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Overexpression of GAP Causes the Delay of NGF-induced Neuronal Differentiation and the Inhibition of Tyrosine Phosphorylation of SNT in PC12 Cells

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Abstract: The GTPase activating protein (GAP) can function both as a negative regulator and an effector of $p21^{ras}$. Overexpression of GAP in NIH-3T3 cells has been shown to inhibit transformation by *ras* or *src*. To investigate the function of GAP in a differentiative system, we overexpressed this protein in the nerve growth factor (NGF)-responsive PC12 cell line. Two-fold overexpression of GAP caused a delay of several days in the onset of NGF- but not FGF-induced neuronal differentiation of PC12 cells. However, the NGF-induced activation or tyrosine phosphorylation of upstream (Trk, PLC-γ1, SHC) and downstream (B-Raf and $p44^{mapk/erk1}$) components of $p21^{ras}$ signalling cascade was not altered by GAP overexpression. Therefore, the change of phenotype induced by GAP was probably not due to GAP functioning as a negative regulator of $p21^{ras}$. Rather, we found that NGF-induced tyrosine phosphorylation of SNT, a specific target of neurotrophin-induced tyrosine kinase activity, was inhibited by GAP overexpression. SNT is thought to function upstream or independent of $p21^{ras}$. Thus in PC12 cells, overexpressed GAP may control the rate of neuronal differentiation through a pathway involving SNT rather than the $p21^{ras}$ signalling pathway.
Key words: GTPase activating protein, neuronal differentiation, nerve growth factor, PC12, SNT.

Nerve growth factor (NGF), a member of the neurotrophin family, is necessary for the development, survival, and maintenance of subpopulations of neurons (Barde, 1989). Most of the studies elucidating the mechanism of action of NGF have been obtained using the rat pheochromocytoma cell line, PC12. NGF induces PC12 cells to differentiate into a neuronal phenotype characterized by the extension of neurites, generation of a sodiun-based action potential, and the cessation of cell division (Greene and Tischler, 1976). NGF binds to two cellular receptors, the Trk tyrosine kinase and $p75^{NGFR}$ (Hempstead *et al.*, 1991; Kaplan *et al.*, 1991a). While the role of $p75^{NGFR}$ in transmitting NGF signals is unclear, Trk has been shown to regulate the timing and extent of NGF-induced neurite outgrowth (Hempstead *et al.*, 1992). Upon binding to its receptor on the plasma membrane, NGF triggers the activation and/or tyrosine phosphorylation of a series of intracellular proteins including PLC-γ1 (Kim *et al.*, 1991), phosphatidylinositol 3-kinase (PI-3 kinase; Soltoff *et al.*, 1992), SHC (Pelicci *et al.*, 1992), $p21^{ras}$ (Qiu *et al.*, 1991), B-Raf (Oshima *et al.*, 1991), and MAP kinases ($p44^{mapk/erk1}$ and $p42^{mapk/erk2}$; Boulton *et al.*, 1991). PLC-γ1, PI-3 kinase, and SHC function upstream of $p21^{ras}$, while B-Raf and MAP kinases reside downstream of $p21^{ras}$ in the NGF signal transduction pathway. While the role of some of these proteins is not known, $p21^{ras}$ has been shown to be important in NGF-induced differentiation of PC12 cells. Expression of oncogenic ras protein induces neuronal differentiation, whereas dominant negative ras protein blocks the effect of NGF (Noda *et al.*, 1985; Szeberenyi *et al.*, 1990). As a small guanine nucleotide binding protein, $p21^{ras}$ can be regulated positively by guanine nucleotide releasing protein (GnRP; SOS in Drosophila, Simon *et al.*, 1991 and hSos1 in human, Chardin *et al.*, 1993) or negatively by GTPase activating protein (GAP; Trahey *et al.*, 1987) and neurofibromin (Xu *et al.*, 1990). Neurofibromin shares some sequence similarity with GAP (Xu *et al.*, 1990). GAP also functions as an effector of $p21^{ras}$; for instance, it potentiates the inhibition of muscarinic atrial potassium channel currents by $p21^{ras}$ (Yatani *et al.*, 1990). It is not known, however, whether neurofibromin can play a role as an effector of $p21^{ras}$, too.

Another protein involved in NGF-induced neuronal

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differentiation is SNT which is present in neurons and PC12 cells (Rabin *et al.*, 1993). SNT is tyrosine phosphorylated rapidly in response to differentiative but not mitogenic factors. SNT is thought to function downstream of Trk but upstream or independent of $p21^{ras}$, because its tyrosine phosphorylation is dependent upon the activation of Trk but not $p21^{ras}$.

In this study, we investigated the function of GAP in PC12 cells. We report that overexpression of GAP in PC12 cells caused a delay in the onset of NGF-induced neuronal differentiation. However, GAP overexpression did not affect the activity of either the downstream or the upstream targets of $p21^{ras}$ but it inhibited NGF-stimulated tyrosine phosphorylation of SNT.

Materials and Methods

Cells

PC12 cells (from R. Kelly, University of California, San Francisco, USA) were cultured at 37℃ in Dulbecco's modified Eagle medium supplemented with 10% horse serum, 5% calf serum, 20 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Transfection

Plasmid pUC101a containing human GAP cDNA (Zhang *et al.*, 1990) was digested with *Eco*R1. The GAP cDNA was subcloned into the vector pCMV-neo which contained a cytomegalovirus promoter and a sequence necessary for G418-resistance. The resulting plasmid (pCMV-neo-GAP) was transfected into PC12 cells using Lipofectin (Gibco/BRL, Gaithersburg, USA) with the slightly modified method of Loeb *et al.* (1991). Briefly, cells of about 50% confluence in a 60 mm tissue culture dish were incubated with a mixture of 10 μg/ml plasmid and 20 μg/ml lipofectin in 2 ml serum-free medium containing 3 μg/ml insulin for 18 h. Cells were provided with an equal volume of serum-containing medium. 24 h after incubation, cells were trypsinized and then transferred into a 150 mm dish. On the next day, G418 (200 μg/ml) was added to the medium. After two weeks, clones resistant to G418 were subcultured into a 24-well plate in medium containing G418 (200 μg/ml).

Cell lysis

Subconfluent cells were washed three times with ice-cold phosphate buffered saline (PBS) and directly lysed with 25 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM sodium vanadate. The cell lysates were centrifuged at 12,000×*g* for 10 min at 4℃. Supernatants were divided into aliquots, immediately frozen in a dry ice-ethanol bath, and stored at −80℃ until use. An aliquot was used to determine protein concentrations by a Bradford protein assay (Bio-Rad, Richmond, USA).

For GAP assays *in vitro*, cells were washed with ice-cold PBS and homogenized in 20 mM MOPS, pH 7.5, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 200 mM sucrose, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM sodium vanadate by sonication as described previously (Li *et al.*, 1992).

Immunoblot analyses and immunoprecipitations

For GAP immunoblot analyses, whole cell lysates (10 μg protein) were electrophoresed on 7.5% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were incubated overnight at 4℃ with anti-GAP (Ellis *et al.*, 1990) or monoclonal anti-phosphotyrosine antibody 4G10 (provided by D. Morrison, NCI-FCRDC) diluted in TBS/0.2% Tween-20. Proteins were detected using enhanced chemiluminescence (ECL) detection as described previously (Rabin *et al.*, 1993).

For immunoprecipitations, cell lysates (150 μg for anti-Erk immunoprecipitation and 2~4 mg for all other immunoprecipitations) were incubated with antibodies against Trk (Hempstead *et al.*, 1992), bovine PLC-γ1 (Suh *et al.*, 1988), human B-Raf (Sithanandam *et al.*, 1990), bovine SHC (Pelicci *et al.*, 1992), or rat $p44^{mapk/erk1}$ (Chen *et al.*, 1992). SNT was precipitated with $p13^{suc1}$ covalently coupled to protein A-agarose as described (Rabin *et al.*, 1993). All incubations were performed for 2 or 3 h at 4℃. Precipitates were collected with protein A-Sepharose, washed three times with NP-40 lysis buffer and one time with water. The immunoprecipitates were boiled in SDS sample buffer (2% SDS, 100 mM DTT, 10% glycerol, and 0.25% bromophenol blue) for 5 min.

B-Raf *in vitro* kinase assay

In vitro kinase activities present in B-Raf immunoprecipitates were assayed as described previously (Stephens *et al.*, 1992).

GAP assay

GTPase activity in the homogenates was assayed by measuring GTP hydrolysis of $p21^{ras}$ as described elsewhere (Berstein *et al.*, 1992; Li *et al.*, 1992). Recombinant $p21^{ras}$ (0.8 μg) was preincubated with 10 μCi of [γ-^{32}P]GTP for 15 min at 30℃ in a final volume of 500 μl of the exchange buffer (20 mM Tris-Cl, pH 7.5, 5 mM EDTA, 1 mM DTT, and 200 μg/ml BSA). At the end of incubation, 9 μl of 1 M $MgCl_2$ was added and the free unincorporated nucleotides were removed by using a NAP-5 column (Pharmacia, Uppsala, Swe-

den) equilibrated with the exchange buffer. Cell lysates (5 μg protein in 25 μg) were mixed with 65 μl of the assay buffer (30 mM Hepes, pH 7.5 and 1.5 mg/ml BSA), and the reaction was started by the addition of 10 μl of $p21^{ras}$-[γ-^{32}P]GTP. After incubation at room temperature for 20 min, the reaction was stopped by adding 0.5 ml of 5% (w/v) Norite. The mixture was centrifuged for 2 min at 12,000×g and the radioactivity in the supernatant was quantified by liquid scintillation counting.

Neurite outgrowth assay

Cells were plated at low densities (5 to 15% confluence) into a 48-well tissue culture plate. The following day, cells were treated with NGF or FGF at a final concentration of 100 ng/ml. For each clone and treatment, about 90~100 cells were counted and the proportion of neurite-bearing cells was determined by counting the number of cells containing the processes of at least one cell diameter in length.

Results

Overexpression of GAP in PC12 cells

In order to study the function of GAP in PC12 cells, we transfected a human GAP cDNA into PC12 cells. After transfection with pCMV-neo-GAP, G418-resistant clones were selected and assayed for GAP expression by immunoblot analyses using anti-human GAP antibody. Among 18 clones selected, two clones (GAP6 and GAP16) were consistently found to express a 2~3 fold higher level of GAP, compared to the control cells (Fig. 1A). When *in vitro* GAP activity was analyzed using purified $p21^{ras}$ (Fig. 1B), the two clones, GAP6 and GAP16 had a two-fold increase in GTPase activity over either the vector-transfected or the non-transfected cells.

Neurite outgrowth

We next determined whether overexpression of GAP has a positive or negative role in the NGF-induced differentiation of PC12 cells. Non-transfected or vector-transfected PC12 cells responded to NGF by extending neurites (Fig. 2). Neurite outgrowth in these cells (defined as neurites of one cell diameter in length) was observed at two days and was seen in most of the cells within 5 days of NGF treatment. GAP6 and GAP 16 cells did not exhibit neurites of discernible size after two days of NGF treatment. Rather, the GAP overex-

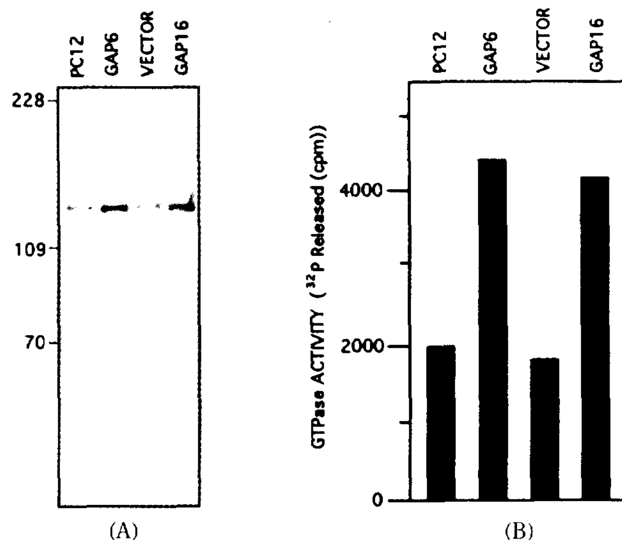

Fig. 1. Expression of human GAP in control, vector-, or GAP-transfected PC12 cells. (A) GAP protein expression. Cell lysates (10 μg protein) were electrophoresed by 7.5% PAGE and probed with GAP antibody. GAP6 and GAP16 are independently isolated clonal cell lines transfected with GAP. Molecular masses are indicated on the left in kilodaltons. (B) GAP activity. Cell lysates were assayed for $p21^{ras}$ GTPase activity as described in the Materials and Methods. Shown is the amount of ^{32}P released from $p21^{ras}$ by GAP activity in the cell lysates.

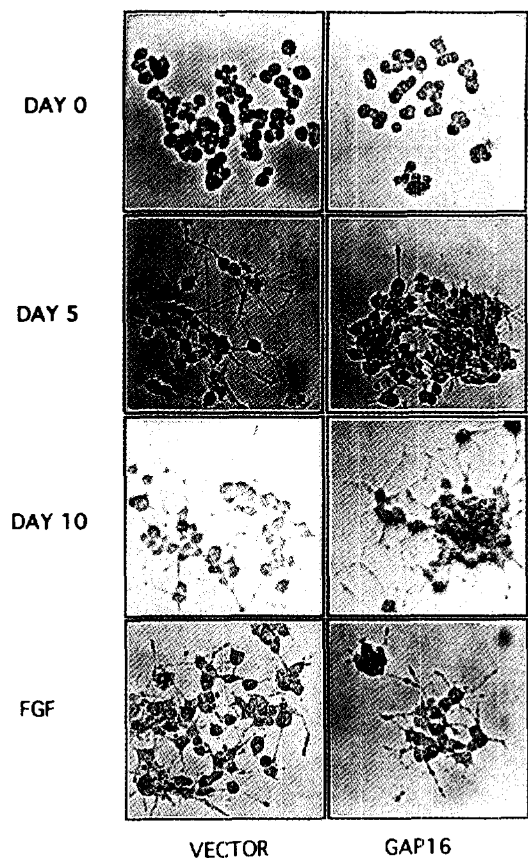

Fig. 2. Delayed neurite outgrowth in GAP-transfected PC12 cells treated with NGF. Cells were seeded at a low density (5% to 15%). The following day, NGF or FGF was added to the medium at the final concentration of 100 ng/ml. Shown are representative photographs of the vector- (VECTOR) or GAP-transfected (GAP 16) cells treated with NGF for 5 or 10 days or FGF for 5 days.

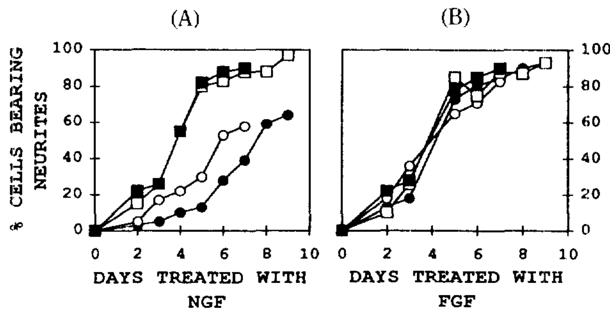

Fig. 3. Quantitation of neurite outgrowth of PC12 (filled square), VECTOR (opened square), GAP6 (opened circle), and GAP16 (filled circle) cells in response to NGF (A) or FGF (B). Neurites of one cell diameter in length were scored as positive.

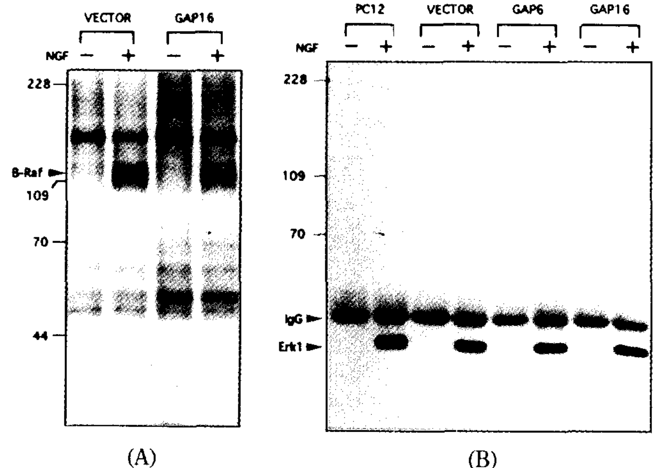

Fig. 4. Activity and phosphorylation state of B-Raf and $p44^{mapk/erk1}$ in PC12 cells overexpressing GAP. Control, VECTOR, GAP6 and GAP16 cells were mock treated (−) or treated (+) with NGF (100 ng/ml) for 5 min. (A) Activity of B-Raf. Cell lysates were immunoprecipitated with B-Raf antibody, electrophoresed by 7.5% PAGE. Immunoprecipitates were incubated in *in vitro* kinase assays to assay B-Raf autophosphorylation. The position of B-Raf is indicated. (B) Tyrosine phosphorylation of $p44^{mapk/erk1}$. Cell lysates were immunoprecipitated with $p44^{mapk/erk1}$ antibody, electrophoresed by 7.5% PAGE, and proteins probed with phosphotyrosine (Ptyr) antibody. The position of $p44^{mapk/erk1}$ and IgG are indicated by the arrowheads.

pressing cells tended to form clumps in the presence of NGF, and neurite outgrowth was first observed after three to five days of NGF treatment. After 10 days of exposure of GAP6 and GAP16 cells to NGF, the length of their neurites was comparable to that of the control cells treated for 5 days with NGF. Quantitative analysis of neurite outgrowth indicated approximately 50% of PC12 and the vector-transfected cells had extended neurites within 4 days of NGF treatment, while it took approximately 6 to 8 days to observe neurites in 50% of GAP6 and GAP16 cells (Fig. 3A). These results indicate that overexpression of GAP delays the neurite outgrowth response of PC12 cells to NGF.

Since fibroblast growth factor (FGF) also promotes the neuronal differentiation of PC12 cells (Togari *et al.*, 1985), we asked whether GAP6 and GAP16 cells would exhibit slower neurite outgrowth responses to FGF. FGF-treated GAP6 and GAP16 cells behaved identically to FGF-treated control cells in their neurite outgrowth responses, although they still had a tendency to form clumps (Fig. 2 and 3B).

Effect of GAP overexpression on B-Raf and Erkl activities

If overexpression of GAP affects $p21^{ras}$ activity, the activity or tyrosine phosphorylation of the downstream proteins of $p21^{ras}$ (B-Raf and p44mapk/erkl) might be expected to be down-regulated. Thus, the activity of B-Raf and tyrosine phosphorylation of $p44^{mapk/erk1}$ were assessed by analyzing the extent of NGF-mediated *in vitro* autophosphorylation of B-Raf (Fig. 4A) and the phosphotyrosine content in the $p44^{mapk/erk1}$ immunoprecipitate (Fig. 4B), respectively. The level of autophosphorylation of B-Raf was similar in both control cells and cells overexpressing GAP. The tyrosine phosphorylation of $p44^{mapk/erk1}$ was also similar in control or GAP-overexpressing cells. These results indicate that B-Raf and $p44^{mapk/erk1}$ activities were not affected by GAP overexpression, and thus suggest that the change in phenotype caused by overexpression of GAP was not due to the modulation of $p21^{ras}$ by GAP.

Effect on tyrosine phosphorylation of Trk, PLC-γ1, and SHC

The delay in NGF-mediated neurite outgrowth responses could also be due to an effect of GAP overexpression on proteins that function upstream of $p21^{ras}$ in NGF-signalling pathways. Following NGF addition to PC12 cells, the tyrosine kinase activity of Trk is rapidly activated (Kaplan *et al.*, 1991b). Trk then mediates the tyrosine phosphorylation and thus the activation of PLC-γ1 (Kim *et al.*, 1991) and SHC (Rozakis-Adcock *et al.*, 1992). The NGF-induced tyrosine phosphorylation of Trk, PLC-γ1, and SHC was the same in control cells, GAP16 cells (Fig. 5) and GAP6 cells (not shown). Thus, overexpression of GAP does not affect the tyrosine phosphorylation of proteins functioning upstream of $p21^{ras}$ in NGF-signal transduction pathways.

Effect of GAP overexpression on SNT phosphorylation

GAP overexpression did not alter the activity or phosphorylation state of several components of the signal transduction pathway involving $p21^{ras}$, suggesting that GAP may delay the differentiation of PC12 cells by acting as an effector of $p21^{ras}$ or by acting independent-

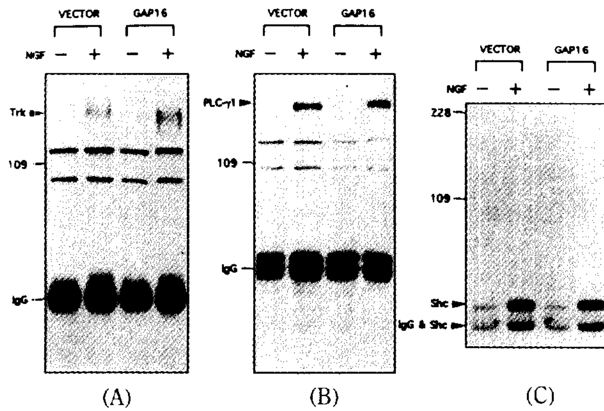

Fig. 5. Tyrosine phosphorylation of Trk, PLC-γ1, and SHC in PC12 cells overexpressing GAP. Control or GAP16 cells were mock treated (−) or treated (+) with NGF for 5 min. Cell lysates were immunoprecipitated with Trk, PLC-γ1, or SHC antibody, electrophoresed by 7.5% PAGE, and proteins probed with Ptyr antibody. (A) Tyrosine phosphorylation of Trk. (B) Tyrosine phosphorylation of PLC-γ1. (C) Tyrosine phosphorylation of SHC. Arrows indicate the 63 kDa and 58 kDa forms of SHC. The positions of Trk, PLC-γ1, SHC, IgG, and molecular masses in kilodaltons are indicated on the left.

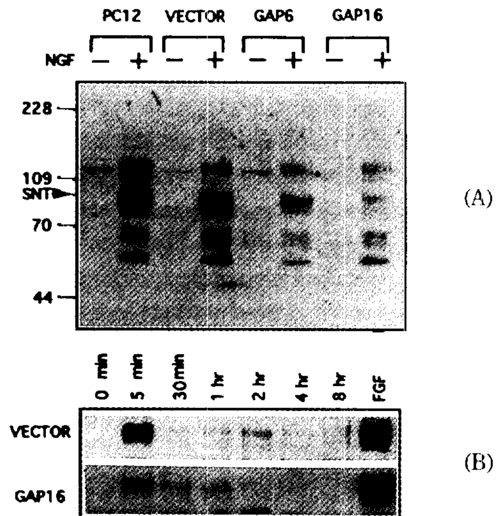

Fig. 6. Tyrosine phosphorylation of SNT in PC12 cells overexpressing GAP. (A) Tyrosine phosphorylation of SNT in control, vector transfected, GAP6, or GAP16 cells. Cells were mock treated (−) or treated (+) with NGF for 5 min. Cell lysates were precipitated with p13^{suc1}-agarose, electrophoresed by 7.5% PAGE, and proteins probed with Ptyr antibody. (B) Time course of SNT tyrosine phosphorylation in vector-transfected and GAP16 cells. Cells were treated with NGF for the indicated times. Cells were also treated with FGF for 5 min.

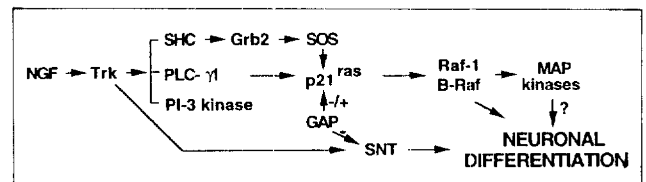

Fig. 7. Model of GAP function in NGF-signalling pathways of PC12 cells. Following NGF addition to cells, the ligand-activated Trk receptor associates with PLC-γ1 and PI-3 kinase. p21^{ras} is activated following SHC/Grb2 association with guanine nucleotide releasing protein (SOS). B-Raf, Raf-1, and MAP kinase activations require p21^{ras} activity. In many systems, GAP suppresses p21^{ras} activity and is an effector of p21^{ras}. GAP also has an effect on SNT. Constitutive overexpression of GAP affects SNT but not B-Raf and MAP kinase. SNT is placed in a signalling pathway parallel to p21^{ras}, because of the independence of SNT tyrosine phosphorylation from p21^{ras} activity. The delaying effect of GAP on NGF-induced neuronal differentiation may be through the inhibition of SNT tyrosine phosphorylation.

ly. A candidate target of GAP activity in PC12 cells is SNT. SNT is rapidly phosphorylated on tyrosine in response to differentiation factors but not mitogens for PC12 cells and neurons. And this phosphorylation depends on the activation of Trk but not p21^{ras} (Rabin *et al.*, 1993). Thus, SNT tyrosine phosphorylation was assayed in control, GAP6, and GAP16 cells treated with NGF. While SNT tyrosine phosphorylation was stimulated by NGF in both control cells and cells overexpressing GAP, the extent of this increase was 5 to 12-fold less in the two GAP-overexpressing cells (Fig. 6A). SNT tyrosine phosphorylation peaked at 5 min after NGF treatment in both control and GAP16 cells (Fig. 6B). Since FGF induced the outgrowth of neurites in control cells and cells overexpressing GAP, we examined the effect of FGF on tyrosine phosphorylation of SNT. FGF stimulated similar levels of SNT tyrosine phosphorylation in control and GAP16 cells (Fig. 6B). These results show a correlation between the effect of GAP on neuronal differentiation and its effect on the extent of SNT phosphorylation.

Discussion

Our results indicate that overexpression of GAP in PC12 cells delays the onset of NGF-mediated neurite outgrowth. The level of GAP overexpression in our PC 12 clones was approximately two-fold. This level of overexpression of GAP in 3T3 cells has been demonstrated to inhibit transformation by *c-ras* (Zhang *et al.*, 1990). Apparently, small increases in the level of GAP expression are enough to produce significant changes in the differentiation and proliferation of cells.

Overexpression of GAP did not affect the NGF-induced activation or phosphorylation of the upstream components of p21^{ras} cascade (B-Raf and p44$^{mapk/erk1}$). These results suggest that the delay in NGF-induced neurite outgrowth by GAP overexpression was not due to an effect of GAP on p21^{ras} activity. There are several explanations for the lack of effect of GAP overexpression on p21^{ras} activity. First of all, more than a two-fold overexpression of GAP might be required to inhibit

$p21^{ras}$ activity and $p44^{mapk/erk1}$ tyrosine phosphorylation. In fact, a high level (100-fold) of GAP overexpression in NIH-3T3 cells has been shown to affect PDGF-induced activation of $p21^{ras}$ (Gibbs *et al.*, 1990) or phorbol ester-induced tyrosine phosphorylation of $p42^{mapk/erk2}$ (Nori *et al.*, 1992). Secondly, in PC12 cells, neurofibromin, another negative regulator and possible effector of $p21^{ras}$ (Xu *et al.*, 1990; Martin *et al.*, 1990) might be in charge of regulating the activity of $p21^{ras}$, while the primary function of GAP might be independent of $p21^{ras}$ or an effector of $p21^{ras}$. Several following observations have indicated that although GAP and neurofibromin are both ubiquitous, one might predominate the other in regulating the activity of $p21^{ras}$. Despite the normal levels of GAP, $p21^{ras}$ was in the active, GTP-bound form in some neurofibrosarcoma cells that lack or have reduced levels of neurofibromin protein (DeClue *et al.*, 1992; Basu *et al.*, 1992), suggesting that neurofibromin, and not GAP, is the predominant regulator of $p21^{ras}$ in these cells. On the other hand, in NIH-3T3 cells, GAP was suggested to be the major regulator of $p21^{ras}$ activity (Zhang *et al.*, 1991). A third possibility is that PC12 cells might develop a homeostasis system to maintain the total GTPase activity for $p21^{ras}$ by down-regulating neurofibromin in response to GAP overexpression.

GAP overexpression also did not affect the NGF-induced tyrosine phosphorylation of the Trk/NGF receptor, SHC, and the Trk substrate, PLC-γ1, indicating that the signal transduction pathway from Trk to $p44^{mapk/erk1}$ appears to be intact in cells overexpressing GAP.

Overexpression of GAP in PC12 cells inhibited the NGF-mediated tyrosine phosphorylation of SNT five to twelve-fold compared to control cells. While the function of SNT in NGF-induced differentiation responses is not known, the co-precipitation (Rabin *et al.*, 1993) of SNT with $p34^{cdc2}$, a cell cycle regulator suggests that SNT may play a role in the regulation of cell cycle events. Here, the reduction of SNT tyrosine phosphorylation in cells overexpresssing GAP correlates with a delay in NGF-mediated neurite outgrowth responses. In PC12 cells which overexpress Trk and thus have accelerated neurite outgrowth in response to NGF, SNT is constitutively tyrosine phosphorylated (Rabin *et al.*, 1993). These results suggest that SNT may contribute to the regulation of the timing of neurite outgrowth responses. However, unlike the effect on NGF-mediated neuronal differentiation and SNT tyrosine phosphorylation, overexpression of GAP did not affect FGF's functions. GAP, therefore, specifically affects only NGF but not FGF signal transduction pathways in PC12 cells.

Our results suggest that GAP is capable of transmitting signals independent of $p21^{ras}$ pathways. Overexpressed GAP might alter the activity of other ras family members that could be involved in NGF responses or influence the activity of the potential GAP effectors, p62 and p190 (Ellis *et al.*, 1990). These proteins contain sequence motifs that suggest functions in transcriptional repression, RNA binding, and small GTP binding protein regulation (Wong *et al.*, 1992; Settleman *et al.*, 1992). The SH2 domains of overexpressed GAP could also compete with tyrosine kinases that phosphorylate SNT for access to SNT. The dependence of NGF-mediated SNT tyrosine phosphorylation on the level of GAP expression, and the apparent independence of SNT tyrosine phosphorylation from $p21^{ras}$ activity, suggest that GAP has distinct biochemical functions in PC12 cells.

References

Barde, Y.-A. (1989) *Neuron* **2**, 1525.

Basu, T. N., Gutmann, D. H., Fletcher, J. A., Glover, T. W., Collins, F. S. and Downward, J. (1992) *Nature* **356**, 713.

Berstein, G., Blank, J. L., Jhon, D.-Y., Exton, J. H., Rhee, S. G. and Ross, E. M. (1992) *Cell* **70**, 411.

Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H. and Yancopoulos, G. D. (1991) *Cell* **65**, 663.

Chardin, P., Camonis, J. H., Gale, N. W., Aelst, L. V., Schlessinger, J., Wigler, M. H. and Bar-Sagi, D. (1993) *Science* **260**, 1338.

Chen, R.-H., Sarnecki, C. and Blenis, J. (1992) *Mol. Cell. Biol.* **12**, 915.

DeClue, J. E., Papageorge, A. G., Fletcher, J. A., Diehl, S. R., Ratner, N., Vass, W. C. and Lowy, D. R. (1992) *Cell* **69**, 265.

Ellis, C., Moran, M., McCormick, F. and Pawson, T. (1990) *Nature* **343**, 377.

Gibbs, J. B., Marshall, M. S., Scolnick, E. M., Dixon, R. A. F. and Vogel, U. S. (1990) *J. Biol. Chem.* **265**, 20437.

Greene, L. A. and Tischler, A. S. (1976) *Proc. Natl. Acad. Sci.* USA **73**, 2424.

Hempstead, B. L., Rabin, S. J., Kaplan, L., Reid, S., Parada, L. F. and Kaplan, D. R. (1992) *Neuron* **9**, 883.

Hempstead, B. L., Kaplan, D., Martin-Zanca, D., Parada, L. F. and Chao, M. (1991) *Nature* **350**, 678.

Kaplan, D. R., Hempstead, B., Martin-Zanca, D., Chao, M. V. and Parada, L. F. (1991a) *Science* **252**, 554.

Kaplan, D. R., Martin-Zanca, D. and Parada, L. F. (1991b) *Nature* **350**, 158.

Kim, U.-H., Fink, D. Jr., Kim, H. S., Park, D. J., Contreras, M. L., Guroff, G. and Rhee, S. G. (1991) *J. Biol. Chem.* **266**, 1359.

Li, B.-Q., Kaplan, D., Kung, H. and Kamata, T. (1992) *Science* **256**, 1456.

Loeb, D. M., Maragos, J., Martin-Zanca, D., Chao, M. and

Greene, L. A. (1991) *Cell* **66**, 961.

Martin, G. A., Viskochil, D., Bollag, G., McCabe, P. C., Crosier, W. J., Haubruck, H., Conroy, L., Clark, R., O'Connell, P., Cawthon, R. M., Innis, M. A. and McCormick, F. (1990) *Cell* **63**, 843.

Noda, M., Ko, M., Ogura, A., Liu, D. G., Amano, T., Takano, T. and Ikawa, Y. (1985) *Nature* **318**, 73.

Nori, M., L'Allemain, G. and Weber, M. J. (1992) *Mol. Cell. Biol.* **12**, 936.

Oshima, M., Sithanandam, G., Rapp, U. R. and Guroff, G. (1991) *J. Biol. Chem.* **266**, 23753.

Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T. and Pelicci, P. G. (1992) *Cell* **70**, 93.

Qiu, M.-S. and Green, S. H. (1991) *Neuron* **7**, 937.

Rabin, S. J., Cleghon, V. and Kaplan, D. R. (1993) *Mol. Cell. Biol.* **13**, 2203.

Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P. G., Schlessinger, J. and Pawson, T. (1992) *Nature* **360**, 689.

Settleman, J., Narasimhan, V., Foster, L. C. and Weinberg, R. A. (1992) *Cell* **69**, 539.

Simon, M. A., Bowtell, D. D. L., Dodson, G. S., Laverty, T. R. and Rubin, G. M. (1991) *Cell* **67**, 701.

Sithanandam, G., Kolch, W., Duh, F.-M. and Rapp, U. R. (1990) *Oncogene* **5**, 1775.

Soltoff, S. P., Rabin, S. L., Cantley, L. C. and Kaplan, D. R. (1992) *J. Biol. Chem.* **267**, 14472.

Stephens, R. M., Sithanandam, G., Copeland, T. D., Kaplan, D. R., Rapp, U. R. and Morrison, D. K. (1992) *Mol. Cell. Biol.* **12**, 3733.

Suh, P.-G., Ryu, S. H., Cho, K. S., Lee, K. Y. and Rhee, S. G. (1988) *J. Biol. Chem.* **263**, 14497.

Szeberenyi, J., Cai, H. and Cooper, G. M. (1990) *Mol. Cell. Biol.* **10**, 5324.

Togari, A., Dickens, G., Kuzuya, H. and Guroff, G. (1985) *J. Neurosci.* **5**, 307.

Trahey, M. and McCormick, F. (1987) *Science* **238**, 542.

Wong, G., Muller, O., Clark, R., Conroy, L., Moran, M. F., Polakis, P. and McCormick, F. (1992) *Cell* **69**, 551.

Xu, G., Lin, B., Tanaka, K., Dunn, D., Wood, D., Gesteland, R., White, R. and Tamanoi, F. (1990) *Cell* **63**, 835.

Yatani, A., Okabe, K., Polakis, P., Halenbeck, R., McCormick, F. and Brown, A. M. (1990) *Cell* **61**, 769.

Zhang, K., DeClue, J. E., Vass, W. C., Papageorge, A. G., McCormick, F. and Lowy, D. R. (1990) *Nature* **346**, 754.

Zhang, K., Papageorge, A. G., Martin, P., Vass, W. C., Olah, Z., Polakis, P. G., McCormick, F. and Lowy, D. R. (1991) *Science* **254**, 1630.