The p16-cyclin D/Cdk4-pRb Pathway as a Functional Unit Frequently Altered in Melanoma Pathogenesis

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ABSTRACT

The p16<sup>ink4a</sup>/CdkN1, D-type cyclins, their partners Cdk4/Cdk6, and pRb constitute a G<sub>1</sub> regulatory pathway commonly targeted in tumorigenesis. Genetic, immunochemical, and functional cell cycle analyses showed abnormalities of this pathway in each of 22 human melanoma cell lines examined. Normal melanocytes and all melanoma lines expressed Cdk4, Cdk6, and cyclins D1 and D3. The tumor suppressors p16<sup>ink4a</sup>/CdkN1 and pRb were lost in 17 and 4 cases, respectively, due to various genetic mechanisms, including transcriptional block of p16 and nonsense mutations of RB1. Ectopic expression of p16 prevented S-phase entry of Rb<sup>+/−</sup> but not Rb-deficient melanoma lines. The SK29-MEL-1 cell line harboring an R24C mutation in Cdk4 expressed wild-type pRb and overabundant p16, the latter preventing endogenous Cdk6 but not Cdk4 from associating with cyclin D1. Microinjection of cyclin D1-neutralizing antibody arrested the SK29-MEL-1 cells in G<sub>1</sub>, whereas p16 did not, indicating that the cyclin D1/Cdk4-R24C complex is required for G<sub>1</sub> progression, and the resistance of the complex to p16 in vivo. These data strongly support the candidacy of Cdk4 as a novel proto-oncogene, providing further evidence for the p16-cyclin D/Cdk4-pRb pathway as a functional unit, and suggest that deregulation of this checkpoint may represent a common step in the multistep progression of sporadic malignant melanomas.

INTRODUCTION

Recent genetic and biochemical investigations of the molecular mechanisms governing the G<sub>1</sub> to S progression in mammalian cells demonstrated a central role for D-type cyclins and their partner kinases Cdk4 and Cdk6 (1–5). The cell cycle-accelerating function of cyclin D-associated kinases appears to be mediated by neutralizing the growth-restraining ability of pRb<sup>−/−</sup> via its phosphorylation in advanced G<sub>1</sub>, a process negatively regulated by p16<sup>ink4a</sup> and other Cdk inhibitors (1–6). pRb is itself a tumor suppressor, the unphosphorylated form of which prevents premature S-phase entry by physically sequestering a series of transcription factors, such as E2F, required to activate S-phase genes (4). Phosphorylation of pRb by cyclin D-Cdk4/6 kinases in G<sub>1</sub> appears to release the captured transcription factors, thereby inducing gene expression and metabolic changes leading to onset of DNA replication. The emerging critical role of the p16-cyclin D/Cdk-pRb-E2F pathway in cell cycle regulation is further supported by frequent aberrations of the individual components of this checkpoint mechanism in human tumors (1–8).

Subsets of both sporadic and familial melanoma cases have recently been shown to harbor mutations in two genes, the products of which are involved in G<sub>1</sub> phase control. First, the p16<sup>ink4a</sup>/CdkN2/MTS1 tumor suppressor gene on chromosome 9p21, frequently lost in a wide range of malignancies (1–14), is deleted or mutated in more than one-half of human sporadic melanomas and cell lines derived therefrom (9, 10, 15), and it appears to be the predisposing mutation in some familial melanoma kindreds (16–19). Second, a small fraction of melanomas harbor a missense mutation (causing an arginine to cysteine change at codon 24, R24C) in the cdk4 gene, potentially activating this positive G<sub>1</sub> regulatory kinase as an oncogene (20, 21).

To assess the role of aberrations within the cyclin D/Cdk-p16-Rb pathway in human melanoma pathogenesis in a broader perspective, we have now examined all the key components of this G<sub>1</sub> checkpoint mechanism by a combination of genetic, immunochemical, and functional approaches in a series of novel melanoma cell lines and, where possible, in the corresponding tumor specimens. Our data show that in all cases examined, at least one of the cell cycle regulators involved in the above pathway is aberrant, consistent with the emerging concept of the cyclin D-Cdk-p16-Rb interplay as a candidate obligatory target in oncogenesis (1–5). In addition, our results from functional analyses extend the evidence for this biochemical pathway as a functional unit, including direct demonstration of the in vivo consequences of the R24C mutation for protein-protein interactions and G<sub>1</sub> regulatory role of Cdk4.

MATERIALS AND METHODS

Cell Culture. The establishment and properties of melanoma cell lines FM3, FM6, FM9, FM28, FM45, FM48, FM60, FM55<sub>1</sub>, FM55<sub>2</sub>, and FM55<sub>3</sub> were described previously (22); the last three lines were established from invasive primary and two s.c. metastatic lesions, respectively, removed from the same patient. The additional melanoma cell lines FM39, FM57, FM58, FM59, FM64, FM70, FM74, FM76, FM77, and FM79 were further established in our laboratory using similar methods. The melanoma cell lines BLM (23) and SK29-MEL-1 (20) were kindly provided by Dr. G. J. Adema (University Hospital, Nijmegen, the Netherlands) and Dr. Pierre van der Bruggen (Ludwig Institute for Cancer Research, Brussels, Belgium). Normal cultured melanocytes (NHME2486) and melanocyte culture medium were purchased from Clonetics (San Diego, CA), and melanocytes were cultured according to the supplier's instructions. Melanoma cell lines were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics. All FM melanoma lines were used at early passages (passages 3–6) corresponding to a total of 5 weeks to 3 months in culture, depending on the cell line. The control human cell lines MCF-7 and BT549 (both breast cancer) and U-2-OS (osteosarcoma) were cultured as described previously (24).

Nucleic Acid Analyses. Detection of homozygous deletions in the p16<sup>ink4a</sup>/CdkN2 gene was performed by PCR analysis of genomic DNA, using previously described conditions and primers (16, 25). RT-PCR for p16 expression was performed as described previously (25). Double-stranded PCR products were directly sequenced using the Thermo Sequenase sequencing kit (Amersham) and a 3<sup>P</sup>-labeled amplification primer as sequencing primer. In the search for the RBI gene mutations, genomic DNA was isolated, and the RBI promoter, the 27 exons including flanking intron sequences, and the polyadenylic acid signal region were all analyzed by direct sequencing of PCR products. Primers were selected essentially as described by Hogg et al. (26) and Lohman et al. (27). PCR was performed using 50 ng of DNA in a 50-μl volume of 1X GeneAmp PCR buffer (Perkin-Elmer Cetus), 0.2 mm dNTP (Pharmacia), 1 unit of AmpliTaq (Perkin-Elmer Cetus), and 25 pmol of each primer (Pharmacia). The amplification conditions included an initial cycle of 10 min at 94°C, 3 min at the annealing temperature (55 and 58°C for exons 6 and 14, respectively), and 3 min at 72°C, followed by 35 cycles of 30 s at 94°C, 30 s at...
annealing temperature, and 1 min at 72°C. In the last cycle, the polymerization step was extended by 10 min. Biotinylated PCR products were sequenced in both directions as described by the manufacturers (Dynabeads M280, Dynal AS, and ABI Prism Sequenase Terminator, Perkin-Elmer Cetus) on an ABI 373 sequencer and compared to the wild-type RB1 (GenBank accession number L11910). To scan for mutations in reverse-transcribed cdk4 mRNA, we established an assay based on PCR, gel-clamping, and DGGE (28). Primers for amplification of a 337-bp fragment covering codons 1–78 were: CCCGCCGCCGCCGGCTCCGCCGCCGC- GCCCTTGGCCGCCGCCGGCTCCGCCGGCTGGAGGTC and CGGTCATGTCCGAGATGTGGC. The theoretical melting map (29) for the amplification product displays a two-domain structure, which is considered optimal for resolution of mutations. PCR amplification was performed with the GeneAmp PCR System 9600 (Perkin-Elmer Cetus) in 25-μl reaction volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.02% gelatin, 5% DMSO, 200 μM each dNTP, 0.8 μM each primer, 500 ng of DNA, and 0.8 unit of Taq DNA polymerase. The amplification protocol consisted of 40 rounds of thermal cycling (94°C for 10 s, 62°C for 10 s, and 72°C for 10 s) and a final incubation step at 72°C for 5 min. Ten μl of the PCR products were loaded onto a 6% polyacrylamide gel containing a gradient of urea and formamide from 35 to 70%, followed by electrophoresis at 160 V for 5 h in 1 × TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA) kept at a constant temperature of 58°C. For quantitative analyses, [α-32P]dATP was included in the PCR, and the DGGE gel was dried and inspected using storage phosphor technology (Molecular Dynamics).

**Immunological Methods.** Mouse Mabs DCS-6, DCS-11 (30, 31), and SD4 (donated by Dr. M. Seto, Aichi Cancer Center, Nagoya, Japan) to cyclin D1; MAB DCS-5 to cyclin D2 (32); MAb DCS-20 (33) and DCS-22 (34) to cyclin D3; MAB 245 to pRb (donated by Dr. W-H. Lee, University of Texas Health Science Center, San Antonio, TX); and MAb DCS-50 to p16 (35) were transcribed cdk4 mRNA, we established an assay based on PCR, GC-clamping, and DGGE (28). Primers for amplification of a 337-bp fragment covering codons 1–78 were: CCCGCCGCCGCCGGCTCCGCCGCCGC- GCCCTTGGCCGCCGCCGGCTCCGCCGGCTGGAGGTC and CGGTCATGTCCGAGATGTGGC. The theoretical melting map (29) for the amplification product displays a two-domain structure, which is considered optimal for resolution of mutations. PCR amplification was performed with the GeneAmp PCR System 9600 (Perkin-Elmer Cetus) in 25-μl reaction volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.02% gelatin, 5% DMSO, 200 μM each dNTP, 0.8 μM each primer, 500 ng of DNA, and 0.8 unit of Tag DNA polymerase. The amplification protocol consisted of 40 rounds of thermal cycling (94°C for 10 s, 62°C for 10 s, and 72°C for 10 s) and a final incubation step at 72°C for 5 min. Ten μl of the PCR products were loaded onto a 6% polyacrylamide gel containing a gradient of urea and formamide from 35 to 70%, followed by electrophoresis at 160 V for 5 h in 1 × TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA) kept at a constant temperature of 58°C. For quantitative analyses, [α-32P]dATP was included in the PCR, and the DGGE gel was dried and inspected using storage phosphor technology (Molecular Dynamics).

**Gene and Protein Transfer.** Cells synchronized in mitosis by nocodazole block and "shake-off" procedure (30) or in G0 by lovastatin treatment for 36 h (36) were microinjected with either the affinity-purified GST-p16 wild-type fusion protein (2 mg/ml), affinity-purified MAb DCS-6 to cyclin D1 (6 mg/ml), or control mouse immunoglobulin as described previously (35), using the AIS microinjection apparatus (Zeiss). Alternatively, cells blocked in mitosis by nocodazole were cotransfected with a modified calcium phosphate method with 25 μg of p16 plus 5 μg of CD20, or a control vector plus the CD20 expression plasmid DNAs as described previously (38). Cell cycle progression of the cells released from the lovastatin block by removal of the drug and addition of fresh medium supplemented with 10% FCS and mevalonate (36) was evaluated by combined in situ double immunofluorescence analysis of BrdUrd incorporation and p16, CD20, or immunoglobulin content as described (31, 35). Each experiment was performed 2–4 times to verify reproducibility.

**RESULTS**

**Abundance and Localization of D cyclins, CDK4/6, p16, and pRb.** Immunoblotting analyses of the p16 and pRb tumor suppressors in total lysates of exponentially proliferating normal human melanocytes showed moderate expression of either protein, in contrast to frequent lack of p16 or absence of pRb found in melanoma cell lines examined in parallel (Fig. 1A; Table 1). The comparison of the two sets of immunoblotting results revealed an inverse correlation between the presence of p16 and pRb among the melanoma lines, and the absence of pRb was accompanied by elevated amounts of p16 (Fig. 1A). Of the 22 melanoma cell lines examined, 4 lacked pRb, and 17 showed undetectable levels of the p16 protein (Table 1). The only cell line with both pRb and p16 clearly detectable by immunoblotting was SK29-MEL-1 (Fig. 1A; Table 1). The spectrum of detectable D-type cyclins included cyclins D1 and D3, analogous in both normal melanocytes and all the melanoma lines investigated (Fig. 1B; Table 1). There was some variation in the abundance of cyclins D1 and D3 from cell line to cell line, with the pRb-deficient lines usually expressing low levels of cyclin D1. In contrast, no obvious correlation was seen between the pRb status and the abundance of Cdk4 or Cdk6 proteins that were detectable in all cell types examined (Fig. 1B; Table 1).
To independently verify the immunoblotting data and to examine the subcellular localization of the G1 regulatory proteins, an immunofluorescence analysis of exponential cultures of each cell type in our panel was carried out using specific antibodies to pRb, p16, cyclin D1, cyclin D2, cyclin D3, and Cdk4. In normal melanocytes, pRb was nuclear; the immunostaining intensity varied moderately from cell to cell (Fig. 2A); the p16 signal was relatively weak and mainly localized to the nuclei but was also detectable in the cytoplasm (Fig. 2B); cyclin D2 was undetectable, in contrast to cyclins D1 and D3, the predominantly nuclear staining of which was extremely variable among the exponentially growing cells (Fig. 2C); and Cdk4 showed granular nuclear staining (Fig. 2D). Among the melanoma cell lines, the immunofluorescence results correlated very well with the immunoblotting data in terms of the presence versus lack of detectable pRb or p16 (see Fig. 3, A—D, and Fig. 4 for examples), pRb being localized exclusively in the nuclei, whereas p16 was localized in both nuclei and cytoplasm. Cyclin D2 was generally undetectable or showed only extremely weak staining in rare cells (not shown), whereas cyclins D1 and D3 displayed highly variable nuclear staining, with cyclin D3 generally detectable in a higher proportion of cells than cyclin D1 (e.g., compare panels E—H of Fig. 3). Cdk4 immunostaining was nuclear with variable intensity, but unlike D cyclins it was detectable in all cells of the exponentially growing melanoma cell populations (Fig. 3, I and J).

In summary, the combined immunoblotting and immunofluorescence analyses of a series of melanoma cell lines frequently revealed abnormalities of the p16 and, less commonly, deficiencies of the pRb tumor suppressor proteins, in addition to a general lack of cyclin D2 and a common, albeit variable, expression of cyclins D1 and D3 and their partner kinases Cdk4 and Cdk6.

Because our finding of the absence of pRb in a subset of the melanoma cell lines was unexpected, we examined paraffin tissue sections from 18 of the 22 original melanoma specimens matching 18 of the 22 used to establish the cell lines, by immunoperoxidase staining. The presence versus lack of detectable pRb or p16 (see Fig. 3, A—D, and Fig. 4 for examples), pRb being localized exclusively in the nuclei, whereas p16 was localized in both nuclei and cytoplasm. Cyclin D2 was generally undetectable or showed only extremely weak staining in rare cells (not shown), whereas cyclins D1 and D3 displayed highly variable nuclear staining, with cyclin D3 generally detectable in a higher proportion of cells than cyclin D1 (e.g., compare panels E—H of Fig. 3). Cdk4 immunostaining was nuclear with variable intensity, but unlike D cyclins it was detectable in all cells of the exponentially growing melanoma cell populations (Fig. 3, I and J).

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 Genetic Analyses of Rbl, p16

Because our study of the proteins participating in the G1 control pathway revealed the common lack of the p16 or pRb tumor suppressor proteins in the melanoma cell lines, we next examined the genetic basis for these aberrations. Homozygous deletions of the p16

gene were detected in 12 of the cell lines by PCR analysis of genomic DNA (Fig. 6A). In all cases, the deletions included all three exons of the gene, and RT-PCR analyses in these cell lines yielded no detectable products (Fig. 6, B and C). In the remaining cell lines, a product was obtained by PCR analysis of reverse-transcribed mRNA, using primers located in exons 2 and 3, respectively. Two of the cell lines, BLM and FM74, showed aberrantly sized products (Fig. 6B), indicating the presence of defects in pre-mRNA splicing. Direct sequencing of the PCR products from the BLM genomic DNA disclosed a G—A transition at the first nucleotide of the splice donor site of intron 2 (data not shown), and sequence analysis of the aberrantly sized

Although p16 status could not be investigated by this approach due to the lack of an antibody suitable for staining of archival formalin-fixed tissues, the immunohistochemistry generally indicated a good correlation with the above analysis of the melanoma cell lines and confirmed that the lack of pRb expression seen in the cell lines occurred already at the stage of the original tumor rather than during the in vitro culture period.

Fig. 3. Immunofluorescence staining of the exponentially proliferating human melanoma cell line FM79 with antibodies against G1 regulatory proteins. A, MAb 245 to pRb; C, MAb DCS-50 to p16; E, MAb DCS-6 to cyclin D1; G, MAb DCS-22 to cyclin D3; I, MAb DCS-35 to Cdk4; B, D, F, H and J, DNA counterstaining with Hoechst of the fields shown in A, C, E, G, and I, respectively, to document the extent of variability of expression. A—J, ×800; A, C, E, G, and I, Texas red detection.

Fig. 4. Lack of pRb and high expression level of p16 in proliferating FM5S melanoma cells as revealed by immunofluorescence. A, MAb 245 to pRb; C, MAb DCS-50 to p16 (note the nuclear and cytoplasmic localization of p16); B and D, Hoechst nuclear counterstaining of the fields shown in A and C, respectively. A—D, ×800; A and C, Texas red detection.

Fig. 5. Immunohistochemical detection of pRb on formalin-fixed, paraffin-embedded sections of human primary melanomas. A, example of a pRb-positive melanoma specimen, from which the FM70 cell line was derived; B, example of a pRb-negative melanoma, from which the FM55p cell line was derived. Note the negative tumor nests contrasting with nuclear positivity for pRb in the adjacent stromal cells. Immunoperoxidase staining using MAb 245 as primary antibody, without nuclear counterstaining. ×400.
RT-PCR product of BLM revealed that the IVS2nt1g → a mutation results in the utilization of a cryptic splice site located in exon 2, 75 bp upstream from the normal donor splice site. The gene defect responsible for aberrant pre-mRNA splicing in FM74 cells has not been identified. Three additional cell lines without homozygous deletion of the \( p16^{ink4a}/CDKN2 \) gene (FM6, FM58, and FM79) showed no product when reverse-transcribed mRNA was analysed by PCR using primers located in exons 1 and 2, respectively, suggesting a complete transcriptional block due to methylation of the CpG island in the 5' end of the gene (12). In conclusion, this analysis of the \( p16^{ink4a}/CDKN2 \) gene identified distinct defects in every melanoma cell line showing lack of the protein (see above and Table 1), thereby confirming that the only cell lines expressing \( p16 \) were those four lacking pRb and the SK29-MEL-1 cells harboring the \( cdk4 \) mutation (Ref. 20; see below).

The paucity of reports on \( RB \) gene mutations in human melanomas and the fact that no detectable pRb could be found in four cell lines from our panel and in the matching clinical melanoma specimens (see above) made the search for the underlying genetic defects of particular interest. We focused on the unique set of three cell lines, FM55, FM55M1, and FM55M2, derived from a primary melanoma and two different metastatic lesions of the same patient, respectively (see "Material and Methods"). Genomic DNA from FM55 was sequenced and found to be heterozygous for two point mutations (Fig. 6D), one converting codon 199 from leucine (TFA) to a stop codon (TAA), the other changing codon 455 from arginine (CGA) to a stop codon (TGA). These results were confirmed by sequencing both DNA strands from the three cell lines, indicating that two independent mutational events within the \( RB1 \) gene occurred relatively early in the original tumor and providing a molecular explanation for the shared lack of pRb in the three cell lines and sections of the primary lesion and both metastases.

Stimulated by the findings of the same \( cdk4 \) gene point mutation, Arg → Cys at position 24 in three independent melanoma cases (20, 21), we also examined all the cell lines of our series for the presence of mutations in the \( cdk4 \) fragment covering codons 1–78 (see "Material and Methods" for details). The DGGE-based analysis clearly
detected the mutation in the SK29-MEL-1 cell line used in the study by Wölffel et al. (20) and revealed a 3-fold excess of the mutant over the wild-type mRNA [Fig. 7A; consistent with the amplification of the former predicted by Wölffel et al. (20)] but failed to detect any cdk4 aberrations in the remaining melanoma cell lines (Table 1).

**Functional Consequences of Aberrant Cdk4, p16, or pRb for G1 Control.** The candidacy of Cdk4 as a proto-oncogene was significantly strengthened by the reports of the R24C mutation in melanomas (20, 21), and the evidence from in vitro experiments that the baculovirus-expressed Cdk4-R24C was much more resistant to inhibition by p16 than the wild-type Cdk4 protein (20). On the other hand, there has been no report as to what are the biochemical and/or biological consequences of the presence of such a Cdk4 mutant protein in living mammalian cells. To address this issue, we first asked what is the composition and relative abundance of the protein-protein complexes involving D-type cyclins, p16, Cdk6, and Cdk4 (both wild-type and the R24C mutant) in the SK29-MEL-1 melanoma cells expressing all these G1 regulators (see Figs. 1A and 7A, Table 1).

Immunoblotting analysis of complexes immunoprecipitated from extracts of exponentially growing SK29-MEL-1 cells showed an abundant p16 protein, complexed to Cdk6 but also to Cdk4 (Fig. 7B). More importantly, there was a clearly detectable complex of cyclin D1 with Cdk4 (and a small amount of cyclin D3 complexed with Cdk4), but not of any D-type cyclin with Cdk6, despite the fact that both these kinases are easily detectable in this cell line (Fig. 7B). This result showed that the abundant p16, possibly together with other inhibitors of the same family (2), is able to sequester all Cdk6 but only a portion of Cdk4, most likely reflecting the ability of the mutant Cdk4-R24C protein to escape from p16 binding (20). If this interpretation were correct, then the Cdk4 protein complexed to cyclin D1 in the SK29-MEL-1 cells would be mostly if not entirely the product encoded by the R24C mutant allele. A predictable, experimentally testable consequence of such situation would be that the G1-activating function of this D1/Cdk4 complex, readily inhibitable by either cyclin D1-neutralizing antibodies or excess exogenous p16 in cell types expressing wild-type Cdk4 (24, 25, 35, 39), could still be prevented by neutralizing antibodies to cyclin D1 but not via p16. To verify this possibility, the SK29-MEL-1 cells were synchronized in early G1 by lovastatin (36). They were then released from the drug and microinjected with either a control immunoglobulin, the MAb DCS-6 to protein complexes involving D-type cyclins, p16, Cdk6, and Cdk4 (both wild-type and the R24C mutant) in the SK29-MEL-1 melanoma

Finally, to demonstrate the functional relevance of the loss of the p16 tumor suppressor in the melanoma lines under study and to verify the mutual interdependence of p16 and pRb, we performed gene transfer experiments with the p16 expression plasmid into the Rb-deficient FM55p cells and three Rb-positive melanoma cell lines, the p16 of which was not expressed due to either homozygous deletion of the gene (FM3), a splice mutation (BLM), or transcriptional block (FM6). As can be seen from Fig. 8F, expression of exogenous p16 efficiently prevented the onset of DNA synthesis in all three melanoma cell lines expressing pRb, whereas this treatment was without any significant effect upon cell cycle progression in the FM55p cells. These results demonstrate that the G1-inhibitory function of p16 in human melanoma cells depends on functional pRb, and they are consistent with the notion that the aberrant loss of p16 expression in Rb-positive melanoma cell lines provides a growth advantage to these cells.

**DISCUSSION**

This study represents the most broadly based analysis of the the p16-cyclin D-Cdk-pRb pathway in human melanomas performed to date, and the results provide several novel pieces of evidence to support the emerging significant role of subverting this G1 checkpoint mechanism in human melanoma pathogenesis.

The first important conclusion from our data is that at least one component of the above pathway is aberrant in each of the 22 human melanoma cell lines examined. None of the previous studies on melanomas could reach such a conclusion, partly due to a selective examination of just one component rather than multiple genes/proteins involved in the G1 control (10, 11, 15, 40—44), partly due to only very recent discoveries of some of the aberrations, such as the transcriptional block of the p16Ink4a/CdkN2 gene (12, 13), which have not been adequately studied in melanomas before (15, 40—42, 44, 45). In fact, one of the novel findings in the present study is the identification of a subset of cases in which the p16 tumor suppressor gene appears to be transcriptionally silenced (3 of 22 cell lines), suggesting that this is an alternative way, apart from the deletions or mutations described before (10, 11, 15—19, 40—42), to eliminate the p16 inhibitor in human melanomas. A critical role of p16 in preventing oncogenic transformation has recently been confirmed by an increased tumor incidence in mice genetically lacking the Ink4a locus, and a potential involvement of p16 in melanocyte function was suggested by a lighter coat pigmentation cosegregating with the Ink4a~−− genotype (46).

Thus, the chromosome region around this locus may be genetically very active in melanocyte differentiation, thereby potentially contributing to its increased fragility in the melanocyte lineage. Whereas the frequent lack of the p16 gene/protein was to be expected based on previous studies, somewhat surprising is our finding of a subset of cases lacking pRb, a result confirmed at the gene and protein levels and verified on the original biopsy material. Although Rb1 gene defects are not commonly detected among melanoma cell lines (43), their involvement in melanoma pathogenesis is suggested by several correlative clinical studies demonstrating that Rb patients and their relatives have a higher frequency of atypical melanocytic nevi and an increased risk of melanoma and that the incidence of melanomas is higher in survivors of the hereditary rather than the sporadic form of Rb (47). Taken together, these data warrant further studies to elucidate the role of Rb1 abnormalities in melanomagenesis.

To judge the significance of our present data for melanoma as a disease, it is crucial to consider two issues. First, the limitation of this study to invasive and metastatic melanomas does not allow any conclusion as to whether the observed aberrations occurred early or late relative to progression of the disease. This question remains to be clarified, but some data showed that at least the p16 expression is commonly preserved in nevi and in situ lesions (15), suggesting that its loss may contribute to progression, rather than initiation of sporadic melanoma development. Second, the possibility of in vitro,
Fig. 8. Effects of exogenous p16 or cyclin D1-neutralizing antibody upon G1→S transition of human melanoma cell lines. A and B, the SK29-MEL-1 cells were microinjected with the GST-p16 protein together with the normal IgG as a marker for successfully injected cells (A, Texas red channel). Most of the injected cells progressed into S phase as revealed by BrdUrd incorporation (arrowheads in B, FITC channel). C and D, the SK29-MEL-1 cells microinjected with the MAb DCS-6 to cyclin D1 (immunoglobulin-positive in C, Texas red channel), were prevented from entering S phase (note the absence of BrdUrd staining in cells marked by arrowheads in D, FITC channel). E, graph showing data from a representative microinjection experiment with synchronized SK29-MEL-1 cells, documenting the significant inhibitory effect upon S phase entry of the cyclin D1-neutralizing antibody (DCS-6), in contrast to little if any effect of the GST-p16 protein, as compared with noninjected cells or those microinjected by control IgG. In control MCF-7 cells synchronized and microinjected in parallel, GST-16 caused a G1 block at least as efficiently as the DCS-6. F, graphs summarizing inhibition of S phase entry (% of BrdUrd-incorporating cells) in various melanoma cells by exogenous wild-type p16 expressed from a transfected CMV-p16 plasmid. The rate of S-phase entry upon transfection of the control plasmid was assessed 24 h poststimulation by BrdUrd incorporation (taken as 100%) and compared with parallel cells transfected by the p16 expression plasmid. Note the correlation of G1 arrest by p16 with the presence of functional pRb. Columns, mean values from three independent experiments; bars, SD.

rather than in vivo, origin of some of the defects found in the cell lines must be considered. Both the Rb1 and Cdk4 (R24C) mutations clearly originated in the primary melanomas in vivo (this study and Ref. 20). In terms of the p16 aberrations, their in vivo origin is supported (although not proven) by the facts that we used only very early passages of the uncloned cell lines and that no traces of wild-type allele were found, implying a homogeneous genetic background, and indirectly, by the accumulating evidence from clinical studies documenting aberrations of the p16

One significant contribution of the present study is the first direct demonstration of the biological impact of the Cdk4-R24C mutation in melanoma cells, best illustrated by the microinjection experiments in which the cyclin D1-neutralizing antibody, but not exogenous p16, could block the G1-S progression. These data show that the SK29-MEL-1 cells are dependent on functional cyclin D1-Cdk complexes, but these complexes are no longer sensitive to inhibition by p16, which is otherwise an even more potent inhibitor of G1 progression than the cyclin D1-neutralizing antibody (25, 35). These data strongly support the candidacy of Cdk4 as a novel proto-oncogene, especially because the biological relevance of the Cdk4 gene amplification identified in subsets of sarcomas and gliomas remains questionable due to coamplification of other proto-oncogenes, such as mdm2, localizing to the same amplicon (5).
Last but not least, our results also strengthen the notion of the p16-cyclin D-Cdk-pRb pathway as a functional unit and a single, complex target of oncogenic alterations. The data relevant to this point include the observation that the aberrations of pRb, p16, and Cdk4 occurred in mutually exclusive subsets of the melanoma lines, a result generally regarded as evidence for participation of the corresponding gene products in a common biochemical mechanism (1–5, 48–52). The mutual interdependence of these regulators is further supported by the lack of any cell cycle inhibition via p16 transfected into the RB-deficient FM53A cells, consistent with analogous results in other cell types (35, 39, 53–55). The G1 arrest caused by ectopic p16 in melanoma cell lines with functional pRb and aberrant lack of p16 indicated their sensitivity to p16 and suggested how p16 loss could have provided a growth advantage to these cells. Apart from its indisputable biological significance, the occurrence of oncogenic mutations within the p16-cyclin D-Cdk-pRb pathway in the majority of, if not all, cases of a particular tumor type may raise a general question as to the potential utility of such information for the management of any particular patient suffering from the disease. Accumulating evidence now suggests that a precise identification of the aberrant component within the G1 pathway may have diagnostic (5, 56), prognostic (57, 58), and potentially therapeutic implications. The biological consequences of deregulating the individual gene products involved appear to be unequal, with the loss of pRb as the central downstream effector of the pathway having a more severe impact upon proliferation control than deregulation of any of the upstream regulators, such as cyclin D1 or p16 (25). It is also becoming clear from the functional studies such as this one that any attempts at gene therapy or other modes of therapy aimed at restoring the derailed G1 control must take into account that, e.g., reintroduction of p16 into cancers deficient in Rb or harboring the R24C mutation in Cdk4 is likely to fail in inducing any positive response, whereas such treatment could be beneficial in tumors with wild-type Rb and deficient for p16 and/or overexpressing cyclin D1 (20, 24, 25, 30, 31, 35, 39, 54, 55, and this study). There is little doubt that a more detailed understanding of the molecular principles governing the cell cycle can help elucidate their subversion in cancer cells and eventually lead to a significant practical impact on the diagnosis and management of a range of hyperproliferative and hypoproliferative diseases.

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