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The *RB*-related gene *Rb2/p130* in neuroblastoma dierentiation and in *B-myb* promoter down-regulation

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Abstract

The retinoblastoma family of nuclear factors is composed of *RB*, the prototype of the tumour suppressor genes and of the strictly related genes *p107* and *Rb2/p130*. The three genes code for proteins, namely pRb, p107 and pRb2/p130, that share similar structures and functions. These proteins are expressed, often simultaneously, in many cell types and are involved in the regulation of proliferation and differentiation. We determined the expression and the phosphorylation of the *RB* family gene products during the DMSO-induced differentiation of the N1E-115 murine neuroblastoma cells. In this system, pRb2/p130 was strongly up-regulated during mid-late differentiation stages, while, on the contrary, pRb and p107 resulted markedly decreased at late stages. Differentiating N1E-115 cells also showed a progressive decrease in B-myb levels, a proliferation-related protein whose constitutive expression inhibits neuronal differentiation. Transfection of each of the *RB* family genes in these cells was able, at different degrees, to induce neuronal differentiation, to inhibit [^{3}H]thymidine incorporation and to down-regulate the activity of the *B-myb* promoter.

Keywords: Rb2/p130; retinoblastoma family; neuroblastoma; differentiation B-myb; E2F

Abbreviations: DMSO, dimethyl sulphoxide; EDTA, ethylenediaminetetraacetic acid, disodium salt; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate; PVDF, polyvinyldifluorene; β-gal, β-galactosidase

Introduction

RB,p107 and *Rb2/p130* are the three genes of the retinoblastoma family. Among these, *RB*, responsible for the susceptibility to retinoblastoma, is the prototype of the tumour suppressor genes, whose inactivation is causally related to cancer (Klein, 1987; Weinberg, 1995; Sang *et al*, 1995). The products of *RB* genes are the phosphoproteins pRb, p107 and pRb2/p130, characterised by a peculiar conformational domain, termed 'pocket', which is involved in most of their functionally relevant protein-protein interactions (Riley *et al*, 1994; Paggi *et al*, 1996). Pocket proteins are negative controllers of cell proliferation (Huang *et al*, 1988; Zhu *et al*, 1993; Claudio *et al*, 1994), in part through their ability to interact with members of the E2F family of transcription factors (Sidle *et al*, 1996). Since growth arrest is a prerequisite for differentiation in many cell types, this might in part explain the involvement of *RB*, *p107* and *Rb2/p130* in differentiative processes and in embryonic development as well (Chen *et al*, 1989, 1995; Coppola *et al*, 1990; Gu *et al*, 1993; Lee *et al*, 1992, 1994; Martelli *et al*, 1994; Jacks *et al*, 1992). Besides E2F sequestering, the role of *RB* in differentiation appears to be exerted through transcriptional regulation of differentiation-specific genes (Chen *et al*, 1996; Rohde *et al*, 1996). Furthermore, cell cycle regulatory proteins which modify pRb phosphorylation and its affinity for E2F play an important role in the differentiation of murine neuroblastoma N1E-115 cells (Kranenburg *et al*, 1995).

Neuroblastoma (NB), the most common non-cranial solid tumour of childhood, arises from the embryonic neural crest cells arrested at an early stage of differentiation (Helman *et al*, 1987). *In vivo* (Everson, 1964) as well as *in vitro* (Tsokos *et al*, 1986), NB cells can undergo neural differentiation, recapitulating the potential of the neural crest. The molecular basis of NB differentiation is largely unknown, but proto-oncogene expression is widely modulated during this process (Schwab, 1993). The expression of *B-myb* is down-regulated during differentiation and its constitutive expression inhibits neuronal differentiation (Raschellà *et al*, 1995). The *B-myb* promoter region contains an E2F site (Lam and Watson, 1993; Lam *et al*, 1995) the mutation of which causes constitutive activation of *B-myb* transcription. In the cell cycle, *B-myb* promoter is repressed in G_0 and early to middle G_1, to become active at the G_1/S boundary (Lam and Watson, 1993). By *in vivo* footprinting experiments, it has been demonstrated that in proliferating cells, where *B-myb* transcription is active, the E2F site in the *B-myb* promoter is not occupied (Zwicker *et al*, 1996). These data indicate that E2F site is important for negative regulation rather than for the activation of *B-myb* transcription. The E2F transcription factors can be complexed with and sequestered by the products of the *RB* family genes (Cao *et al*, 1992; Shirodkar *et al*, 1992; Cobrinik *et al*, 1993; Beijersbergen *et al*, 1994; Ginsberg *et*

al, 1994; Hijmans *et al*, 1995; Johnson, 1995; Sala *et al*, 1996a) that negatively modulate E2F activity. Taken together, these observations suggest that *B-myb* could be a target for negative regulation by the *RB* gene family during neural differentiation. In addition, it has been recently shown that *MYCN* overexpression and the heterozygous deletion of *RB* act synergistically in inducing NB in a transgenic model (Weiss *et al*, 1997), stimulating studies on the involvement of the *RB* genes in the transformation process leading to NB.

In the attempt to unravel some of the molecular mechanisms underlying neural differentiation, we analyzed the role of the pocket genes in inhibiting NB cell proliferation, in promoting NB cell differentiation and in controlling *B-myb* promoter activity.

Results

Expression of pRb, p107, pRb2/p130 and B-myb during neuronal differentiation

Western blot analysis indicated that pRb, p107, pRb2/p130 and B-myb expression was clearly modulated during DMSO-induced differentiation process of N1E-115 NB cells (Figure 1). In untreated cells (Figure 1, 0 h), the bands corresponding to pRb and pRb2/p130 had a microheterogeneous pattern due to the presence of species with different degrees of phosphorylation (Chen *et al*, 1989; Beijersbergen *et al*, 1995; Baldi *et al*, 1995). Microheterogeneity was less evident for p107. After 2 h of DMSO treatment (Figure 1, 2 h), pRb, p107 and pRb2/p130 displayed a hyperphosphorylated, slow-migrating band which for pRb2/p130 became the only detectable form. From 2 to 8 h, pRb2/p130 was detected as a faint, hyperphosphorylated band, while from 24 to 96 h the amount of this protein increased dramatically, with a progressive accumulation of an underphosphorylated, fast-migrating form. On the other hand, the amount of pRb and p107 remained high up to 48 h, drastically falling at 96 h, when pRb was mostly underphosphorylated. B-myb levels decreased during DMSO-induced differentiation, reaching the nadir at 96 h. This finding was consistent with the changes of *B-myb* mRNA and protein levels detected in differentiating human NB cells (Raschellà *et al*, 1995, 1996). Normalisation was done by measuring β-actin levels with a specific monoclonal antibody.

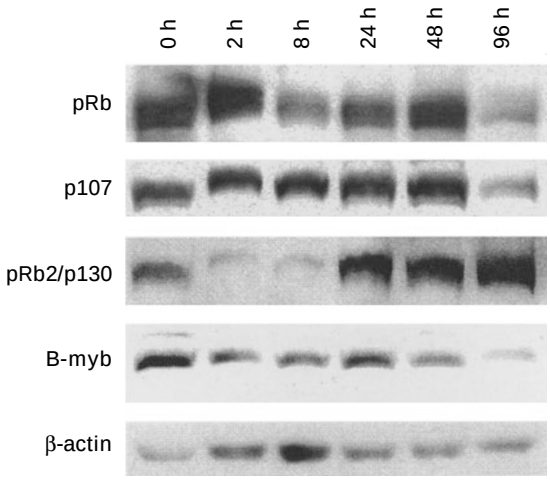

Figure 1 Pattern of RB family protein and B-myb expression during DMSO-induced differentiation of N1E-115 neuroblastoma cells. Cells were harvested at the indicated time points and Western blot analysis for pRb, p107, pRb2/p130 and B-myb expression was done after protein separation by 6% SDS–PAGE. Normalisation was carried out using an anti-β-actin antibody

Induction of neuronal differentiation in N1E-115 cells by genes of the RB family

To test whether overexpression of the pocket proteins would be sufficient to induce NB cell differentiation, N1E-115 cells were transfected with the *lacZ* marker vector in combination with pcDNA3 or the expression vectors for *RB*, *p107* and *Rb2/p130* (Figure 2A–D). The effects of *RB* expression were used

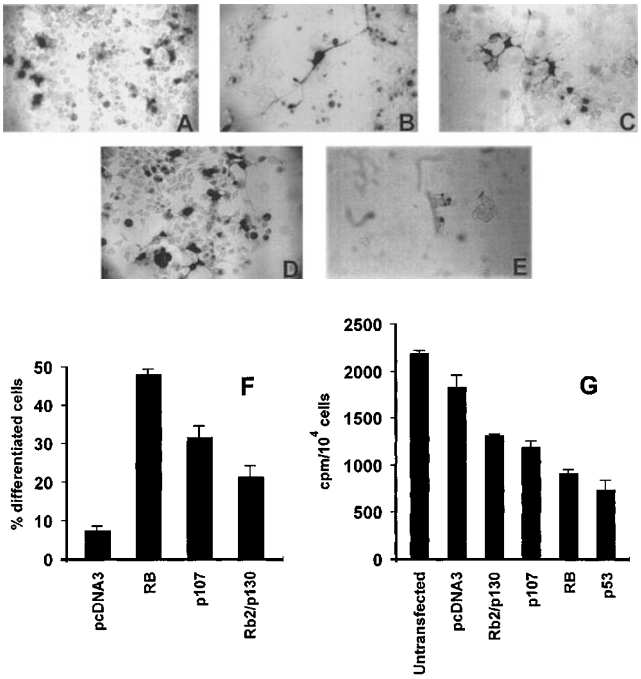

Figure 2 Effect of the *RB* family genes and *p53* on morphology, differentiation and growth suppression of transfected N1E-115 neuroblastoma cells. (**A** to **E**:) morphology of N1E-115 cells after transfection of pcDNA3 vector or of vectors containing *RB*, *P107*, *Rb2/p130* and *p53* genes, respectively. Dark cells are transfected cells, co-expressing lacZ vector and stained for β-gal. (**F**) graphic representation of the effects of the expression of the *RB* genes on the neuronal differentiation of N1E-115 cells. Cells were cotransfected with the empty vector pcDNA3 or with the expression vectors coding for *RB*, *p107* and *Rb2/p130* (indicated in the panel as pcDNA3, RB, p107 and Rb2/p130, respectively), together with an expression plasmid coding for β-gal. Four days after transfection, cells were stained for the β-gal activity and a minimum of two hundred positive cells was observed in each experiment. Cells were scored as differentiated when neuritic emissions exceeded the length of the cellular body. Each bar in the graph represents the mean of three independent experiments $\pm$ S.D. (**G**) growth analysis by [^{3}H]thymidine incorporation. N1E-115 cells were untreated or transfected with expression vectors encoding *Rb2/p130*, *p107*, *RB* and *p53* (indicated as Untransfected, pcDNA3, Rb2/p130, p107, RB and p53, respectively) and pulse-labeled with [^{3}H]thymidine. Incorporation was done and detected as described in Materials and Methods. Each bar in the graph represents the mean of three independent experiments $\pm$ S.D.

as a positive control, since it has been shown to cause neuronal differentiation in N1E-115 cells (Kranenburg *et al*, 1995). Analysis of *p107*- and *Rb2/p130*-transfected cells (dark in Figure 2, panels C and D, respectively) was indicative of the onset of differentiation- related morphologic changes, such as cell flattening and neurite extension. As expected (Kranenburg *et al*, 1995), *RB*-transfected cells clearly showed a more completely differentiated phenotype (Figure 2, panel B). Cells transfected with the empty pcDNA3 vector were unchanged (Figure 2A). To assess whether a strong growth suppressive stimulus was able *per se* to induce NB differentiation, cells were also transfected with *p53*. Transfected cells exhibited morphologic changes, such as cell shrinkage and nuclear fragmentation, reminiscent of an apoptotic phenotype (Figure 2E). Figure 2F, shows the percentage of differentiated cells (neurite outgrowth longer than the diameter of the cell body) among those transfected with *RB* family genes. *RB* was the most efficient differentiation inducer. Cells transfected with each of the three *RB* family genes were also analyzed by indirect immunofluorescence for the expression of neurofilaments (NF) 160 kDa and β-tubulin. The changes in expression, summarised in Table 1, were consistent with the induction of a differentiated phenotype (Tsokos *et al*, 1986; Portier *et al*, 1982). After immunodetection, the same fields were scored for β-gal positivity. Together, these data demonstrate that the overexpression of all *RB* family genes in N1E-115 cells was sufficient to trigger both morphological and biochemical changes specific for neuronal differentiation.

Thymidine incorporation was measured after transfection of N1E-115 cells with each of the *RB* family genes, *p53* or with the empty vector (Figure 2G). Among the *RB* family genes, *RB* was the most active in inhibiting DNA synthesis, followed by *p107* and *Rb2/p130*. The effect of *p53* was not significantly different from that of *RB*. In each experiment, transfection efficiency assessed by β-gal determination was approximately 30% (not shown) and was comparable in all cases. Thus, the overall inhibitory effect should be estimated accordingly.

Regulation of B-myb promoter activity by RB family genes

Next, we assessed whether the *RB* family genes might regulate the promoter activity of the *B-myb* gene. Cotransfections were carried out in exponentially growing N1E-115 cells using the reporter vector pGLHB-myb wt, in which part of the human *B-myb* promoter containing a functional E2F site was cloned (Lam *et al*, 1995), and the expression vectors for *RB*, *P107* or *Rb2/p130*. As a control, the effect of *p53* overexpression, which decreases the levels of *B-myb* mRNA (Lin *et al*, 1994), perhaps acting as an inhibitor of *B-myb* transcription, was also analyzed. Luciferase assays were carried out in N1E-115 cells grown in basal conditions. Figure 3A shows that *B-myb* promoter activity was decreased more than fourfold by *RB*, more than sixfold by *p107* and more than twofold by *Rb2/p130*. The most potent inhibition (more than 20-fold) was brought about by *p53*. Thus, transfection of *p107*, *RB* and *Rb2/p130* inhibited the activity of the human *B-myb* promoter, with a decreasing efficiency. Similar inhibitory effects were also measured in cells treated with DMSO for 48 h (not shown). Next, we asked if the effects of the pocket proteins on the *B-myb* promoter were dependent upon the presence of the E2F site. To this end, the reporter vector pGLHB-myb (mut), bearing a mutation within the E2F consensus site (Lam *et al*, 1995), was used. Figure 3B, shows that, in the case of the *RB* gene, the inhibitory effect was strictly dependent on the presence of a wild-type E2F binding site. On the other hand, the suppressive effect induced by *p107* and, to a lower extent, by *Rb2/p130* was

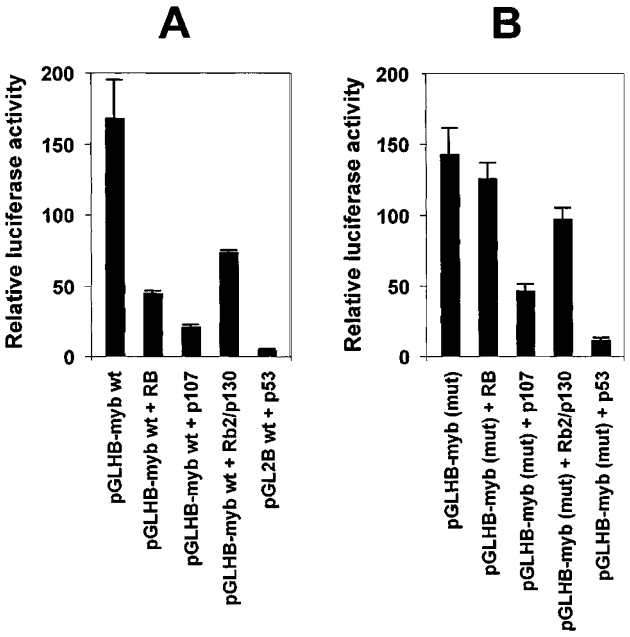

Figure 3 Effect of the *RB* family genes and *p53* on *B-myb* promoter activity. Exponentially growing N1E-115 cells were cotransfected with pGLHB-myb wt (**A**) or pGLHB-myb (mut) (**B**) with the *RB* family genes or *p53* expression plasmids indicated in each panel. Luciferase activity is given relative to that of the promoterless luciferase plasmid pGL2-basic (assigned a value of unity). Results are given as the average $\pm$ S.D. of four independent experiments

Table 1 Immunofluorescence of transfected N1E-115 cells

	Untransfected	pcDNA3	RB	p107	Rb2/p130
Neurofilaments 160 kDa	+/−	+/−	+	+	+
β-tubulin	++	++	+/−	+/−	+/−

Microscopic observations were carried out scoring and photographing at least 200 cells in two different sets of experiments. After immunofluorescence detection, the plates were stained for β-gal activity and the same fields were observed and photographed. Changes in the intensity of immunofluorescence were referred only to the transfected (β-gal positive) cells.

less dependent upon the presence of an intact E2F binding site. Luciferase activity from vector pGLHB-myb (mut) resulted strongly repressed by *p53*.

Since pocket proteins associate with different affinities with the members of the E2F family, we tested whether overexpression of the *E2F4* gene product, a preferential partner for p107 and pRb2/p130 (Ginsberg *et al*, 1994; Beijersbergen *et al*, 1994; Vairo *et al*, 1995), could influence the inhibitory activity of *RB* proteins. *E2F4* lacked a transactivation effect on the *B-myb* promoter, but, when cotransfected with *Rb2/p130*, inhibited the *B-myb* promoter more than *Rb2/p130* alone (data not shown).

Discussion

Despite the remarkable structural and functional similarities among the members of the *RB* family, they do not exert overlapping functions, as suggested by the distinct phenotype associated with targeted disruption of *RB*, as compared to those of the *p107*, *Rb2/p130* and *p107-Rb2/p130* double knock-out mice (Lee *et al*, 1992; Jacks *et al*, 1992; Cobrinik *et al*, 1996). The aim of our work was to analyze, in the same cellular context, the expression of the three members of the *RB* family genes and their role in differentiation control and in modulating the promoter activity of *B-myb*, a gene involved in the regulation of proliferation and differentiation in various cell types (Sala and Calabretta, 1992; Raschellà *et al*, 1995).

In DMSO-induced differentiation of N1E-115 neuroblastoma cells (Figure 1), pRb2/p130 strongly accumulated at late stages of differentiation while pRb and p107 levels were markedly decreased. Quantitative determination could, however, underestimate underphosphorylated pRb, since this form is more strongly associated with the nuclear matrix and is more difficult to extract in the presence of non-ionic detergents (Mittnacht and Weinberg, 1991; Mancini *et al*, 1994). However, differentiation was associated with accumulation of underphosphorylated pRb, which is active in inducing growth suppression. It is worth to note that, at early stages after induction of differentiation (2 h), all three pocket proteins displayed an hyperphosphorylated, fast-migrating band, which was absent in asynchronous cells (0 h). Being these hyperphosphorylated forms inactive as cell cycle negative regulators, it could be argued that N1E-115 cells could need to undergo one or more complete mitotic cycles before completing the differentiation process. This is the case of confluent 3T3-L1 mouse adipocytes induced to differentiation (Richon *et al*, 1997).

Over-expression of all retinoblastoma family members was associated with a decreased [^{3}H]thymidine incorporation and with induction of differentiation in N1E-115 cells, both according to morphological (neurites outgrowing, cell flattening) and to immunohistochemical criteria. In fact, all the three pocket genes were able to elicit a decrease in the expression of β-tubulin and an increase in the levels of NF 160 kDa. NF 160 kDa determination was chosen as a marker for specific neuronal differentiation, because it marks an intermediate level of differentiation, between the expression of NF 68 kDa and that of NF 200 kDa (Bennett and DiLullo, 1985).

We were concerned about the possibility that the induction of differentiation by the pocket genes could be secondary to the inhibition of proliferation. Thus, we asked whether a strong growth suppressive stimulus was able *per se* to induce neuroblastoma cell differentiation. Accordingly, N1E-115 cells were transfected also with *p53*, a gene with well known growth and tumour suppressor properties, but structurally and functionally different from genes of the *RB* family (Harris, 1996). *p53* growth arrest gave rise to peculiar morphological changes which did not have any of the characteristics of neural differentiation but, rather, were reminiscent of an apoptotic phenotype. No further analysis was performed to verify if *p53* overexpression resulted in apoptosis of N1E-115 cells, but induction of apoptosis in tumour cell lines by wild-type *p53* is a well-known phenomenon (Elledge and Lee, 1995; Liebermann *et al*, 1995; Harris, 1996). The different behaviour of *p53* in comparison to that of *RB* family genes did not seem to be caused by a more potent growth suppressive effect exerted by *p53*, since growth analysis did not show significant differences in the effects of *p53* and *RB* (see Figure 2). Thus, in this model system, it appears that *p53* induced cell death, while the growth inhibition caused by *RB* genes was rather associated with neuronal differentiation. *RB* exogenous expression was the most efficient in inducing differentiation, numerically and for the extent of the morphological changes.

It is likely that pocket genes act on many cellular targets to induce differentiation. We investigated possible effects at the transcriptional level by assessing whether the *RB* family genes could regulate the promoter activity of the *B-myb* gene. The functional interrelationship between *RB* genes and *B-myb* was analyzed for several reasons. *B-myb* expression is down-regulated during differentiation of various cell types, including NB (Bies *et al*, 1996; Raschellà *et al*, 1995, 1996). We recently demonstrated that *B-myb* expression is transcriptionally regulated in neuroblastoma differentiation (Raschellà *et al*, 1996). A decrease in B-myb protein expression was also observed in N1E-115 cells induced to differentiate with DMSO (Figure 1), which might represent a prerequisite for differentiation. Noteworthy, the decrease of B-myb expression at late differentiation stages did correlate with an increase of the underphosphorylated active forms of the pocket proteins, albeit in the case of pRb and p107 the total amount of protein was reduced. Finally, the *B-myb* promoter contains a functional E2F-binding site (Lam and Watson, 1993; Lam *et al*, 1995). Accordingly, we evaluated the effect of overexpression of the pocket family genes in modulating the activity of the *B-myb* promoter in N1E-115 cells, where they were able to induce differentiation. As a control, the effect of *p53* was also analyzed. Indeed, pocket genes overexpression was capable to decrease the basal luciferase activity of the construct containing the *B-myb* promoter. In the case of the *RB* gene, the effect was strictly dependent on the presence of a wild-type E2F binding site, whereas the suppressive effect induced by *p107* and, to a lower extent, by *Rb2/p130* was less dependent upon the presence of an intact E2F binding site. This suggests that the effect of *p107* and *Rb2/p130* might be due to interaction

with and inactivation of positive regulator(s) of *B-myb* transcription. Noteworthy, it has been recently reported that the p107 protein binds to B-myb and suppresses its autoregulatory activity in SAOS-2 human osteosarcoma cells (Sala *et al*, 1996b). In the light of the detrimental effect of *p53* overexpression in N1E-115 cells, the role of this gene in controlling *B-myb* promoter activity during differentiation seems unlikely.

In a recent report, we describe that LAN-5 human NB cells, induced to differentiate with retinoic acid, undergo changes in pocket protein expression, which overlap those here described for the N1E-115 cells. Moreover, pRb2/p130 amount and its binding to the *B-myb* promoter as E2F-pRb2/p130 complex increase throughout the differentiation process, where E2F4 is up-regulated and undergoes changes in sub-cellular distribution (Raschellà *et al*, 1997).

Taken together, our findings show, for the first time, that *p107* and *Rb2/p130* induced differentiation and confirm the reported ability of *RB* to promote growth inhibition and differentiation in NB cells (Kranenburg *et al*, 1995). In addition, we propose that, in our model system, *RB* and *p107* could be more substantial in the onset of the differentiation process, while, on the other hand, *Rb2/p130* could play a major role in maintaining the differentiated state. Moreover, the effect on *B-myb* promoter activity by the *RB* family genes are consistent with differences in the mechanisms involved in inducing growth arrest and differentiation in NB cells. In fact, while *RB*-induced repression of *B-myb* promoter activity was strictly dependent on the presence of an intact E2F site, this was not the case for *p107*- and *Rb2/p130*-dependent effects. Our data emphasise the need to take into account the role of active cofactors, such as E2F family members, and their interplay with the *RB* family gene products for a proper evaluation of the contribution of each pocket protein in the regulation of complex cellular processes, such as growth and differentiation.

Materials and Methods

Cell line

The murine neuroblastoma cell line N1E-115 (Kimhi *et al*, 1976; Larcher *et al*, 1992) was maintained in culture and induced to differentiate with DMSO as described (Kranenburg *et al*, 1995).

pRb, p107, pRb2/p130 and B-myb analysis in Western blotting

Cells (5×10^5) were lysed in 50 mM Tris-HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 0.1% Triton X-100, 1 mM PMSF and 10 μg/ml leupeptin. After centrifugation, supernatants were transferred to a fresh tube and electrophoresis sample buffer was added. SDS–PAGE and protein transfer to a PVDF membrane were done as already described (Baldi *et al*, 1995). pRb was detected using the G3-245 monoclonal antibody (PharMingen, San Diego, CA); p107 levels were determined using a rabbit polyclonal antibody (sc-318, Santa Cruz Biotechnology, Inc. Santa Cruz, CA); polyclonal antibodies for pRb2/p130 and B-myb detection were described elsewhere (Pertile *et al*, 1995; Raschellà *et al*, 1996); anti-β-actin JLA20 monoclonal antibody was purchased from Oncogene Science Inc, Cambridge, MA. Bands were detected using the ECL reagents (Amersham, Milan, Italy) for chemiluminescence.

Transfections and luciferase assays

Transient transfections were performed as described (Kranenburg *et al*, 1995). Expression vectors and the amounts used were: lacZ marker vector (2 μg) (Clontech, Palo Alto, CA); pCMVRB (10 μg), pCMVp107 (10 μg) and pCMVRb2/p130 (10 μg) (Claudio *et al*, 1994); pCMVp53 (10 μg) (Noble *et al*, 1992). Luciferase reporter vectors were: pGL2-basic (Promega, Madison, WI), pGLHB-myb wt and pGLHB-myb (mut) (4 μg) (Lam *et al*, 1995). pcDNA3 (Invitrogen, San Diego, CA) was used for controls and for balancing the load of DNA. Assays were carried out 36 h after transfection as described (Lam and Watson, 1993), using the Luciferase Assay kit (Promega, Madison, WI). Samples were quantified using a Lumac Biocounter 2500 luminometer (Lumac, Landgraaf, The Netherlands).

Immunofluorescence

Cells were fixed in methanol/acetone (25:75) for 20 min at -20°C and processed as described (Raschellà *et al*, 1995). Monoclonal antibodies anti-neurofilaments 160 kDa and anti-β-tubulin (Sigma-Aldrich, Milan, Italy) were used at 1:50 and 1:200 dilution, respectively.

Growth analysis

N1E-115 cells were transfected with the expression vectors for *RB*, *p107*, *Rb2/p130*, *p53* and with the empty vector pcDNA3. Thirty-six hours later, cells were pulse-labelled for 150 min with 3.7 kBq/ml of [^{3}H]thymidine (2.59–3.33 TBq/mmol, DuPont de Nemours, NEN Products, Cologno Monzese, Milan, Italy) and counted in a Beckman scintillation counter (Beckman Instruments Inc., Fullerton, CA).

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References

Baldi A, De Luca A, Claudio PP, Baldi F, Giordano GG, Tommasino M, Paggi MG and Giordano A (1995) The Rb2/p130 gene product is a nuclear protein whose phosphorylation is cell cycle regulated. J. Cell. Biochem. 59: 402–408

Beijersbergen RL, Carlée L, Kerkhoven RM and Bernards R (1995) Regulation of the retinoblastoma protein-related p107 by G_1 cyclin complexes. Genes Dev. 9: 1340–1353

Beijersbergen RL, Kerkhoven RM, Zhu L, Carlée L, Voorhoeve PM and Bernards R (1994) E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. Genes Dev. 8: 2680–2690

Bennett GS and DiLullo C (1985) Expression of a neurofilament protein by the precursors of a subpopulation of ventral spinal cord neurons. Dev. Biol. 107: 94 – 106

Bies J, Hoffman B, Amanullah A, Giese T and Wolff L (1996) B-Myb prevents growth arrest associated with terminal differentiation of monocytic cells. Oncogene 12: 355 – 363

Cao L, Faha B, Dembski M, Tsai LH, Harlow E and Dyson N (1992) Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. Nature 355: 176 – 179

Chen PL, Riley DJ, Chen-Kiang S and Lee WH (1996) Retinoblastoma protein directly interacts with and activates the transcription factor NF-IL6. Proc. Natl. Acad. Sci. USA 93: 465 – 469

Chen PL, Riley DJ and Lee WH (1995) The retinoblastoma protein as a fundamental mediator of growth and differentiation signals. Crit. Rev. Eukaryot. Gene Expr. 5: 79 – 95

Chen PL, Scully P, Shew JY, Wang JY and Lee WH (1989) Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell 58: 1193 – 1198

Claudio PP, Howard CM, Baldi A, De Luca A, Fu Y, Condorelli G, Sun Y, Colburn N, Calabretta B and Giordano A (1994) p130/pRb2 has growth suppressive properties similar to yet distinctive from those of retinoblastoma family members pRb and p107. Cancer Res. 54: 5556 – 5560

Cobrinik D, Lee MH, Hannon G, Mulligan G, Bronson RT, Dyson N, Harlow E, Beach D, Weinberg RA and Jacks T (1996) Shared role of the pRB-related p130 and p107 proteins in limb development. Genes Dev. 10: 1633 – 1644

Cobrinik D, Whyte P, Peeper DS, Jacks T and Weinberg RA (1993) Cell cycle-specific association of E2F with the p130 E1A-binding protein. Genes Dev. 7: 2392 – 2404

Coppola JA, Lewis BA and Cole MD (1990) Increased retinoblastoma gene expression is associated with late stages of differentiation in many different cell types. Oncogene 5: 1731 – 1733

Elledge RM and Lee WH (1995) Life and death by p53. 17: 923 – 930

Everson TC (1964) Spontaneous regression of cancer. Ann. NY Acad Sci. 114: 721 – 735

Ginsberg D, Vairo G, Chittenden T, Xiao Z-X, Xu G, Wydner KL, DeCaprio JA, Lawrence JB and Livingston DM (1994) E2F-4, a new member of the E2F transcription factor family, interacts with p107. Genes Dev. 8: 2665 – 2679

Gu W, Schneider JW, Condorelli G, Kaushal S, Mahdavi V and Nadal Ginard B (1993) Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell 72: 309 – 324

Harris CC (1996) Structure and function of the p53 tumor suppressor gene: Clues for rational cancer therapeutic stratagies. J. Natl. Cancer Inst. 88: 1442 – 1455

Helman LJ, Thiele CJ, Linehan WM, Nelkin BD, Baylin SB and Israel MA (1987) Molecular markers of neuroendocrine development and evidence of environmental regulation. Proc. Natl. Acad. Sci. USA 84: 2336 – 2339

Hijmans EM, Voorhoeve PM, Beijersbergen RL, Van 't Veer LJ and Bernards R (1995) E2F-5, a new E2F family member that interacts with p130 in vivo. Mol. Cell. Biol. 15: 3082 – 3089

Huang HJ, Yee JK, Shew JY, Chen PL, Bookstein R, Friedmann T, Lee EY and Lee WH (1988) Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. Science 242: 1563 – 1566

Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA and Weinberg RA (1992) Effects of an Rb mutation in the mouse. Nature 359: 295 – 300

Johnson DG (1995) Regulation of *E2F-1* gene expression by p130 (Rb2) and D-type cyclin kinase activity. Oncogene 11: 1685 – 1692

Kimhi Y, Palfrey C, Spector I, Barak Y and Littauer UZ (1976) Maturation of neuroblastoma cells in the presence of dimethylsulfoxide. Proc. Natl. Acad. Sci. USA 73: 462 – 466

Klein G (1987) The approaching era of the tumor suppressor genes. Science 238: 1539 – 1545

Kraneburg O, Scharnhorst V, van der Eb AJ and Zantema A (1995) Inhibition of cyclin-dependent kinase activity triggers neuronal differentiation of mouse neuroblastoma cells. J. Cell Biol. 131: 227 – 234

Lam EW, Bennett JD and Watson RJ (1995) Cell-cycle regulation of human B-myb transcription. Gene 160: 277 – 281

Lam EW and Watson RJ (1993) An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. EMBO J. 12: 2705 – 2713

Larcher JC, Basseville M, Vayssiere JL, Cordeau-Lossouarn L, Croizat B and Gros F (1992) Growth inhibition of N1E-115 mouse neuroblastoma cells by c-myc or N-myc antisense oligodeoxynucleotides causes limited differentiation but is not coupled to neurite formation. Biochem. Biophys. Res. Commun. 185: 915 – 924

Lee EYHP, Hu N, Yuan S-SF, Cox LA, Bradley A, Lee W-H and Herrup K (1994) Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. Genes Dev. 8: 2008 – 2021

Lee EY, Chang CY, Hu N, Wang YC, Lai CC, Herrup K, Lee WH and Bradley A (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis [see comments]. Nature 359: 288 – 294

Liebermann DA, Hoffman B and Steinman RA (1995) Molecular controls of growth arrest and apoptosis: p53-dependent and independent pathways. Oncogene 11: 199 – 210

Lin D, Fiscella M, O'Connor PM, Jackman J, Chen M, Luo LL, Sala A, Travali S, Appella E and Mercer WE (1994) Constitutive expression of B-*myb* can bypass p53-induced Waf1/Cip1-mediated G_1 arrest. Proc. Natl. Acad. Sci. USA 91: 10079 – 10083

Mancini MA, Shan B, Nickerson JA, Penman S and Lee WH (1994) The retinoblastoma gene product is a cell cycle-dependent, nuclear matrix-associated protein. Proc. Natl. Acad. Sci. USA 91: 418 – 422

Martelli F, Cenciarelli C, Santarelli G, Polikar B, Felsani A and Caruso M (1994) MyoD induces retinoblastoma gene expression during myogenic differentiation. Oncogene 9: 3579 – 3590

Mittnacht S and Weinberg RA (1991) G1/S phosphorylation of the retinoblastoma protein is associated with an altered affinity for the nuclear compartment. Cell 65: 381 – 393

Noble JR, Willetts KE, Mercer WE and Reddel RR (1992) Effects of exogenous wild-type p53 on a human lung carcinoma cell line with endogenous wild-type p53. Exp. Cell Res. 203: 297 – 304

Paggi MG, Baldi A, Bonetto F and Giordano A (1996) Retinoblastoma protein family in cell cycle and cancer: A review. J. Cell. Biochem. 62: 418 – 430

Pertile P, Baldi A, De Luca A, Bagella L, Virgilio L, Pisano MM and Giordano A (1995) Molecular cloning, expression, and developmental characterization of the murine retinoblastoma-related gene Rb2/p130. Cell Growth Differ. 6: 1659 – 1664

Portier MM, Croizat B and Gros F (1982) A sequence of changes in cytoskeletal components during neuroblastoma differentiation. FEBS Lett. 146: 283 – 288

Raschellà G, Negroni A, Pucci S, Amendola R, Valeri S and Calabretta B (1996) B-myb transcriptional regulation and mRNA stability during differentiation of neuroblastoma cells. Exp. Cell Res. 222: 395 – 399

Raschellà G, Negroni A, Sala A, Pucci S, Romeo A and Calabretta B (1995) Requirement of B-*myb* function for survival and differentiative potential of human neuroblastoma cells. J. Biol. Chem. 270: 8540 – 8545

Raschellà G, Tanno B, Bonetto F, Amendola R, Battista T, De Luca A, Giordano A and Paggi MG (1997) Retinoblastoma-related protein pRb2/p130 and its binding to the B-*myb* promoter increase during human neuroblastoma differentiation. J. Cell. Biochem, 67: 293 – 303

Richon VM, Lyle RE and McGehee RE, Jr. (1997) Regulation and expression of retinoblastoma proteins p107 and p130 during 3T3-L1 adipocyte differentiation. J. Biol. Chem. 272: 10117 – 10124

Riley DJ, Lee EYHP and Lee W-H (1994) The retinoblastoma protein: More than a tumor suppressor. Annu. Rev. Cell Biol. 10: 1 – 29

Rohde M, Warthoe P, Gjetting T, Lukas J, Bartek J and Strauss M (1996) The retinoblastoma protein modulates expression of genes coding for diverse classes of proteins including components of the extracellular matrix. Oncogene 12: 2393 – 2401

Sala A and Calabretta B (1992) Regulation of BALB/c 3T3 fibroblast proliferation by B-myb is accompanied by selective activation of cdc2 and cyclin D1 expression. Proc. Natl. Acad. Sci. USA 89: 10415 – 10419

Sala A, Casella I, Bellon T, Calabretta B, Watson RJ and Peschle C (1996a) B-*myb* promotes S phase and is a downstream target of the negative regulator p107 in human cells. J. Biol. Chem. 271: 9363 – 9367

Sala A, De Luca A, Giordano A and Peschle C (1996b) The retinoblastoma family member p107 binds to B-MYB and suppresses its autoregulatory activity. J. Biol. Chem. 271: 28738 – 28740

Sang N, Baldi A and Giordano A (1995) The roles of tumor suppressors pRb and p53 in cell proliferation and cancer. Mol. Cell. Diff. 3: 1 – 29

Schwab M (1993) Amplification of N-myc as a prognostic marker for patients with neuroblastoma. Semin. Cancer Biol. 4: 13 – 18

Shirodkar S, Ewen ME, DeCaprio JA, Morgan J, Livingston DM and Chittenden T (1992) The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. Cell 68: 157 – 166

Sidle A, Palaty C, Dirks P, Wiggan O, Kiess M, Gill RM, Wong AK and Hamel PA (1996) Activity of the retinoblastoma family proteins, pRB, p107, and p130, during cellular proliferation and differentiation. Crit. Rev. Biochem. Mol. Biol. 31: 237 – 271

Tsokos M, Scarpa S, Ross RA and Triche TJ (1986) Differentiation of human neuroblastoma recapitulates neural crest development - Study of morphology, neurotransmitter enzymes, and extracellular-matrix proteins. Am. J. Pathol. 128: 484 – 496

Vairo G, Livingston DM and Ginsberg D (1995) Functional interaction between E2F-4 and p130: Evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. Genes Dev. 9: 869 – 881

Weinberg RA (1995) The retinoblastoma protein and cell cycle control. Cell 81: 323 – 330

Weiss WA, Aldape K, Mohapatra G, Feuerstein BG and Bishop JM (1997) Targeted expression of *MYCN* causes neuroblastoma in transgenic mice. EMBO J. 16: 2985 – 2995

Zhu L, van den Heuvel S, Helin K, Fattaey A, Ewen ME, Livingston DM, Dyson N and Harlow E (1993) Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. Genes Dev. 7: 1111 – 1125

Zwicker J, Liu NS, Engeland K, Lucibello FC and Müller R (1996) Cell cycle regulation of E2F site occupation in vivo. Science 271: 1595 – 1597