, 21). Comparison of this sequence motif with other protein kinases reveals homology with a number of RTKs (22–27). Studies carried out largely with wild type and mutated insulin, IGF and c-Met/HGF receptors indicate that these sites are important for initial ligand-induced autophosphorylation and that they are required for efficient signal transduction and promotion of biological responses (28–36).

A physical model that begins to account for the roles of the three conserved kinase domain tyrosines is provided by the crystal structure of the non-phosphorylated insulin receptor kinase domain (37). This structure indicates that the three kinase domain tyrosines lie near the catalytic core of the kinase within an “activation loop” that prevents binding of ATP as well as physical approximation of the amino and carboxyl lobes of the kinase domain. The activation loop appears to be stabilized in this inhibitory conformation by interactions of Tyr-1162 (the homologue of Tyr-674 in TRK) with residues near the catalytic core of the kinase. This interaction also inhibits basal kinase activity by acting as a pseudosubstrate. Furthermore, it

For many growth factors, ligand-mediated activation of receptor tyrosine kinases (RTKs) is the first essential step leading to biological responses (1, 2). Nerve growth factor (NGF) and other neurotrophins bind to and activate particular members of the Trk receptor tyrosine kinase family (3, 4); NGF binds to and activates TRKA (5, 6). The pheochromocytoma-derived PC12 cell line (7) has been highly useful for studying NGF signal transduction and its molecular mechanisms of action. PC12 cells respond to NGF by undergoing neuronal differentiation, survival in serum-free medium, cell hypertrophy, and regulation of numerous biochemical markers (8). NGF induces rapid tyrosine phosphorylation of TrkA and consequent phosphorylation and activation of signal transduction components including Shc, extracellular signal-regulated kinases (ERKs), phosphatidylinositol 3′-kinase (PI3′K), phospholipase C γ-1 (PLC-γ), and Shc (29, 30). Activation of these signal transduction molecules culminates in downstream signaling events, including the induction of immediate early and late genes (10, 11).

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was suggested that ligand-promoted phosphorylation of activation loop tyrosines induces movement of the loop away from the catalytic core to permit efficient kinase activation.

Although studies on activation loop tyrosines in several systems collectively suggest that they function to regulate RTK activation, the roles of individual tyrosines are unclear and are subject to some inconsistency within the literature (29, 38–45). For example, tyrosine to phenylalanine mutation in the insulin receptor at the residue equivalent to TRK Tyr-670 in one case was reported to have no effect on in vitro kinase activity (35), whereas in several other studies the same mutation was reported to reduce in vitro kinase activity by approximately 80% (39) or more (41). It also appears that there may be differences between the roles of each of the activation loop tyrosines in the various receptors (34, 38, 39, 44). Moreover, past studies have indicated that mutation of individual activation loop tyrosines may selectively abolish some receptor-mediated biological responses while sparing others, although the mechanistic basis for these observations is unknown. In the present study, we have examined the activation loop tyrosines in the TRKA receptor with respect to their roles in regulating signaling and biological responses to NGF. To achieve this, we mutated the tyrosines singly and in all combinations and have stably expressed receptors containing these mutations in PC12nnr5 cells at various levels of expression. Our findings are consistent with a major role for tyrosines 670, 674, and 675, not only in regulating overall catalytic activity of the receptor but also in governing site-specific receptor autophosphorylation.

**MATERIALS AND METHODS**

**Construction of TRKA Mutants and Production of pLNC(TRKA)s—**

TRKA mutants were constructed using single strand mutagenesis (Bio-Rad). Briefly, full-length TRKA was used to generate antisense template, and sense oligonucleotides were used to introduce the point mutations. For double and triple mutations, oligonucleotides spanning all mutated sites were used. In the multiple site mutation constructions, restriction site cassettes containing each zone mutation (e.g. Y490F, Y751F, etc.) were shuttled between the autophosphorylation loop backbone and then sequenced for confirmation. The mutant TRKs were then cloned into the pLNCX5 retroviral expression vector using HindIII and XhoI. The resulting constructs were then transfected into 293 cells (46), and cells resistant to G418 (500 µg/ml, Life Technologies, Inc.) were selected, and the total population of cells was propagated in G418 (200 µg/ml). Cellular mutantant from these cells was used to infect PC12nnr5 cells (described below).

**Cell Culture and NGF Treatment—**

PC12 cells and PC12nnr5-derived mutants were grown on collagen-coated tissue culture plates in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated donor horse and 5% fetal bovine serum (both from JRH Biosciences), as described previously (7). Retroviral infections were carried out by adding 0.2–1 ml of virus-containing supernatant, recovered from packaging cell lines to 50% confluent PC12nnr5 cell cultures (47). Transfections (used in generating cell lines bearing expression vectors containing Y490F, Y751F, and Y785F superposed on triple activation loop mutants) were carried out by electroporation as described previously (47, 49). Immunoprecipitated samples were washed three times in TNGT (20 mM Tris, pH 7.5, 10 mM NaCl, 10 µg/ml aprotinin, 5 mM ZnCl2, 2 mM phenylmethylsulfonyl fluoride, and 1 µg/ml leupeptin), and the lysates were clarified by ultracentrifugation after a 15-min incubation on ice, all as described previously (48). Samples normalized for total protein content were subjected to immunoprecipitation with the indicated antibodies as described previously (15, 48, 49). Protein A-Sepharose beads were from Pharmacia Biotech Inc., and antiserum against She and PLCγ-1 were from UBI. Anti-pan Trk (203) and anti-phosphotyrosine (4G10) were used as described previously (47, 48). Immunoprecipitated samples were washed three times in TNGT (20 mM Tris, pH 7.5, 10% glycerol, 0.1% Triton X-100, and 150 mM NaCl) and subjected to SDS-polyacrylamide gel electrophoresis followed by electrophotore transfer to nitrocellulose (48). Western blotting was conducted with the indicated antibodies using the protocol of Amersham Life Sciences, Inc., followed by visualization by enhanced chemiluminescence (Amersham Corp.). For quantification, data were scanned and analyzed using NIH Scion Image v1.55 software. Blots were reprobed with antibody after stripping in 2% SDS, 100 mM 2-mercaptoethanol, and 62.5 mM Tris, pH 6.8, and rocking at room temperature for ≥16 h.

ERK tyrosine phosphorylation was assessed by subjecting clarified lysates (150 µg of protein) to SDS-polyacrylamide gel electrophoresis and Western blotting with anti-pan-Trk antibodies. Anti-ERK C-16 antibody was from Santa Cruz Biotechnology. PI3K activity and SNT tyrosine phosphorylation were assessed as described previously (49–51). Measurements of radiolabeled products in the PI3-K assay were made using a Molecular Dynamics PhosphorImager.

**Northern Blotting—**

Cells treated with NGF for 0, 30, 60, and 120 min were washed three times with ice-cold phosphate-buffered saline, and total RNA was extracted using Tri-Reagent (MRC). Purified RNA was quantified, and 20-µg samples were subjected to Northern blotting as described previously (14, 49, 52). Blots were UV cross-linked and hybridized with probes to c-fos, c-jun, tis 1, tis 8, and tis 11 as described previously (49, 53).

**RESULTS**

**Generation of Cell Lines Stably Expressing TRKs Mutated at Activation Loop Autophosphorylation Sites—**

To study the biological importance of the tyrosine autophosphorylation sites in the activation loop of TRK, constructs of the human receptor were mutated from tyrosine to phenylalanine at residues 670, 674, and 675, both singly and in all combinations. These and wild type (WT) TRK were cloned into the pLNCX5 retroviral expression vector using HindIII and XhoI. The result constructs were then transfected into 293 cells (46), and cells resistant to G418 (500 µg/ml, Life Technologies, Inc.) were selected, and the total population of cells was propagated in G418 (200 µg/ml). Cellular mutantant from these cells was used to infect PC12nnr5 cells (described below).

**Correlation between Mutant Receptor Expression Levels and NGF-promoted Neuritogenesis—**

Cell lines representative for a spectrum of WT and mutant TRK levels were chosen for each of the constructs on the basis of analyses carried out by receptor immunoprecipitated followed by quantitative immunoblotting with Trk antiserum (Table I). Expression in each line was compared with that of the PC12nnr5-T14 cell line that expresses wild type TRK at levels approximately 3–5-fold higher than PC12 cells (49, 52). Expression of the mutant and wild type TRKs ranged from equal to that of PC12nnr5-T14 wild type cell cultures to 20–50-fold higher. These cell lines were then assessed for NGF-induced neuritogenesis. In general, neurite outgrowth in the cell lines bearing mutant TRKs reached maximal levels by 8 h after NGF exposure (data not shown), which is comparable with previous observations for PC12nnr5-T14 cells and other TRK over-expressed PC12nnr5 cell lines (15, 52, 55).

Comparison of the maximal neuritogenic responses in cell lines expressing comparable levels of each of the different TRK mutants revealed dramatic differences. For example, following a vertical path in Fig. 1D at the level of the TRK expression corresponding to 10-fold that in PC12nnr5-T14 cells shows that none of the multiply-mutated TRKs confer NGF-dependent...
neuritogenesis, whereas the Y675F receptors confer responsiveness in 10–15% of cells, and Y670F and Y674F receptors confer responsiveness in 50% of cells. Moreover, when neuritogenic extent was correlated over a range of receptor expression levels, dose-response relationships became apparent (Fig. 1). These plots revealed several important points. First, receptor expression levels significantly affect biological responsiveness (especially evident for the WT, Y670F, and Y674F TRKs) and therefore must be considered when interpreting assessed phenotypes. Second, each of the activation loop mutations impairs receptor function to at least some degree. This is demonstrated by the shifts in dose-responsiveness to NGF for cells expressing mutant TRKs as compared with cells expressing wild type TRK (Fig. 1, D and E). Even those mutant TRKs conferring the greatest neurite outgrowth responses (i.e. Y670F and Y674F) had to be expressed at levels 3–5-fold higher than present in PC12nnr5-T14 cells to achieve significant neuritogenesis. Third, there exists a hierarchy for restoration of neuritogenesis as a function of receptor over-expression in the various TRKs: WT > Y670F ≈ Y674F >> Y675F >> YY670/674FF ≈ YY670/675FF >> YY674/675FFF ≈ YY670/674/675FFF. Overall, the degree of rescue achievable shows that the mutants fall into three general groupings (Fig. 1D). The WT, and Y670F and Y674F TRKs are each capable of mediating near-complete levels of neuritogenesis, although the two mutant TRKs require higher expression to achieve this. The second group of receptors (Y675F, YY670/674FF, and YY670/675FFF), even when greatly over-expressed, mediate only partial restoration of neurite outgrowth. An important point is that the degree of impairment achieved by the Y675F mutation is much greater than that caused by the other two single mutations, Y670F and Y674F. The third group includes YY674/675FFF and YY670/674/675FFF. These lack the ability to mediate neuritogenesis, irrespective of expression level. Collectively, these observations indicate that TRKs mutated at different activation loop tyrosines have differing capacities to functionally rescue NGF-dependent responses. This is due to both the type of mutation assessed and the level to which it is expressed.

**NGF-inducible Tyrosine Autophosphorylation of Activation Loop Mutant TRK Receptors and Assessment of NGF-induced Signaling through She, the ERKs, SNT, and Immediate Early Genes**—Our neuritogenesis studies revealed that for partially compromised receptors, the expression levels of TRK receptors can significantly influence the degree to which they mediate biological responses. To control for this, we therefore assessed additional parameters in cell lines expressing comparable levels of different mutant receptors, as well as in cell lines expressing different levels of each individual TRK mutant. Two cell lines were selected for each of the constructs, one expressing receptors at approximately 5-fold, and the second at approximately 10–25-fold higher than expressed by PC12nnr5-T14 cells. Cell lines with TRK expressed at lower levels were not assessed for biochemical responses, due to their lack of neuritogenic responses (Fig. 1).

To assess NGF-promoted Trk tyrosine autophosphorylation, cultures were treated with or without 100 ng/ml NGF for 5 min. Lysates were normalized for protein content, subjected to immunoprecipitation with Trk antibody, and immunoprecipitates subjected to Western blotting and sequentially probed with anti-phosphotyrosine and anti-Trk (Fig. 2). All of the TRK mutants showed NGF-inducible tyrosine phosphorylation, including those in which all three of the activation loop tyrosines were replaced with phenylalanine (Fig. 2A). However, while autophosphorylation was evident with all of the mutants, it was clearly reduced as compared with wild type Trk (as judged by qualitative ratios of anti-phosphotyrosine to anti-Trk signals, even after taking into consideration the numbers of mutated tyrosines). Moreover, the deficits in NGF-induced autophosphorylation of the TRK mutants were not rescueable by receptor over-expression (Fig. 2B) nor were they attributable to altered kinetics of activation when assessed over a time course of several hours (data not shown). Furthermore, the levels of
receptor autophosphorylation did not correlate well with the capacity of the TRK mutants to mediate neurite outgrowth. For instance, although the single mutants (Y670F, Y674F, and Y675F) showed comparable levels of NGF-stimulated TRK tyrosine autophosphorylation, as noted above, TRK Y675F was much less effective in mediating neuritogenesis.

Trk activation by NGF in PC12 cells and PC12nnr5 cells expressing wild type and mutated TRKs. NGF-promoted neuritogenesis was assessed as described under "Materials and Methods" for PC12nnr5 cells expressing the indicated relative levels of wild type and mutated TRK receptors. The values reported are averages (± S.E.) derived from three independent experiments and were determined at a 1-week time point. The identity of the various constructs are indicated in A–D and the particular lines are listed in Table I. E, is included to aid in interpretation of D at TRK expression levels equal to or below 10-fold that found in PC12nnr5-T14 cells. A, □, wild type; ○, Y670F; △, Y675F; B, ◦, Y674F; □, YY670/674FF; ◊, YY670/674/675FFF. C, ●, YY670/675FF; ●, YY674/675FF; D, □, wild type; ○, Y670F; ◦, Y674F; △, Y675F; ◊, YY670/674FF; ●, YY670/675FF; ◊, YY674/675FF; ○, YY670/674/675FFF. E, □, wild type; ○, Y670F; ◦, Y674F; △, Y675F; ◊, YY670/674FF; ●, YY670/675FF; ◊, YY674/675FF; ○, YY670/674/675FFF.
dependent Shc tyrosine phosphorylation (15, 60). ERK activation is downstream of Shc and Ras activation and has been implicated as a necessary component of the mechanism by which NGF promotes neurite outgrowth in PC12 cells (15, 49, 58, 59, 61–67). Immunoprecipitation and Western immunoblotting were used to assess NGF-promoted Shc tyrosine phosphorylation at a 5-min time point in cell lines expressing wild type and activation loop mutants of TRK. Observations generally correlated with those found for TRK autophosphorylation and neuritogenesis in that single mutants were significantly more functional than combination mutants and that even single mutants required over-expression (as compared with WT receptors) to yield significant Shc phosphorylation (Fig. 3 A). In addition, as anticipated, lines with 10–25-fold over-expression showed greater activation than those with 5-fold over-expression (data not shown). The one case in which Shc phosphorylation and neuritogenesis potential did not correlate well was for the Y675F mutant which was about equal in activity to Y670F and Y674F but significantly less effective in mediating neurite outgrowth.

Studies to assess NGF-promoted tyrosine phosphorylation of the ERK kinases in lines with WT and mutant TRKs were carried out at the 5-min time point by subjecting cell lysates to Western immunoblotting. NGF-stimulated ERK tyrosine phosphorylation signal intensities closely paralleled the neuritogenic behavior of the lines (Figs. 1 and 3B). A time course study (up to 10 h) with lines expressing the singly mutated receptors revealed that the Y675F construct showed both lower signal intensity and significantly shorter signal duration when compared with WT, Y670F, and Y674F TRKs (data not shown). This further supports the correlation between neuritogenic activities of the receptors and capacity to stimulate ERK tyrosine phosphorylation.

PC12 cells, or PC12nnr5 cells expressing WT TRK, undergo NGF-dependent tyrosine phosphorylation of the p13 suc1 agaose associating tyrosine-phosphorylated nuclear localized protein (SNT), which appears to be a differentiation-specific signaling molecule in these cells (49, 51). A recent study (49) further supports a role for SNT in PC12 cell neuronal differentiation by establishing that deletion of a conserved juxtamembrane motif (KFG) in TRK selectively renders it incapable of activating the pathway leading to phosphorylation of SNT and that PC12nnr5 cells expressing these mutant receptors are unable to promote neuritogenesis in response to NGF. Although SNT phosphorylation requires the KFG motif (49), it is not dependent on phosphorylation of Tyr-490 or Tyr-785. To determine if the activation loop mutations in TRK affect the NGF-induced tyrosine phosphorylation of SNT, cell lines expressing wild type and activation loop mutant receptors were assessed for this parameter both before and after treatment with NGF for 5 min. As illustrated in Fig. 4, all activation loop mutations impair SNT tyrosine phosphorylation in response to NGF, and very high over-expression of mutant receptors (e.g., YY670/674FF) can rescue NGF-promoted SNT phosphorylation to levels near those observed in wild type PC12 cells. Comparison of these data with those in Fig. 1 reveals that SNT phosphorylation was detectable in all lines capable of NGF-stimulated neurite outgrowth and was undetectable in lines incapable of neuritogenesis. This correlation is consistent with a required role for SNT phosphorylation in promotion of neurite outgrowth. In addition, these findings indicate that although SNT tyrosine phosphorylation does not require autophosphorylation of TRK Tyr-490 and Tyr-785, it is dependent on autophosphorylation of the activation loop tyrosines.

Stimulation of Trk and downstream signaling pathways by NGF promotes the induction of a number of immediate early
genes (68, 69). To determine the effects of the activation loop Trk mutations and consequent alterations in intracellular signaling on induction of these genes, cell lines expressing wild type or mutant TRKs were treated with NGF for 0, 30, 60, and 120 min and assessed by Northern blotting for levels of c-fos, c-jun, tis 1 (NGFI-B), tis 8 (NGFI-A), and tis 11 mRNA. These experiments revealed that the extent of immediate early gene induction generally paralleled neuritogenic potential and ERK activation. Thus, at over-expression levels 5–10-fold that of PC12nnr5-T14 cells, the single mutants all exhibited NGF-promoted levels of gene inductions comparable with those of PC12nnr5-T14 cells; the YY670/674FF and YY670/675FF bearing cell lines showed induction only when the receptors were over-expressed by 10–20-fold relative to the level of TRK expressed in PC12nnr5-T14 cells; the YY674/675FF lines do not respond unless expressed to levels over 30-fold higher than PC12nnr5-T14 cells; and the YYY670/674/675FFF lines that we have assessed had no detectable responses. The one anomaly was the YY675F lines that exhibited immediate early gene responses similar to those of lines with the other single activation loop mutations but which showed relatively poor neuritogenesis.

**NGF-mediated Tyrosine Phosphorylation of PLC\_γ-1 Is Abrogated in Cells Expressing TRK Activation Loop Mutant Receptors—**Autophosphorylation of TRK at Tyr-785 results in binding, tyrosine phosphorylation, and activation of PLC\_γ-1 (14, 15, 18, 70). To assess the effect of activation loop mutations on NGF-promoted tyrosine phosphorylation of PLC\_γ-1, lysates were prepared from cells expressing wild type Trk (PC12, PC12nnr5-T14 and PC12nnr5-WT.13) and NGF-treated cells as described under “Materials and Methods.” PLC\_γ-1 was sequentially probed in Western blot experiments with anti-phosphotyrosine and anti-PLC\_γ-1 antibodies. As shown in Fig. 5, NGF-induced PLC\_γ-1 tyrosine phosphorylation was undetectable, or greatly diminished, in all activation loop mutant cell lines tested, including those with high receptor over-expression and those that mediate robust neurite outgrowth. In contrast, NGF treatment of cells expressing wild type Trk resulted in PLC\_γ-1 tyrosine phosphorylation. Therefore, any mutation of the activation loop tyrosines renders TRKA highly impaired for tyrosine phosphorylation of PLC\_γ-1.

To determine if this impairment was attributable to abrogated interaction between PLC\_γ-1 and TRK activation loop tyrosines, we assessed the ability of these proteins to co-immunoprecipitate. Samples prepared as above were subjected to immunoprecipitation with anti-PLC\_γ-1 antibody, Western blotting, and detection of PLC\_γ-1 tyrosine phosphorylation. As shown in Fig. 4, PLC\_γ-1 was co-immunoprecipitated with SHC and ERK1/2 in all activation loop mutant cell lines tested, including those with high receptor over-expression and those that mediate robust neurite outgrowth. In contrast, PLC\_γ-1 was not co-immunoprecipitated with SHC and ERK1/2 in wild type Trk expressing cells. Therefore, any mutation of the activation loop tyrosines renders TRKA highly impaired for tyrosine phosphorylation of PLC\_γ-1.

**NGF-induced tyrosine phosphorylation of Shc and the ERK kinases is decreased but not abrogated by mutation of TRK activation loop tyrosines.** A, PC12 cells, PC12nnr5-T14 (T14) cells, and expression-matched activation loop mutant cell lines expressing TRK at levels approximately 5-fold that of PC12nnr5-T14 cells were NGF-treated, or not, for 5 min, and detergent-solubilized lysates were prepared as described under “Materials and Methods.” Samples normalized for protein were subjected to anti-Shc immunoprecipitation followed by serial immunoblotting with anti-phosphotyrosine (4G10) and anti-Shc. B, lysates prepared identically to those in A were subjected to Western immunoblotting as described under “Materials and Methods,” and blots were serially probed with 4G10 and anti-ERK 1 (C-16) antibodies.

**NGF-induced tyrosine phosphorylation of SNT is impaired but not abolished by mutation of TRK activation loop tyrosines.** Clarified lysates were prepared from control and NGF-treated cells as described under “Materials and Methods.” SNT was recovered from samples containing equal levels of total proteins using p13suc1-Sepharose and was subjected to Western immunoblotting with anti-phosphotyrosine (4G10). Samples are from PC12, PC12nnr5-T14 (T14), WT.13, and expression-matched activation loop mutant cell lines expressing TRK at levels 10–25-fold that of PC12nnr5-T14.
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NGF-induced PLCγ-1 activation is abrogated by mutation of activation loop tyrosines. NGF-treated and control cells were lysed and total proteins were prepared as described under “Materials and Methods.” Samples normalized for protein were subjected to immunoprecipitation with anti-PLCγ-1, and the immunoprecipitates were analyzed by Western immunoblotting using anti-phosphotyrosine (4G10) and anti-PLCγ-1 as probes. Samples are from PC12, PC12nnr5-T14 (T14), and expression-matched activation loop mutant cell lines expressing TRK at levels 5-fold (A) or 10–25-fold (B) that of PC12nnr5-T14 cells.

NGF-mediated Induction of PI3K Activity Is Undetectable in Cells Expressing TRK Activation Loop Mutant Receptors—Phosphatidylinositol 3'-kinase (PI3K) activity is an additional signaling enzyme that is activated by NGF in PC12 cells (50) and implicated as a mediator of both neuritogenesis and survival (71, 72). The mechanism of PI3K activation is not clear, but recent findings appear to implicate tyrosine 490 as a major determinant of NGF-mediated PI3K activation (70). PI3K activity was assessed in vitro in cell lines expressing wild type and activation loop mutant TRKs treated for 5 min with NGF. As illustrated in Fig. 6, PI3K activity was induced by NGF 2–3-fold in PC12 cells, 6–10-fold in PC12nnr5-T14 cells, and 3–6-fold in the PC12nnr5-WT.13 cell line. In contrast, no PI3K activity was reproducibly induced by NGF treatment in any of

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the cell lines expressing the mutant TRKs, including those with very high levels of receptor over-expression. Thus, as in the case of PLC-γ-1, all of the activation loop mutations, including those affecting single tyrosines, abolished detectable NGF-induced PI3′K activation. An additional effect revealed by our analyses was that, on a per mg of cell protein basis, the basal levels of PI3′K activity in those lines with highest over-expression of mutant receptors were consistently elevated 3–10-fold when compared with basal levels in PC12nnr5-T14 cells (data not shown). The latter observation raises the possibility that very high over-expression of mutated TRKs may lead to at least some degree of constitutive PI3′K activation.

**Mutation of Any TRK Activation Loop Tyrosine Compromises Autophosphorylation of Tyr-785**—To extend our experiments on autophosphorylation and activation of signaling molecules, we generated and examined cell lines expressing TRK mutants in which the activation loop triple mutant was combined with the latter are the TRK tyrosine autophosphorylation sites not contained in the activation loop and are responsible for binding and/or activating Shc, PI3′K, and PLC-γ-1. Initial characterization of these cell lines revealed that, like the triple mutants, they failed to modulate both NGF-promoted ERK activation and neuritogenesis (data not shown). As shown in Fig. 7, analysis of the various lines indicated that basal and NGF-promoted tyrosine autophosphorylation of the mutant receptors were maintained by those mutants that retained Tyr-490 and were absent in those that had this site mutated, despite the simultaneous presence of Tyr-785 and Tyr-751. Previous work has demonstrated that the V490F mutation alone in otherwise unmodified hTRK receptors does not impair TRK tyrosine autophosphorylation, either overall or at Tyr-785 (15, 73) and thus is not a first step in the TRK autophosphorylation mechanism. The present findings indicate that the tyrosine autophosphorylation observed in the triple activation loop TRK mutants occurs solely at the Tyr-490 position. This observation, taken together with receptor autophosphorylation and PLC-γ-1 signaling data, is consistent with the prediction that mutation of any of the activation loop tyrosines disrupts TRK autophosphorylation at Tyr-785.

**DISCUSSION**

The purpose of the present study was to characterize the roles of the putative autophosphorylation activation loop tyrosines in the TRK receptor. This was achieved by mutating these tyrosines singly, and in all combinations, and then examining the signaling properties of each mutant receptor when stably expressed at various levels in PC12nnr5 cells that lack endogenous Trk. The use of PC12-derived cells permitted us to carry out our investigation in a background that displays neuronal phenotypic properties that include neurite outgrowth.

**Level of Mutant TRK Expression Affects Responsiveness to NGF**—An important feature here is that TRK-transfected cell lines were studied over a range of receptor expression. Examining NGF-induced responses in this manner provided means to take TRK protein expression into account and revealed that each of the tyrosines in the activation loop plays an important role in regulating NGF responsiveness. In many cases, the presence and degree of functional impairment induced by particular receptor loop mutations could be masked by over-expression. Thus, lines expressing several of the activation loop mutant receptors exhibited wild-type responses, and functional impairment of the mutant receptors was detected only by comparing dose-response curves for cell lines expressing mutant and non-mutant receptors. Such findings raise a general cautionary note for studies in which mutant receptors are functionally evaluated by exogenous introduction into cell lines; in these cases, expression level is likely to be an important determinant of apparent preservation of function.

**Activation Loop Mutations Affect Total Receptor Autophosphorylation**—Our findings demonstrate that mutation of any one of the three activation loop tyrosines in TRK results in substantial and essentially equal reduction of NGF-mediated receptor tyrosine autophosphorylation. Likewise, further decreases in TRK tyrosine autophosphorylation occur with the combined mutation of any two tyrosines, and an additional reduction occurs in response to mutation of the third tyrosine. Autophosphorylation impairment appears to be independent of both receptor expression level and autophosphorylation kinetics. Additionally, the decrements in autophosphorylation do not appear to be attributable simply to reduction of the number of available substrate tyrosine residues, because the relative loss of TRK tyrosine phosphorylation is not proportional to the number of sites mutated, especially with regard to single tyrosine mutants. One potential contribution to the dramatic loss of tyrosine autophosphorylation during the transition from WT to single activation loop tyrosine mutant is provided by our finding that tyrosine 785 is not autophosphorylated in activation loop mutant receptors. Observations with other RTKs, and particularly the insulin receptor, indicate that the extent of autophosphorylation correlates with activation of kinase activity (1, 34, 74–79). Therefore, an additional major factor in the reduction of autophosphorylation observed in the mutated TRK receptors appears to be impairment of receptor kinase activity necessary for efficient autophosphorylation.

Mutations homologous to those studied here have been carried out with several other RTKs, and they appear to show some variation from one case to the next with respect to their...
FIG. 7. NGF-inducible autophosphorylation of activation loop mutant TRK receptors occurs at tyrosine 490. PC12mnr5-derived cell lines expressing mutated receptors were assessed for NGF-promoted TRK tyrosine autophosphorylation. Receptor mutations present in various lines are indicated in the figure. Cells were treated with NGF, or not, and cell lysates were prepared as described under "Materials and Methods." Samples containing equal amounts of total cell protein were subjected to immunoprecipitation with anti-pan-Trk (203) and sequentially immunoblotted with anti-phosphotyrosine (4G10) and anti-Trk (203).
retained only in receptors with an intact Tyr-490 (Fig. 7), therefore indicating that Tyr-785 was not being phosphorylated. This potential mechanism recapitulates previous studies in which TRK tyrosine 785 was mutated to phenylalanine, which eliminated phosphorylation at this position, and consequently abolished binding and activation of PLCγ-1 (14, 18). Taken together, our observations indicate that mutation of any of the activation loop tyrosines selectively abrogates tyrosine phosphorylation of TRK-785 and, consequently, binding and activation of PLCγ-1.

Although loss of PI3K activation would have a similar mechanistic explanation if association of this enzyme with TRK was mediated via phospho-Tyr-751 (60, 82), recent findings suggest that TRK-evoked PI3K activation is mediated by Tyr-490 (70). In this case, the differential loss of PI3K activity, *via a vis* the partially preserved Shc tyrosine phosphorylation, could be explained by a difference in the threshold of TRK kinase activity necessary for generation of a functional and biochemically detectable signal. For example, if PI3K activity is dependent upon Shc-mediated activation of Ras (83, 84), then the reduction of Shc tyrosine phosphorylation observed for the single mutants could completely impair PI3K activation by falling below a given critical level, while simultaneously maintaining partial activation of other signaling molecules downstream of Ras (e.g. ERKs). In agreement with this idea of a threshold effect, wild type PC12 cells show relatively poor NGF-mediated PI3K activation, and TRK over-expression results in substantially enhanced responses. An alternative suggestion for the lack of PI3K activation is provided by recent findings of a feedback mechanism in which phosphatidylinositol kinase products block the association of phosphatidylinositol kinases with tyrosine-phosphorylated proteins/RTKs (85). We found that basal PI3K activity was elevated in cell lines highly over-expressing activation loop mutant TRKs, which is consistent with the production of phosphatidylinositol kinase products in the absence of NGF. It is possible that these products block association of the p85 subunit of PI3K with TRK, even in the presence of NGF, thereby blocking NGF-dependent increases in PI3K activity.

The differential consequences that activation loop tyrosine mutations have on autophosphorylation of specific sites in the TRK receptor may also pertain to other RTKs. Tavare and Dickens (91) presented evidence that mutation of the site homologous to TRK Tyr-674 in the insulin receptor leads to abolition of autophosphorylation at two C-terminal tyrosine residues. Although effects on autophosphorylation at other sites were not reported in this case, it is of interest that autophosphorylation of insulin receptor substrate 1, which binds to insulin receptors toward the N-terminal side of the kinase domain, is only partially impaired in response to mutation of the tyrosine equivalent to TRK Tyr-670 (38, 39). Differential, site-specific effects on autophosphorylation may also account for the observation that mutation of the insulin receptor site equivalent to TRK Tyr-670 abolishes insulin-stimulated thymidine incorporation but not activation of glycogen synthetase (41).

Despite loss of signaling through PI3K and PLCγ-1, TRK receptors with single activation loop mutations were still able to mediate differentiative responses to NGF. This is consistent with past observations that PLCγ-1 tyrosine phosphorylation is not required for neurite outgrowth (14, 73). Our present results extend these observations, for both PLCγ-1 and PI3K, to NGF-inducible downstream signaling responses such as induction of immediate early genes. It has been reported that wortmannin, an inhibitor of PI3K, blocks NGF-stimulated neurite outgrowth (71). If such effects are entirely due to PI3K inhibition, then our findings suggest that the observed elevated basal PI3K activity reported for over-expressing activation loop mutant cell lines, rather than NGF-stimulated PI3K activity, is sufficient for neuritic outgrowth. Alternatively, the wortmannin used in previous studies may have inhibited the activity of other proteins, in addition to PI3K, required for this response. The presence of biological responses to NGF in cells bearing receptors mutated at activation loop tyrosines must be interpreted with respect to those pathways that although impaired are partially functional. At present, this includes the Shc/Ras pathway and the pathway defined by the TRK AKF mutation that involves SNT. Past findings have indicated that both of these pathways may play roles in neuritogenesis and immediate early gene regulation in PC12 cells (15, 49, 73). This is consistent with findings here in which neurite outgrowth and gene regulation generally correlate with the extent of NGF-stimulated Shc and SNT tyrosine phosphorylation. Nevertheless, there were several apparent exceptions to this observation. One was that for the Y675F mutants, neuritogenic responses and ERK activation were relatively weak even when Shc tyrosine phosphorylation was strong. This suggests that there may be an additional TRK/Tyr-675-regulated element that plays a role in the signaling between Shc and ERKs. A second discrepancy was that the triple activation loop TRKs appeared capable of autophosphorylating Tyr-490 but showed little if any phosphorylation of Shc. One possible interpretation of this is that the kinase activity in these mutants is sufficient to autophosphorylate the receptor and thereby promote binding of Shc but is impaired with respect to phosphorylation of this substrate; such a mechanism appears to occur in the TRK Y751F mutant.³

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³ X. Peng, M. E. Cunningham, R. M. Stephens, D. R. Kaplan, and L. A. Greene, manuscript submitted for publication.
core and C-terminal tyrosine residues. One model to account for this would be that after the activation loop tyrosines are phosphorylated, each interacts with specific nearby basic residues to stabilize a conformation that promotes full activation of the receptor kinase, including access to the C-terminal region. In this model, autophosphorylation of the activation loop tyrosines would function both to relieve inhibition of kinase activity as well as to stabilize a new, optimally active conformation.

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Matthew E. Cunningham, Robert M. Stephens, David R. Kaplan and Lloyd A. Greene

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