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Interplay between the antimetastatic *nm23* and the Retinoblastoma-related *Rb2/p130* genes in promoting neuronal differentiation of PC12 cells

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Abstract

Increasing evidence indicates that the *nm23* genes, initially documented as suppressors of metastasis progression, are involved in normal development and differentiation. We have shown previously that the murine *nm23* gene enhances pheochromocytoma PC12 cells responsiveness to NGF by accelerating cell growth arrest and neurite outgrowth. The present study was aimed at elucidating the mechanisms by which *nm23* controls cell proliferation and promotes neuronal differentiation. We demonstrated that *nm23* modulates the expression of the *Rb2/p130* gene, a negative regulator of cell cycle progression also implicated in the maintenance of the differentiated state. Furthermore, we showed that *nm23*-H1 mutants, defective in inhibiting the invasive phenotype, downregulate *Rb2/p130* expression and inhibit NGF-induced PC12 cell differentiation. In synthesis, our results provide first evidence of interplay between the *nm23* and the *Rb2/p130* genes in driving PC12 cells neuronal differentiation and suggest that the antimetastatic and the differentiative *nm23* functions can have similar features. *Cell Death and Differentiation* (2001) **8,** 470–476.

Keywords: *nm23*; metastasis; retinoblastoma gene family; *Rb2/p130*; cell cycle; growth arrest; pheochromocytoma PC12 cell line; neuronal differentiation

Abbreviations: NDPK, nucleoside diphosphate kinase; NGF, nerve growth factor; CAT, chloramphenicol acetyltransferase; β-gal, β-galactosidase; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

Introduction

The *nm23* gene family includes two murine and six human genes[1] (and references therein).[2] The first, referred to as *nm23*-M1, was isolated on the basis of its reduced expression in highly metastatic murine melanoma cell lines, as compared with their nonmetastatic counterparts.[3] Afterward, a crucial role in blocking metastasis progression has been suggested for the murine *nm23*-M1 and the human *nm23*-H1 genes that share 88% nucleotide identity and encode proteins 95% identical[1,4] (and references therein). Nevertheless, in tumours such as childhood neuroblastoma, a high expression of *nm23* positively correlates with tumor aggressiveness.[5,6] Moreover, a serine 120 to glycine mutation in the Nm23-H1 sequence, that impairs proper protein folding,[7] occurs in aggressive childhood neuroblastoma.[8] Noteworthy, the mutant *nm23*-H1^{S120G} cDNA transduced into human breast carcinoma cells, fails to inhibit cell motility,[9] a fundamental component of the metastatic phenotype. The inhibition of cell motility is also abrogated by the transduction of the mutant *nm23*-H1^{P96S} cDNA.[9] This mutant reproduces the *killer of prune* (k-*pn*) mutation of the *awd* gene, the *nm23* homologue in *Drosophila melanogaster*.[10] The *awd*$^{k-pn}$ mutation is lethal in the genetic context of the null mutation of the *prune (pn)* gene involved in the eye pigment synthesis.[11]

The *nm23* genes encode nucleoside diphosphate kinases (NDPK), exameric enzymes that catalyse the exchange of γ-phosphates between tri- and diphosphonucleosides.[12] The reaction involves the transient phosphorylation of a conserved histidine, namely histidine 118 in the human enzymes.[13] The NDPK activity does not correlate with the suppression of the metastatic phenotype.[14]

Several observations suggest an involvement of the *nm23* genes in development and differentiation[1] (and references therein). Specifically, their action is mainly relevant to the functional differentiation of epithelia and neural tissues.[15–17] We have demonstrated previously that the overexpression of *nm23*-M1 in rat pheochromocytoma PC12 cells enhances susceptibility to nerve growth factor (NGF)-induced sympathetic neuronal cell differentiation by inhibiting proliferation and stimulating neurite outgrowth.[18] Upon NGF treatment, stable *nm23*-M1 PC12 transfectants undergo growth arrest, express cytoskeletal proteins specific to neuritogenesis and display branching neurites within 4 days, whereas parental PC12, as well as control transfectants, require at least 8 days. Stable antisense *nm23*-M1 PC12 transfectants, instead, show a marked increase in the proliferative rate and are inhibited in undergoing differentiation.

Growth arrest being a prerequisite for cell differentiation, genes that negatively regulate the transition from the G1 to the S-phase of the cell cycle are involved in such a phenomenon. A crucial role is played by those belonging

to the family of the retinoblastoma (*RB*) gene, the prototype of the oncosuppressors[19,20] (and references therein). Their products, pRB, p107 and pRb2/p130, are phosphoproteins that control the function of transcription factors such as those of the E2F family mainly responsible for the S-phase entry[21] (and references therein). Moreover, the *RB* family genes regulate embryonic development and differentiation[22] (and references therein). As far as *Rb2/p130* is concerned, it has been suggested that it might play a major role in maintaining the differentiated state, due to the ability of the gene product to form (with the E2F family members) stable complexes at late stages of the differentiation process.[23–27] Nevertheless, *Rb2/p130* overexpression is able to induce differentiation of neuroblastoma cells.[28] High expression levels of pRb2/p130 are detectable in terminally differentiated skeletal muscle and nervous tissue.[29] In PC12 cells, NGF treatment increases pRb2/p130 endogenous expression levels and *Rb2/p130* overexpression is able *per se* to trigger the onset of differentiation.[30]

In this scenario, in order to elucidate the mechanisms by which the modulation of the antimetastatic *nm23* gene influences PC12 cells proliferation and differentiation, we approached, in such model system, the analysis of the negative regulators of the cell cycle G1/S transition. The results herein shown provide the first evidence of interplay between *nm23* and *Rb2/p130* in committing PC12 cells to a more sensitive state for neuronal differentiation.

Results

Expression of the RB family proteins in stable sense and antisense *nm23*-M1 PC12 transfectants

PC12 cells exposed to NGF stop dividing within 7–8 days and gradually develop the phenotype of sympatethic neurons.[31,32] Stable *nm23*-M1 PC12 transfectants, in the presence of NGF, rapidly accumulate in the G0/G1 phase of the cell cycle undergoing early morphological differentiation after 4 days of treatment. On the contrary, stable antisense *nm23*-M1 PC12 transfectants, upon NGF treatment, continue to proliferate without differentiating.[18] In this model system we investigated the expression and postranslational modifications of the RB proteins, because of their role as negative regulators of the cell cycle G1/S transition. Protein analysis was performed on cell lysates from different control, sense and antisense stable *nm23*-M1 PC12 transfectants,[18] both cycling and NGF-treated. Figure 1 shows Western blots chosen as representative of the experiments performed with the different clones. In cycling control and sense transfectants the bands corresponding to pRb, pRb/p130 and p107 displayed, as expected, a microheterogeneous pattern due to the presence of species with different degrees of phosphorylation. In cycling antisense transfectants the bands corresponding to the RB proteins displayed a majority of hyperphosphorylated slow-migrating forms. After 4 and 8 days of NGF-treatment, pRb and p107 showed a similar increase in the hypophosphorylated fast-migrating forms in both control and sense transfectants. The pattern of pRb2/p130 did not change between 4- and 8-day NGF-treated control transfectants, instead a dramatic increase in the protein level, in its underphosphorylated form, was detected in NGF-treated sense transfectants. The increase in pRb2/p130 expression was more evident after 4 days of NGF treatment coincident with the acquisition of the differentiated phenotype by the stable sense *nm23*-M1 PC12 transfectants.[18] NGF-treated antisense transfectants showed a microheterogeneous pattern of all the RB proteins in agreement with their continuous proliferative activity.[18]

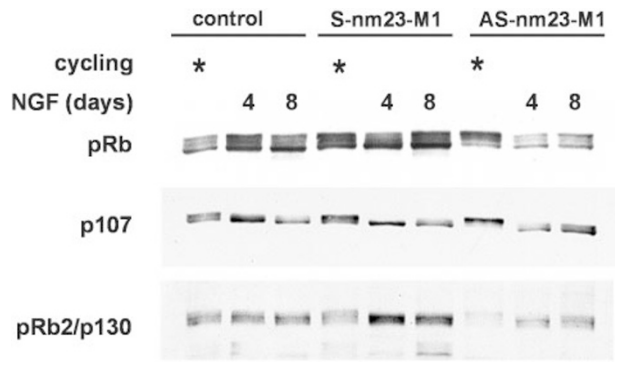


Figure 1 Western blot analysis of the RB pocket proteins in cycling and NGF-treated control, sense (S-*nm23*-M1) and antisense (AS-*nm23*-M1) PC12 transfectants. Aliquots of cell lysates containing 30 μg of proteins were resolved by 6% SDS–PAGE. The analysis shown is representative of at least three experiments per each detection, performed with different clones

Regulation of the *Rb2/p130* promoter activity by *nm23*-M1 in PC12 cells

The marked increase in pRb/p130 expression observed in stable *nm23*-M1 PC12 transfectants upon NGF treatment, prompted us to investigate whether it was due to an increase in the transcription of the *Rb2/p130* gene. For this purpose, PC12 cells were cotransfected with the plasmid containing the minimal promoter region of the *Rb2/p130* gene, encompassing 201 nucleotides prior to the translation codon, upstream the CAT reporter gene (hereinafter referred to as −201 CAT construct),[30] together with either the empty control vector (pcDNA3), or the plasmid encoding *nm23*-M1 (pcDNA3-S-*nm23*-M1),[18] or the plasmid containing the *nm23*-M1 coding region in the antisense orientation pcDNA3-AS-*nm23*-M1.[18] Figure 2 shows the histogram corresponding to the CAT activity detected 2 days after the transfection with each couple of vectors in growing condition (Figure 2A) and upon NGF treatment (Figure 2B). The cotransfection of *nm23*-M1 increased the CAT activity by about 35 and 50% in cycling and in NGF-treated cells, respectively as compared to the basal CAT activity. On the contrary, the cotransfection of antisense *nm23*-M1 inhibited the basal CAT activity by about 45 and 20%, respectively. These data indicate that in PC12 cells *Rb2/p130* transcription can be modulated by different *nm23* levels.

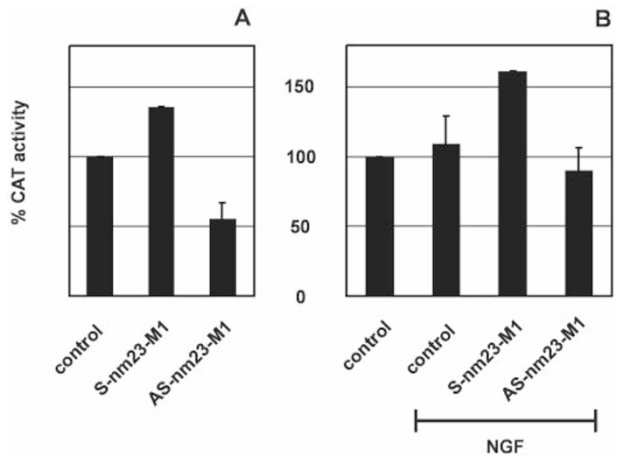


Figure 2 Modulation of *RB2/p130* promoter activity by *nm23*-M1 in PC12 cells. PC12 cells were cotransfected with 6 μg of the -201 CAT construct plus 12 μg of pCDNA3 (control), or pCDNA3-S-*nm23*-M1, or pCDNA3-AS-*nm23*-M1 plus 3 μg of pCMV-β-*gal*. Following transfection, cells were maintained in growth medium (**A**) or NGF-treated (**B**) for 48 h. Afterward, cell extracts, normalised for β-galactosidase activity, were assayed for CAT activity. Histograms in (**A**) and (**B**) show the CAT activities detected for each condition relative to the control activity that was arbitrarily assigned value 100. Data represent the average of five experiments, each in duplicate, for each condition

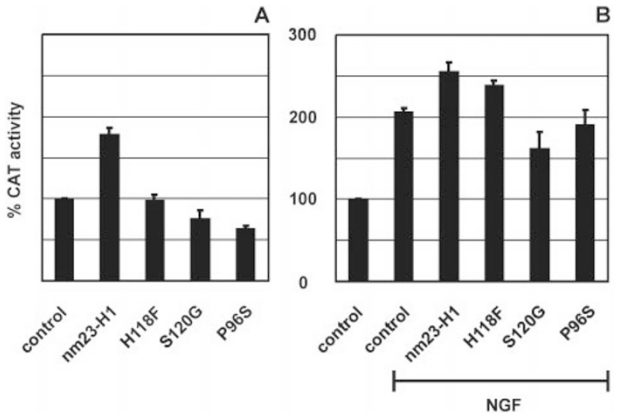


Figure 3 Modulation of *RB2/p130* promoter activity by *nm23*-H1 and *nm23*-H1 mutants in PC12 cells. PC12 cells were cotransfected with 6 μg of the -201 CAT construct plus 12 μg of pCMV (control), or pCMV-*nm23*-H1, or pCMV-H118F, or pCMV-S120G, or pCMV-P96S plus 3 μg of pCMV-β-*gal*. Following transfection, cells were maintained in growth medium (**A**) or NGF-treated (**B**) for 48 h. Afterward, cell extracts, normalised for β-galactosidase activity, were assayed for CAT activity. Histograms in (**A**) and (**B**) show the CAT activities detected for each condition relative to the control activity that was arbitrarily assigned value 100. Data represent the average of five experiments, each in duplicate, for each condition

Regulation of *Rb2/p130* promoter activity and endogenous pRb2/p130 levels by *nm23*-H1 and *nm23*-H1 mutants in PC12 cells

In a first attempt to identify in the *nm23* sequence the residues required for the induction of *Rb2/p130* transcription, we also investigated the effect of different mutants of the human *nm23*-H1 gene on the *Rb2/p130* promoter as compared with wild-type *nm23*-H1. PC12 cells were cotransfected with the -201 CAT construct together with either the empty control vector (pCMV) or the plasmid containing the *nm23*-H1 coding region (pCMV-*nm23*-H1)[9] or one of the following plasmids containing mutant *nm23*-H1 cDNAs: pCMV-*nm23*-H1^{H118F} (hereinafter referred to as pCMV-H118F), pCMV-*nm23*-M1^{S120G} (hereinafter referred to as pCMV-S120G), pCMV-nm23-H1^{P96S} (hereinafter referred to as pCMV-P96S).[9] Histidine 118 is involved in Nm23 NDPK activity;[13] serine 120 is the site of the mutation found in human neuroblastoma;[8] and proline 96 corresponds to the site of the *k-pn* mutation of the *Drosophila awd* gene.[11] Figure 3 shows the histograms corresponding to the CAT activity detected two days after the transfection with each couple of vectors in cycling condition (Figure 3A) or upon NGF treatment (Figure 3B). In cycling cells the cotransfection of *nm23*-H1 increased the CAT activity of 70% above the basal level, whereas the H118F mutant had no effect. The S120G and the P96S mutants inhibited the basal CAT activity of about 25 and 35%, respectively (Figure 3A). In NGF-treated cells the cotransfection of *nm23*-H1 increased the CAT activity of 25% above the control level. The H118F mutant, that was ineffective in cycling cells, increased the CAT activity of about 20%. The S120G and the P96S mutants, instead, inhibited the CAT activity of 20 and 10%, respectively (Figure 3B). We also investigated the effects of *nm23*-H1 and its mutants on *Rb2/p130* translation in PC12 cells. Lysates from PC12 cells transiently transfected with either *nm23*-H1 or each of the different mutants were normalised on the basis of an equivalent expression of the endogenous and exogenous Nm23 proteins, as evaluated by Western blotting (not shown), and then analyzed for the expression of endogenous pRb2/p130. Figure 4 shows that the levels of the *Rb2/p130* gene product detected in cycling and in NGF-treated PC12 transfectants 2 days after the transfection, were strongly inhibited by the S120G and P96S mutants, as also indicated by the reported relative density values.

Inhibition of PC12 cells differentiation by *nm23*-H1 S120G and P96S mutants

We also verified the effect on NGF-induced PC12 differentiation elicited by the expression of the *nm23*-H1 mutants, as compared with wild-type *nm23*-H1. PC12 cells were cotransfected with the plasmids containing *nm23*-H1 or the mutants in combination with a β-galactosidase reporter construct, as a transfection marker, and cultured in NGF containing medium. Four days after transfection, cells were stained for the β-galactosidase activity and a minimum of 200 positive cells were observed per each experiment. Cells were scored as differentiated when neuritic length exceeded at least twofold the cell body. Figure 5 shows the morphology of the PC12 cells transfected with the control pCMV vector, the pCMV-*nm23*-H1, the pCMV-H118F, the pCMV-S120G, and the pCMV-P96S plasmids upon 4-day NGF treatment. The histogram represents the percentage of differentiated cells observed for each type of transfection. It appears evident

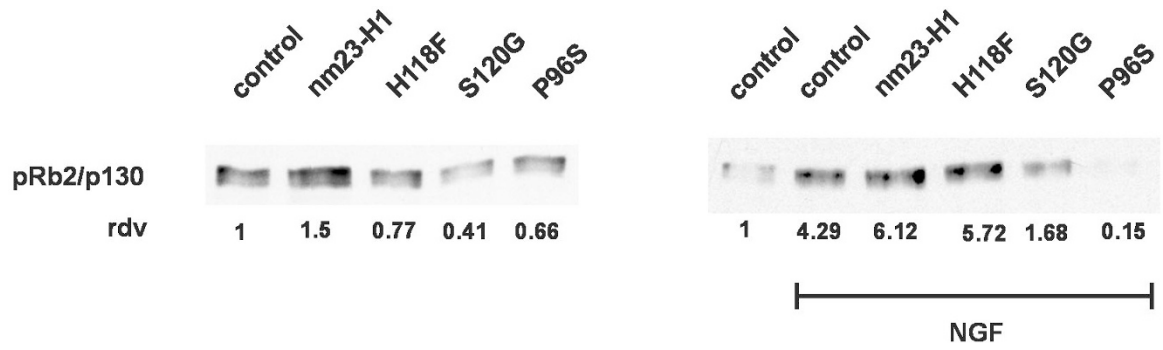


Figure 4 Modulation of *Rb2/p130* translation by *nm23*-H1 and *nm23*-H1 mutants in PC12 cells. Western blot analysis of pRb2/p130 in transient PC12 transfectants. PC12 cells were transfected with 25 μg of pCMV (control), or pCMV-*nm23*-H1, or pCMV-H118F or pCMV-S120G, or pCMV-P96S. Following transfection, cells were maintained in growth medium (left) or NGF-treated for 48 h (right). Afterward, aliquots of cell lysates, normalised for endogenous and exogenous Nm23 protein expression, were resolved by 6% SDS – PAGE. Relative density values (rdv) of the detected bands are reported. The analysis shown is representative of at least three experiments, performed with different transfectants

that *nm23*-H1, as its murine cognate *nm23*-M1,[18] enhanced neuronal differentiation of PC12 cells and that the NDPK-defective H118F mutant was similarly able to promote neuritogenesis. On the contrary, the S120G and P96S mutants inhibited NGF induced differentiation.

Discussion

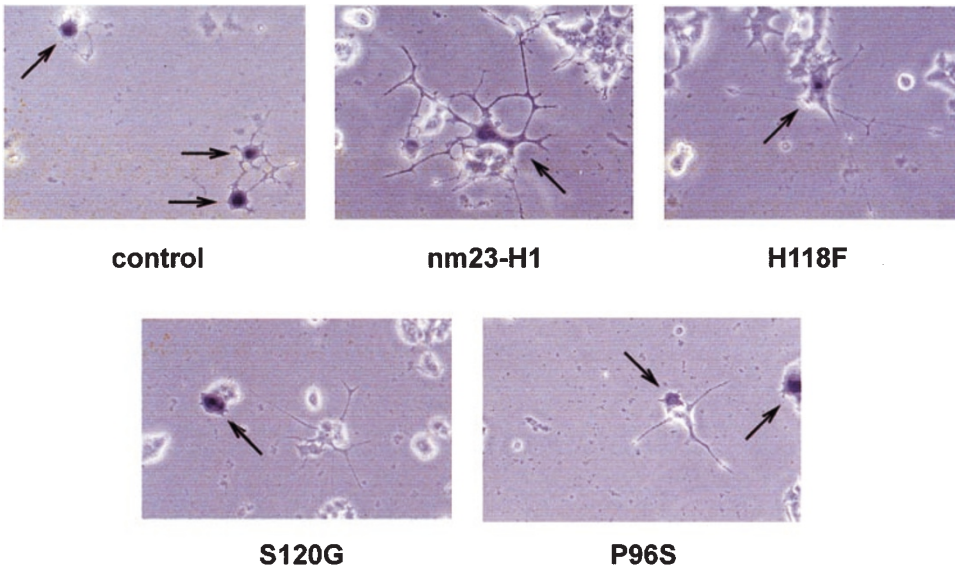
We have demonstrated previously that the murine antimetastatic *nm23* gene is able to promote NGF-induced differentiation of PC12 cells.[18] Cell cycle withdrawal is mandatory for differentiation in many cell types. Accordingly, NGF-treated PC12 cells overexpressing *nm23* rapidly accumulate into the G0/G1 phase.[18] The present study was aimed at establishing the link between the *nm23* gene and the regulatory machinery of cell cycle during PC12 neuronal differentiation.

The genes of the retinoblastoma family, namely *RB, p107* and *Rb2/p130* are negative regulators of the transition between G1- and S-phases of the cell cycle and are crucial effectors in embryo development and cell differentiation. Their products are phosphoproteins that in the hypophosphorylated form modulate the function of several nuclear transcription factors mainly of the E2F family.

We investigated the expression of the members of the *RB* family in PC12 clones stably transduced with either sense or antisense *nm23-M1* cDNA.[18] Upon NGF treatment, as expected, a general increase in the dephosphorylation of the RB proteins was observed in control and sense *nm23*-M1 PC12 transfectants. In contrast, in the antisense *nm23*-M1 transfectants the three RB proteins displayed a pattern of hyperphosphorylated forms. The hyperphosphorylated forms being inactive as negative regulators of the cell cycle, this observation reflected the high proliferative activity shown by the antisense *nm23*-M1 PC12 transfectants despite the NGF treatment.[18] Moreover, beside the increased dephosphorylation, a striking accumulation of pRb/p130 was also evident in the sense *nm23*-M1 PC12 transfectants.

Since NGF treatment of PC12 cells upregulates the endogenous Nm23[18] and pRb2/p130[30] proteins, and *Rb2/p130* overexpression activates the PC12 differentiation program[30], we investigated whether the modulation of the expression of *nm23*-M1 in PC12 cells could result into a modulation of the *Rb2/p130* promoter. Our results indicated that in the PC12 model, the activity of the *Rb2/p130* promoter was modulated with relation to the expression levels of *nm23*-M1. The stimulation of *Rb2/p130* transcription, occurring upon NGF treatment of PC12 cells, was clearly enhanced by the transfection of *nm23*-M1 and, on the contrary, inhibited by the transfection of the antisense *nm23*-M1 cDNA. Moreover, the human *nm23*-H1 was effective in stimulating the activity of the promoter of *Rb2/p130* and the synthesis of the gene product as well.

The synthesis of nucleoside triphosphates does not appear to support the role of *nm23* in the suppression of the metastatic phenotype.[14] We verified the effect of the NDPK defective H118F mutant on *Rb2/p130* transcription and on PC12 differentiation. The mutant was still effective in increasing the *Rb2/p130* promoter activity upon NGF treatment, even if at a lesser extent than wild-type *nm23*-H1. Accordingly, the H118F mutant was as able as wild-type *nm23*-H1 to up-regulate the endogenous expression of pRb2/p130 and to promote NGF-induced differentiation in PC12 cells. These results indicate that the regulation of the NGF-induced differentiation mediated by *nm23* does not require the NDPK activity of the *nm23* gene product, thus resembling what observed as far as cell motility inhibition,[14] a parameter that well correlates with the metastatic potential of tumour cells. These data are in contrast with those described by other authors that reported a suppression of NGF- and dibutyryl cyclic AMP-induced differentiation of a PC12 sub-cell line.[33] They showed a slight decrease of neurite outgrowth upon transient transfection of a NDPK defective mutant and a marked inhibition of differentiation in stably transfected clones, as compared to wild-type NDPK transfection. The discrepancy between the results might be partly due to a clonal peculiarity of the PC12 sub-cell line and to a shorter treatment period prior to evaluating the morphological differentiation. An inhibitory effect on the *Rb2/p130* promoter activity was instead



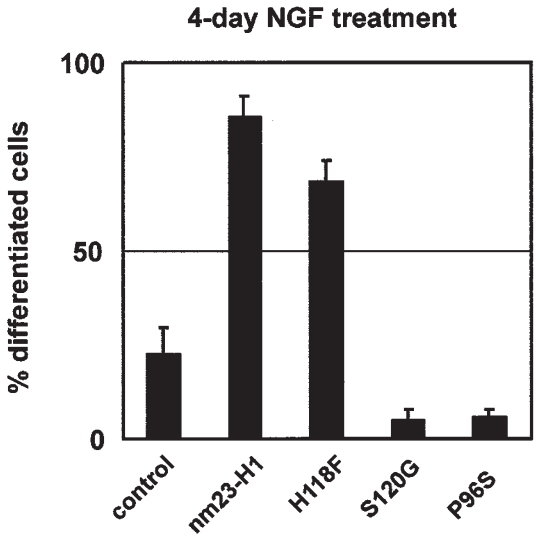


Figure 5 Effects of *nm23*-H1 and *nm23*-H1 mutants on NGF-induced neurite outgrowth in PC12 cells. PC12 cells were cotransfected with 20 μg of pCMV (control), or pCMV-*nm23*-H1, or pCMV-H118F, or pCMV-S120G, or pCMV-P96S plus 4 μg of pCMV-β-*gal*. After 4 day-NGF treatment, cells were stained for β-galactosidase activity. Two hundred positive cells per transfection were scored as differentiated when neuritic length exceeded at least twofold the cell body. Arrows indicate β-galactosidase positive cells. Data in the histograms represent the mean of three experiments, each in duplicate, for each condition

consequent to the expression of the S120G and P96S mutants. In agreement, the expression of PC12 endogenous pRb2/p130 was downregulated. Furthermore, a striking inhibition of NGF-induced PC12 differentiation was observed as comparing the morphology, upon NGF treatment, of S120G and P96S transfectants with control PC12 transfectants.

The serine 120 to glycine mutant NDPK retains the enzymatic activity, instead protein stability to denaturation is greatly compromised[34] leading to an incorrect protein folding.[7] Moreover, the mutation alters subunits interaction, resulting in the preferential formation of dimers rather than examers, relatively to the wild-type, which may affect interaction with other cellular factors.[34] The proline 96 to serine mutation mimics the *k-pn* mutation of *awd*, the *Drosophila nm23* homologue. The *k-pn* mutation alters protein assembly[35] and the structure of the so-called k-pn loop that is important for NDPK subunits interaction and plays an important role in the formation and stability of the examers.[35,36] Noteworthy, our data indicated serine 120 and proline 96 to be of crucial importance for Nm23-H1 to promote PC12 differentiation and to up-regulate *Rb2/p130*

expression. Since both mutations affect subunits interaction, it can be postulated that the regulatory function exerted by the Nm23-H1 protein in cell differentiation strictly requires the formation of high affinity examers that allow the interaction with other factors. In view of this and considering that Nm23-H1 has not been demonstrated as a transcription factor, it can be supposed that the effect on *Rb2/p130* promoter activity, observed upon *nm23*-H1 transfection of PC12 cells, is mediated by the interaction of the Nm23 protein with regulators of *Rb2/p130* transcription. It is conceivable that the expression of the S120G and P96S *nm23*-H1 mutant cDNAs impairs proper protein complexes assembly resulting in the inhibition of the differentiative process and of *Rb2/p130* expression. Moreover, taking into account the effects on cell motility observed upon S120G or P96S cDNA transfection,[9] our results suggest that *nm23* antimetastatic and differentiative functions share a common pathway.

In synthesis, the present study, aimed to verify a putative interplay between *nm23* and the negative regulators of the cell cycle, demonstrated the *Rb2/p130* gene as a partner in driving neuronal differentiation of PC12 cells. The importance of *Rb2/p130* in PC12 cells differentiation as an effector downstream the AP-2 differentiation program, has been demonstrated.[30] AP-2 is a transcription factor implicated in embryogenesis[37,38] and in the ectodermal differentiation program.[39,40] It can be argued that *nm23* enhances the susceptibility of PC12 cells to NGF-induced differentiation by promoting the onset of the differentiation program that involves AP-2 and *Rb2/p130*. Of note, we have herein shown that the transfection of the AS-*nm23*-M1 cDNA into PC12 cells, besides having an inhibitory effect on NGF-induced differentiation,[18] also downregulates *Rb2/p130* promoter activity. In this context, in order to better define the specific role of *nm23* in driving the neuronal differentiation program, it will be of relevance to investigate whether the antimetastatic gene operates in a synergism with AP-2 in positively regulating *Rb2/p130* transcription and translation.

Materials and Methods

Cell lines

PC12 cells were the original described by Greene and Tischler.[32,33] Stable control, sense and antisense *nm23*-M1 PC12 transfectants were as described.[18] Cell lines were grown in RPMI 1640 (Bio Whittaker) supplemented with 10% heat-inactivated horse serum (Hyclone Laboratories) and 5% foetal bovine serum (Hyclone Laboratories). NGF treatment was performed as previously described[18] in RPMI 1640 supplemented with 1% horse serum and 100 ng/ml NGF.

Western blot analysis

Cell lysis and SDS–PAGE were performed as previously described.[41] To avoid quantitative errors due to protein degradation, equal quality of cell lysates was verified by staining duplicate gels with Coomassie brilliant blue G-250 (Bio-Rad).[18] After protein electroblotting to nitrocellulose,[41] complete transfer was verified by staining nitrocellulose with Ponceau S (Sigma).[18] Immunodetection was performed by enhanced chemiluminescence (ECL, Amersham). Primary antibodies were the followings: anti-pRb, anti-pRb2/p130, anti-p107 (all from Santa Cruz Biotechnology, Inc.), anti-GST-Nm23-M1[41] anti-Nm23-H1 (Transduction Laboratories).

Plasmids

Plasmids were the followings: pcDNA3 vector (Invitrogen Corp.); pcDNA3-S-*nm23*-M1 and pcDNA3-AS-*nm23*-M1 plasmids containing the full-length *nm23*-M1 coding region in sense and antisense orientation, respectively;[18] pCMV vector (Invitrogen Corp.); pCMV-*nm23*-H1 containing the full-length *nm23*-H1 coding region;[9] pCMV-*nm23*-H1^{H118F}, pCMV-*nm23*-H1^{S120G}, pCMV-*nm23*-H1^{P96S} containing the H118F, the S120G and the P96S *nm*23-H1 mutant cDNAs, respectively;[9] -201 CAT construct containing the minimal promoter region of the *Rb2/p130* gene cloned in the pSV0t2-CAT vector;[30] pCMV-β-*gal* plasmid expressing the β-galactosidase reporter.[30]

Transient transfections

PC12 cells (2×10^6) were transfected by electroporation at 300 V and 500 μF using a Bio-Rad Gene Pulser apparatus connected to a Bio-Rad Pulse Controller unit achieving about 30–35% transfection efficiency. When required, the electroporated PC12 cells were plated on collagen coated plates and NGF-treated as above.

CAT assays

The plasmids were individually cotransfected with the -201 CAT construct and the pCMV-β-*gal* plasmid into PC12 cells. After 48 h, cell extracts were normalised based on β-galactosidase activity and assayed for CAT activity as already described.[42]

Differentiation assays

The plasmids were individually cotransfected with the pCMV-β-gal plasmid. The transfected cells were NGF-treated for 4 days, stained for β-galactosidase activity and observed under a phase-contrast microscope.

Acknowledgements

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