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16. For example, such lineages included NB1-1 [(*5, 10*), *n* = 8], NB3-1 [(*14*), Fig. 1A, *n* = 4], NB4-2 [(*3, 14*), Fig. 1B, *n* = 11], and MP2 [J. B. Thomas, M. J. Bastiani, M. Bate, C. S. Goodman, *Nature* **310**, 203 (1984), *n* = 9].
17. Such lineages included ventral unpaired median neurons (VUM) (*n* = 8), MP1 (*n* = 7), unpaired median interneurons (UMI) (*n* = 5), median neuroblast (MNB) (*n* = 2), and midline glia (MG) (*n* = 16); for a description of the midline lineages, see (*6*).
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19. Dorsal nPCs taken from the midline-specific marker strain P[3.7 *sim-lacZ*] (*21*) did not express the marker when transplanted into ventral midline positions. Expression of *lacZ* in this strain is driven by the *sim* early promotor, which is first activated in the cellular blastoderm (*21*). Thus, upon transplantation into the midline at the gastrula stage the *sim* early promotor does not seem to be sufficiently activated in dorsal nPCs. This result suggests that early *sim* expression (until gastrulation) is dispensable for the generation of morphologically normal midline clones.
20. Such clones included VUM (*n* = 28), MP1 (*n* = 4), UMI (*n* = 7), MNB (*n* = 1), and midline glia (*n* = 6).
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22. As opposed to *X55*, *sim-lacZ* is already expressed in the midline progenitors before gastrulation (*21*). This, and the fact that dorsal nPCs from the same P[3.7*sim-lacZ*] strain do not express the marker upon transplantation into the midline at gastrulation (*19*), suggest that positive staining of the donor cells is the result of cell-autonomous expression or perdurance of the marker.
23. K. Lüer and G. M. Technau, data not shown.
24. For example, such clones included NB1-1 (*n* = 10), NB3-1 (Fig. 1A, *n* = 4), NB4-2 (Fig. 1B, *n* = 2), and MP2 (*n* = 14).
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32. Cells were removed (at stage 7) from the midline of *sim-lacZ* donors and transplanted into the dorsolateral neuroectoderm of mutant hosts. Because of autonomous expression or perdurance of β-galactosidase, the progeny of the donor cell can be unamibiguously identified in late host embryos. Homozygous mutant hosts are characterized by fusion of the longitudinal axon bundles and collapse of the axon scaffold [J. B. Thomas, S. T. Crews, C. S. Goodman, *Cell* **52**, 133 (1988)]. The preparation was double-stained with the glia-specific reversed polarity (repo) antibody 4α3 [D. A. Halter *et al.*, *Development* **121**, 317 (1995)]; the staining showed irregular distribution of many glial cells. In Fig. 1, A to D, left panels, and in Fig. 1, E and F, different focal planes were combined with the use of the Photoshop program on a Macintosh computer.
33. We thank A. Brand, A. Prokop, and J. Urban for comments on the manuscript, C. Klämbt and S. Crews for the *X55*, *sim-lacZ*, and *sim*^{H9} strains, and C. Rickert for assistance with the artwork. Supported by a grant from the Deutsche Forschungsgemeinschaft (G.M.T.).

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A p16^{INK4a}-Insensitive CDK4 Mutant Targeted by Cytolytic T Lymphocytes in a Human Melanoma

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A mutated cyclin-dependent kinase 4 (CDK4) was identified as a tumor-specific antigen recognized by HLA-A2.1–restricted autologous cytolytic T lymphocytes (CTLs) in a human melanoma. The mutated CDK4 allele was present in autologous cultured melanoma cells and metastasis tissue, but not in the patient's lymphocytes. The mutation, an arginine-to-cysteine exchange at residue 24, was part of the CDK4 peptide recognized by CTLs and prevented binding of the CDK4 inhibitor p16^{INK4a}, but not of p21 or of p27^{KIP1}. The same mutation was found in one additional melanoma among 28 melanomas analyzed. These results suggest that mutation of CDK4 can create a tumor-specific antigen and can disrupt the cell-cycle regulation exerted by the tumor suppressor p16^{INK4a}.

Tumor antigens have been demonstrated in murine tumors induced by chemicals and ultraviolet (UV) light (*1*). CTLs mediate the rejection of these experimental tumors, and their target antigens have been identified in some instances (*2*). Human tumors have also been examined for equivalent antigens (*3*). In the human melanoma model of patient SK29(AV), the response of blood-derived lymphocytes to autologous cultured tumor cells has been studied over a long period (*4, 5*). Some autologous tumor-reactive CTL clones were broadly cross-reactive and were found to recognize melanocyte differentiation antigens such as tyrosinase and Melan-A/MART-1 (*6*). However, HLA-A2–restricted CTLs against a third antigen, called SK29-C, lysed only autologous tumor cells but did not recognize autologous Epstein-Barr virus–transformed B lymphocytes or a panel of allogeneic HLA-A2–positive melanoma cell lines (*5*). Three HLA-A2–restricted CTL clones recognizing SK29-C [CTLs requiring antigen

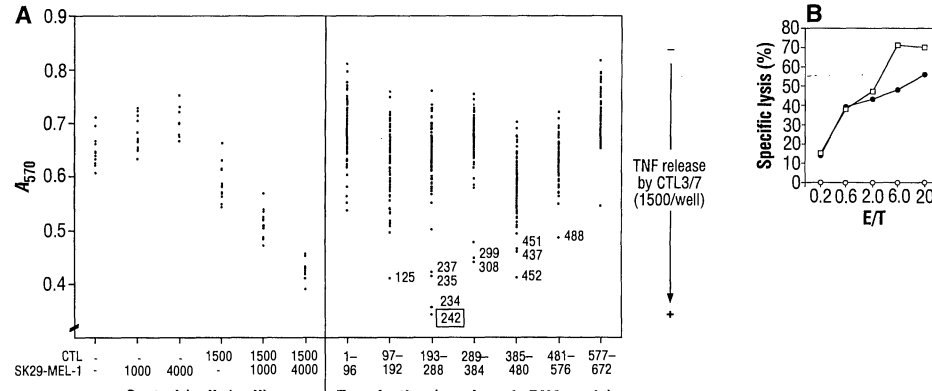

Fig. 1. Identification of cDNAs encoding the melanoma antigen SK29-C. (**A**) A cDNA library from SK29 melanoma cells in vector pcDNAI/Amp (Invitrogen) (*6*) was divided in pools of about 200 bacterial colonies. Plasmid DNA from 672 cDNA pools was cotransfected with HLA-A*0201 (inserted with pcDNAI/ Amp) into COS-7 cells, and transfectants were screened for antigen expression with CTL3/7 (*7*) as described in (*6*). TNF production by CTLs was assessed by measurement of the supernatant cytotoxicity to WEHI 164 clone 13 (W13) cells in a colorimetric assay (*6*). Each point indicates the screening result with a single cDNA pool. Positive pools, confirmed in an independent experiment, are indicated by their number. As a control, production of TNF by CTLs was measured in the presence of increasing numbers of SK29-MEL-1 cells. A_{570}, absorbance at 570 nm. (**B**) The cDNA clone C11.1 was cloned from pool 242. Allogeneic melanoma cells were cotransfected with HLA-A*0201 and C11.1 by electroporation and were tested for susceptibility to lysis by CTL anti-C (CTL5/76) (*7*). Data of a 4-hour, ^{51}Cr release assay are shown. E/T, effector-to-target ratio. Targets were autologous melanoma cells SK29-MEL-1 (open squares), allogeneic melanoma cells MZ2-MEL-2.2–A2.1 (open circles) cotransfected by electroporation with HLA-A*0201 and the hygromycin B resistance gene (*27*), and MZ2-MEL-2.2–A2.1-C11.1 cells (closed circles) additionally cotransfected with C11.1 and the neomycin resistance gene. The ^{51}Cr release assay, electroporation of melanoma cells, and cell culture conditions were as described (*5*).

SK29-C (CTL anti-C)] were isolated from lymphocytes separated from the patient's blood in different years and were used in our experiments here (*7*).

Using the COS transfection approach for cloning T cell–defined antigens (*6*), we identified SK29-derived complementary DNA (cDNA) pools that induced the production of tumor necrosis factor (TNF) by CTL anti-C after cotransfection with HLA-A*0201 (Fig. 1A). Positive pool 242 was cloned. Four of 1920 clones derived from pool 242 induced TNF production (*8*), and one of them, C11.1, was chosen for further experiments. C11.1 also conferred recognition by CTLs in direct cytotoxicity testing when stably transfected into allogeneic melanoma cells expressing HLA-A2.1 (Fig. 1B). This result largely excluded the possibility that the high-level replication of C11.1 in COS cells generated an artificial CTL target that would not be produced under moderate expression conditions.

Clone C11.1 contained a cDNA insert [1331 base pairs (bp)] (*9*) whose longest open reading frame encoded a 303–amino acid protein. This protein is identical in sequence to human cyclin-dependent kinase 4 (CDK4) except for an arginine (R) to cysteine (C) replacement at position 24 produced as a result of a cytosine to thymine (C → T) transition (*9*, *10*). Accordingly, this mutation was named R24C and the resultant mutant protein was named CDK4-R24C.

To determine if the R24C mutation was a somatic mutation, we compared CDK4 gene sequences amplified by polymerase chain reaction (PCR) from normal lymphocytes and from cultured melanoma cells of the patient. The sequence obtained with lymphocyte DNA contained a CGT codon (arginine) at position 24 (Fig. 2A), whereas the sequence obtained from melanoma cell DNA contained both CGT (arginine) and TGT (cysteine) codons at position 24, which suggests that the patient's melanoma cells carried both the wild-type and the R24C alleles (Fig. 2B). Both the mutant and the wild-type alleles were transcribed in SK29 melanoma cells (*8*). Genomic DNA was extracted from paraffin sections of a lymph node metastasis of patient SK29(AV), and CDK4 fragments around codon 24 were amplified. Direct sequencing of PCR fragments revealed that the R24C mutation was also present in the patient's metastatic tumor tissue (Fig. 2C); the amplified DNA was subsequently cloned, and after sequencing, it was found that 14 of 16 clones carried the R24C mutation, whereas the others were identical to the wild-type sequence. The predominance of the mutated CDK4 allele in the metastatic tissue may have resulted from amplification of CDK4-R24C. Amplification of the gene encoding CDK4 is a relatively frequent alteration in sarcomas and gliomas (*11*).

Altogether, these results indicate that R24C is a somatic mutation. The C → T transition that produced the R24C mutation occurred at a dipyrimidine site (Fig. 2). This type of mutation is typically caused by UV irradiation (*12*), a key etiologic factor in melanoma. The R24C mutation might therefore have occurred early in tumorigenesis. The same R24C mutation was found in one of 28 other melanomas that have been surveyed so far (*13*).

To determine if the R24C mutation was

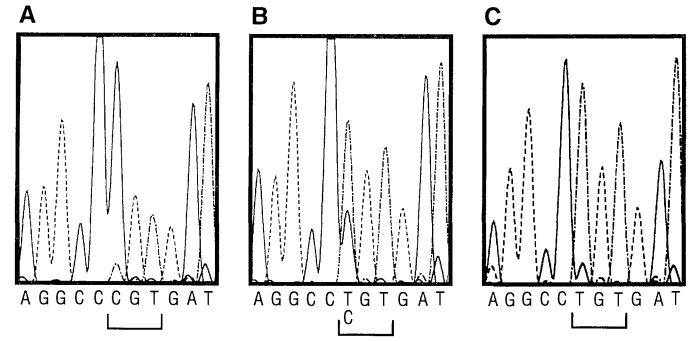

Fig. 2. A CDK4 allele of SK29 melanoma with a missense mutation in codon 24 (bracketed). Genomic DNA was extracted from (**A**) the patient's Epstein-Barr virus–transformed B lymphocytes (SK29-EBV-B) and from (**B**) cultured melanoma cells (SK29-MEL-1). The cell line SK29-MEL (previously named SK-MEL-29) had been derived from a lymph node metastasis in 1975 (*4*). SK29-MEL-1 is a clone of the SK29-MEL cell line. PCR reactions were performed on extracted DNA with primers 5′-TTGAATTCGCCGCCATGGCTACCTCTCGA (primer C2) and 5′-AATCTAGAGCCGCCTTGATCGTTTCGGCT (primer C3), allowing amplification of codons 1 to 117 (CDK4-specific primer sequences are underlined). Fragments amplified with these primers from both cell lines were 520 bp in length, as compared to the 353 bp expected from the CDK4 cDNA sequence; the increased length was due to the presence of an intron (*26*). (**C**) Genomic DNA was extracted as described (*25*) from paraffin sections of a lymph node metastasis surgically removed from the patient's left axilla in 1978 (*4*). A 120-bp CDK4 fragment spanning codons 6 to 43 was amplified with primers 5′-CGATATGAGCCAGTGGCTGAAATTGGT and 5′-TCCTCCTCCATTGGGGACTCTCACACT. No DNA was amplified when PCR was performed with primers 5′-CGATATGAGCCAGTGGCTGAAATTGGT (sense) and Sp6 (antisense) under conditions that allowed detection of 0.04 pg of C11.1 DNA, thus excluding contamination from the C11.1 plasmid (*8*). PCR fragments were purified with the QIAquick PCR purification kit (Qiagen) and directly sequenced with an automated sequencing device (Applied Biosystems ABI 373A).

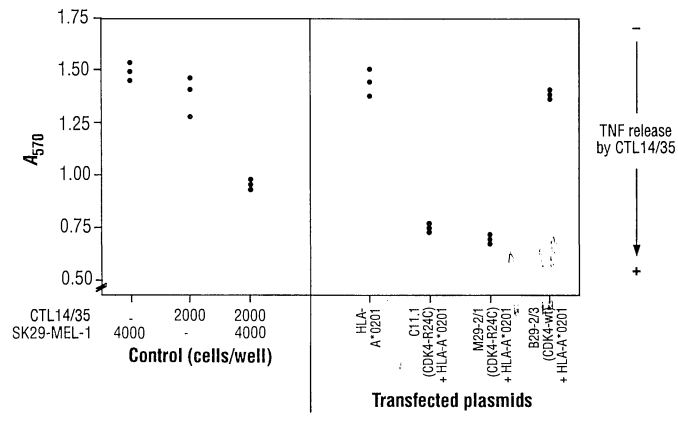

Fig. 3. Transfection of COS cells with mutant and wild-type CDK4 cDNA fragments and assay for recognition by CTL anti-C. CDK4 cDNA fragments spanning codons 1 to 117 were amplified by PCR after reverse transcription of RNA from the patient's lymphocytes and melanoma cells and cloned in expression vector pcDNAI/Amp (*28*). COS cells were transfected by the DEAE-dextran method, and the TNF assay was performed as shown in Fig. 1A. The cDNA clone M29-2/1 was derived from RNA of SK29-MEL-1, and its sequence was identical to that of clone C11.1 (CDK4-R24C). The cDNA clone B29-2/3 was derived from RNA of Epstein-Barr virus–transformed B lymphocytes, and its sequence was identical to that of wild-type CDK4 (*8*). COS-7 cells were cotransfected with plasmids C11.1, M29-2/1, or B29-2/3 (200 ng of each per well) and HLA-A*0201 (inserted in pcDNAI/Amp, 200 ng per well) or with HLA-A*0201 alone. Transfectants were tested for their ability to induce TNF production by CTL anti-C (CTL 14/35; 1500 cells/well) (*7*). Each point indicates the result of a TNF assay with a single transfected COS cell population. As a control, production of TNF in the presence of various numbers of SK29-MEL-1 cells was measured. Data were confirmed in five independent experiments.

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associated with CTL recognition, we transfected mutated or wild-type CDK4 cDNA fragments together with HLA-A*0201 DNA into COS-7 cells. Whereas cDNAs encoding CDK4-R24C (M29-2/1) conferred recognition by CTL anti-C, those encoding wild-type CDK4 (B29-2/3) did not (Fig. 3). This result suggests that Cys^{24} either is part of the peptide antigen recognized by CTL anti-C or influences peptide production. The selective recognition of CDK4-R24C was consistent with our earlier specificity analysis for CTL anti-C on autologous and allogeneic HLA-A2–positive target cell lines (5).

To identify the CTL target peptide, we generated CDK4-R24C cDNA fragments by PCR, cotransfected them with HLA-A*0201 DNA into COS cells, and tested the transfectants for CTL recognition. These results (8) showed that the CTL target peptide was encoded by codons 22 to 33. Synthetic peptides corresponding to amino acids 22 to 32 of mutated (R24C) and wild-type (wt) CDK4 were tested for recognition by CTL anti-C. The two peptides, 22-32^{R24C} and 22-32^{wt}, sensitized target cells equally well against CTL lysis (Fig. 4). This finding was inconsistent with our earlier observation that wild-type cDNA fragments did not confer CTL recognition after transfection (Fig. 3). However, a clear difference between mutated and wild-type peptides was observed for decapeptides 23-32^{R24C} and 23-32^{wt} with respect to target sensitizing activity. Peptide 23-32^{R24C} induced half-maximal lysis at a concentration of 10 nM and was about two or three orders of magnitude more efficient than its wild-type homolog (Fig. 4).

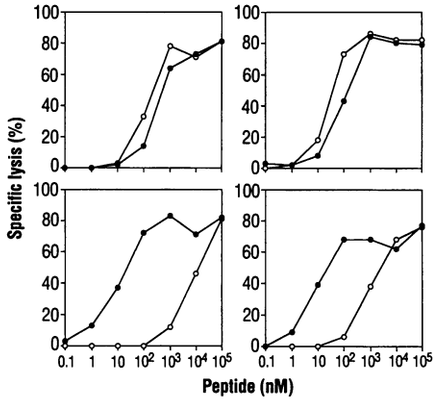

Fig. 4. Recognition of synthetic CDK4 peptides by CTL anti-C. Assays were performed essentially as described (*27*). T2 cells (*29*) were labeled with $Na(^{51}Cr)O_4$ and then incubated at a concentration of 2000 cells per well for 90 min with the indicated concentrations of peptides in the presence of human β_2-microglobulin (10 μg/ml; Sigma). CTL anti-C [CTL3/7 (**left panels**) and CTL14/35 (**right panels**)] (*7*) was added at an effector:target ratio of 40:1. Chromium release was measured after 6 hours. Amino acid sequences were as follows. **Top panels**: open circles, KARDPHSGHFV; closed circles, KACDPHSGHFV. **Bottom panels**: closed circles, ACDPHSGHFV; open circles, ARDPHSGHFV; changes in the residues are underlined. (Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; F, Phe; G, Gly; H, His; K, Lys; P, Pro; R, Arg; S, Ser; and V, Val.) The data are the means of duplicate samples from one experiment and were confirmed in six independent experiments. Peptides were synthesized by a standard solid-phase method with a multiple peptide synthesizer (Abimed 422). The purity of the peptides was determined by analytical reversed-phase high-performance liquid chromatography and proved to be at least 80% pure (UV, 214 nm). Their integrity was determined on a Lasermat mass spectrometer (Finnigan MAT).

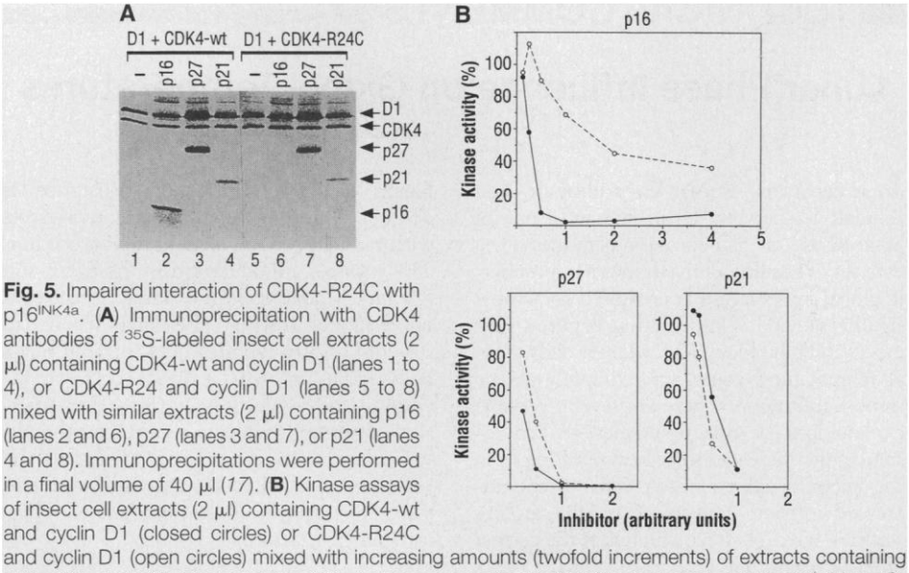

Fig. 5. Impaired interaction of CDK4-R24C with $p16^{INK4a}$. (**A**) Immunoprecipitation with CDK4 antibodies of ^{35}S-labeled insect cell extracts (2 μl) containing CDK4-wt and cyclin D1 (lanes 1 to 4), or CDK4-R24 and cyclin D1 (lanes 5 to 8) mixed with similar extracts (2 μl) containing p16 (lanes 2 and 6), p27 (lanes 3 and 7), or p21 (lanes 4 and 8). Immunoprecipitations were performed in a final volume of 40 μl (*17*). (**B**) Kinase assays of insect cell extracts (2 μl) containing CDK4-wt and cyclin D1 (closed circles) or CDK4-R24C and cyclin D1 (open circles) mixed with increasing amounts (twofold increments) of extracts containing p16, p27, and p21. Final mixtures (10 μl) were assayed for their ability to phosphorylate glutathione-S-transferase fused to an Rb deletion fragment containing residues 373 to 928 (Rb large pocket) as in (*17*). The total amount of insect cell extract in each mixture was compensated for by addition of a similar extract containing the baculoviral polyhedrin protein. The amount of the different inhibitors is expressed in arbitrary units: one unit corresponds to 1 μl of p16 extract, 0.5 μl of p27 extract, and 4 μl of p21 extract. Quantification was done in a Fuji Phosphorimager, and percentages refer to the activity in the absence of added inhibitors. The absolute activities of CDK4-wt and cyclin D1 and CDK4-R24C and cyclin D1 in the absence of added inhibitors were similar.

The affinity of exogenously added peptides for HLA-A2.1 correlates with their ability to induce the expression of HLA-A2.1 on 0.174×CEM.T2 cells (T2) (*14*). We compared the two decapeptides in this assay and found that peptide 23-32^{R24C} increased HLA-A2.1 expression on T2 cells to an extent comparable to that of known high-affinity peptides, whereas peptide 23-32^{wt} had no effect (8). No significant lysis of target cells was induced by peptides 22-31^{R24C} and 24-32^{R24C} (8). We conclude that Ala^{23} and Val^{32} serve as the NH_2- and COOH-terminal anchor residues, respectively, as in other HLA-A2.1 binding peptides (*15*), and that the R24C mutation in CDK4 enhances binding to HLA-A2.1 for peptide 23-32. Differential peptide binding affinity might account for the resistance of cells expressing wild-type CDK4 to lysis by CTL anti-C. It is also possible that in contrast to the R24C-derived peptide, the wild type–derived peptide is not presented on the cell surface, which would imply that the R24C mutation affects peptide processing or transport. A comparable situation has been discussed for the CTL-defined antigen P35B in mouse mastocytoma P815 (*16*).

To characterize the effect of the R24C mutation on CDK4 function, we expressed CDK4-R24C in insect cells. Cell extracts from cultures metabolically labeled with [^{35}S]methionine and coinfected with baculoviruses encoding cyclin D1 and CDK4-R24C were mixed with similar extracts from insect cells expressing $p16^{INK4a}$, p27, or p21, all known inhibitors of CDK4 (*17*, *18*). Immunoprecipitation with antibodies to CDK4 showed that CDK4-R24C formed stable complexes with cyclin D1, p27, and p21, but not with $p16^{INK4a}$ (Fig. 5A). In kinase assays, both CDK4-wt and CDK4-R24C were enzymatically active in the presence of cyclin D1, and both were inhibited by p27 and p21 (Fig. 5B). In contrast, CDK4-R24C was considerably less sensitive to inhibition by $p16^{INK4a}$ than was the wild-type enzyme (Fig. 5B). Similarly, $p15^{INK4b}$, a member of the INK4 family of inhibitors (19), did not form a stable complex with CDK4-R24C and did not effectively inhibit its kinase activity (*20*). These results indicate that CDK4-

R24C is selectively impaired in its interaction with $p16^{INK4a}$ and $p15^{INK4b}$ and suggest that Arg^{24} is directly involved in binding to $p16^{INK4a}$ and $p15^{INK4b}$.

The cell cycle regulatory pathway that involves the retinoblastoma protein (Rb), cyclin D1, $p16^{INK4a}$, and CDK4 has been implicated in tumorigenesis (*21*). In particular, $p16^{INK4a}$ can inhibit cell proliferation and oncogenic transformation of cultured cells (*22*). Inactivation of the gene encoding $p16^{INK4a}$ is common in some tumor cell lines and primary tumors and is responsible for genetic predisposition to melanoma (*23*). Mutation of CDK4 at positions that disrupt its interaction with $p16^{INK4a}$ may constitute a mechanism to subvert this regulatory pathway in tumor cells. It seems plausible that, aside from its antigenicity, the expression of CDK4-R24C contributed to malignant transformation in melanoma SK29(AV). Antigens derived from oncogenic proteins are ideally suited as targets of tumor rejection responses because tumorigenesis is likely to depend on the continued expression of the antigen. Indeed, CTLs against viral oncoproteins have been demonstrated to elicit rejection response and protective immunity to virally induced murine tumors (*24*). It remains to be proven that human tumor-specific antigens like CDK4-R24C can constitute targets for rejection response in vivo. However, it should be noted that patient SK29(AV) has been free of detectable disease since 1978 (*7*).

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13. DNA was extracted from four allogeneic melanoma lines and from paraffin-embedded melanoma tumor samples of 24 patients as described (*25*). A 270-bp CDK4 genomic fragment was amplified with primers 5′-ATGGCTACCTCTCGATATGAGCCAGTG (codons 1 to 9) and 5′-AGGCTGTCTTTTCCCTTTACTCCCCA [binding to intron sequence (*26*)]. By using an exon-intron primer pair, contamination with cDNA was excluded. Amplified CDK4 fragments were purified and directly sequenced as shown in Fig. 2. DNA sequences from 27 of 28 melanomas were identical to that of wild-type CDK4. One melanoma tumor carried both CGT (arginine) and TGT (cysteine) codons at position 24 and therefore contained an R24C allele (*8*).
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26. The intron amplified with CDK4 primers C2 and C3 (see Fig. 2A) from genomic DNA was interspersed between codons 73 and 74. Thus, the mutation in codon 24, described herein, does not affect splicing.
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28. Total RNA was extracted from SK29-MEL-1 and SK29-EBV-B cells as described [P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987)]. RNA (5 μg) was transcribed with the Superscript preamplification kit (Gibco-BRL) with oligo(dT) primers. Single-stranded cDNA served as a template for PCR with CDK4 primers C2 and C3 (Fig. 2A). Primers contained restriction sites for enzymes Eco RI (C2) or Xba I (C3) in their nonspecific nucleotide regions. PCR products were digested with Eco RI and Xba I and were then cloned in the corresponding sites of vector pcDNAI/Amp (Invitrogen) as described (*27*). Clones B29-2/3 (CDK4-wt) and M29-2/1 (CDK4-R24C) were sequenced and used for transfection.
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TECHNICAL COMMENTS

Lunar Phase Influence on Global Temperatures

In a report by Robert C. Balling Jr. and Randall S. Cerveny about the influence of lunar phase on planetary temperature (*1*), a data set of satellite-derived, lower tropospheric global temperature is presented showing a significant 0.02 K modulation between new moon and full moon. The warmest daily global temperatures occur approximately at the time of full moon. There are several possible explanations of this phenomenon. For instance, the increased solar load resulting from the moon's reflection—as well as the increased infrared emission from the moon's surface—give an effect which is of the correct order of magnitude (*1*).

There is another factor contributing to the global temperature modulation. Earth and moon circulate around their common center of gravity, the barycenter. At full moon the sun, Earth, and moon lie approximately on a line and the Earth is nearest to the sun. As the moon weights 1/81 of the Earth and the Earth-moon distance is 384,000 km, the Earth moves in a circle with radius 4700 km around the barycenter. This induces a modulation in the Earth-sun distance equal to 9400 km. Consider a naïve model in which the Earth absorbs all sunlight and re-radiates it as infrared black body radiation, with balance between incoming and outgoing radiation. The intensity of sunlight received varies as the inverse Earth-sun distance squared, and the intensity of black body radiation varies as the temperature to the fourth power. Thus, for small variations

$$dT/T = -dR/(2R)$$

where T is the temperature and R is the Earth-sun distance. For R = 150,000,000 km and a mean temperature of 269 K this leads to 0.008 K temperature modulation.

Inspection of figure 1 of the report (*1*) shows that the data are noisy. There is some