Mutations associated with familial melanoma impair p16^{INK4} function

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Cell division is controlled by a series of positive and negative regulators which act at sequential points throughout the cell cycle. Disturbance of these checks could contribute to cancer by allowing excessive cell proliferation. The point in G1 at which cells irrevocably commit to DNA synthesis is controlled by protein complexes consisting of cyclin-dependent kinases (CDK4 or CDK6) and cyclins (D1, D2 or D3). These complexes are inhibited by low molecular weight proteins, such as p16^{INK4} (refs 1,2), p15^{INK4B} (ref. 3) and p18 (ref. 4). Deletion or mutation of these CDK-inhibitors could lead to unchecked cell growth, suggesting that members of the p16^{INK4} family may be tumour suppressor genes. The recent detection of p16^{INK4} (*MTS1*) mutations in familial melanoma kindreds[5,6], many human tumour cell lines[7,8], and primary tumours[9,10] is consistent with this idea. Previously, we described eight germline p16^{INK4} substitutions in 18 familial melanoma kindreds[5]. Genetic analyses suggested that five mutations predisposed carriers to melanoma, whereas two missense mutations had no phenotypic effect. We now describe biochemical analyses of the missense germline mutations and a single somatic mutation detected in these families. Only the melanoma-predisposing mutants were impaired in their ability to inhibit the catalytic activity of the cyclin D1/CDK4 and cyclin D1/CDK6 complexes *in vitro*. Our data provide a biochemical rationale for the hypothesis that carriers of certain p16^{INK4} mutations are at increased risk of developing melanoma.

Familial melanoma is a genetically heterogeneous disease, with approximately half of the families showing evidence of linkage to chromosome 9p21 (refs 11,12). Germline p16^{INK4} mutations have been detected in twelve families in which the occurrence of melanoma is linked to 9p21 (refs 5,6). These mutations include a nonsense mutation (Arg50Ter), a splice donor site mutation (IVS2+1), and six missense mutations (Ile41Thr, Asn63Ser, Arg79Pro, Gly93Trp, Val118Asp and Ala140Thr; see Table 1). The Ile41Thr and Ala140Thr variants are most likely to be polymorphisms unrelated to the development of melanoma since they were detected in normal controls. However, the nonsense, splice donor and three of the missense mutations (Arg79Pro, Gly93Trp and Val118Asp) were highly correlated with melanoma in 11 families, and probably play a part in melanoma development. The role of the Asn63Ser mutation could not be evaluated by linkage analysis[5]; this mutation was only weakly associated with melanoma with a penetrance of 27% as compared to an average penetrance for the other mutations of 85%, but we could not formally classify it as a benign polymorphism as there were no unaffected spouses carrying the variant and we did not detect it in 72 normal controls. In addition to the germline mutations, we have also identified a somatic mutation (Pro73Leu) in a melanoma from a family member who had inherited the Arg79Pro germline mutation.

The only known targets of p16^{INK4} are the cyclin D-dependent kinases, CDK4 and CDK6 (refs 1–4). As it is not known which of these CDKs is important in the growth of melanocytes, we examined whether the missense mutations described above had an effect on the ability of p16^{INK4} to inhibit the activity of cyclin D1/CDK4 and cyclin D1/CDK6. The wild-type and mutant p16^{INK4} genes were fused to the glutathione-S-transferase (GST) gene, and purified GST fusion proteins were tested for their ability to inhibit cyclin D1/CDK4 or cyclin D1/CDK6 complexes present in insect cell lysates. Equal amounts of lysate were mixed with increasing amounts of the GST–p16^{INK4} fusion proteins, and the mixtures assayed for their ability to phosphorylate another fusion protein consisting of GST and the 'large pocket' of the retinoblastoma susceptibility protein (GST-Rb)[1,3].

Wild-type GST–p16^{INK4} fusion protein inhibits the kinase activity of cyclin D1/CDK4 and cyclin D1/CDK6 (Fig. 1). Compared to wild-type p16^{INK4}, an eight-fold excess of the melanoma-associated mutant Val118Asp was required for inhibition of cyclin D1/CDK4 activity, and failed to inhibit cyclin D1/CDK6 activity completely. Two other germline mutations had an even more severe effect, as an eight-fold excess of the Arg79Pro and Gly93Trp mutants failed to inhibit completely cyclin D1/CDK4 or cyclin D1/CDK6 activity. The somatic mutant, Pro73Leu, is equally debilitated in its capacity to inhibit cyclin D1/CDK4 or cyclin D1/CDK6 activity. On the other hand, the Ala140Thr polymorphism inhibited cyclin D1/CDK4 and cyclin D1/CDK6 activity as well as wild-type. The Ile41Thr and the Asn63Ser substitutions had slightly reduced (~two-fold) capacity to inhibit both cyclin D1/CDK complexes. The data suggest that the Asn63Ser change is indeed a polymorphism, like Ile41Thr, given its small effect on the assayed function *in vitro*.

The reduced inhibitory activity of the mutant p16^{INK4}

Table 1 Inhibition of cyclinD1/CDK4 and cyclin D1/CDK6 activity by mutantGST– p16^{INK4} proteins

	CDK inhibition relative to wild-type		Melanoma-specific mutation (M) or polymorphism (P)	
Mutation	CyclinD1/CDK4	Cyclin D1/CDK6	Genetic Analysis[a]	Functional Analysis[b]
Ile41Thr	0.5	0.5	P	P
Asn63Ser	0.5	0.5	P/M	P
Pro73Leu[c]	<0.12	<0.12	NA	M
Arg79Pro	<0.12	<0.12	M	M
Gly93Trp	<0.12	<0.12	M	M
Val118Asp	0.12	<0.12	M	M
Ala140Thr	1.0	1.0	P	P

[a]Described in ref. 5; [b]this paper. [c]A somatic mutation detected in a tumour from an individual who inherited the Arg79Pro mutation. NA, not applicable. Each protein was tested twice and representative values are shown.

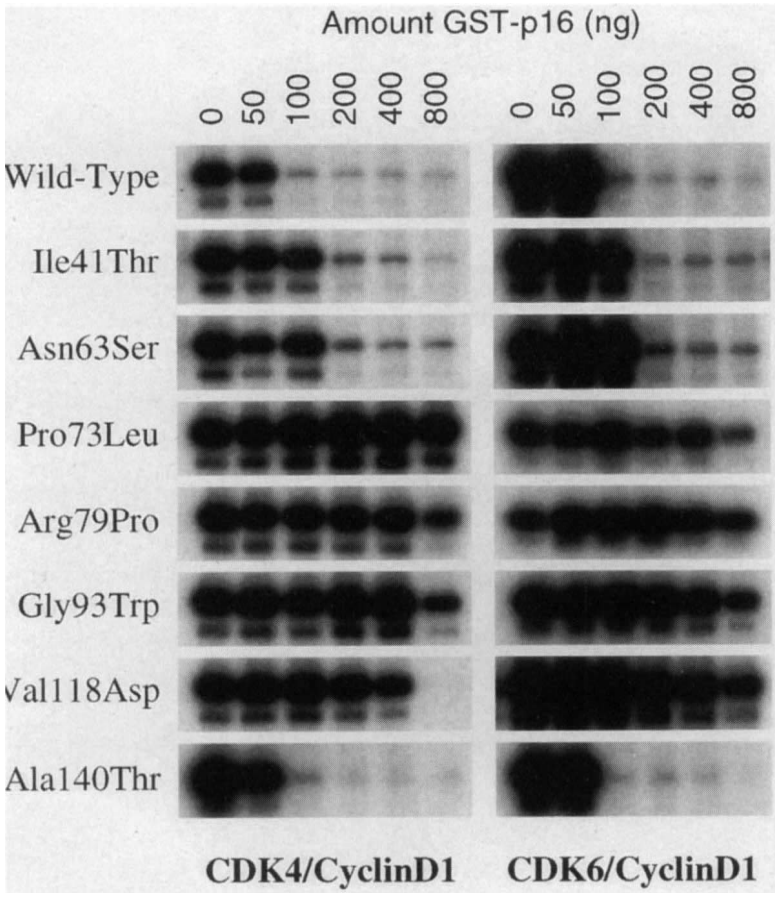

Fig. 1 Familial melanoma-specific p16^{INK4} mutations affect its ability to inhibit cyclin D1/CDK4 and cyclin D1/CDK6 activity. Extracts from baculovirus-infected Sf9 insect cells expressing cyclin D1 and CDK4 or cyclin D1 and CDK6 were mixed with 0, 50, 100, 200, 400, or 800 ng of wild-type or the 7 mutant GST-p16^{INK4} fusion proteins. The mixtures were assayed for their ability to phosphorylate the GST-Rb fusion protein as described in Methodology.

proteins could be due either to their inability to bind stably with the cyclin D/CDK complex, or to a defect in a subsequent step (such as inducing a conformational change that inactivates the kinase). To address this issue, the wild-type and mutant GST-p16^{INK4} fusion proteins were tested for binding to cyclin D1/CDK4 complexes present in insect cell lysates. Equal amounts of the fusion proteins were mixed with equal amounts of insect cell lysates containing cyclin D1/CDK4 metabolically labelled with ^{35}S-methionine. The fusion proteins were then purified using glutathione-sepharose, and bound proteins were resolved on SDS-acrylamide gels and detected by flurography (Fig. 2). Only two labelled proteins of ~33 and ~38 kD co-purify with the GST-p16^{INK4} fusions. The smaller ~33 kD protein is identified as CDK4 because it comigrates with CDK4 translated *in vitro*. The larger ~38 kD protein appears to bind non-specifically to GST (or to glutathione-sepharose); because of its size, we suspect it is Cyclin D1. As can be seen, CDK4 co-purifies with wild-type GST-p16^{INK4} or with fusions bearing the three polymorphisms (Ile41Thr, Asn63Ser and Ala140Thr). In comparison, little or no CDK4 co-purifies with the fusion proteins bearing the melanoma-specific germline mutations (Arg79Pro, Gly93Trp and Val118Asp) or the

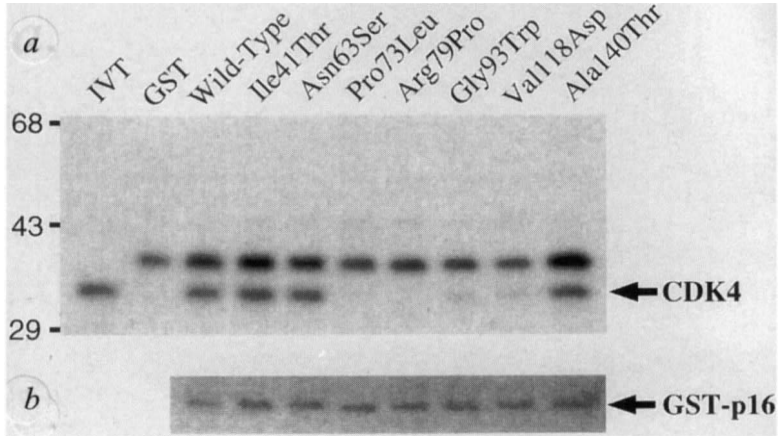

Fig. 2 p16^{INK4} mutants fail to bind stably to the cyclin D1/CDK4 complex. *a*, Equal amounts of insect cell lysates containing ^{35}S-methionine labelled cyclin D1/CDK4 complexes were mixed with approximately 400 ng of GST or GST- p16^{INK4} fusions. Proteins bound to GST/GST- p16^{INK4} were purified using glutathione sepharose, resolved on a 10% SDS-acrylamide gel and detected by fluorography. The lane marked IVT contains CDK4 translated *in vitro* in the presence of ^{35}S-methionine. Numbers on the left indicate molecular weight markers in kilodaltons. We estimate that 40% of the input CDK4 bound to GST–p16^{INK4}. *b*, Prior to fluorography, the gel shown in (*a*) was stained with Coomassie Blue to ascertain that equivalent amounts of GST–p16^{INK4} fusion proteins were recovered from the binding reaction.

somatic Pro73Leu mutation. These observations indicate that the weak inhibition of cyclin D1/CDK4 kinase activity by the mutants is most likely is due to their poor binding to the cyclin D1/CDK4 complex. Interestingly, the amount of cyclin D1 that co-purifies with the GST-p16^{INK4} fusions increases if CDK4 is also co-purified (compare the intensity in Fig. 2 of the Cyclin D1 band in lanes with CDK4 with that in lanes without CDK4), suggesting that GST- p16^{INK4} forms a ternary complex with cyclin D1/CDK4. Further experiments will be needed to test this idea rigorously. Given that the mutants are equally deficient in their ability to inhibit cyclin D1/CDK6 activity, we expect that they will also bind unstably to cyclin D1/CDK6.

In familial melanoma, linkage has been reported to both chromosome 1p36 (refs 13,14) and 9p21 (refs 11,12,15,16). Among the 18 families we have examined, six show strong evidence of linkage to 9p21, clearly three show no evidence of linkage to 9p21, and nine remain equivocal. p16^{INK4} mutations have been detected in five of the six 9p21-linked families[5]. These include a nonsense mutation (Arg50Ter), two missense mutations that impaired inhibition of cyclin D1/CDK4 and cyclin D1/CDK6 (Val118Asp and Gly93Trp), and an undefined mutation that prevents p16^{INK4} transcription (unpublished data). p16^{INK4} mutations were also detected in four of the nine families with inconclusive linkage data. These mutations include a splice donor site mutation, and two missense mutations that reduced inhibition of cyclin D1/CDK4 and cyclin D1/CDK6 (Gly93Trp and Arg79Pro). No mutations were detected in the three families that showed no evidence of linkage to 9p21. Therefore, almost all of the 9p21-linked melanoma kindreds that were examined have demonstrable functional defects in p16^{INK4}.

Two tumour cell lines derived from members of these families have been examined for additional p16^{INK4} mutations. One tumour showed loss of the wild type allele, and retention of the Val118Asp allele; in the other a novel Pro73Leu somatic mutation accompanied the germline Arg79Pro mutation[5]. Therefore the development of these tumours is consistent with the idea that p16^{INK4} is a tumour suppressor gene because they have both inherited a non-functional p16^{INK4} allele and lost the wild-type allele by a secondary event. Furthermore, the occurrence of a somatic, single amino acid substitution (Pro73Leu) in a patient with a germline Arg79Pro mutation suggests that large deletions, including p16^{INK4} and p15^{INK4B}, are not required for the development of familial melanoma.

Methodology

Construction of GST-p16^{INK4} fusion proteins. Wild-type p16^{INK4} was amplified from placental mRNA and cloned into the Bluescript SK vector (Stratagene). Sequence analysis confirmed complete sequence homology through the coding region to those listed in GenBank (U12818, U12819 and U12829), including an additional 24 bp at the 5' end not present in the published sequence[1]. Mutant p16^{INK4} clones were constructed by PCR-directed mutagenesis[17], and sequenced on both strands to confirm that they had only the desired mutation. The wild-type and mutant p16^{INK4} genes were then fused to the GST gene in the vector pGEX-5X-3 (Pharmacia). Fusion proteins were prepared as described (Pharmacia), except that the expression of proteins was induced with 0.4 mM IPTG and was allowed to proceed for ~12 h at 24 °C.

CDK inhibition assays. Baculoviral lysates were prepared and kinase assays performed essentially as described[1,3]. Briefly, 5 µl of lysate was mixed with the indicated amount of p16^{INK4} fusion protein in 30 µl of kinase buffer (20 mM Tris, pH 8.0, 10 mM $MgCl_2$, 1 mM EGTA) for 30 min at 30 °C. Following incubation, ~250 ng of purified GST-Rb and 5 µCi of γ-^{32}P-ATP were added, and the mixture was incubated at 30 °C for 15 min. Subsequent processing was done exactly as described[3]. Prior to autoradiography, the gels were stained with Coomassie Blue to check that equivalent amounts of GST-Rb fusion protein were recovered from the kinase assay.

Cyclin D1/CDK4 binding assays. Sf9 insect cell lysates containing ^{35}S-methionine labelled cyclin D1/CDK4 were prepared as described[1]. For the binding reaction, approximately 400 ng of the GST-p16^{INK4} fusion or GST protein was mixed with an aliquot (5 µL) of the lysate in a final volume of 30 µl. Following incubation at 30 °C for 30 min, the mixture was diluted to 250 µl with IP buffer (50 mM Tris, pH 7.5, 150 mM NaCl, and 0.5% NP-40) and Glutathione Sepharose beads (bed volume 12.5 µl) were added. The GST fusion proteins were allowed to bind to the Sepharose at room temperature for 60 min., and the beads were then washed three times with 1 ml IP buffer. Finally, bound proteins were released by boiling in SDS sample buffer. Proteins were resolved on a 10% SDS acrylamide gel, and detected by flurography. Prior to treatment with the fluor, the gel was stained with Coomassie Blue to ascertain that equivalent amounts of GST– p16^{INK4} fusions were recovered from the binding reaction.

Acknowledgements

K.R. thanks Tony Poteete for helpful discussions. R.S.S. is a Fellow of the Howard Hughes Medical Institute.

Received 8 March; accepted 21 March 1995.

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