Oncogene (2002) 21, 1739–1749

www.nature.com/onc

Glial cell line-derived neurotrophic factor induces proliferative inhibition of NT2/D1 cells through RET-mediated up-regulation of the cyclin-dependent kinase inhibitor $p27^{kip1}$

Gustavo Baldassarre[1], Paola Bruni[2], Angelo Boccia[3], Giuliana Salvatore[3], Rosa Marina Melillo[3], Maria Letizia Motti[3], Maria Napolitano[1], Barbara Belletti[1], Alfredo Fusco[3], Massimo Santoro[3] and Giuseppe Viglietto*[,3]

[1]Istituto Nazionale Tumori, via M. Semmola, 80131, Naples, Italy; [2]BIOGEM, c/o Dipartimento di Biochimica e Biotecnologie Mediche, Facolt' di Medicina e Chirurgia, Universit' Federico II, Via S. Pansini 5, 80131, Naples, Italy; [3]Centro di Endocrinologia ed Oncologia Sperimentale del CNR, c/o Dipartimento di Biologia e Patologia Cellulare e Molecolare, Universit' di Napoli 'Federico II', via S. Pansini 5, 80131 Naples, Italy

Growth factors of the glial cell line-derived neurotrophic factor (GDNF) family control the differentiation of neuronal cells of the central and peripheral nervous systems. Intracellular signalling of these growth factors is, at least in part, mediated by activation of the RET receptor tyrosine kinase. Here, we demonstrate that GDNF triggering inhibits the proliferation of the embryonal carcinoma cell line NT2/D1. This anti-proliferative effect is accompanied by down-regulation of the SSEA-3 antigen, a marker typical of undifferentiated NT2/D1 cells. We show that these effects are mediated by activation of RET signalling. The block of RET by a kinase-deficient dominant negative mutant impairs GDNF-dependent growth inhibition, whereas the adoptive expression of a constitutively active RET, the RET-MEN2A oncogene, promotes effects similar to those exerted by GDNF. We show that RET signalling increases the expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$ in NT2/D1 cells. Both DNA synthesis inhibition and SSEA-3 down-regulation are prevented if $p27^{kip1}$ expression is blocked by an antisense construct, which demonstrates that RET-triggered effects are mediated by $p27^{kip1}$.
Oncogene (2002) **21,** 1739–1749. DOI: 10.1038/sj/onc/1205226

Keywords: GDNF; RET; p27; embryonal carcinoma

Introduction

The GDNF protein family consists of four members: glial cell line-derived neurotrophic factor (GDNF), neurturin (NTN), persephin (PSP) and artemin (ART). These neurotrophins play a crucial role in the differentiation, proliferation and survival of neurons of the peripheral and central nervous system (Airaksinen *et al*., 1999). The GDNF proteins signal through a multi-component receptor complex consisting of ligand-binding GDNF family receptors, i.e. α-subunits (GFRα) and RET receptor tyrosine kinase. GFRαs are glycosyl-phosphatidylinositol (GPI)-linked polypeptides; to date, four GFRα proteins, termed GFRαs1, 2, 3 and 4 have been identified. GFRα1, 2, 3 and 4 bind predominantly GDNF, NTN, ART and PSP, respectively. RET functions as a common intracellular signal-transducing component in conjunction with each of the GFRαs (Airaksinen *et al*., 1999). GFRα also plays a role in recruiting RET to lipid rafts, an event that is required for effective signalling (Tansey *et al*., 2000). GFRασ co-receptors may signal in the absence of RET; GDNF induces the phosphorylation of the cyclic AMP response element-binding protein (CREB) and c-fos transcription in neuronal precursors that express GFRα1 but not RET (Trupp *et al*., 1999).

The GDNF-GFRαs-RET system is crucial for the development of neuronal cells. Hirschsprung's disease, which is characterized by the lack of enteric innervation to the hindgut, arises from mutations that inactive RET (Pasini *et al*., 1995; Carlomagno *et al*., 1996). Knock-out mice lacking GDNF, RET or GFRα1 fail to develop enteric neurons (Rosenthal, 1999). RET triggering induces survival and differentiation of neuroepithelial cell types (Califano *et al*., 1995; Jing *et al*., 1996; De Vita *et al*., 2000). However, despite the body of information on the mechanisms of RET-mediated signalling (van Weering and Bos, 1998), the molecular pathways that trigger RET-mediated neuronal differentiation remain obscure.

Inhibition of the progression through the cell cycle is a pre-requisite for cells to enter a program of terminal differentiation. Indeed, neuronal cells respond to neurotrophic factors, such as nerve growth factor (NGF), by undergoing growth arrest and, then, proceeding to differentiation (van Grunsven *et al*., 1996). Nerve growth factor suppresses cell proliferation by reducing the activity of cyclin-dependent kinases (CDK) (Park *et al*., 1996). CDK activity is modulated

*Correspondence: G Viglietto;
E-mail: gvigliet@tin.it or viglietto@sun.ceos.na.cnr.it
Received 28 August 2001; revised 5 December 2001; accepted 5 December 2001

by the binding of positive (cyclins) and negative (CDK inhibitory proteins, CKI) regulatory subunits. Whereas the cellular level of CDK proteins varies little through the cell cycle, a rapid increase in cyclins and CKI subunits leads to progression and arrest of the cell cycle, respectively (Sherr and Roberts, 1999). The CKI subunits $p21^{cip1}$ and $p27^{kip1}$ play a pivotal role in arresting cell in the G1 phase of the cell cycle (Sherr and Roberts, 1999).

NTERA-2 clone D1 (NT2/D1) cells display characteristics of committed neuronal progenitors. NT2/D1 cells arrest growth and differentiate into post-mitotic neurons upon treatment with retinoic acid (RA) (Andrews, 1984). Retinoic acid-differentiated NT2/D1 cells elaborate processes that differentiate into axons and dendrites (Pleasure *et al*., 1992; Pleasure and Lee, 1993; Lee and Andrews, 1986; Simeone *et al*., 1990). Furthermore, RA-treated NT2/D1 cells down-regulate expression of the SSEA-3 antigen and up-regulate the A2B5 antigen (Andrews *et al*., 1990; Chen *et al*., 1989; Baldassarre *et al*., 1997, 1999). NT2/D1 cells transplanted into immunodeficient mice have been used as an *in vivo* model to study the formation and remodelling of the developing central nervous system (Miyazono *et al*., 1996).

We report that GDNF induces DNA synthesis inhibition and reduced expression of the SSEA-3 marker in NT2/D1 cells. These effects are mimicked by the expression of the RET-MEN2A oncogene, but are blocked by a dominant-interfering RET mutant. $p27^{kip1}$ is a pivotal mediator of the effects induced by RET since inhibition of RET-dependent $p27^{kip1}$ accumulation impaired proliferative inhibition and SSEA-3 down-regulation in NT2/D1 cells.

Results

GDNF reduces growth rate and SSEA-3 antigen expression in NT2/D1 cells

NT2/D1 cells were treated in parallel with GDNF (2 and 20 ng/ml) or RA (10 μM) for 7 days and the effects of both compounds on cell proliferation were determined by evaluating cell number at different time points. Growth rate was lower in GDNF- or RA-treated as compared with untreated cells (Figure 1a). Similar results were obtained by calculating the rate of bromodeoxyuridine (BrdU) incorporation, a measure of DNA synthesis. BrdU incorporation was significantly lower in cells treated with GDNF (20 ng/ml) for 3, 5 and 7 days (46, 33 and 32%, respectively), *versus* 60% for untreated cells). Inhibition of GDNF-mediated DNA synthesis was minor compared with that induced by RA (15% of cells incorporated BrdU after 7 days of RA treatment; data not shown). A representative microscopic field is shown in Figure 1b.

In theory, GDNF may suppress NT2/D1 cell growth by arresting cell cycle progression or by increasing cell death. We therefore used flow cytometry to investigate the mechanism whereby GDNF suppressed growth in NT2/D1 cells. Asynchronously proliferating NT2/D1 cells were treated for 4, 7 and 15 days with 20 ng/ml of GDNF, labelled with propidium iodide and analysed with FACScan. Typically, 44% of proliferating NT2/D1 cells were in the S phase compartment, whereas upon GDNF treatment, the percentage of cells in G1 increased and the percentage of cells in the S phase compartment was reduced (29% of S phase cells at 4 days of treatment, 26% of S phase cells at 7 days and 22% at 15 days of treatment, respectively). Results are summarized in Table 1 and a representative experiment is reported in Figure 1c.

We next analysed the effects exerted by GDNF on the differentiation of NT2/D1 cells. Most NT2/D1 cells express the SSEA-3 antigen (64%, on average) but not the A2B5 antigen. The differentiation induced by RA consists in the loss of SSEA-3 expression and in the appearance of A2B5 (Andrews *et al*., 1990; Chen *et al*., 1989; Baldassarre *et al*., 1997, 1999). By indirect immunofluorescence (a representative field is shown in Figure 1d), we found that GDNF treatment (20 ng/ml for 7 days) down-regulated the expression of SSEA-3; after GDNF stimulation only 28% of cells remained positive for SSEA-3 expression (Table 1). However, unlike RA, GDNF did not up-regulate A2B5 or induce neurite extensions (not shown).

Subsequently, we determined GDNF-induced RET phosphorylation in NT2/D1 cells by immunoprecipitating the RET protein and staining the immunoblot with anti-phosphotyrosine antibodies. Treatment of NT2/D1 cells with 100 ng/ml of GDNF for 10 min promptly induced RET phosphorylation (Figure 1e). RET stimulation by GDNF was likely mediated by the GFRα1 co-receptor, because GFRαs1 expression was detected in NT2/D1 cells by RT–PCR (not shown).

Interestingly, GDNF treatment also reduced the tumorigenicity of NT2/D1 cells (not shown). These findings indicate that GDNF, by reducing the rate of proliferation and the expression of the SSEA-3 antigen, reproduces in part the effects exerted by RA on NT2/D1 cells.

GDNF-triggered growth inhibition of NT2/D1 cells is mediated by RET activation

We asked whether RET activation was necessary for GDNF to inhibit NT2/D1 cell growth. We first investigated whether the impairment of RET signalling affected the antiproliferative activity exerted by GDNF. To this aim, we engineered a truncated RET mutant, RET(ΔK) (Figure 2a). This truncated form of RET lacked the cytoplasmic catalytic domain; by dimerizing with the wild type RET, RET(ΔK) was predicted to prevent GDNF-induced RET transphosphorylation. The presence of a C-terminal tag (FLAG) discriminated the transfected RET(ΔK) from endogenous RET. In turn, RET(ΔK) does not interact with the anti-RET polyclonal antibody, which is directed against the RET kinase domain (see Materials and methods). COS-7 cells were transfected with the

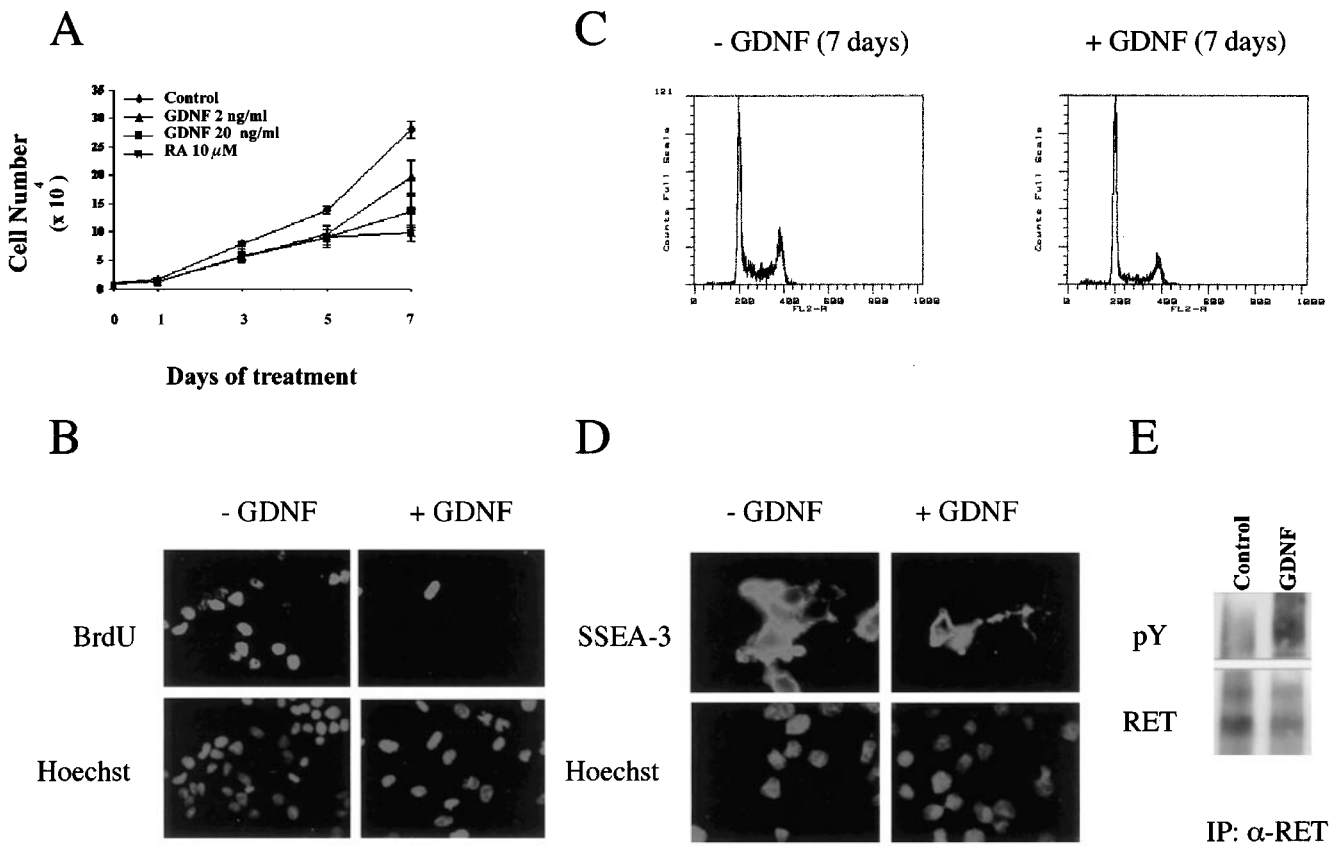

Figure 1 Effects of GDNF on NT2/D1 cells. (**a**) Growth curves of NT2/D1 cells treated with GDNF (2 and 20 ng/ml) or with RA (10 μM). (**b**) Upper panel, BrdU incorporation of untreated (left) or 7-day GDNF-treated (right) NT2/D1 cells; lower panel, Hoechst staining of cell nuclei. (**c**) NT2/D1 cells were treated for 7 days with GDNF (20 ng/ml), collected and stained with propidium iodide; labelled cells were analysed with FACScan using CELL-FIT program. A representative experiment is reported. (**d**) Upper panel, SSEA-3 expression of untreated (left) or 7-day GDNF-treated (right) NT2/D1 cells; lower panel, Hoechst staining of cell nuclei. (**e**) Western blot analysis of RET protein phosphorylation following 10 min of GDNF treatment of NT2/D1 cells. RA, retinoic acid; BrdU, bromodeoxyuridine; GDNF, glial cell lene-derived neutrophic factor

empty vector (LTR), RET(ΔK), RET and both RET and RET(ΔK) at different molar ratios (1 : 1 and 1 : 2); in all cases GFRα1 was co-transfected. Cells were treated with GDNF and lysed; protein extracts were immunoprecipitated with anti-RET followed by immunoblot with anti-phosphotyrosine. In the absence of RET(ΔK) expression, GDNF triggered RET phosphorylation (Figure 2b). RET(ΔK) reduced GDNF-mediated phosphorylation of RET when the two plasmids were transfected at a 1 : 1 ratio. At a 1 : 2 ratio RET phosphorylation was almost abrogated (Figure 2b), demonstrating that RET(ΔK) dominantly interferes with wild type RET stimulation. An immunoblot with anti-tag antibodies was performed to ascertain RET(ΔK) expression. Thus, to obtain cells that were unable to activate the RET tyrosine kinase, the RET(ΔK) mutant was transfected into NT2/D1 cells. Six G418 resistant clones that expressed the transfected construct, as shown by Western blot using anti-tag antibodies (Figure 2c), were selected. The growth rate of three representative transfected clones, i.e. NT2-RET(ΔK)3, NT2-RET(ΔK)5 and NT2-RET(ΔK)8 in response to GDNF was compared with the growth rate of one vector-transfected clone used as a control. Flow cytometry showed that after 7 days of GDNF treatment (20 ng/ml), 29% of vector-transfected NT2/D1 cells were in S phase, whereas about 40% of NT2/D1-RET(ΔK) cells were in the S phase compartment (Table 1 and Figure 2d). The analysis of BrdU incorporation yielded similar results (not shown). As expected, GDNF failed to induce SSEA-3 down-regulation in NT2/D1-RET(ΔK) cells (the average results of four experiments are shown in Table 1). Figure 2e shows a representative field.

Subsequently, we investigated whether RET activation was not only necessary but also sufficient to inhibit the growth of NT2/D1 cells. To this aim, we transfected a constitutively activated RET-MEN2A mutant (RETC634Y) into NT2/D1 cells (Figure 3a). The C634Y mutation induces dimerization and constitutive activation of RET receptor mediated by the formation of aberrant disulfide-bonds (Santoro *et al*., 1995). NT2/D1 cells were transiently transfected with RET-MEN2A or with the empty vector together with a pEGFP plasmid that encodes the autofluorescent eukaryotic green fluorescent protein, to identify transfected cells. In this case we used the rate of BrdU incorporation as a measure of cell growth inhibition

Table 1 Effects of GDNF on the growth and differentiation of NT2/D1, NT2-RET (ΔK) and NT2-p27AS cells

Cell line[a]	*FACS analysis (−GDNF)*	*FACS analysis (+GDNF)*[b]	*SSEA-3*[c] *(−GDNF)*	*SSEA-3 (+GDNF)*
NT2/D1	G1 36 S 44 G2 20	G1 55 S 26 G2 19	64±7.7	28±5.5
NT2-LTR	G1 38 S 42 G2 20	G1 53 S 29 G2 18	68±7.6	26±4.9
NT2-RET(ΔK)3	G1 38 S 43 G2 19	G1 40 S 41 G2 19	60±5.1	61±9.4
NT2-RET(ΔK)5	G1 33 S 47 G2 20	G1 42 S 42 G2 16	68±7.4	73±9.3
NT2-RET(ΔK)8	G1 34 S 44 G2 22	G1 37 S 41 G2 22	64±7.2	70±9
NT2-p27AS2.4	G1 31 S 46 G2 23	G1 31 S 49 G2 20	62±9.2	58±10
NT2-p27AS2.5	G1 38 S 44 G2 18	G1 38 S 40 G2 22	not done	61±5.5
NT2-p27AS2.6	G1 37 S 46 G2 17	G1 33 S 46 G2 21	not done	73±9.5

[a]The data reported are referred to parental and vector-, RET(ΔK)- or p27AS-transfected NT2/D1 cells. Two representative clones out of at least six analysed for each transfection are shown for each transfection. [b]GDNF was administered for 7 days at the concentration of 20 ng/ml. [c]SSEA-3 expression is reported as the percentage of cells positive with respect to total cell number. The data represent the mean (±s.d.) of four different experiments in which at least 500 cells were counted

exerted by activated RET. Cells were plated onto coverslips and 48 h after transfection they were processed for indirect immunofluorescence to determine BrdU incorporation rate and SSEA-3 expression. At least 200 transfected (GFP-positive) cells were evaluated. Similarly to GDNF treatment, transfection of the RET-MEN2A mutant reduced BrdU incorporation: on average 56% of vector-transfected NT2/D1 cells incorporated BrdU, whereas only 23% of RET-MEN2A NT2/D1 cells incorporated BrdU (Table 2).

Furthermore, RET-MEN2A suppressed SSEA-3 expression (Table 2), although, like GDNF, it did not induce A2B5 up-regulation (not shown). Conversely, the kinase defective RET(ΔK) failed to induce growth arrest and SSEA-3 down-regulation; instead, it determined a slight increase of SSEA-3 levels compared with vector-transfected cells (Table 2). Figure 3b shows a representative experiment: control LTR plasmid did not modify SSEA-3 expression (first column), whereas RET-MEN2A induced a loss of SSEA-3 expression (second column, the green-transfected cell did not stain red). SSEA-3 down-regulation was not observed in NT2/D1 cells transfected with RET(ΔK) mutant (third column, the green transfected cells are also red). Interestingly, consistent with the growth arresting effect, we were unable to select NT2/D1 cell clones stably expressing the activated RET-MEN2A mutant (not shown).

These findings demonstrate that activation of the RET pathway in NT2/D1 cells is sufficient to exert effects similar to those elicited by GDNF and that the functional integrity of the RET pathway is required for GDNF-induced growth inhibition and SSEA-3 down-regulation.

GDNF and RET-MEN2A up-regulate $p27^{kip1}$ expression in NT2/D1 cells

The growth and differentiative effects exerted by RA or other differentiative compounds in NT2/D1 cells are mediated by up-regulation of the cyclin-dependent kinase inhibitor $p27^{kip1}$ and subsequent inhibition of CDK2 activity (Baldassarre *et al*., 1999, 2000). Therefore, we investigated whether GDNF induces $p27^{kip1}$ expression. GDNF induced a threefold increase in $p27^{kip1}$ protein levels at day 7 of treatment (Figure 4a), but it did not affect the expression of cyclins and CDK or the expression of the other members of the Kip/cip family of inhibitors ($p21^{cip1}$ and $p57^{kip2}$) (not shown). To determine whether GDNF induced p27 expression at the mRNA or protein level, we performed RT–PCR analysis at a low number of cycles (23) with p27 specific primers. As internal control, we used primers that amplified GAPDH. RT–PCR analysis demonstrated that GDNF did not induce an increase in the steady-state levels of p27 mRNA in NT2/D1 cells (Figure 4b), which suggests that most p27 accumulation after GDNF treatment results from post-transcriptional mechanisms.

GDNF treatment resulted in a greater amount of $p27^{kip1}$ associated to CDK2 (2.3- and 3.2-fold at 5 and 7 days of treatment, respectively) (Figure 4c) and to CDK4 (1.4- and 2.7-fold at 5 and 7 days respectively) (Figure 4d) compared with cycling cells, as assessed by co-immunoprecipitation; the corresponding kinases activities were proportionally reduced (Figure 4c,d, lower panels).

Suppression of $p27^{kip1}$ expression impairs GDNF/RET-mediated proliferative inhibition

We sought to obtain more direct evidence that $p27^{kip1}$ could be a downstream effector of the GDNF/RET signalling system. To this aim, we used the antisense methodology to suppress GDNF- and RET-MEN2A-induced expression of $p27^{kip1}$. We generated NT2/D1-p27AS cell clones by transfecting a pCMV vector carrying the $p27^{kip1}$ cDNA in the antisense orientation. We selected several neomycin-resistant clones and chose three independent clones for further studies (NT2/D1-p27AS2.6, p27AS2.4 and p27AS2.8); these clones are described elsewhere (Baldassarre *et al*., 1999, 2000). GDNF failed to induce $p27^{kip1}$ up-regulation in

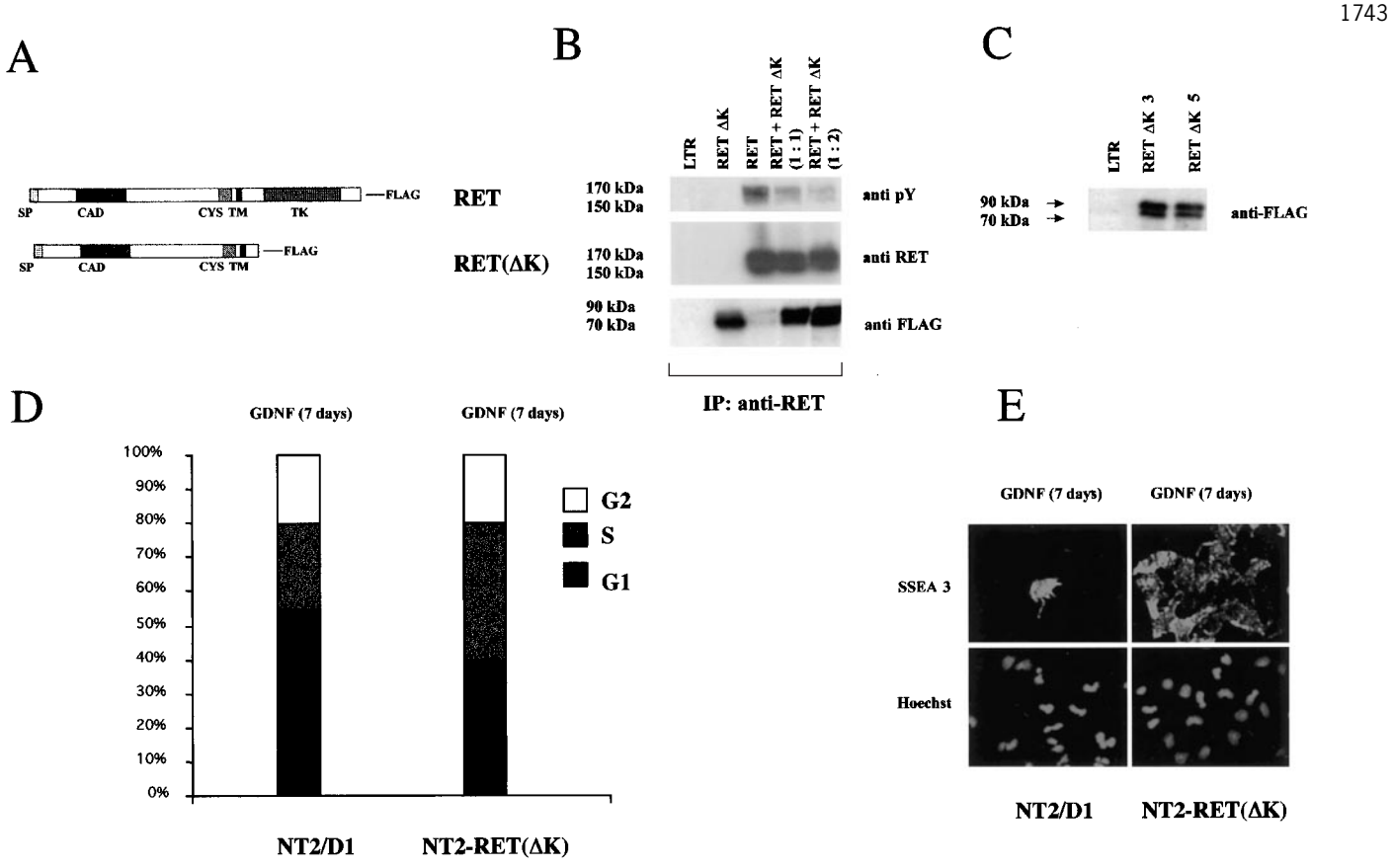

Figure 2 Effects of GDNF in NT2/D1 cells are mediated by RET. (**a**) Schematic representation of the wild type or the kinase-dead C-terminal tagged RET(ΔK) construct lacking the TK domain. (**b**) COS-7 cells were co-transfected with GFRα1 and the indicated plasmid combinations. Cells were treated with GDNF and lysed; subsequently, proteins were immunoprecipitated with anti-RET antibodies directed against the RET TK domain and assayed for tyrosine phosphorylation. Expression of RET(ΔK) was assessed with anti-FLAG antibodies. Molecular weights of wild type and truncated RET products are indicated. (**c**) Expression of the RET(ΔK) plasmid in stably transfected NT2/D1 cells by Western blot using anti-FLAG epitope antibodies. (**d**) Effects of GDNF treatment on cell cycle distribution of NT2/D1 cells expressing RET (NT2-RET(ΔK)). NT2/D1 or NT2-RET(ΔK) cells were treated for 7 days with GDNF (20 ng/ml), collected and stained with propidium iodide; labelled cells were analysed with FACScan using the CELL-FIT program. A representative experiment is reported. (**e**) Effects of GDNF on SSEA-3 expression in NT2/D1 cells transfected with RET(ΔK): a representative field. Upper panel, SSEA-3 expression of untreated (left) or 7-day GDNF-treated (right) NT2/D1 cells; lower panel, Hoechst staining of cell nuclei

p27AS cell clones (a representative clone is shown in Figure 5a). The analysis of growth rate by flow cytometry and BrdU incorporation demonstrated that while GDNF reduced the rate of DNA synthesis in parental cells, it failed to do so in cells in which $p27^{kip1}$ up-regulation had been prevented.

NT2/D1 cells or $p27^{kip1}$-antisense clones (NT2/D1-p27AS2.4, p27AS2.5 and p27AS2.6) were treated with 20 ng/ml of GDNF for 7 days, labelled with propidium iodide and analysed with FACScan. NT2/D1 cells showed 44% of cells in the S phase compartment, whereas upon GDNF treatment, cells accumulated in G1 (26% of S phase cells at 7 days and 22% at 15 days of treatment, respectively). Conversely, in all three $p27^{kip1}$-antisense clones analysed, a larger fraction of cells remained in the S-phase compartment (49, 40, 46% respectively). The results are summarized in Table 1 and a representative experiment is shown in Figure 5c. Similar results were obtained with the analysis of BrdU incorporation (not shown).

GDNF-induced SSEA-3 down-regulation (28% of GDNF-treated NT2/D1 cells stained for SSEA-3) was impaired if $p27^{kip1}$ expression was suppressed (58 and 73% of p27AS2.4 and p27AS2.6 cells stained for SSEA-3, respectively) (Table 1 and Figure 5c). Finally, the RET-MEN2A construct failed to induce $p27^{kip1}$ and to reduce BrdU incorporation and SSEA-3 expression when transiently transfected into NT2/D1-p27AS cells (Figure 6). The results are summarized in Table 2. Thus, the failure to up-regulate $p27^{kip1}$ leads to the failure of NT2/D1 cells to respond properly to the growth-arresting and differentiating signals elicited by RET.

GDNF up-regulates $p27^{kip1}$ expression in different cellular model of neuronal differentiation

We also investigated whether the GDNF/RET system regulated the expression of $p27^{kip1}$ in other cellular model of neuronal differentiation. As a cellular model

we used the rat pheochromocytoma cells PC12 stably transfected with a plasmid encoding the GFRα1 glycosyl phosphatidylinositol-linked GDNF co-receptor (the PC12-GFRα1 cells), and the human neuroepithelioma SK-N-MC cells engineered to express RET and GFRα1 (SK-N-MC/RET/GFRα1 cells). These stable cell lines are described elsewhere (Pelicci *et al*., submitted). Both PC12-GFRα1 cells and SK-N-MC/RET/GFRα1 cells potently responded to 10 min of GDNF treatment (100 ng/ml) as demonstrated by prompt RET autophosphorylation (Figure 7c,d). As in the case of NT2/D1 cells, activation of RET induces growth inhibition in SK-N-MC cells (van Puijenbroek *et al*., 1997) and terminal differentiation in PC12-GFRα1 cells (Pelicci *et al*., submitted). Western blot analysis of $p27^{kip1}$ expression in SK-N-MC/RET/GFRα1 and PC12-GFRα1 cells indicated that GDNF treatment induced accumulation of $p27^{kip1}$ protein in parallel with growth inhibition (Figure 7a,b).

These results indicate that the up-regulation of $p27^{kip1}$ protein levels represents a common molecular target whereby the GDNF/RET system regulates the growth and the terminal differentiation of cells that differentiate along the neuronal pathway.

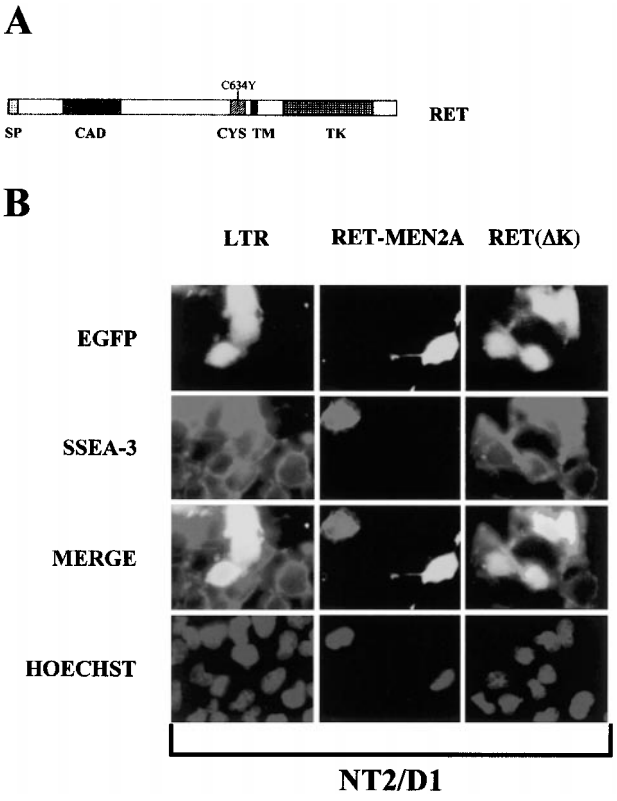

Figure 3 RET-MEN2A induces growth arrest and SSEA-3 down-regulation in NT2/D1 cells. (**a**) Schematic representation of the RET-MEN2A protein. (**b**) The empty LTR vector, RET-MEN2A and RET(ΔK) were co-transfected with pEGFP in NT2/D1 cells as indicated on the top of each column. Upper row, green fluorescence emitted by EGFP reveals transfected cells; second row, SSEA-3 expression in transfected cells; third row, merging of EGFP and SSEA-3; fourth row, Hoechst staining of cell nuclei. Representative fields are shown. Images were taken with a 100× Neo-Achromat Zeiss objective

Discussion

Neuronal differentiation is closely coupled to cessation of cell proliferation, suggesting that the antimitotic activity of neurotrophic factors may be necessary for differentiation. Although it is well-established that ligands of the GDNF family commit neuronal cells to differentiate, the molecular pathways mediating this effect are largely unknown.

In this study we have used the human NT2/D1 cell line as a model system to study the effects exerted by GDNF. NT2/D1 cells exhibit the properties of pluripotential stem cells and terminally differentiate into neurons upon treatment with RA (Andrews *et al*., 1990). We report that GDNF induces growth inhibition and a corresponding down-regulation of the proliferation-associated SSEA-3 antigen in NT2/D1 cells. Remarkably, GDNF reduces the growth of NT2/D1 cells *in vitro* and *in vivo*. Treatment of NT2/D1 cells with GDNF markedly reduced the fraction of NT2/D1 cells in the S phase and tumorigenicity in nude mice. However, GDNF treatment did not drive NT2/D1 cells to a complete differentiated phenotype: upon GDNF treatment, NT2/D1 cells neither developed neuronal processes nor up-regulated the neuron-specific A2B5 marker.

The effects exerted by GDNF in NT2/D1 cells are mediated by activation of RET kinase; indeed, RET

Table 2 Effects of RET mutants on the growth and differentiation of NT2/D1 and NT2-p27AS cells

Plasmids[a]	*Assay*	*NT2/D1*	*NT2/D1 CMV*	*NT2/D1 p27AS2.4*	*NT2/D1 p27AS2.6*
LTR	BrdU	56±7	58±9.6	58±5	65±5
LTR	SSEA-3[c]	68±7.6	65±5	68±8	67±8
RET-MEN2A	BrdU[b]	23±4	26±4	40±5.2	38±7
RET-MEN2A	SSEA-3[c]	22±7.5	25±7	46±6.8	42±6
RET (ΔK)	BrdU[b]	63±7	63±5	68±7	64±8
RET (ΔK)	SSEA-3[c]	78±5.2	77±7.7	71±8	71±5

[a]Cells were transiently transfected with the indicated plasmids and with the pEGFP vector. Forty-eight hours after transfection, cells were either analysed for BrdU incorporation or for expression of surface antigens. Results are the average (±s.d.) of three experiments in which at least 200 GFP-positive cells were counted. [b]BrdU uptake is expressed as a percentage of positive cells with respect to the total transfected cells. [c] SSEA-3 expression was evaluated as the percentage of positive cells with respect to the total transfected cells

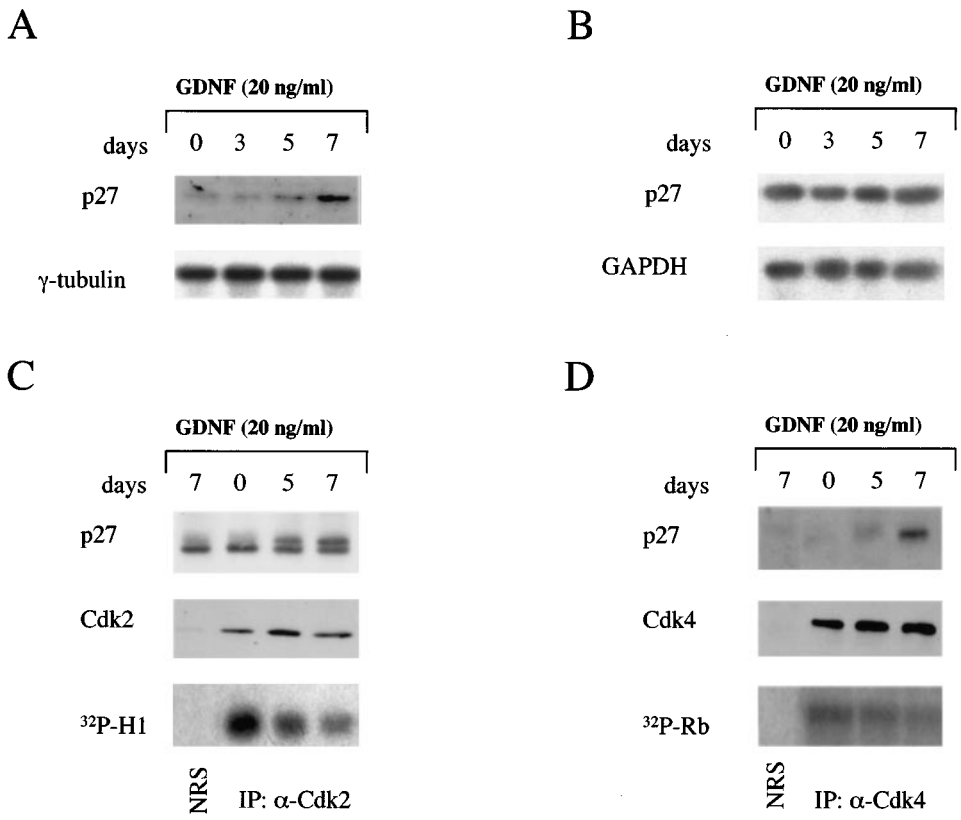

Figure 4 GDNF induces $p27^{kip1}$ up-regulation in NT2/D1 cells. (**a**) Upper panel: Western blot analysis of $p27^{kip1}$ protein levels in exponentially proliferating (lane 0) or GDNF-treated (days 3, 5, 7) NT2/D1 cells. Lower panel: Western blot analysis of γ-tubulin was used for normalization. (**b**) Upper panel: RT–PCR analysis of $p27^{kip1}$ mRNA levels in exponentially proliferating (lane 0) or GDNF-treated (days 3, 5, 7) NT2/D1 cells. Lower panel: RT–PCR analysis of GAPDH mRNA levels was used for normalization. (**c**) Lysates from cycling or GDNF-treated NT2/D1 cells were immunoprecipitated with anti-CDK2 antibodies. Upper panel, $p27^{kip1}$ association to CDK2; middle panel, CDK2 levels in the corresponding immunoprecipitates; lower panel, CDK2 activity using recombinant Histone H1 as substrate. (**d**) Lysates from cycling or GDNF-treated NT2/D1 cells were immunoprecipitated with anti-CDK4 antibodies. Upper panel, $p27^{kip1}$ association to CDK4; middle panel, CDK4 levels in the corresponding immunoprecipitates; lower panel, CDK4 activity using recombinant GST-RB peptide as substrate. As a control non-immune rabbit serum (NRS) was used for immunoprecipitation. Red Ponceau staining of the filters was performed in every experiment to ensure uniform protein loading

signalling is both necessary and sufficient to exert both growth inhibition and SSEA-3 down-regulation. These effects are markedly reduced if RET activity is suppressed by a dominant negative mutant; conversely, the constitutively active RET-MEN2A allele mimicks the effects exerted by exogenously administered GDNF.

Previous work from our laboratory has shown that the CDK inhibitor $p27^{kip1}$ is a key factor for the physiology of NT2/D1 cells at a critical switch point where growth-arrest is followed by differentiation (Baldassarre *et al*., 1999, 2000). Here we show that $p27^{kip1}$ is also a pivotal effector of GDNF/RET-mediated signalling. In fact, $p27^{kip1}$ expression was up-regulated by RET; as a result, $p27^{kip1}$ associated with cyclin-CDK complexes and impaired their kinase activity. On the other hand, expression of an antisense construct for $p27^{kip1}$ virtually abrogated the effects of GDNF and/or RET-MEN2A on cell cycle progression. These findings are consistent with the notion that high levels of $p27^{kip1}$ protein are associated with terminally differentiated neurons (Durand *et al*., 1997, 1998). Neuronal differentiation has been correlated with loss of CDK activity (Yan and Ziff, 1995; Park *et al*., 1996). Inhibition of CDK activity is mediated by up-regulation of CDK inhibitors in neuroblastoma cells treated with differentiating agents (Kranenburg *et al*., 1995). Our results indicate that in NT2/D1 cells, RET-mediated up-regulation of $p27^{kip1}$ is likely responsible for the loss of CDK activity. Interestingly, activation of the RET pathway induces the expression of $p27^{kip1}$ protein in parallel with growth inhibition also in other model systems (such as the human neuroepithelioma SK-N-MC cells and the rat pheochromocytoma cells). These results strongly suggest that this cyclin-dependent kinase inhibitor represents a common molecular target whereby the GDNF/RET system regulates the growth and the terminal differentiation of cells that differentiate along the neuronal pathway.

Similarly, the differentiative response of PC12 cells to NGF depends on growth arrest mediated by a decline in the activity of G1 CDK. In this model

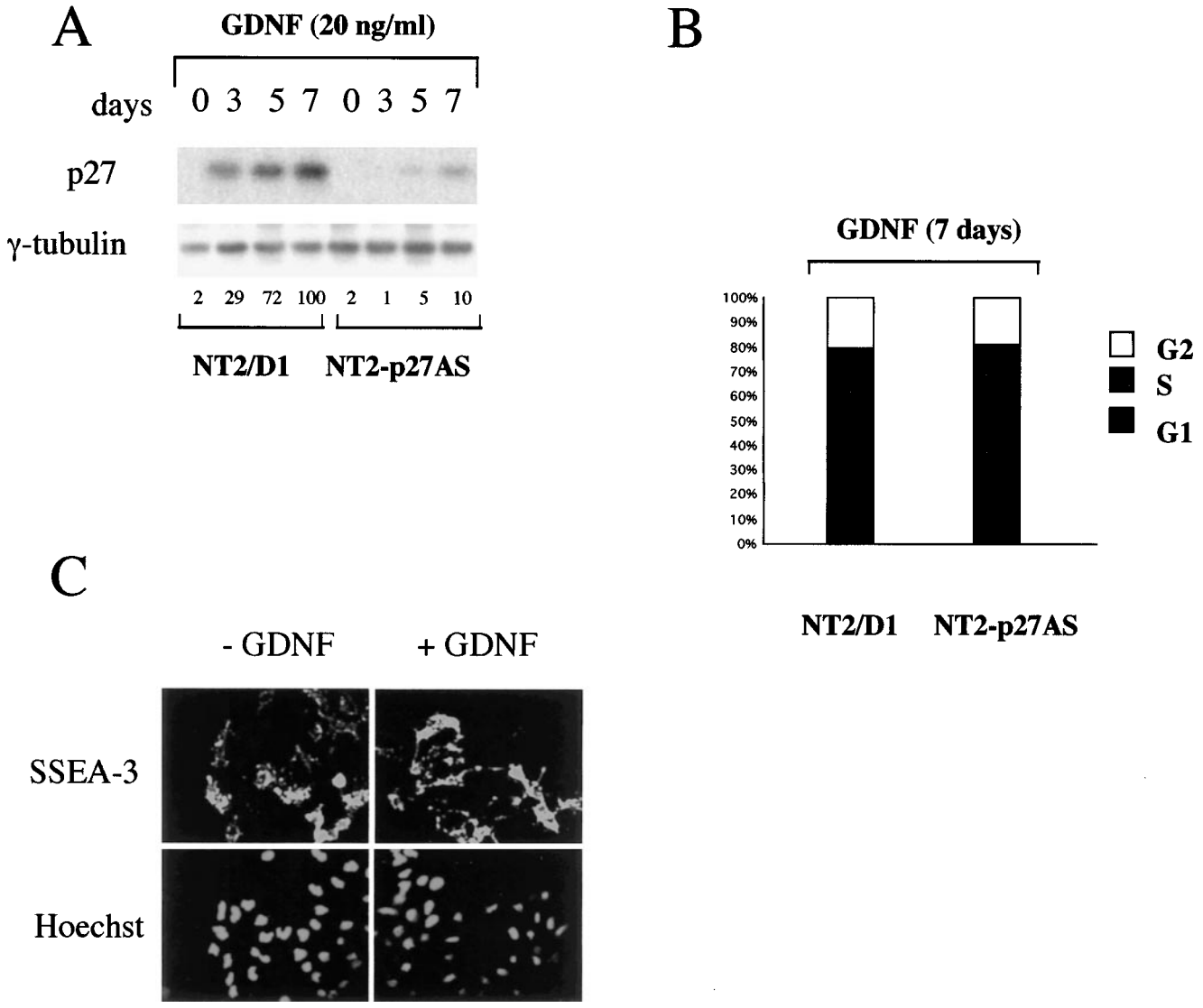

Figure 5 $p27^{kip1}$ is required for GDNF-induced growth arrest and SSEA-3 down-regulation. (**a**) Western blot analysis of $p27^{kip1}$ in NT2/D1 or NT2/D1-p27AS cells treated with GDNF for 0, 3, 5 or 7 days. (**b**) Effects of GDNF treatment on cell cycle distribution of NT2/D1 cells expressing p27 antisense construct (NT2-p27AS). NT2/D1 or NT2-p27AS cells were treated for 7 days with GDNF (20 ng/ml), collected and stained with propidium iodide; labelled cells were analysed with FACScan using the CELL-FIT program. A representative experiment is reported. (**c**) Upper panel, SSEA-3 expression of GDNF-treated NT2/D1-p27AS cells; lower panel, Hoechst staining of cell nuclei. Images were taken with a 100× Neo-Achromat Zeiss objective

system, another cyclin-dependent kinase inhibitor of the cip/Kip family, $p21^{cip1}$ rather than $p27^{kip1}$, mediates growth arrest and differentiation in response to NGF (Billon *et al*., 1996; Erhardt and Pittman, 1998; Qu *et al*., 1998; van Grunsven *et al*., 1996; Yan and Ziff, 1995). Thus it appears that the cyclin-dependent kinase inhibitors of the cip/Kip family play a critical role in the regulation of growth and terminal differentiation induced by neurotrophins.

The pathway(s) leading to $p27^{kip1}$ accumulation after RET stimulation remain(s) to be established. It has been demonstrated that NGF-triggered $p21^{cip1}$ accumulation is mediated by transcriptional activation of the $p21^{cip1}$ promoter (Marshall, 1995; Billon *et al*., 1996). In contrast, several lines of evidence indicate that $p27^{kip1}$ levels are regulated by RA at the post-transcriptional rather than transcriptional levels in neuroblastoma (Borriello *et al*., 2000) and in NT2/D1 cells (Baldassarre *et al*., 2000). The currently accepted model indicates that $p27^{kip1}$ is phosphorylated on threonine 187 and is targeted to ubiquitin-mediated degradation at the G1/S transition (Carrano *et al*., 1999; Montagnoli *et al*., 1999). Our results suggest that in NT2/D1 cells, the expression of $p27^{kip1}$ is essentially regulated at post-transcriptional level. In fact, neither RA nor GDNF increases steady-state levels of $p27^{kip1}$ mRNA. This conclusion is in agreement with the observation that in NT2/D1 cells the ubiquitin/proteasome pathway is involved in the degradation of the $p27^{kip1}$ protein.

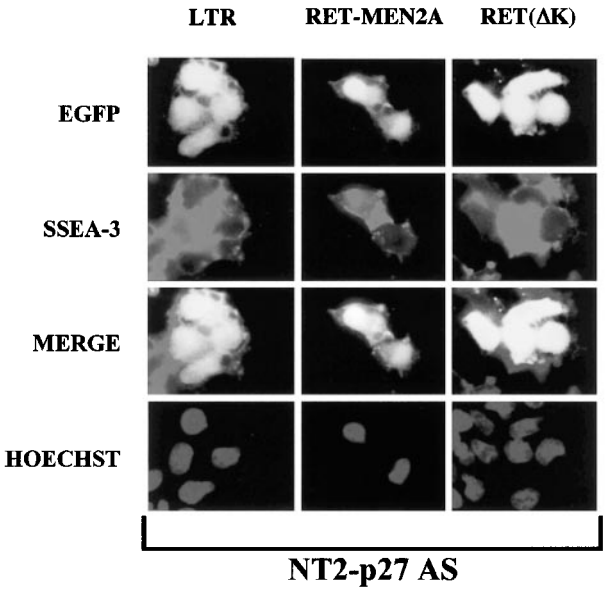

Figure 6 $p27^{kip1}$ is required for RET-MEN2A-induced growth arrest and SSEA-3 down-regulation. LTR, RET-MEN2A and RET(ΔK) plasmids were co-transfected with pEGFP in NT2/D1-p27AS cells as indicated on the top of each column. Upper row, green fluorescence emitted by EGFP reveals transfected cells; second row, SSEA-3 expression in transfected cells; third row, merging of EGFP and SSEA-3; fourth row, Hoechst staining of cell nuclei. Images were taken with a 100× Neo-Achromat Zeiss objective

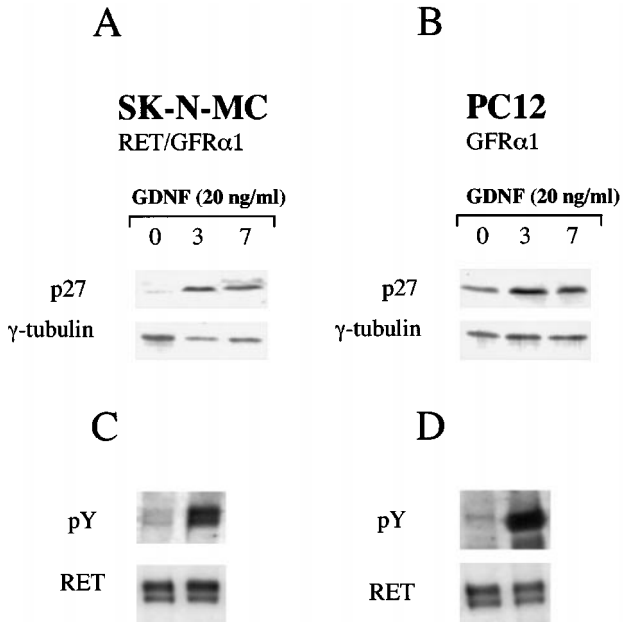

Figure 7 GDNF up-regulates $p27^{kip1}$ expression in human neuroepithelioma SK-N-MC and rat pheochromocytoma PC12 cells. (**a**) Upper panel: Western blot analysis of $p27^{kip1}$ protein levels in exponentially proliferating (lane 0) or GDNF-treated (days 3 and 7) SK-N-MC cells engineered to express RET and GFRα1 (SK-N-MC/RET/GFRα1); lower panel: Western blot analysis of γ-tubulin was used for normalization. (**b**) Upper panel: Western blot analysis of $p27^{kip1}$ protein levels in exponentially proliferating (lane 0) or GDNF-treated (days 3 and 7) PC12 cells engineered to express GFRα1 (PC12/GFRα1); lower panel: Western blot analysis of γ-tubulin was used for normalization. In both cases, GDNF treatment induced rapid RET phosphorylation (**c** and **d**)

In conclusion, our results suggest that the GDNF/RET pathway induces proliferative inhibition in NT2/D1 cells and that $p27^{kip1}$ represents a key molecular factor in GDNF-dependent pathways regulating growth and differentiation.

Materials and methods

Plasmids and antibodies

The RET plasmid encoding the MEN2A-associated mutant (C634Y) cloned in the LTR vector is described elsewhere (Santoro *et al*., 1995). The RET(ΔK) mutant was generated by recombinant PCR by deleting the entire kinase domain of RET (residues 719–1072) and fusing the product in-frame at the C-terminal with the FLAG octapeptide (DYKDDDDK). The mutation was confirmed by DNA sequencing and the tagged RET(ΔK) mutant was cloned in the LTR vector. The expression vector encoding the GFRα1 construct was a gift from Dr S Jing). Polyclonal Ret antibodies were raised against the kinase domain of the protein (Santoro *et al*., 1995). Anti-phosphotyrosine antibodies (4G10) were from Upstate Biotechnology Inc. (Lake Placid, NY, USA). The other antibodies were obtained from Santa Cruz (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) (CDK4), Pharmingen (San Diego, CA, USA), and Transduction Laboratories (Lexington, KY, USA) (anti-p27 and anti-CDK2).

Cell culture and treatments

The NT2/D1 cell line was grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% of heat inactivated foetal calf serum (FCS), 4 mM glutamine, 100 U/ml penicillin and 10 μg/ml streptomycin (GIBCO–BRL, PA, USA). NT2/D1 cells were transfected as described (Baldassarre *et al*., 1999). COS-7 cells were grown in DMEM supplemented with 10% FCS and transfected with the calcium phosphate technique. GDNF was from Alomone labs (Jerusalem, Israel) and was solubilized in serum-free DMEM.

Immunoprecipitation and kinase assay

Cells were scraped and lysed in cold NP-40 buffer (0.5% NP-40, 50 mM HEPES pH 7.2; 50 mM NaCl, 5 mM EDTA) supplemented with a cocktail of protease inhibitors. Proteins were separated and transferred to nitrocellulose filters membranes (Hybond C, Amersham Inc., Bucks, UK) and revealed by the specific antibody using enhanced chemiluminescence (ECL, Amersham Inc.). For immunoprecipitations, 500 μg of proteins were immunoprecipitated twice with 1–2 μg of the indicated antibodies for 60 min at 4°C. Immunoprecipitated proteins were collected on protein A/G-Sepharose (Santa Cruz), resolved on polyacrylamide denaturing gels, transferred to nitrocellulose filters and incubated with primary antibodies. One-tenth of immunoprecipitates were resuspended in kinase buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium ortovanadate, 1 mM DTT, 7.5 mM $MgCl_2$, 50 mM ATP, 10 μCi of [γ-^{32}P]dATP) supplemented with 2 μg

of histone H1 (Upstate Biotechnology) or 1 μg of GST-pRB (Santa Cruz Inc.) and incubated for 15 min at 30°C. Reactions were stopped and radioactive phosphate incorporation was determined by SDS–PAGE. Analysis of the dried gel was performed using a Phosphorimager (GS-525 Biorad) interfaced with a Hewlett-Packard computer.

Immunofluorescence analysis

Bromodeoxyuridine (BrdU) was added to the culture medium to a final concentration of 10 mM and the labelling procedure was carried out for 1 h. BrdU incorporation assay was performed using the BrdU labelling and detection kit (Boehringer Mannheim Biochemicals). To analyse the expression of SSEA-3 and A2B5, cells were incubated with primary antibody followed by rhodamine-conjugated anti-mouse IgGs. Fluorescence was analysed by an epifluorescent microscope that discriminates between rhodamine (SSEA-3 and A2B5) and green fluorescent protein (EGFP).

Flow-cytometric analysis

NT2/D1 cells were analysed for DNA content and expression of cell surface antigens as previously described (Baldassarre *et al*., 1999). Cells were collected and washed in PBS. DNA was stained with propidium iodide (PI) (50 μg/ml) and analysed with FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) interfaced with a Hewlett-Packard computer (Palo Alto, CA, USA). Cell cycle analysis was performed by CELL-FIT program (Becton Dickinson). Detection of SSEA-3 or A2B5 mAbs was performed as previously described (Baldassarre *et al*., 1999).

RNA extraction and RT–PCR

Total RNA was extracted by RNAzol (Tel-Test, Inc., Friendswood, TX, USA), according to standard procedures (Sambrook *et al*., 1989). Five micrograms of total RNA, digested with RNAse-free DNAse, were reverse transcribed using random exanucleotides as primers (100 mM) and 12 units of AMV reverse transcriptase (Promega). Five microliters of cDNA were amplified in a 25 μl reaction mixture containing 1 U of Taq DNA polymerase (Boehringer), 0.4 mM dNTP, 2.0 mM $MgCl_2$, 0.2 mM of each primer. The PCR amplification was performed for 23 cycles (94°C for 30 min, 55°C for 30 min and 72°C for 30 min), using the Protocol Thermal Cycler (AMS Biotechnology). Primers were designed to specifically amplify the transcripts of the mouse and human *p27* gene (forward primer: 5′-ATGT-CAAACGTGCGAGTGTC-3′; reverse primer 5′-TTACGT-TTGACGTCTTCTGAGGCC-3′). In addition, a set of primers specific for the GAPDH gene (forward primer: 5′-ACATGTTCCAATATGATTCC-3′ and reverse primer: 5′-TGGACTCCACGACGTACTCAG-3′) was added to each reaction as internal control for the amount of cDNA tested. The PCR products were separated on 1.5% agarose gel, and transferred to nylon membranes. The membranes were hybridized with a full-length $p27^{kip1}$ cDNA fragment according to standard procedures (Sambrook *et al*., 1989). The random priming procedure was used for labelling (Sambrook *et al*., 1989). The relative level of $p27^{kip1}$ was assessed by comparison with the level of GAPDH in the same sample and quantified by Phosphorimager densitometric scanning. The values were normalized with the corresponding value of GAPDH expression.

Acknowledgements
This work was supported by the Associazione Italiana Ricerca sul Cancro (AIRC), the Progetto Finalizzato Biotecnologie of the CNR, the Progetto Biotecnologie 5% of the Consiglio Nazionale delle Ricerche (CNR), and MURST. We are indebted to Dr PW Andrew for kindly providing the differentiation-specific antibodies and to Jean Ann Gilder for editing the text. G Baldassarre, A Boccia, ML Motti and P Bruni are supported by fellowships from the Fondazione Italiana per la Ricerca sul Cancro (FIRC).

References

Airaksinen MS, Titievsky A and Saarma M. (1999). *Mol. Cell. Neurosci.,* **13,** 313–325.
Andrews PW. (1984). *Dev. Biol.,* **103,** 285–293.
Andrews PW, Nudelman E, Hakomori S and Fenderson BA. (1990). *Differentiation,* **43,** 131–138.
Baldassarre G, Romano A, Armenante F, Rambaldi M, Paoletti I, Sandomenico C, Pepe S, Staibano S, Salvatore G, De Rosa G, Persico MG and Viglietto G. (1997). *Oncogene,* **15,** 927–936.
Baldassarre G, Barone MV, Belletti B, Sandomenico C, Bruni P, Spiezia S, Boccia A, Vento MT, Romano A, Pepe S, Fusco A and Viglietto G. (1999). *Oncogene,* **18,** 6241–6251.
Baldassarre G, Boccia A, Bruni P, Sandomenico C, Barone MV, Pepe S, Angrisano T, Belletti B, Fusco A and Viglietto G. (2000). *Cell Growth Differ.,* **11,** 517–526.
Billon N, van Grunsven LA and Rudkin BB. (1996). *Oncogene,* **13,** 2047–2054.
Borriello A, Pietra VD, Criscuolo M, Oliva A, Tonini GP, Iolascon A, Zappia V and Della Ragione FD. (2000). *Oncogene,* **1,** 51–60.
Califano D, Monaco C, de Vita G, D'Alessio A, Dathan NA, Possenti R, Vecchio G, Fusco A, Santoro M and de Franciscis V. (1995). *Oncogene,* **11,** 107–112.
Carlomagno F, De Vita G, Berlingieri MT, de Franciscis V, Melillo RM, Colantuoni V, Kraus MH, Di Fiore PP, Fusco A and Santoro M. (1996). *EMBO J.,* **15,** 2717–2725.
Carrano AC, Eytan E, Hershko A and Pagano M. (1999). *Nat Cell Biol.,* **1,** 193–199.
Chen C, Fenderson BA, Andrews PW and Hakomori S. (1989). *Biochemistry,* **28,** 2229–2238.
De Vita G, Melillo RM, Carlomagno C, Visconti R, Castellone MD, Bellacosa A, Billaud M, Fusco A, Tsichlis PN and Santoro M. (2000). *Cancer Res.,* **60,** 3916–3920.
Durand B, Fero ML, Roberts JM and Raff MC. (1998). *Curr. Biol.,* **8,** 431–440.
Durand B, Gao FB and Raff M. (1997). *EMBO J.,* **16,** 306–317.
Erhardt JA and Pittman RN. (1998). *Oncogene,* **16,** 443–451.
Jing S, Wen D, Yu Y, Holst PL, Luo Y, Fang M, Tamir R, Antonio L, Hu Z, Cupples R, Louis JC, Hu S, Altrock BW and Fox GM. (1996). *Cell,* **85,** 1113–1124.
Kranenburg O, Scharnhorst V, Van der Eb AJ and Zantema A. (1995). *J. Cell Biol.,* **131,** 227–234.
Lee VM and Andrews PW. (1986). *J. Neurosci.,* **6,** 514–521.
Marshall CJ. (1995). *Cell,* **80,** 179–185.

Miyazono M, Nowell PC, Finan JL, Lee VM and Trojanowski JQ. (1996). *J. Comp. Neurol.,* **376,** 603–613.
Montagnoli A, Fiore F, Eytan E, Carrano AC, Draetta GF, Hershko A and Pagano M. (1999). *Genes Dev.,* **13,** 1181–1189.
Park DS, Farinelli SE and Greene LA. (1996). *J. Biol. Chem.,* **271,** 8161–8169.
Pasini B, Borrello MG, Greco A, Bongarzone I, Luo Y, Mondellini P, Alberti L, Miranda C, Arighi E, Bocciardi R, Seri M, Barone V, Radice MT, Romeo G and Pierotti MA. (1995). *Nat. Genet.,* **10,** 35–40.
Pleasure SJ and Lee VM. (1993). *J. Neurosci. Res.,* **35,** 585–602.
Pleasure SJ, Page C and Lee VM. (1992). *J. Neurosci.,* **12,** 1802–1815.
Qu Z, Wolfraim LA, Svaren J, Ehrengruber MU, Davidson N and Milbrandt J. (1998). *J. Cell Biol.,* **142,** 1075–1082.
Rosenthal A. (1999). *Neuron,* **22,** 201–203.
Sambrook J, Fritsch EF and Maniatis T. (1989). *Molecular Cloning: A laboratory manual.* 2nd edn. Cold Spring Harbor Laboratory Press: New York.
Santoro M, Carlomagno F, Romano A, Bottaro DP, Dathan NA, Grieco M, Fusco A, Vecchio G, Matoskova B, Kraus MH and di Fiore P. (1995). *Science,* **267,** 381–383.
Sherr CJ and Roberts JM. (1999). *Genes Dev.,* **13,** 1501–1512.
Simeone A, Acampora D, Arcioni L, Andrews PW, Boncinelli E and Mavilio F. (1990). *Nature,* **346,** 763–766.
Tansey MG, Baloh RH, Milbrandt J and Johnson Jr EM. (2000). *Neuron,* **25,** 611–623.
Trupp M, Scott R, Whittemore SR and Ibanez CF. (1999). *J. Biol. Chem.,* **274,** 20885–20894.
van Grunsven LA, Billon N, Savatier P, Thomas A, Urdiales JL and Rudkin BB. (1996). *Oncogene,* **12,** 1347–1356.
van Puijenbroek AA, van Weering DH, van den Brink CE, Bos JL, van der Saag PT, de Laat SW and den Hertog J. (1997). *Oncogene,* **14,** 1147–1157.
van Weering DH and Bos JL. (1998). *Rec. Res. Cancer Res.,* **154,** 271–281.
Yan GZ and Ziff EB. (1995). *J. Neurosci.,* **15,** 6200–6212.