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Inhibition of pRb phosphorylation and cell-cycle progression by a 20-residue peptide derived from p16^CDKN2/INK4A

Robin Fåhraeus*, Jesús M. Paramio†‡, Kathryn L. Ball*, Sonia Laín† and David P. Lane*

Background: The *CDKN2/INK4A* tumour suppressor gene is deleted or mutated in a large number of human cancers. Overexpression of its product, p16, has been shown to block the transition through the G_1/S phase of the cell cycle in a pRb-dependent fashion by inhibiting the cyclin D-dependent kinases cdk4 and cdk6. Reconstitution of p16 function in transformed cells is therefore an attractive target for anti-cancer drug design.

Results: We have identified a 20-residue synthetic peptide – corresponding to amino acids 84–103 of p16 – that interacts with cdk4 and cdk6, and inhibits the *in vitro* phosphorylation of pRb mediated by cdk4–cyclin D1. The amino-acid residues of p16 important for its interaction with cdk4 and cdk6 and for the inhibition of pRb phosphorylation were defined by an alanine substitution series of peptides. In normal proliferating human HaCaT cells and in cells released from serum starvation, entry into S phase was blocked by the p16-derived peptide when it was coupled to a small peptide carrier molecule and applied directly to the tissue culture medium. This cell-cycle block was associated with an inhibition of pRb phosphorylation *in vivo*.

Conclusions: These results demonstrate that a p16-derived peptide can mediate three of the known functions of p16: firstly, it interacts with cdk4 and cdk6; secondly, it inhibits pRb phosphorylation *in vitro* and *in vivo*; and thirdly, it blocks entry into S phase. The fact that one small synthetic peptide can enter the cells directly from the tissue culture medium to inhibit pRb phosphorylation and block cell-cycle progression makes this an attractive approach for future peptidometic drug design. Our results suggest a novel and exciting means by which the function of the p16 suppressor gene can be restored in human tumours.

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Background

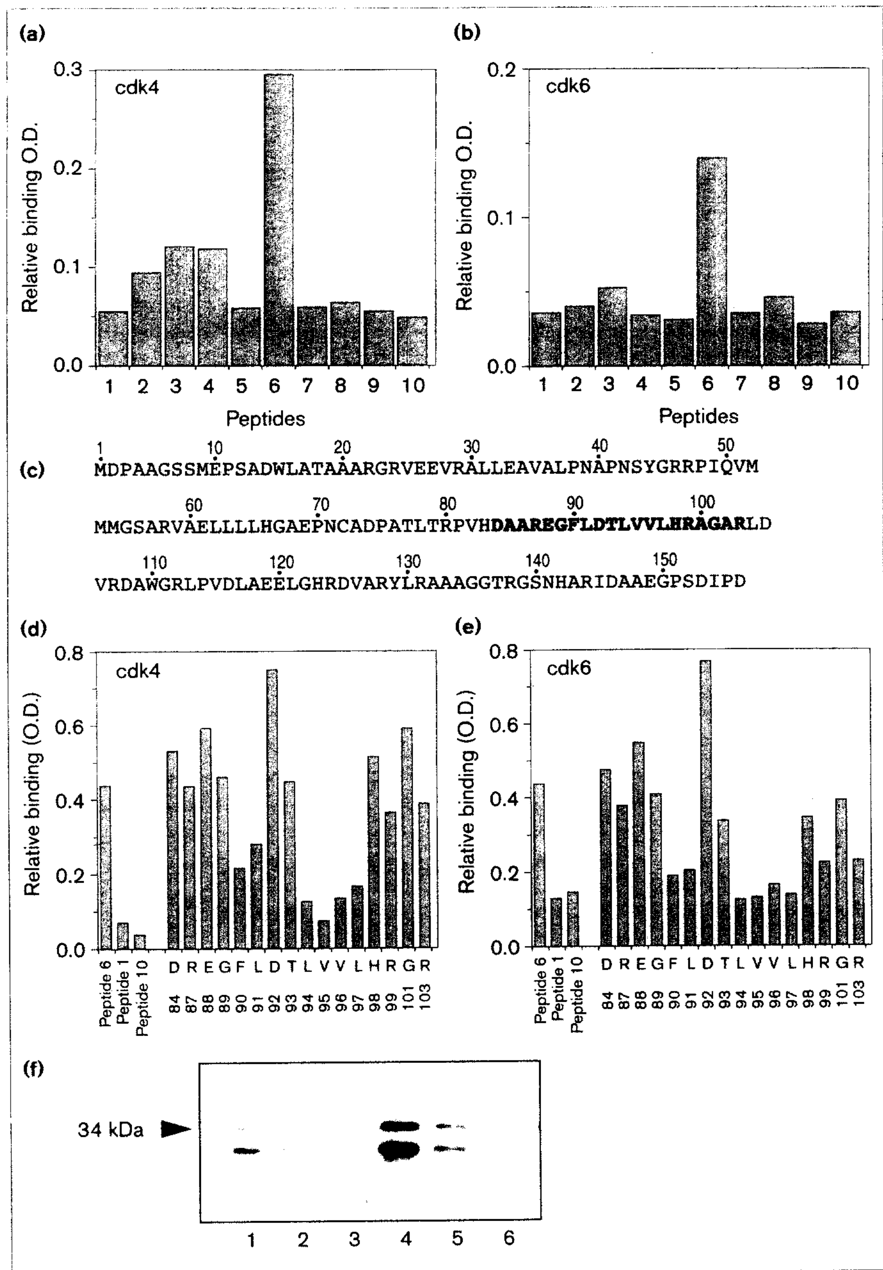
Phosphorylation of the retinoblastoma (*Rb*) gene product, pRb, by members of the cyclin-dependent kinase (cdk) family is an important step in the cell's commitment to undergo mitosis. This step is regulated in the later part of the G1 phase of the cell cycle at what is known as the restriction point [1]. The cdks are key regulatory enzymes through which both positive- and negative-acting signal transduction pathways merge. Mitogenic stimulation induces the formation of an active complex between the D-type cyclins and cdk4 or cdk6 that promotes the phosphorylation of pRb in late G1 phase. These kinases are also the targets for cell growth inhibitory signals arising from contact inhibition, growth factor starvation or transforming growth factor-β. The inhibitory signals can block kinase activity through different members of the two rapidly enlarging families of p16-like and p21/KIP cdk inhibitors that interfere either directly with the kinases or with the cyclin–kinase complexes [2]. The kinase inhibitory proteins can block cell proliferation; accordingly, two of these proteins — p21 and p16 — have been associated with tumour suppressor-like activity. However, unlike the gene encoding p21, which is indirectly linked to tumour suppression activity through its transcriptional induction by p53, the *CDKN2* gene is deleted or mutated in a large number of human tumours [3–9] and germ line mutations in *CDKN2* are associated with an increased risk of developing melanoma [3,4]. Deletions or mutations in the genes encoding cdk inhibitors are, however, not the only mechanism by which the pRb regulatory pathway can be perturbed in tumour cells: mutations in the *Rb* gene itself or elevated levels of cyclin D or cdk4 have been reported in other tumours [10–12].

Results

Interaction between a p16-derived peptide and cdk4/cdk6

Because binding to cdk4 and cdk6 is thought to be an important mechanism by which p16 acts as a tumour suppressor, we wanted to identify and study the region of p16 that is involved in these interactions. Small peptides have previously been used as powerful tools to identify regions of proteins mediating different biological activities and protein–protein interactions [13–17]. We therefore synthesized a series of overlapping 20-residue peptides that

Figure 1

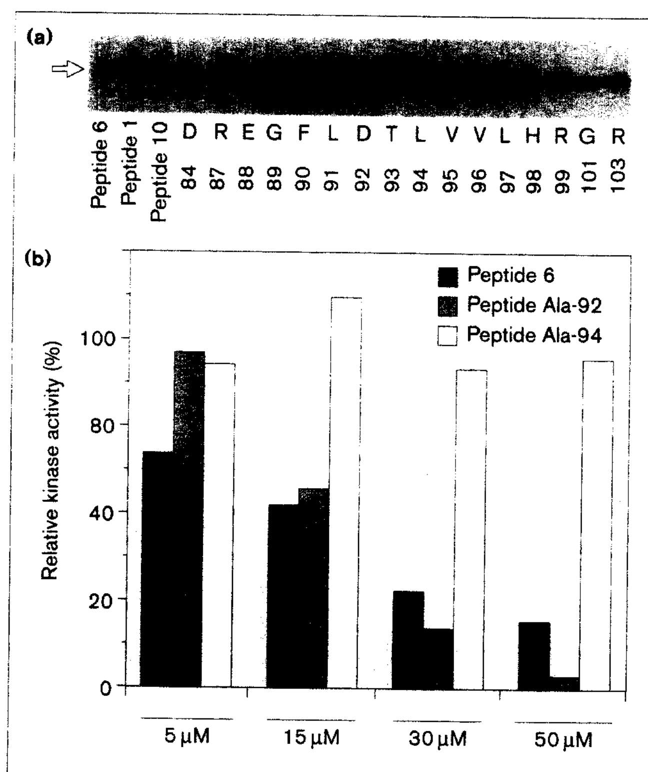


Interaction of p16-derived synthetic peptides with cdk4 and cdk6. **(a,b)** Relative binding of the p16-derived peptides to *in vitro* expressed cdk4 and cdk6. **(c)** The amino-acid sequence of p16; the region corresponding to peptide 6 (amino acids 84–103) is highlighted. **(d,e)** Similar binding profile of a series of alanine substitution mutations of peptide 6 to *in vitro* translated cdk4 and cdk6. The amino-acid residues of peptide 6 substituted by alanine are indicated and the relative amount of cdk4 and cdk6 precipitated by each peptide is shown. **(f)** Sf9 insect cell lysates containing cdk4 were incubated with the following biotinylated peptides: peptide 6 (lanes 1 and 4), peptide 1 (lanes 2 and 5) and peptide 10 (lanes 3 and 6). Extracts containing cyclin D1 were added before (lanes 1–3) or after (lanes 4–6) the peptides. The complexes were precipitated with streptavidin-coated agarose beads. The lower of the cdk4 bands is related to the extraction method (see Materials and methods).

spanned the p16 amino-acid sequence. Biotinylated peptides were coupled to streptavidin–agarose beads and tested for their ability to form a stable interaction with ^{35}S-labelled cdk4 and cdk6 expressed in rabbit reticulocyte lysates. Figure 1a,b shows that peptide 6, which corresponds to amino-acids 84–103 of p16 (Fig. 1c), was able to deplete both cdk4 and cdk6 from the reticulocyte lysates.

A series of alanine substitution mutations of this peptide, in which individual amino acids were replaced by alanine residues, revealed that substitution of the hydrophobic amino acids in the region between residues 90–97 decreased the ability of the peptide to bind to both cdk4 and cdk6. Interestingly, the Ala-92 peptide, in which the aspartic acid at position 92 was substituted with alanine, had a significantly increased affinity for both kinases (Fig. 1d,e). Because p16 can compete with cyclin D for binding to cdk4 (see Discussion) we wanted to know whether the interaction between peptide 6 and cdk4 could be affected by the assembly of the cdk4–cyclin D1 complex. We used extracts prepared from Sf9 insect cells expressing cdk4 and cyclin D and showed that if peptide 6 was incubated

Figure 2



(a) Inhibition of the phosphorylation of *E. coli* expressed and purified full-length pRb protein. p16-derived wild-type peptides (peptides 1, 6 and 10) or the alanine substitution series of peptides were tested for their ability to interfere with pRb phosphorylation in lysates prepared from Sf9 insect cells expressing cdk4 and cyclin D1 (see Materials and methods). The amino-acid residues of peptide 6 substituted with alanine are indicated and the levels of pRb phosphorylation in the presence of each peptide are shown. **(b)** The effect of increasing amounts of peptides 6, Ala-92 and Ala-94 on pRb phosphorylation. At a concentration of 50 μM, Ala-92 almost completely blocks pRb phosphorylation by cdk4–cyclin D.

with extracts containing cdk4 prior to the addition of cyclin D1-containing extracts, it could form a stable complex with cdk4. However, peptide 6 no longer seemed to form a stable complex with cdk4 if the extracts containing cyclin D1 and cdk4 were mixed before the addition of the peptide (Fig. 1f).

Peptide inhibition of pRb phosphorylation *in vitro*

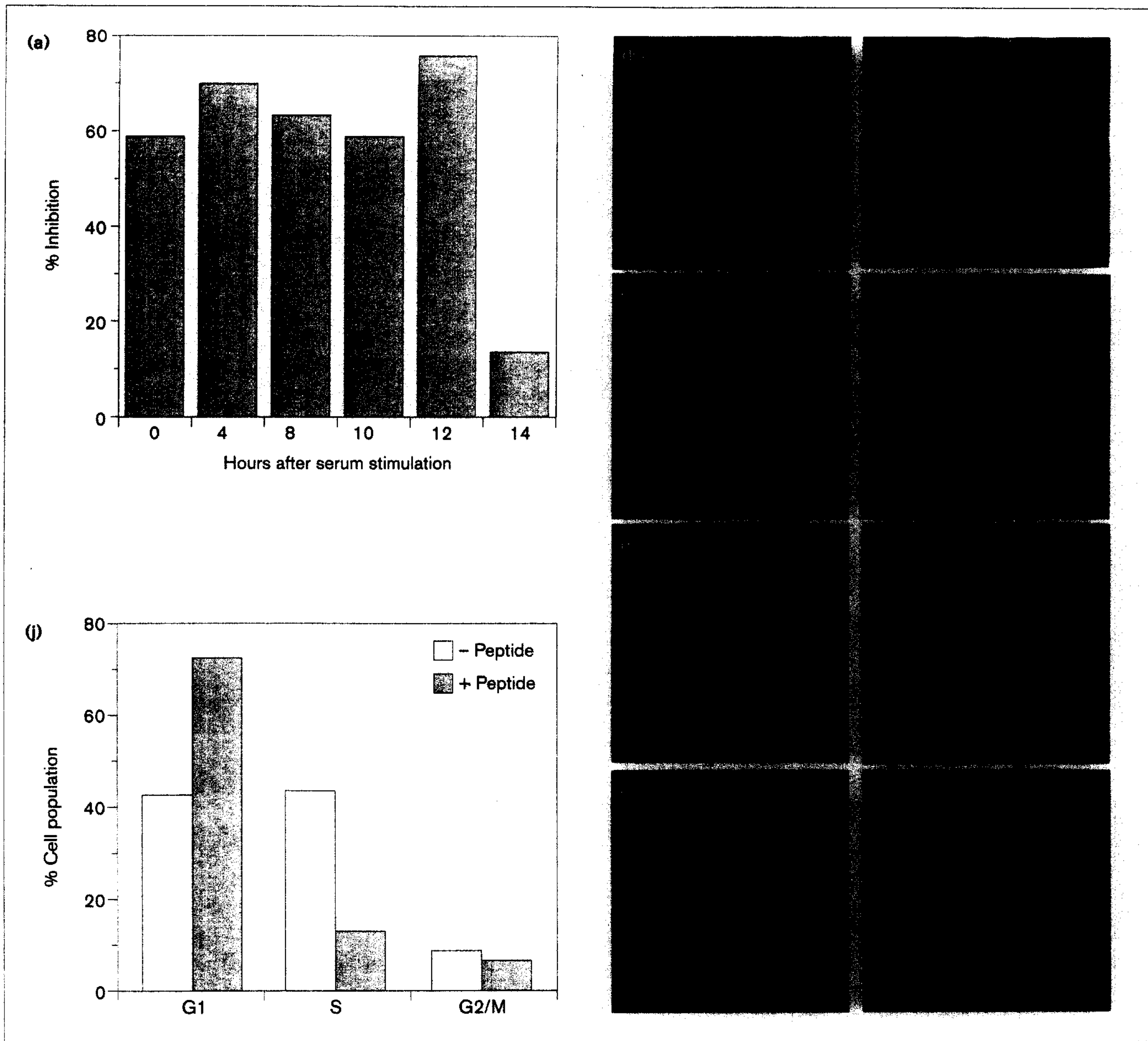
In order to study the functional significance of the interaction between the p16 peptides and the cdks we asked whether the p16-derived peptides, and the series of alanine-substitution mutations of peptide 6, affected the phosphorylation of pRb *in vitro*. Peptides were incubated with lysate prepared from Sf9 insect cells infected with a cdk4-expressing baculovirus, and subjected to further incubation in the presence of purified recombinant pRb, radiolabelled ATP and lysate prepared from Sf9 cells expressing cyclin D1. Figure 2 shows that peptide 6 significantly decreased pRb phosphorylation, which correlated with its ability to bind to cdk4 and cdk6 (Fig. 1a,b); in contrast, peptides 1 and 10, which were unable to precipitate cdk4 or cdk6, had no effect on pRb phosphorylation (only results from peptides 1, 6 and 10 are shown). A correlation was also observed between the ability of the various peptides in the alanine-substitution series to bind to cdk4 and cdk6 and the extent to which they inhibited cdk4–cyclin D1 kinase activity (Figs 1d,e and 2a). Most significantly, substitution of amino acids in the hydrophobic region of peptide 6, located between residues corresponding to positions 90–97 of the full-length p16 protein, resulted in a clear decrease in the inhibition of pRb phosphorylation. Interestingly, the Ala-92 peptide was more potent than peptide 6 in its inhibition of cdk4 kinase activity, reflecting its increased ability to bind to cdk4 and cdk6.

A dilution series revealed that when the Ala-92 peptide and peptide 6 were present at a concentration of 50 μM, pRb phosphorylation was almost completely blocked. In contrast, replacement of the leucine residue at position 94 with alanine (peptide Ala-94) completely inactivated the inhibitory function of peptide 6 in this assay (Fig. 2b). The correlation between enhanced binding of the Ala-92 peptide and its greater efficiency as a kinase inhibitor is provocative and suggests that further variants of the peptide 6 sequence might possess greater inhibitory activity. A similar inhibition of pRb phosphorylation was observed when the peptides were mixed with lysates prepared from insect cells co-infected with cdk4- and cyclin D1-expressing baculoviruses (data not shown). The region of p16 represented by peptide 6 is identical to the corresponding domain of the kinase inhibitor p15 [18–20], and is conserved in the closely related p18 [19] and p19 [21,22] inhibitors. Point mutations in the p16 gene have been found in tumours from familial and primary melanomas as well as in tumours from the oesophagus and the bladder. Some of these mutants are clustered in, or near, the region encompassed by peptide 6, and have been shown to have lost their ability to inhibit cell proliferation and pRb phosphorylation [7,23,24].

Inhibition of S-phase entry and pRb phosphorylation *in vivo*

As the overexpression of p16 in cultured cells can block S-phase entry [24,25], we wanted to determine whether peptide 6 could affect cell proliferation. A 16 amino-acid region of the Antennapedia homeodomain (known as Penetratin), which has been shown to translocate through biological membranes in a rapid and energy-independent fashion [26], was coupled to peptide 6 and used as a carrier; this was added to the tissue culture medium of serum-starved human keratinocyte-derived HaCaT cells. Figure 3 shows that when 0.5 μM carrier-linked p16 peptide was added to the medium at the same time or up to 12 hours after the addition of serum, the number of

Figure 3



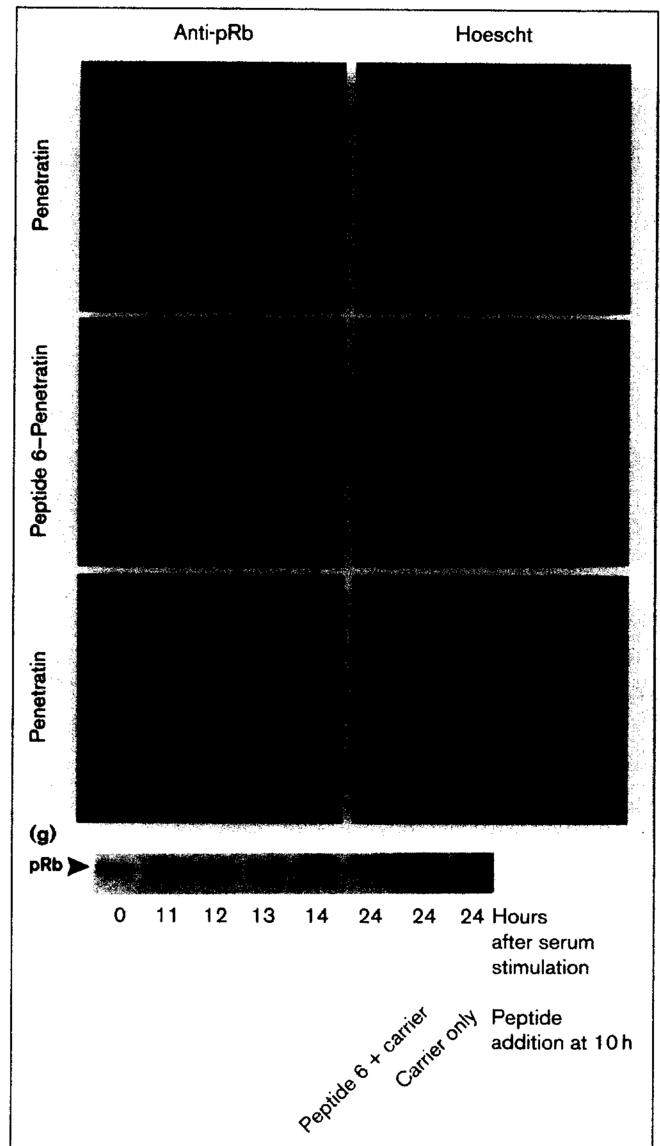
Peptide 6 coupled to the Penetratin carrier molecule inhibits S-phase entry in HaCaT cells. Cells were synchronised in G0 phase by serum starvation for 72 h before serum and 10 μM BrdU were added. **(a)** At the indicated time points after serum stimulation, 0.5 μM peptide 6 coupled to the Penetratin carrier molecule was added to the tissue culture medium. The data presented show percentage inhibition of cells entering S-phase after incubation with carrier-linked peptide 6 in relation to cells incubated with serum only. **(b,d,f,h)** DNA synthesis in S-phase cells assayed by BrdU labelling; **(c,e,g,i)** the same fields of cells stained with Hoescht. The percentage of cells that incorporated BrdU after release from serum starvation was 71 % at 24 h (f,g) and 14 % at 3 h (h,i). The number of cells incorporating BrdU at 24 h was significantly reduced when peptide 6 coupled to the Penetratin carrier molecule was added at 12 h (b,c) compared to 14 h (d,e). No effect on DNA synthesis could be observed with Penetratin only (not shown). **(j)** Fluorescence-activated cell sorting (FACS) analysis of normal proliferating HaCaT cells showing an increase of the number of cells in G1-phase and a decrease of the number of cells in S phase in the total cell population 20 h after treatment with a 36-residue peptide containing the amino-acid sequences of peptide 6 and Penetratin. (a), (b–i) and (j) each present results from one representative experiment.

cells entering S phase was reduced dramatically, as assayed by bromodeoxyuracil (BrdU) incorporation 24 hours after the addition of serum. However, when the coupled peptide was added to the medium 14 hours after the release of serum starvation, the number of cells entering S phase was the same as that seen in cells not treated with peptide. This suggests that the effect of the peptide is limited to a rather narrow window in the cell cycle

corresponding to the later part of G1 phase. This period includes the restriction point, where serum stimulation and protein synthesis are no longer required to ensure entry into S phase; this point has been suggested to be the critical time for pRb phosphorylation [1,27]. The accumulation of normal proliferating HaCaT cells in G1 phase seen after the addition of 25 μM of peptide suggests that the peptide only blocks S-phase entry and is not active during other parts of the cell cycle (Fig. 3c). It should be pointed out that the concentration of peptide required to induce G1 arrest in proliferating cells was considerably higher than what was needed to inhibit S-phase entry in serum-starved cells. The expression of cyclin D1 and cdk4 did not seem to be altered after addition of the peptide to the cells (data not shown).

When starved cells were incubated with the carrier-linked p16 peptide or the carrier molecule alone 10 hours after addition of serum, we observed a difference in pRb extractability when analyzed 23 hours after serum addition. This difference in extractability may result from hyperphosphorylated pRb having a lower affinity for the nuclear compartment than the hypophosphorylated subtypes, so it can be extracted from the nucleus by using a hypotonic buffer containing Triton X-100 [28]. Approximately 60 % of the cells incubated with carrier-linked peptide 6 stained with an anti-pRb monoclonal antibody, compared with only 14 % of the cells that had been incubated with the carrier only (Fig. 4a–d). This observation was confirmed by western immunoblot analysis of whole HaCaT cell extracts treated in a similar fashion (Fig. 4g). The results show that the number of cells with hypophosphorylated pRb increased significantly 23 hours after serum stimulation when carrier-linked peptide 6 was added up to 10 hours after the addition of serum. The data also imply that the inhibition of cdk4–cyclin D activity observed in baculovirus-infected Sf9 cell extracts (Fig. 2) takes place *in vivo*. The consistent inhibition of S-phase entry after the addition of the coupled peptides between 0–12 hours shows that the effect of the peptide is persistent and it suggests that the carrier-linked peptide is not rapidly degraded in the cells.

Figure 4



Phosphorylation of pRb *in vivo*. **(a,c,e)** Cells stained with anti-pRb monoclonal antibody; **(b,d,f)** the same field of cells stained with Hoescht. HaCaT cells were serum-starved for 72 h before serum stimulation; 8 h after serum addition cells were treated with 0.5 μM Penetratin (a,b,e,f) or 0.5 μM Penetratin-linked peptide 6 (c,d). Cells were then treated with extraction buffer containing Triton X-100 (a–d) or left untreated (e,f) before fixation. The amount of phosphorylated pRb was estimated at 23 h by determining pRb extractability (a,c) compared with a non-extracted staining (e). **(g)** The status of pRb phosphorylation in whole-cell extracts prepared from HaCaT cells as determined by western blot analysis using IF8. Cells were starved for 72 h before the addition of serum and harvested at the indicated time points. pRb phosphorylation can be detected at 14 h as indicated by the altered migration of the upper band on the gel which represents pRb. Peptide 6 coupled to Penetratin, or Penetratin alone, was added at 10 h as indicated. In the presence of carrier-linked peptide 6, pRb remains hypophosphorylated for up to 24 h after serum stimulation.

Discussion

The results presented here demonstrate that a 20-residue synthetic peptide corresponding to amino acids 84–103 of the p16 protein can mimic the essential biochemical and biological properties described for the full-length wild-type p16 protein. Most important is the discovery that the carrier-linked peptide has the capacity to inhibit the proliferation of serum-starved HaCaT cells after direct addition to the tissue culture medium. This cell-cycle arrest could be seen from the point at which they were released from serum starvation to a point before 14 hours. At this latter stage, the cells normally pass the restriction point — that is, when pRb becomes hyperphosphorylated and the cells

are committed to entering mitosis. However, in the presence of the p16 peptide, pRb remains mainly unphosphorylated 24 hours after serum stimulation, suggesting that the effect of the peptide on S-phase entry could be accounted for by its inhibition of pRb phosphorylation in G1 phase. Our biochemical data support this hypothesis. We have shown that cyclin D1 interferes with the formation of the complex between cdk4 and peptide 6 *in vitro* (cyclin D1 also disrupts the interaction between full-length p16 and cdk4 [29]), which suggests that peptide 6 alters protein–protein interactions in the pRb phosphorylation complex. This idea is further strengthened by the correlation between the ability of the alanine substitution series of peptides to precipitate cdk4 and cdk6 and their ability to inhibit pRb phosphorylation *in vitro*. Because our observations demonstrate that the biochemical effect of p16 can be mimicked by a small peptide, they suggest that the inhibitory effect of the full-length p16 protein is mediated by its interaction with cdk4 and cdk6. This effect could be achieved through direct competition between p16 and cyclin D1 for binding to cdk4/cdk6 or by conformational alterations caused by these interactions. The results presented here on the competition between peptide 6 and cyclin D for binding to cdk4 do not rule out either of these alternatives, but they do suggest that the interaction between cyclin D and cdk4 will effect binding of the cdk4 inhibitory domain of p16, which is in line with previous observations and indicates that the interaction between peptide 6 and cdk4 is specific [29].

The sequence included in peptide 6 has similarities with those of central ankyrin-like repeats [20,30]. Both the aspartic acid and the hydrophobic residues demonstrated to be important for the interaction between peptide 6 and cdk4/cdk6 and for the inhibition of pRb phosphorylation lie within an 8 amino-acid consensus sequence suggested to form an α-helix, with highly conserved hydrophobic residues involved in protein–protein interactions. Our observation that substituting the hydrophobic amino acids (corresponding to positions 94–97 of p16) with alanines decreases the affinity of peptide 6 for the two kinases fits well with this model. If this is the case, then the increased binding of the peptide to cdk4 and cdk6 resulting from substitution of the aspartic acid at position 92 with alanine could reflect an unfavourable presentation of this aspartic acid (with regard to the hydrophobic residues) in the original peptide structure. This hypothesis will, however, have to be confirmed by future structural studies of the p16 peptide by itself, and in the presence of cdk4 or cdk6. Interestingly, even though most of the mutations in p16 detected so far that result in inactivation of the kinase inhibitory effect have been reported to be clustered within, or close to, the region corresponding to peptide 6, they are also found in other regions of the p16 sequence. Our observations suggest that all of these mutations might share one common effect in that they, by different means, inactivate the p16 functional domain corresponding to peptide 6.

We have described a method for studying the inhibition of pRb phosphorylation during G1 phase and blocking entry into S phase in tissue culture cells. However, even more interesting is the possibility of applying this method to blocking pRb phosphorylation in living tissues where the regulation of pRb phosphorylation is defective. Evidence for the importance of controlling pRb phosphorylation during the cell cycle is accumulating rapidly [11,12]. A large number of tumours have been detected in which the cells lack functional p16 or overexpress either cyclin D or cdk4, making the signalling pathway for pRb phosphorylation a common target for tumour-cell development. Our results show that it is possible to target and inhibit the enzymes that phosphorylate pRb *in vivo*, and thereby compensate for such defects in the tumour cells. Interference with complex formation between cdk4/6 and cyclin D demonstrated by the p16-derived peptide makes this complex an attractive target for future cancer drug design. Previous results have shown that small peptides can be both effective and specific inhibitors of cell-transforming mechanisms and can be used as models for drug design [14–16]. For example, peptides derived from the region of Ras that interacts with other proteins can block the biological function of Ras upon microinjection. Furthermore, the carboxy-terminal CAAX box of Ras that is the donor site for the farnesyl protein transferase (FPTase) has been used to design peptidometic drugs that inhibit FPTase activity and selectively inhibit the proliferation of *ras*-transformed cells both in tissue culture and in live animal models [31]. Our results constitute the basis for a model for future work aimed at replacing specific suppressor gene function with possible therapeutic applications.

Conclusions

The results presented here show that the known functions of the full-length p16 tumour suppressor can be mimicked by a 20-residue synthetic peptide, corresponding to amino acids 84–103 of the p16 protein. Thus, this peptide can form stable complexes with the cyclin D-dependent kinases cdk4 and cdk6, inhibit their ability to phosphorylate pRb *in vitro*, and block cell-cycle progression through G_1/S phase. We have also demonstrated that the effect of this peptide on passage through G_1/S phase is associated with an inhibition of pRb phosphorylation *in vivo*, and that the peptide is only active at a time point prior to pRb phosphorylation. These results are interesting from several different points of view. Firstly, it is the first time that it has been demonstrated that the function of a tumour suppressor gene product can be mimicked by a small peptide. Secondly, the approach we have used is useful for direct intracellular studies of *in vitro* active peptides in many different biological fields. Finally, because at least some, if not all, of the known tumour suppressor

functions of p16 can be incapsulated in one 20-residue peptide, this is an attractive target for future peptidometic drug design aimed at cell-cycle inhibition and anti-cancer treatment.

Materials and methods

Peptide precipitation

A series of 20-residue peptides with 5 amino-acid overhangs (that is, with a 15 amino-acid overlap), corresponding to the entire sequence of p16 (apart from the first 8 amino-terminal residues), was synthesized. Each peptide contained a SGSG linker at the amino terminus to which a biotin group was coupled. The series of alanine substitution mutations of peptide 6 was synthesized in the same way. The peptides were coupled to streptavidin immobilized on agarose beads and washed four times in PBS before incubation for 1 h on ice with rabbit reticulocyte lysate (Promega) containing [^{35}S]methionine-labelled cdk4 or cdk6. The beads were washed four times in 1.2 × PBS containing 0.2 % Triton X-100; SDS loading buffer was then added to the samples, which were then applied to 12 % SDS–polyacrylamide gels. The gels were exposed to an autoradiography film and the bands corresponding to cdk4 and cdk6 were analyzed by densitometry. cdk4 precipitated from Sf9 insect cells with the p16 peptides was immunoblotted and stained using a monoclonal antibody directed against cdk4 monoclonal antibody (Santa Cruz). The variations in the intensity of the Hoescht staining are only due to the quality of the photographs and do not reflect the pRb extraction methods used in this assay.

pRb phosphorylation in vitro

Sf9 insect cells infected with human cdk4-expressing baculovirus were lysed in a buffer containing 10 mM Hepes pH 7.4, 10 mM NaCl, 1 mM EDTA and 0.5 mM PMSF. Peptides (at a final concentration of 25 μM) were incubated with 3 μl Sf9 cell extract in a buffer containing 50 μM Hepes pH 7.4, 10 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM NaF and 1 mM Na_3VO_4. The mixture was incubated for 60 min on ice, after which 3 μl Sf9 lysate containing human cyclin D, prepared as above, was added together with 0.6 μg of purified recombinant full-length pRb protein and 2.5 μM α-[^{32}P]ATP in a final concentration of 50 mM ATP. This mixture was incubated for 10 min at 30 °C, after which the reaction was terminated by the addition of SDS loading buffer. Samples were then subjected to SDS–PAGE on 8 % gels. The gels were either exposed to autoradiographic film or the levels of pRb phosphorylation were estimated by Phosphoimaging.

Cell-cycle inhibition

A cysteine residue was added to the carboxyl terminus of peptide 6 and used for coupling to the 16-residue Penetratin peptide of the Antennapedia homeodomain (Appligen) by means of a disulphide bond. A 36-residue peptide, including peptide 6 and the Penetratin sequence, was synthesized and used for the FACS analysis of normal proliferating HaCaT cells. Cells were seeded on coverslips prior to starvation for 72 h in Dulbecco's modified Eagle's medium (DMEM) without foetal calf serum (FCS). After this period the medium was substituted with DMEM containing 10 % FCS and BrdU. The coupled peptides were added at different time points after serum stimulation. The number of cells entering S phase was determined by estimating the number of cells incorporating BrdU at 24 h. Cells were fixed on coverslips in acetone:methanol (1:1), incubated in 1 M HCl for 30 min, washed six times in PBS and then incubated with an anti-BrdU monoclonal antibody and a Texas Red-conjugated secondary antibody. Samples were then mounted in Mowiol containing Hoescht. At least six different areas (including at least 50 cells per area) on three different coverslips were counted for each single experiment which was repeated at least twice.

pRb phosphorylation in vivo

Hyperphosphorylated pRb was extracted from cells cultured on coverslips by treating the cells with hypotonic buffer containing 0.1 % Triton X-100 prior to fixation in acetone/methanol (1:1) [28]. Fixed cells were incubated for 1 h with anti-pRb monoclonal antibody IF8, washed three times in PBS and incubated for 45 min with Texas Red-conjugated secondary antibody before the coverslips were mounted in Mowiol containing Hoescht. For western immunoblot analysis, cells were lysed in RIPA buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 1.0 % NP-40, 0.5 % DOC, 0.1 % SDS, 0.1 mM PMSF and 1 mM aprotinin for 30 min at 4 °C. The protein concentrations were determined before the samples were boiled in SDS loading buffer, run on 8 % SDS–polyacrylamide gels and transferred to a nitrocellulose membrane. The filters were first incubated with the anti-pRb monoclonal antibody IF8 before incubation with a secondary antibody conjugated to horseradish peroxidase (DAKO) and developed with ECL (Amersham).

Acknowledgements

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