Mitogen-activated protein kinases control p27/Kip1 expression and growth of human melanoma cells

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The mitogen-activated protein kinases (MAPKs) extracellular signal-regulated protein kinase (ERK)1 and ERK2, involved in regulating cell growth and differentiation, are constitutively active in A375 and WM239 human melanoma cells. Using PD098059, an inhibitor of MAPK kinase (MEK), we investigated the role of persistently activated ERK1/2 in cell growth. The inhibition of MAPK activity induced a dose-dependent growth arrest in G₀/G₁ phase. Correspondingly, we observed the up-regulation of the cyclin-dependent kinase (Cdk) inhibitor p27/Kip1 and hypophosphorylation of the retinoblastoma protein.

Further studies showed that PD098059 treatment significantly decreased Cdk2 kinase activity, most probably owing to an augmented level of p27/Kip1 associated with cyclin E–Cdk2 complexes. The accumulation of p27/Kip1 protein in A375 cells was attributed to its increased stability. Our findings suggest that constitutively active ERK1/2 kinases contribute to the growth of melanoma cells by negative regulation of the p27/Kip1 inhibitor.

Key words: PD098059, proliferation, protein stability.

INTRODUCTION

The mitogen-activated protein kinases (MAPKs) comprise a family of protein serine/threonine kinases mediating signals from a variety of extracellular stimuli. The mitogenic activation of extracellular signal-regulated protein kinase (ERK)1 and ERK2 kinases involves their threonine phosphorylation as well as tyrosine phosphorylation by the dual-specificity MAPK kinases also called MAP kinase/ERK kinase (MEK)1/2. The MEKs in turn are phosphorylated and activated by Raf, which initiates the signalling after its binding to the activated GTP-bound Ras protein. On activation, MAPKs translocate to the nucleus, where they phosphorylate and activate various transcription factors controlling the expression of their respective target genes, and are most frequently involved in the regulation of cell growth and differentiation [1]. Constitutive activation of mitogenic pathways is generally thought to contribute to cell transformation. The expression of constitutively active forms of MEK1/2 kinases was reported to cause transformation of murine fibroblasts and induce tumour formation in nude mice [2]. So far no naturally occurring constitutively active mutated forms of MAPK and MEK have been found. However, several upstream components of the MAPK cascade (Ras, Raf) and its downstream targets (Jun, Fos) are products of proto-oncogenes, emphasizing the relevance of this signalling pathway for promoting cell growth [3].

Several studies have suggested the involvement of the constitutively active MAPK cascade in melanoma development. The overexpression of activated H-ras in cultured human melanocytes changed their phenotype into one resembling transformed melanoma cells [4]. Activating ras mutations were observed in approx. 20% of primary melanomas and at even higher rates in metastatic and recurrent melanomas [5,6], suggesting a role of activated Ras in promoting progression of the disease. The importance of the

Ras signalling cascade for melanoma growth was further underlined by a recent study in a transgenic mouse model: it was shown that oncogenesis and maintenance of melanoma required the continuous presence of activated Ras [7].

Here we investigate the relevance of constitutively active MAPKs in human melanoma cells and show that their inhibition leads to G₀/G₁ arrest and to up-regulation of the inhibitor of cyclin-dependent kinases (Cdks) p27/Kip1.

EXPERIMENTAL

Cells and reagents

A human A375 malignant melanoma cell line established from a solid tumour was purchased from A.T.C.C. (CRL-1619). Human primary WM902b and metastatic WM239, WM9 malignant melanoma cells were obtained from Dr R. S. Kerbel (Sunnybrook Health Science Center, Toronto, Canada). Human Mel Inv metastatic melanoma cells were kindly provided by Dr A. Bosserhoff (Department of Pathology, RWTH Aachen, Germany). Cells were grown in RPMI 1640 medium supplemented with 5% (for general culture) or 1% (for growth assays) (v/v) fetal calf serum, 50 µg/ml penicillin and 100 µg/ml streptomycin in a humidified air/CO₂ (19:1) atmosphere. PMA, puromycin, MG132, U0126 and PD098059 were purchased from Calbiochem (Bad Soden, Germany).

Growth inhibition assay

Viable cells (3×10^3) were seeded in triplicate into 96-microwell plates; PD098059 or vehicle only (DMSO) was added to the medium at various concentrations. After 4 days of culture, an

Abbreviations used: Cdk, cyclin-dependent kinase; ERK, extracellular signal-regulated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase (MAP kinase/ERK kinase); pRb, retinoblastoma protein.

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XTT {sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis-(4-methoxy-6-nitro)benzenesulphonic acid hydrate} colorimetric assay (Roche, Mannheim, Germany) was performed as described previously [8]. The percentage growth inhibition was calculated in relation to the growth of control cells.

DNA staining and flow cytometry

For the analysis of DNA content, 5×10^5 cells were stained with propidium iodide by using the CycleTEST Plus DNA Reagent Kit (Becton Dickinson, Heidelberg, Germany) in accordance with the manufacturer's protocol. Fluorescence data were collected with a FACScalibur (Becton Dickinson) and further analysed using the ModFit 2.0 software (Verity Software, Topsham, ME, U.S.A.).

Immunoblotting analysis

Cells were lysed on the dish in lysis buffer A containing 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% SDS, 20 mM Tris/HCl, pH 7.2, 158 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4 , 1 mM PMSF, 5 $\mu\text{g}/\text{ml}$ aprotinin and 5 $\mu\text{g}/\text{ml}$ leupeptin. Lysates were further cleared by centrifugation at 12000 *g* and the protein concentration was determined with the Bio-Rad protein assay reagent (Munich, Germany). Equal amounts of protein were used for SDS/PAGE gels, transferred to PVDF membrane (GelmanSciences, Dreieich, Germany) and probed with the respective antibodies. The antibodies used were polyclonal phospho-specific anti-(p44/42 MAPK) antibodies (threonine-202/tyrosine-204; New England Biolabs, Schwalbach, Germany), monoclonal anti-p21 antibodies (SX118; PharMingen, Heidelberg, Germany), polyclonal anti-ERK1 (C-16), anti-ERK2 (C-14), anti-Cdk2 (M2), anti-Cdk4 (H-303), anti-(cyclin E) (C-19), anti-p18 (N-20), anti-p27 (C-19), anti-(retinoblastoma protein) (anti-pRb) (C-15) and monoclonal anti-(cyclin D1) (R-124) antibodies (Santa Cruz Biotechnology, Heidelberg, Germany). Detection and quantification of signals generated by the enhanced chemiluminescence method (Amersham, Braunschweig, Germany) was performed on the Lumi-Imager with LumiAnalyst Software (Roche).

Immunoprecipitation

Before immunoprecipitation, cells were extracted in lysis buffer B containing 0.5% Nonidet P40, 50 mM Hepes, pH 7.5, 150 mM NaCl, 25 mM β -glycerophosphate, 15 mM pyrophosphate, 25 mM NaF, 5 mM EGTA, 1 mM EDTA, 1 mM Na_3VO_4 , 1 mM PMSF, 5 $\mu\text{g}/\text{ml}$ aprotinin and 5 $\mu\text{g}/\text{ml}$ leupeptin. After adjustment to equal protein concentrations, lysates were incubated overnight at 4 °C with 1 μg of polyclonal antibodies against human cyclin E (C-19) or against human Cdk4 (H-303) (Santa Cruz Biotechnology). The immunoprecipitates were later collected with Protein A-Sepharose, washed three times with lysis buffer and analysed further by SDS/PAGE. Western blot analysis was performed with polyclonal anti-p27 antibodies (C19); after stripping, the blot was redeveloped with polyclonal anti-Cdk2 antibodies (M2) or anti-Cdk4 antibodies (H-303) (Santa Cruz Biotechnology). The detection of cyclin E was not possible, probably owing to co-migration of cyclin E with the heavy chains of the anti-(cyclin E) antibodies.

Immunocytochemistry and confocal fluorescence microscopy

Cells grown to subconfluence on coverslips were fixed in 2% (w/v) paraformaldehyde at room temperature for 20 min. After

being washed three times with PBS, the cells were permeabilized with 0.2% (v/v) Triton X-100 and incubated first with polyclonal anti-p27 antibodies (C19; Santa Cruz Biotechnology) and then with secondary FITC-conjugated antibodies. The fluorescence images were observed with a laser-scanning microscope LSM 510 (Zeiss, Halbergmoos, Germany), with an Apochromat 63 $\times$ objective lens, 488 nm laser excitation and a 505 nm bandpass emission filter.

Histone H1 kinase assay

Histone H1 kinase assays were performed as described by Hermeking et al. [9]. In brief, the anti-Cdk2 (M2) immunoprecipitates prepared from 150 μg of lysate protein were incubated for 20 min at 30 °C in 20 μl of kinase buffer [20 mM MgCl_2 /10 mM EGTA/40 mM Hepes (pH 7.0)] with 1 μg of histone H1 (Roche) and 1 μl of 300 mCi/mmol [γ - ^{32}P]ATP (Amersham). After stopping the reaction with 2 $\times$ SDS sample buffer, phosphorylated proteins were resolved by SDS/PAGE [12% (w/v) gel] and signals were analysed with a Storm 840 PhosphorImager with the ImageQuant software (Molecular Dynamics).

RNA isolation and Northern blot analysis

Total RNA was isolated with the RNeasy kit from Qiagen (Hilden, Germany). RNA (5 μg) was denatured, size-fractionated by electrophoresis in a 1% (w/v) agarose/formaldehyde gel and transferred to a GeneScreen Plus nylon membrane (DuPont NEN Research Products, Boston, MA, U.S.A.). Detections of p27 mRNA were performed with human p27 cDNA labelled with [α - ^{32}P]dATP with the use of a random primer labelling kit (Roche). After stripping of the blot, hybridization with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was used to control the loading and transfer of RNA. Signals were quantified with a Storm 840 PhosphorImager with the ImageQuant software (Molecular Dynamics) and normalized to the GAPDH values.

RESULTS

Constitutive activation of the MEK/MAPK pathway in A375 cells

We have shown previously that the specific activation of tyrosine phosphatase SHP-2, often acting as an adaptor to the Ras/MAPK pathway, did not affect the growth behaviour of transfected human A375 melanoma cells [8]. By testing the activity state of ERK1 and ERK2 MAPKs in Western blot analysis with antibodies recognizing specifically the tyrosine-phosphorylated forms of both proteins, we observed elevated levels of phosphorylation, predominantly of ERK2 and to a much smaller extent that of ERK1. The intensity of the phosphospecific bands did not decline on serum starvation of the cells for 24 h (Figure 1A). Treating the cells with serum or PMA did not significantly increase MAPK phosphorylation. Thus ERK1 and ERK2 MAPKs seem to be constitutively active in A375 melanoma cells.

To investigate the contribution of MEK to the pronounced ERK1/ERK2 phosphorylation, we used PD098059, a highly specific inhibitor of MEK activation [10]. Incubation of A375 cells with PD098059 dose-dependently decreased the MAPK activation. In concentrations exceeding 5 μM , ERK1/ERK2 phosphorylation was diminished to undetectable levels without any major effect on the total level of MAPK protein (Figure 1A). Similar to A375 cells, we found constitutively activated and not further PMA-inducible ERK1 and ERK2 also in another human metastatic melanoma cell line, WM239, but not in other primary

Figure 1 MAPKs are constitutively tyrosine-phosphorylated in human A375 and WM239 melanoma cells

A375 (A) and WM239 (B) cells starved for 24 h in medium without serum were treated thereafter for 30 min with fetal calf serum (10%, v/v), PMA (100 nM) or PD098059 at the indicated concentrations. Equal amounts of total cell lysates (50 μ g of protein) were separated by SDS/PAGE [8.75% (w/v) gel] followed by a Western blot analysis with phospho-specific anti-p44/42 MAPK antibodies. After being stripped, the blot was redeveloped with anti-ERK1 and/or anti-ERK2 antibodies to demonstrate equal loading. The positions of a molecular mass marker (in kDa) and of ERK1 and ERK2 are indicated.

(WM902b) and metastatic (WM9 and Mel Inv) melanoma cell lines (Figure 1B, and results not shown). As expected, the activities of MAPKs in WM239 cells could be decreased by treatment with PD098059 at concentrations above 10 μ M. These results indicate that constitutive activation of MAPK in human melanoma cells is the result of a constant positive regulation by members of Ras/MAPK pathway located further upstream.

PD098059 inhibits the growth of A375 cells

We analysed the effect of PD098059 on the proliferation of A375 and WM239 cells (Figure 2A). PD098059 at concentrations ranging from 5 μ M to 20 μ M exerted a dose-dependent growth inhibitory effect on the cells within 4 days of incubation. Unexpectedly, low inhibitor concentrations seemed to promote the proliferation of A375 slightly but not that of WM239 cells. Nevertheless, the extent of growth inhibition induced by higher concentrations of PD098059 correlated with the decrease in ERK1/ERK2 phosphorylation demonstrated in Figure 1.

To characterize this effect further, we performed a cell cycle analysis by measuring the DNA content of A375 cells incubated with 10 μ M PD098059 for different periods (Figure 2B). A strong growth-inhibitory effect was visible after 24 h, with a significant decrease in the number of dividing cells. After 48 h almost 99% of the cells were arrested in the G_0/G_1 phase. A longer incubation with the inhibitor further increased the ratio of sub- G_1 events from less than 1% to over 7% of all counted events, indicative of apoptotic cells. WM239 cells reacted to the inhibition of MEK in a similar way (results not shown). PD098059 therefore induces a G_0/G_1 arrest in both tested human melanoma cell lines and, secondarily, with greatly prolonged incubation times might additionally decrease cell survival.

Figure 2 Effect of PD098059 on the growth of human melanoma cells

(A) A375 cells (■) or WM239 (□) (initial density 3000 cells per well) were cultured for 4 days in the presence of PD098059 at different concentrations. Growth was assessed by an XTT test; values were recalculated in relation to controls treated with vehicle only [0.05% (v/v) DMSO]. Error bars represent the S.D. for triplicate samples. (B) For cell cycle analysis, cells were incubated in medium with or without the addition of 10 μ M PD098059 for the durations indicated. After staining of DNA with propidium iodide, fluorescence was measured with a FACScalibur (Becton Dickinson), and the data were quantified further with ModFit 2.0 software (Verity Software). Sub- G_1 values were excluded from the calculation.

Inhibition of MAPKs by PD098059 leads to the up-regulation of p27/Kip1 protein

To explain the effect of PD098059 on G_0/G_1 arrest we examined the protein levels of negative regulators of cell cycle progression, the Cdk inhibitors. Incubation of A375 and WM239 cells with PD098059 resulted in the accumulation of p27/Kip1 protein (Figures 3A and 3B). In A375 cells the effect became detectable after 24 h, when p27/Kip1 protein amounts were increased 2.5-fold above the respective control levels, reached a maximum

Figure 3 MEK inhibition results in up-regulation of p27/Kip1 expression in human melanoma cells

Stimulation-dependent accumulation of p27/Kip1 protein in A375 (A, C–E) and WM239 (B) cells. (A–D) Western blot analysis of cells treated with 10 μ M PD098059 (A, B, D) or 1 μ M U0126 (C) in comparison with controls incubated with vehicle only [0.05% (v/v) DMSO] for the durations indicated. Equal amounts of total cell lysates (50 μ g of protein) were separated by SDS/PAGE on 6% (for Rb detection) or 12.5% (for p27 and ERK2 detection) gels. Blots were developed with anti-Rb, anti-p27, anti-p21, anti-p18 and anti-(cyclin D1) antibodies. After being stripped, blots were redeveloped with anti-ERK2 antibodies to demonstrate equal loading. The positions of molecular mass markers (in kDa) and of hyperphosphorylated and hypophosphorylated pRb (pRbP and pRb respectively), Cdk inhibitors, cyclin D1 and ERK2 are indicated. (E) Nuclear localization of p27/Kip1 in A375 cells. Indirect fluorescence of cells stained intracellularly with anti-p27 antibodies, revealed with FITC-conjugated secondary antibodies and observed with a laser-scanning microscope with a Zeiss ApoChromat objective (63 $\times$) and identical measurement parameters in both control and PD098059-treated cells.

after 48 h (5-fold increase) and stayed elevated for up to 72 h of stimulation. The level of a control protein, ERK2, remained unchanged. Apparently the time course of p27/Kip1 protein accumulation correlates well with the abrogation of cell growth (Figure 2). A similar effect of the p27/Kip1 up-regulation coinciding with a strong G₀/G₁ growth arrest was efficiently induced also by U0126, another inhibitor of ERK activation structurally unrelated to PD098059 (Figure 3C, and results not shown).

The protein level of other Cdk inhibitors present in A375 cells, p18/INK4c and p21/Cip1, did not significantly change in the course of PD098059 treatment (Figure 3D). Because A375 cells lack the expression of both p15/INK4b and p16/INK4a genes [11] and the protein levels of two other inhibitors, p19/INK4d and p57/Kip2, were hardly detectable, p27/Kip1 might be a specific target of MAPK-dependent regulatory pathways. It is also worth noting that the expression of cyclin D1, which reportedly can be regulated by ERK kinases in a variety of cell types [3,12,13], remained unchanged on incubation with PD098059 (Figure 3D).

The function of p27/Kip1 as an inhibitor of cell cycle progression depends on its proper nuclear localization [14]. As determined by indirect immunofluorescence, p27/Kip1 resided almost exclusively in nuclei of untreated A375 cells and the inhibition of MAPK activity strongly enhanced the observed nuclear signals (Figure 3E). Thus it seems plausible that augmented amounts of nuclear p27/Kip1 could negatively influence the activity of Cdk, which are responsible for the inactivation of pRb. pRb, a key regulator of the cell cycle, is inactivated by its hyperphosphorylation, thereby allowing cell cycle progression. As expected, in PD098059-treated A375 cells, the accumulation of p27/Kip1 was paralleled by the disappearance of hyperphosphorylated pRb (Figure 3A). It decreased to the lowest level after only 24 h of incubation with PD098059 and remained low. Interestingly, after 72 h of treatment we also observed a decrease in the total level of Rb protein.

Decrease in Cdk2 kinase activity correlates with the association of p27/Kip1

To test whether the observed effect of the hypophosphorylation of Rb protein could result from the decreased activity of Cdk2 kinase, the major target of p27/Kip1 inhibition, we performed a histone H1 kinase assay. As shown in Figure 4(A), abrogation of MAPK signalling had a strong inhibitory effect on phosphorylation of the Cdk2-dependent histone H1. The Cdk2 activity dropped below 80% of the initial value after 6 h of incubation with PD098059 and decreased to approx. 30% of its normal level after 18 h. Therefore the maximal decrease in Cdk2 activity correlates with accumulation of p27/Kip1. In addition, we found increased amounts of p27/Kip1 at relatively stable levels of Cdk2 that could be co-precipitated with cyclin E after 18 h of PD098059 treatment (Figure 4B). However, we were unable to detect the presence of p27/Kip1 in anti-Cdk4 immunoprecipitates (Figure 4B).

PD098059 increases p27/Kip1 protein stability in A375 cells

The up-regulation of p27/Kip1 on inhibition of MEK/MAPK signalling seems to be controlled post-transcriptionally because no significant changes in the amount of p27/Kip1 mRNA were observed in A375 cells treated with PD098059 for up to 24 h (Figure 5A). We examined whether inhibition of proteasome function in A375 cells could counteract the negative influence of the MAPK pathway on p27/Kip1 expression and lead to its accumulation. As shown in Figure 5(B), the proteasome inhibitor MG132 was able to up-regulate the p27/Kip1 protein level even more efficiently than PD098059 during the same incubation period. These results suggest that the abundance of p27/Kip1 in A375 cells can be regulated post-translationally by the ubiquitin–proteasome system. Indeed, the stability of p27/Kip1 protein in A375 cells was significantly enhanced by addition of the MEK inhibitor (Figure 5C). After blocking of translation with puromycin for 3 h the level of p27/Kip1 remained almost unchanged

Figure 4 Inhibition of Cdk2 kinase activity correlates with the association of p27/Kip1 with cyclin E-Cdk2 complex

(A) Cdk2 immunoprecipitates prepared from lysates of A375 cells treated with 10 μ M PD098059 or with vehicle only [0.05% (v/v) DMSO] for the indicated durations were used for a kinase assay with histone H1 as a substrate. After quantification of the signals with the Storm 840 PhosphorImager (Molecular Dynamics), values from different time points were recalculated in relation to respective controls. (B) Association of p27/Kip1 with complexes of Cdk2 immunoprecipitated (IP) with anti-(cyclin E) or with anti-Cdk4 antibodies from A375 cells treated for 18 h with 10 μ M PD098059 or with vehicle only (0.05% DMSO). The immunoprecipitates from cell lysates containing equal concentrations of proteins were resolved by SDS/PAGE, blotted and developed with anti-p27, anti-Cdk2 and anti-Cdk4 antibodies. The position of a molecular mass marker is indicated (in kDa).

in PD098059-treated cells, whereas it decreased by approx. 70% in untreated controls. Similar results were obtained with cycloheximide, another inhibitor of translation (results not shown). We conclude that MAPKs regulate the level of p27/Kip1 in A375 melanoma cells by promoting protein degradation.

DISCUSSION

The results of our study provide evidence for a negative effect of constitutively active MAPKs on the expression of p27/Kip1 in human melanoma cells. Using the inhibitor PD098059 we have found that suppression of the permanent activity of ERK1/2 results in a strong nuclear accumulation of p27/Kip1 protein and correlates with a potent inhibition of cell growth. Additionally, the inhibition of MAPK signalling strongly decreased the kinase activity of Cdk2, which in complex with cyclin E or cyclin A constitutes the major target of inhibition by p27/Kip1 [15,16]. As expected, we also found increased amounts of p27/Kip1 associated with cyclin E-Cdk2 but not with cyclin D-Cdk4 complexes after treatment with PD098059.

The up-regulation of p27/Kip1 in A375 cells treated with PD098059 does not seem to occur at the level of its transcription, because we failed to detect any increase in the levels of p27/Kip1 mRNA. In contrast, we have observed an enhanced stability of p27/Kip1 protein. Thus the MAPK pathway seems to be involved in the negative control of this universal Cdk inhibitor by augmenting its degradation, thereby presumably supporting unrestricted cell growth. The negative regulation of p27/Kip1 levels by post-translational mechanisms, mainly by its increased proteolysis, seems to be a more general phenomenon, triggered for instance by growth factor stimulation [12,17]. A similar effect has been reported for murine NIH 3T3 fibroblasts stably transfected with an inducible c-Raf-1-oestrogen receptor cDNA [13]. Stimulation of the Raf/MAPK pathway abrogated p27/Kip1 protein expression. In contrast, the experiments with an analogous conditionally active c-Raf-1 protein in small-cell lung cancer cells yielded opposite results [18]. Raf activation in these cells was accompanied by a marked induction of p27/Kip1 and growth inhibitory effects. The specific cellular context might therefore critically influence the biological outcome of the MAPK signalling.

p27/Kip1 seems to be the only Cdk inhibitor that is down-regulated by mitogenic stimuli [17]. Accordingly, we were unable to detect any PD098059-dependent increase in protein expression of other cell-cycle inhibitors present in A375 and WM239 cells, such as p18/INK4c, p19/INK4d, p21/Cip1 or p57/Kip2. Although we cannot so far unequivocally determine whether the up-regulation of p27/Kip1 is a cause of the observed growth arrest, several reports suggest a role for p27/Kip1 in the control of proliferation of melanoma cells. Recently, the accumulation of p27/Kip1 has been demonstrated in melanoma cells during growth inhibition induced by interleukin 6 and oncostatin M or fibrillar collagen [8,19,20]. In contrast with the INK4 family of inhibitors (p15, p16, p18, p19), p27/Kip1 is rarely lost via genetic aberrations in tumours. Expression of p27/Kip1 was found to be high in normal melanocytes and in benign naevi, whereas in malignant melanomas the expression pattern was heterogeneous [21]. The level of p27/Kip1 significantly correlated with tumour thickness in the nodular subtype of primary melanoma but not in the superficially spreading subtype. Furthermore, the complete loss of p27/Kip1 expression had a potential prognostic value as an indicator of early relapse of the disease [21]. Interestingly, the nodular subtype of melanoma was previously shown to be associated with activating mutations in the *ras* gene [6,22]. The eventual inverse correlation between the expression of mutated Ras and the inhibitor p27/Kip1 could well be explained by the involvement of a constitutively active Ras-Raf-MAPK pathway in the negative regulation of p27/Kip1 expression. Further studies will be necessary to prove the prognostic potential of these two events, namely *ras* mutations and down-regulation of p27/Kip1 levels, as combined markers of melanoma aggressiveness.

Our results demonstrate a very potent growth-inhibitory action of PD098059 on human melanoma cells. So far, the effects of this specific inhibitor of MEK1 kinase have been studied in a murine B16 melanoma cell line. Englaro et al. [23] observed that blocking the mitogen-dependent activation of ERK1/2 kinases triggers the differentiation of murine B16 melanoma cells, promoting cell dendricity and melanogenesis. In our model cell lines the abrogation of constitutive activation of MAPKs arrested cell growth in the G₀/G₁ phase, which correlated in time with the accumulation of p27/Kip1 protein but did not lead to major

Figure 5 Abrogation of constitutive MEK/MAPK signalling does not up-regulate p27/Kip1 mRNA levels but increases its protein stability

(A) Northern blot analysis of p27/Kip1 and GAPDH (loading control) expression in A375 cells treated with 10 μ M PD098059 or with vehicle only [0.05% (v/v) DMSO] for the durations indicated. (B) Immunoblot analysis of p27/Kip1 expression in A375 cells treated for 18 h with 10 μ M PD098059, 5 μ M MG132 or vehicle only. The position of a molecular mass marker is indicated (in kDa). (C) A representative Western blot analysis of p27/Kip1 protein levels in human A375 melanoma cells (upper panel) and its quantification (lower panel). Cells were treated for 24 h with 10 μ M PD098059 (■) or with vehicle only (□); puromycin was then added and total cellular lysates were prepared at the indicated times. Western blot detection with anti-p27 was performed as described in the legend to Figure 3. The graph shows the quantification of p27/Kip1 signals with a Lumi-Imager (Roche), presented as percentages of the respective controls at the initial time points.

changes in cell dendricity or melanogenesis (results not shown). In addition, we have observed increased rates of cell death on long-term incubation with PD098059. A plausible explanation for this late effect could be provided by the postulated role of overexpressed p27/Kip1 as an apoptosis-promoting factor [17]. It could also result from partial degradation of Rb protein, which reportedly triggers the p53 apoptotic pathway [24], or from decreased expression of anti-apoptotic genes such as bcl-2,

which was up-regulated in human melanoma cell lines transformed with the N-Ras oncogene [25].

Our finding that constitutive activation of MAPKs in melanoma cells is involved in the down-regulation of p27/Kip1 expression agrees well with the hypothesis that deregulation of p27/Kip1 might contribute to melanoma development [19,21]. The pharmacological approach targeting the Ras–Raf–MAPK pathway might represent a sensible way to inhibit melanoma growth. As reported recently, the chemical antagonist of Ras, farnesylthiosalicylic acid can efficiently restrain melanoma growth *in vitro* as well as in mice with severe combined immunodeficiency [26]. The efficient growth-inhibitory action of specific inhibitors of ERK1/2 activation underlines the potential of novel therapeutic approaches targeting members of the Ras–Raf–MAPK pathway in the treatment of human melanoma. Moreover, our study emphasizes the role of p27/Kip1 as a plausible key player in the control of melanoma cell proliferation.

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