Inhibition of mammalian cell proliferation by genetically selected peptide aptamers that functionally antagonize E2F activity

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The p16-cyclin D-pRB-E2F pathway is frequently deregulated in human tumors. This critical regulatory pathway controls the G1/S transition of the mammalian cell cycle by positive and negative regulation of E2F-responsive genes required for DNA replication. To assess the value of the transcription factors E2Fs as targets for antiproliferative strategies, we have initiated a program aiming to develop inhibitors targeting specifically these proteins *in vitro* and *in vivo*. The cellular activity of E2F is the result of the heterodimeric association of two families of proteins, E2Fs and DPs, which then bind DNA. Here, we use a two hybrid approach to isolate from combinatorial libraries peptide aptamers that specifically interact with E2Fs DNA binding and dimerization domains. One of these is a potent inhibitor of E2F binding activity *in vitro* and in mammalian fibroblasts, blocks cells in G1, and the free variable region from this aptamer has the same effect. Our experiments argue that the variable region of this aptamer is structured, and that it functions by binding E2F with a motif that resembles a DP heterodimerization region, and blocking E2F's association with DP. These results show that cell proliferation can be inhibited using genetically-selected synthetic peptides that specifically target protein-protein interaction motifs within cell cycle regulators. These results also emphasize the critical role of the E2F pathway for cell proliferation and might allow the design of novel antiproliferative agents targeting the cyclin/CDK-pRB-E2F pathway.

Keywords: E2F transcription factors; peptidic inhibitors; cell proliferation; DNA binding and dimerization domains

Introduction

E2F transcription factors were originally characterized as cellular proteins activated by the viral oncoprotein E1A and needed for adenovirus gene expression. Since then, E2Fs have come to be recognized as central players in the control of animal cell cycle, differentiation and transformation (Sardet *et al.*, 1997; Slansky and Farnham, 1996). The cellular activity of E2F is the result of the heterodimeric association of two families

of proteins, E2Fs (E2F1 to 6) and DPs (DP1 to 2) (Sardet *et al.*, 1997; Slansky and Farnham, 1996). All E2Fs share a highly conserved DNA binding domain (DB) encompassing a stretch of basic residues, along with overlapping helix-loop-like and putative leucine-zipper-like domains (Sardet *et al.*, 1995). Binding of E2Fs to DNA requires, however, additional flanking conserved regions necessary for heterodimerization with DP proteins, including a conserved heptad repeat with an hydrophobic residue at position 7 and a domain termed 'the marked box' with no predictable secondary structure (Vidal *et al.*, 1996). The C-terminal region of all E2Fs, except E2F6, is a transactivation region (TA) rich in serines and acidic residues (Sardet *et al.*, 1997; Slansky and Farnham, 1996). The C-terminal 18 residues of this TA are necessary and sufficient to bind to the pRB tumor suppressor protein family (pRB, p107 and p130, also called 'pocket proteins') (Sardet *et al.*, 1995; Helin *et al.*, 1992; Shan *et al.*, 1996). Interaction with pocket proteins inhibits E2Fs/DPs-dependent transactivation and may also inhibit transcription factors bound close to the E2Fs (Weintraub *et al.*, 1995). Thus, E2Fs regulate gene expression positively when they are bound on DNA as E2F/DP heterodimers, and negatively when bound to pocket proteins (Sardet *et al.*, 1997; Slansky and Farnham, 1996; Weinberg, 1995).

The E2F/DP association with the pRB family is controlled by the cyclin/cdk (D/k4, E/k2, A/k2)-dependent phosphorylation of the 'pocket proteins' and of E2F/DP. This control exerted by cyclin/cdks directly links the cell-cycle machinery to gene transcription, and as such, defines a critical regulatory pathway that gates cell cycle progression through positive and negative regulation of E2F-regulated genes. Most E2F-regulated genes are periodically expressed during the cell cycle, they encode proteins that either directly control cell-cycle progression from G0/G1 into S phase or function in metabolic processes linked to DNA replication (Sardet *et al.*, 1997; Slansky and Farnham, 1996). Indeed, E2F's DNA binding sites (TNTCG/CCGC) were found to be critical in the promoters of genes that encode cell cycle regulatory proteins (cyclin A, cyclin E, cyclin D1, p107, pRB, cdc6, hsorc1, E2F1 and E2F2), DNA synthesis enzymes (DHRR, TK, DNA pol α), and cellular proto-oncogenes (c-*myc*, N-*myc*, *erb*-B and B-*myb*).

The role of these target genes in cell proliferation is indicated by the fact that ectopic expression of different components of E2F complexes has a profound effect on cell cycle progression. Depending on the genetic background of the cells, directed E2F expression can

drive serum starved G0-arrested cells through G1 into S and lead to cellular transformation or apoptosis (Johnson *et al.*, 1995; Qin *et al.*, 1994; Shan and Lee, 1994; Kowalik *et al.*, 1993; De Gregori *et al.*, 1995; Duronio and O'Farrel, 1995; Lukas *et al.*, 1996). Conversely, inhibition of E2F activity with dominant negative E2F and DP mutants, transfected E2F binding sites, and DP related peptides, inhibits cell growth (Mann and Jones, 1996; Dobrowolski *et al.*, 1994; Morishita *et al.*, 1995; Wu *et al.*, 1996; Bandara *et al.*, 1993). Similarly, overexpression of pRB, p107 or p130 results in the arrest of cells in the G1 phase of the cell cycle (Goodrich *et al.*, 1991; Sherr 1996).

Consistent with this picture, mammalian tumors typically show a number of distinct genetic aberrations in this Cyclin/CDK-pocket protein/E2F pathway that might cause a deregulation of E2F activity. These include translocations and amplifications affecting cyclin and cdk genes, loss of function mutations of the cdk inhibitor p16^{ink4} (Sherr, 1996), and loss of pRB function in a variety of human tumors (Weinberg, 1995; Sherr, 1996). These facts show that E2Fs are key signal transducers whose deregulation is required for tumor development.

Here, we used a recently developed strategy (Colas *et al.*, 1996; Gyuris *et al.*, 1993) to isolate random peptides that specifically inhibited cellular E2F. We first used a two-hybrid approach to isolate thioredoxin-20mer peptide aptamers that interacted with the E2F1 DB and dimerization domains. Among these anti-E2F aptamers was one that strongly inhibited E2F activity *in vitro* and in mammalian fibroblasts. The variable region of this aptamer was also a potent inhibitor. Our results suggest that this peptide may be structured, and that it inhibits E2F activity by blocking E2F/DP association through a region that resembles a DP1 motif that heterodimerizes with E2F. Moreover, we show that this novel E2F inhibitor strongly inhibits cell proliferation by blocking cells in G1. Our results show that peptide aptamers and peptides will make useful molecular tools to selectively inhibit steps in the E2F pathway.

Results

Genetic selection of peptide aptamers that recognize the E2F DNA binding and heterodimerization domains

To isolate peptides that interfere with E2F activity, we used a genetic screen in yeast (Fields and Song, 1989) to select peptide sequences that bind to functional domains highly conserved among members of the E2F proteins family. We used the DNA binding and DP heterodimerization domains of E2F1 as baits in a yeast two hybrid assay to screen a combinatorial prey library of constrained 20-residue peptides displayed in the active loop of *E. coli* thioredoxin (Trx) molecule (chimeric proteins, here called aptamers or Apt) (Gyuris *et al.*, 1993; Colas *et al.*, 1996). We selected six positive interactors (which we termed Apt1-Apt6) from 5×10^5 bait-prey co-transformants. These anti-E2F1 aptamers were isolated and retested in similar two hybrid assays for interaction with: (i) a Cdc2 bait used as negative control; (ii) other members of the E2F family, including baits containing the DNA binding

and DP1 heterodimerization domains of E2F4 and E2F5. As shown in Figure 1a, all six aptamers interact equivalently with E2F1, E2F4 and E2F5 whereas they do not react with an unrelated protein such as Cdc2. This result suggests that these aptamers recognize epitopes conserved among these different E2Fs.

The sequence of the variable region of these aptamers is shown in Figure 1b. The variable regions do not show more than random sequence similarity. We also compared their sequences with those of known proteins present in data libraries, focusing on putative similarities with known E2F-interactors such as DP1 and adenovirus E4 proteins. By contrast with previous reports, one notable similarity between a variable region and a naturally occurring protein was revealed by this analysis, a short stretch of four amino-acids (WIGL) present in Apt5 and in the heterodimerization domain of DP1 proteins (see also Figure 4).

Anti-E2F aptamers Apt4 and Apt5 inhibit E2F binding to DNA in vitro

In order to assess whether the interacting aptamers can alter E2F functions, we performed a bandshift disruption assay to check whether GST-recombinant aptamers (GST-Apt) interfered with binding of *in vitro* translated E2F1/DP1, E2F4/DP1 and E2F5/DP1 to an E2F site. As shown in Figure 2a we tested four aptamers in this assay. Among these, GST-Apt4 and GST-Apt5 were the most potent in blocking the binding of the three E2F/DP heterodimers to this site, whereas other GST-Apts had weak or no effects. Moreover, this inhibitory effect was concentration dependent (not shown) and depended on the variable regions; it was not observed with control recombinant GST-Trx (Figure 2a).
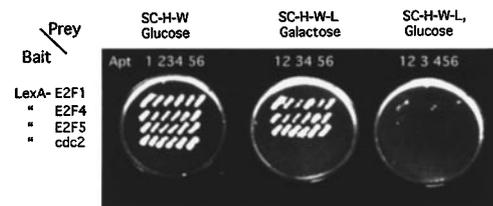

Figure 1 Isolation of anti-E2F aptamers. (**a**) Aptamers (1–6) selected upon screening of a peptide library with E2F1. Aptamers were checked for specific interaction with different LexA-containing baits (Materials and methods) that bore Cdc2 (a control) or the E2F1, E2F4 and E2F5 DNA binding and heterodimerization domains. Yeast reporter strains carrying these different baits, growing on permissive plates (SC -W -H, Glucose) were replica plated onto SC -W -H -L galactose plates as above, to select for Galactose inducible leucine prototrophy. (**b**) Sequences of the variable regions of these anti-E2F aptamers
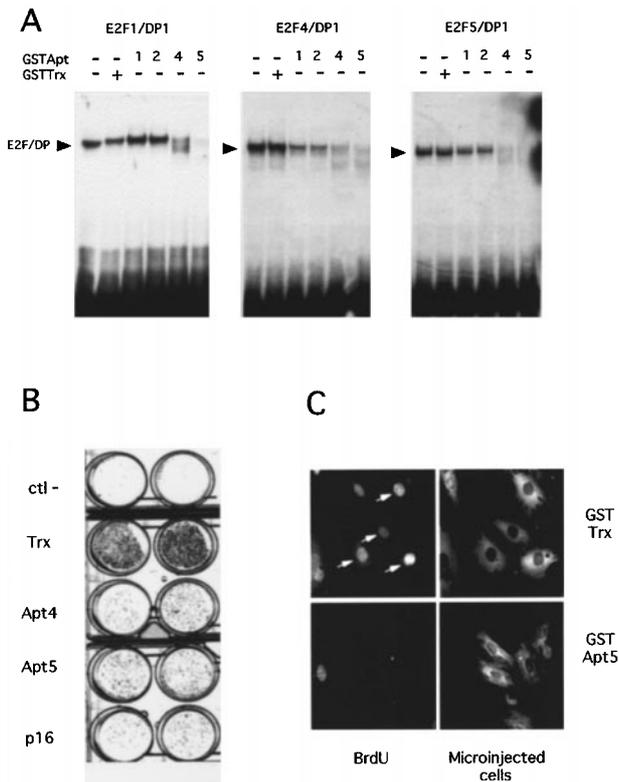
Figure 2 Effect of apt5 on E2F binding and cell growth. (**a**) Anti-E2F aptamers inhibit DNA binding by recombinant E2Fs. We incubated ^{32}P-labelled probe that bore an E2F consensus DNA binding site (E2wt) with various *in vitro* translated E2F/DP1 proteins in the presence or absence of GST-Trx or GST-Apts (final concentration, 125 nM). (**b**) Apt4 and Apt5 inhibit the proliferation of CCL39 fibroblasts. A colony formation assay was performed in CCL39 cells transfected with CMV-driven aptamers or p16^{INK4A}, in the presence or absence (CTL-) of a selectable marker. (**c**) Microinjection of GST-Apt5 prevents hs68 fibroblasts from entering S phase. BrdU incorporation (left panels) was monitored in synchronized cells microinjected with either GSTTrx (control) or GSTApt5. Fluorescent cells in the right panels represent microinjected cells (Materials and methods)

Apt4 and Apt5 inhibit the proliferation of mammalian cells

Since E2F is required for progression through the cell cycle, we asked whether Apt4 and Apt5 could alter proliferation of mammalian cells in culture. For this purpose we designed two assays. First, we performed colony formation assays in the Chinese hamster fibroblast cell line CCL39 transfected with pCDNA3-Apt4 and -Apt5 expression vectors. A representative result of these assays is shown in Figure 2b. Compared with cells transfected with plasmids that directed the synthesis of Trx, the number and size of CCL39 colonies were strongly decreased in dishes where cells had been transfected with Apt4 and Apt5. Scanning analysis showed that compared to cells transfected with Trx (100%), growth of cells transfected with Apt4 and Apt5 was 43% and 36% respectively. As a positive control to validate this test, we also transfected CCL39 cells with the cyclin-dependent kinase inhibitor p16^{INK4A}, which blocks mammalian cells in G1, acting upstream of E2F in the cyclin/cdks-pRB-E2F pathway (see introduction). As shown in the lower panel of Figure 2b, pCDNA3p16 also reduced colony formation

in CCL39 (growth of these cells was 28% compared to Trx used as control), with a level of reduction similar to that obtained with the E2F-interacting aptamers.

We focussed on Apt5 to perform a second type of growth assay with this aptamer, assessing whether it could block the G0-S cell cycle progression of hs68 primary human fibroblasts in culture. Purified GST-Apt5 protein was microinjected in serum-starved G0/G1-synchronized hs68 cells restimulated to grow with serum. Figure 2c shows that cells injected with the E2F-interacting aptamer were viable but, as monitored by BrdU incorporation, failed to initiate DNA synthesis, showing that more than 80% of the injected cells were blocked in G0 or G1. Collectively, these results suggest that Apt4 and Apt5 are growth inhibitors that impede entry into S by inhibiting E2F.

Pept5, a 20 mer-synthetic peptide corresponding to the variable region of Apt5, also inhibits E2F function in vitro *and inside cells*

In the assays performed thus far, the variable regions of the peptide aptamers were constrained conformationally by their fusion to the thioredoxin moiety. To determine whether the unconstrained 20-mer variable regions alone would display the same effects than the full length aptamers, we synthesized a 20-mer peptide (pept5) that carried the Apt5 variable region and tested its capacity to interfere with E2F as described above. Figure 3a demonstrates that it does, Pept5, like Apt5, blocked the binding of the recombinant E2F1/DP1, E2F4/DP1 and E2F5/DP1 heterodimers to E2F sites in EMSAs. By contrast with Apt5, the unconstrained pept5 seems to be more efficient on E2F1 than on the related E2F4 and E2F5. A ten-fold greater concentration of Pept5 (5 μM) was needed to completely block the E2F4/DP1 and E2F5/DP1 interactions than to block the E2F1/DP1 interaction (500 nM). Thus, while it is not completely specific, pept5 may provide a basis for designing other peptides or peptido-mimetic molecules that target individual members of the E2F protein family.

We then assessed the effects of Pept5 on cellular E2F activity by transfecting CCL39 cells with pept5 or a control peptide (PeptCtl) using lipofectamine (Hayashida *et al.*, 1996). Whole cell extracts were prepared from these peptide-transfected CCL39 cells and were tested for the presence of E2F binding activity (Figure 3b). EMSAs performed on control cells in the presence of wild type (E2wt) and mutant (E2mut) oligonucleotides as well as antibody (data not shown) demonstrated that the two major E2F complexes detected in control cells corresponded to 'free' E2F (E2F/DP) heterodimers and to pocket protein-associated E2F, referred to as 'a' and 'b' complexes, respectively (Figure 3b). By contrast, the 'a' and 'b' complexes were barely detected in Pept5-transfected cells (Figure 3b). These facts suggest both 'free' and pRB-associated E2F complexes were lacking in these cells (Figure 3b). We believe that this inhibitory effect is specific for E2F activity since the same extracts did not show a significant variation in the specific DNA binding activity of an unrelated transcription factor, ATF2 (data not shown).

Two other lines of experiment support these results. First, E2F-dependent transactivation was diminished in

A

E2F1/DP   E2F4/DP   E2F5/DP

B

C
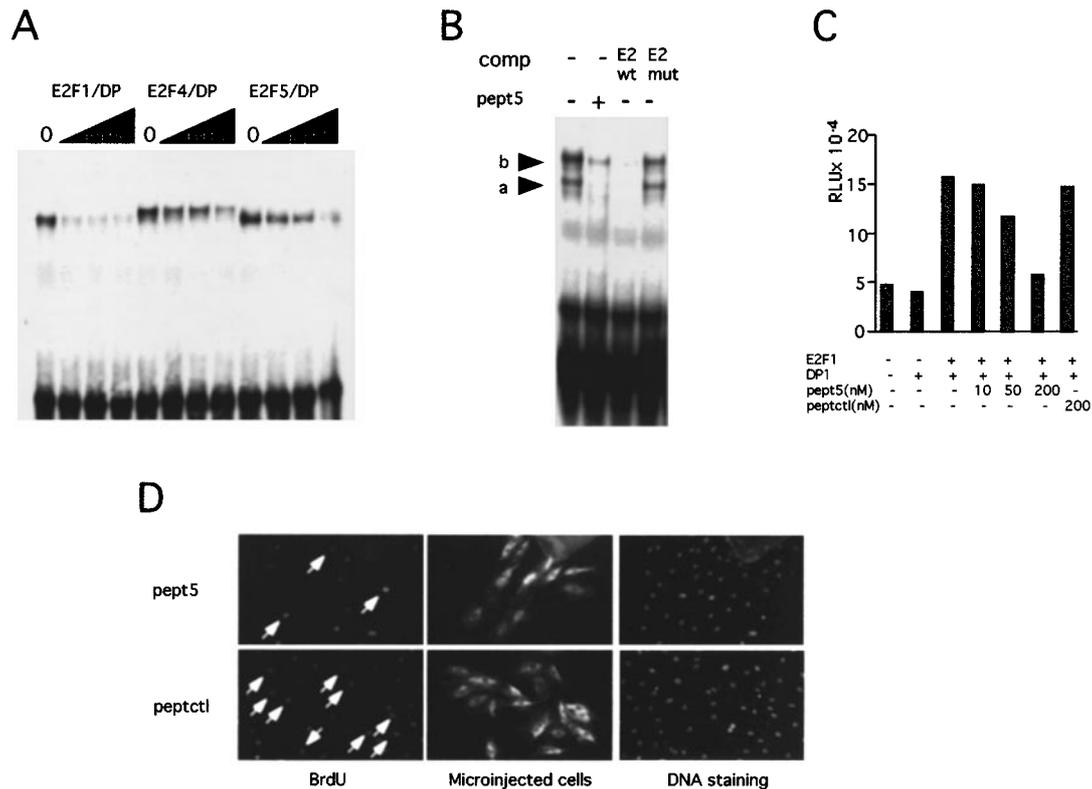
Figure 3 Pept 5, the variable region of Apt5, also inhibits E2F activity. (**a**) Pept5 inhibits DNA binding recombinant E2Fs. EMSAs were performed as described in Figure 2a except that synthetic pept5 was added at increasing concentration into reaction buffer (final concentration: 0, 0.5, 1 and 5 μM). (**b**) CCL39 cells transfected with pept5 demonstrate reduced E2F activity. EMSAs were performed as described in Figure 2a with 10 μg of cell extract prepared from control- or Pept5-transfected cells. The peptide concentration in cell culture medium was 200 nM. The 'a' (free E2F/DP heterodimer) and 'b' (pocket protein-associated E2F complexes) were observed in the presence of a 100-fold molar excess of E2mut cold probe but not in the presence of the same amount of E2wt competitor. (**c**) Pept5 inhibits E2F-dependent reporter expression. Relative luciferase activity (RLU) was measured in CCL39 cells transfected with 3XE2F-LUC and various combinations of pCMV-E2F1, pCMV-DP1, pept5 or control peptide (peptctl), as indicated. Each result is representative of two independent experiments performed in duplicate. (**d**) Microinjected pept5 keeps hs68 fibroblasts from entering S. Experiments were performed as described in Figure 2c except that cells were injected with synthetic peptides (pept5 or peptctl). Cells in the right panels are shown after DNA staining in the right panels

Pept5-transfected cells. As shown in Figure 3c, Pept5 inhibited the activity of a co-transfected E2F-responsive reporter gene (3XE2F-LUC) in a concentration dependent manner, while an unrelated control peptide had no effect. Second, the peptide itself can delay or block entry into S. As shown on Figure 3d, microinjection of this peptide in hs68 human fibroblasts resulted in a similar phenotype as that observed with GST-Apt5: among the cells microinjected with Pept5 25% were able to reenter S phase as judged by incorporation of BrdU, while this percentage doubled when using PeptCtl.

Apt5 and pept5 mimic a portion of DP1 that binds E2F

In order to investigate the mechanism underlying E2F inhibition by Apt5/pept5, we tested whether the WIGL sequence in these peptides and in helix 3 of the DP heterodimerization domain (35 and Figure 4a) was needed for binding E2F proteins. To address this point, we mutated the WIGL motif in human DP1 and tested its ability to heterodimerize with E2F1, as judged by the ability of the heterodimer to bind E2F sites in an EMSA. Indeed, E2F1 and DP1 alone bind DNA very poorly, whereas E2F1/DP1 heterodimers bind DNA with high efficiency. Here, we used equivalent levels of

in vitro translated wild type (DP1) and mutant DP1 (DP1m) (tested by Western blot analyses, data not shown) in EMSAs, together with E2F1 (Figure 4b). Extracts that contained DP1m demonstrated a significantly reduced DNA binding capacity as compared to those containing DP1, suggesting that DP1m has a reduced capacity to heterodimerize with E2F1. Consistent with this *in vitro* experiment, in comparison with DP1, DP1m had a decreased ability to synergize with E2F1 in transactivating a 3XE2FLUC-reporter construct in CCL39 cells (Figure 4c). Thus, these data suggest that Apt5 and pept5 compete with DP1 by dimerizing with E2Fs through their WIGL motif (Figure 4d).

Discussion

Experimental alteration of the E2F/pRB pathway has a profound effect on cell cycle progression (Sardet *et al.*, 1997; Slansky and Farnham, 1996; Sherr, 1996), and it has been proposed that deregulation of this pathway may be an obligatory step in the genesis of a fully transformed phenotype. For this reason, the ability to experimentally target E2F activity *in vivo* is of great interest. Toward this goal, several studies have been
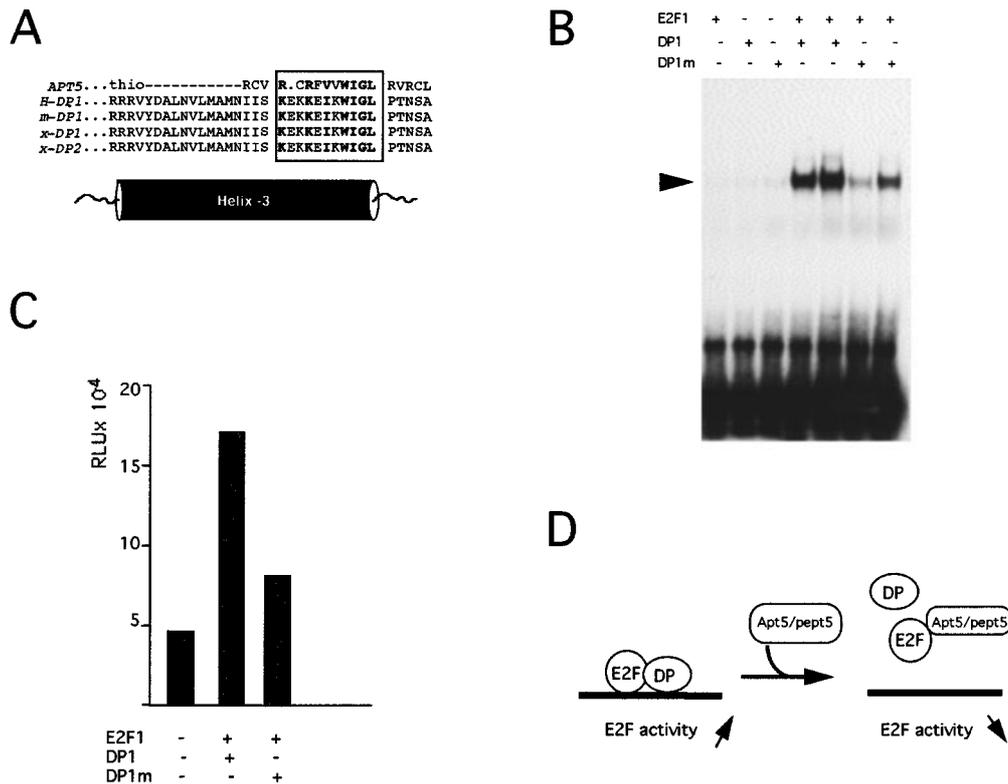
Figure 4 Mechanism of action of pept5. (**a**) Apt5, pept5 sequences contain a motif present in the evolutionary conserved helix 3 region of DP proteins, a domain involved in E2F/DP heterodimerization (h: human, m: mouse and x: xenopus). (**b**) mutagenesis within the WIGL sequence DP1 shared with pept5 reduces heterodimerization-dependent E2F1 binding activity. E2F-specific EMSAs were performed with various combinations of *in vitro* translated E2F1, DP1 and DP1m (WIGL mutant), as indicated. Two concentrations 1× and 3× of DP1 and DP1m were used. (**c**) DP1m has a diminished capacity to stimulate E2F-dependent transactivation. Relative luciferase activity was measured on CCL39 cells transfected with a reporter construct 3XE2F-LUC and various combinations of E2F1, DP1 and DP1m, as indicated. (**d**) Cartoon showing that Apt5/pept5 inhibits E2F activity by blocking E2F/DP association through a motif that resembles a DP motif involved in E2F/DP heterodimerization

performed, with oligonucleotide decoys that carry consensus E2F binding sites) (Morishita *et al.*, 1995), and with peptides bearing various domains of the E2F heterodimeric partner DP that inhibit E2F/DP hetero-dimers formation (Bandara *et al.*, 1997). Here, we describe a novel approach to inhibit E2Fs in cells. In these experiments, we isolated random-sequence pep-tides from a yeast genetic screen that are potent inhibitors of E2F binding activity *in vitro* and *in vivo*. In at least one case, that of Apt5/pept5, inhibition resulted from the direct interaction between this peptide and E2F, which likely prevented the formation of transcriptionally competent E2F/DP heterodimers.

In these experiments the aptamer's variable region is sufficient for activity. Interestingly, the effective concentration of Apt5 is in the same range than the effective concentration of anti-Cdk2 aptamers pre-viously isolated (Colas *et al.*, 1996) and 10 times lower than that of anti-Ras aptamers (Xu and Brent, 1997). Moreover, in our hands, there is only a 10-fold difference between the effective concentration of the aptamer (Apt5) and the unconstrained peptide (pept5), whereas, in the case of a recently developed aptamer that binds to cdk2 and inhibits its kinase activity, the IC_{50} was in the nanomolar range and the corresponding unconstrained variable region in the micromolar range (Cohen *et al.*, 1998). Our observations are consistent with the idea that the variable region of Apt5/Pept5 is

structured, so that there is free energy lost to entropy when this region binds the E2F target. Although in the cell the peptide maybe not as tightly structured as in the active site of trxA because when cells were blocked in G1 using GST-Apt5 the effect was stronger compared to Pept5. This supposition can be directly tested with NMR. If they can be isolated routinely, aptamers with these affinities will greatly contribute to an understanding of intracellular protein function.

Pept5 contains a discrete motif (WIGL) that occurs in the region of DP required for its heterodimerization with E2F. Strikingly, this four amino acid motif is conserved in all DP molecules from Human to Xenopus. Mutagenesis of this WIGL sequence in DP1 strongly inhibited DP-dependent E2F activity, thus demonstrating that our genetic screen selected an important functional motif. These results are at odds with those recently presented by Bandara *et al.* (1997) who found that synthetic peptides encompassing the WIGL motif in DP1 sequence had no significant effect on E2F activity. To explain this discrepancy, we cannot evoke differences in peptide concentrations since they were in the same range in both studies. More likely, other amino-acids surrounding the WIGL motif in pept5 might be important for binding of E2F and/or might impose an internal constraint on the WIGL motif not present in the native DP1 sequence used by Bandara *et al.* Undoubtedly, comparison of

the NMR structure of Pept5 with DP will be useful to establish this point.

As pept5 and Apt5 were potent inhibitors of E2F DNA binding *in vitro*, we tested their capacity to block cellular E2F *in vivo*. When introduced into fibroblasts, these molecules blocked formation of the E2F/DP and E2F/DP-pocket proteins complexes competent to bind DNA. Consistent with their effects on E2F activity and with previous observations showing that E2F activity is required for the G1/S transition (Sardet *et al.*, 1997; Slansky and Farnham, 1996; Sherr, 1996), transfected or microinjected pept5 and Apt5 inhibited the ability of fibroblasts to enter S phase. A recent work based on the development of such aptamers using two hybrid technology showed that it is possible to target drosophila Dmcdk2 *in vivo*. This aptamer provokes during organogenesis adult eye defects typical of those caused by cell cycle inhibition (Kolonin and Finley, 1998). This result and our present work show that cell proliferation can be inhibited using genetically-selected peptides that target with a high affinity protein-protein interaction motifs within cell cycle regulators (Morishita *et al.*, 1995; Wu *et al.*, 1996; Fahraeus *et al.*, 1996). Such peptides may facilitate design of new antiproliferative drugs that target the cyclin/CDK-pRB-E2F pathway.

Materials and methods

DNA and yeast manipulation

Bait constructs: We inserted a *Bam*HI PCR fragment encompassing the conserved DNA binding and dimerization domains of E2F1 (a.a. 126–302), E2F4 (a.a. 16–184) and E2F5 (a.a. 49–185) into the *Bam*HI site of the yeast expression vector pEG202 (25). Primers used for these constructions are E2F1s cgggatcctatcacgctatgagacctca, E2F1as cgggatccggtctcctcagggcacag, E2F4s and E2F5s cgggatcctaagcaggcacgagaa, E2F4as cgggatcctgcctccttgttcaccag, E2F5as cgggatcccgactctttatttataag. We introduced a library that directed the synthesis of Thioredoxin-constrained aptamers (Colas *et al.*, 1996) into EGY48 that contained the E2F1 bait (Gyuris *et al.*, 1993). DNA preparation from yeast were performed according to standard procedures (Finley and Brent, 1996). 5×10^5 transformants screened directly onto galactose plates for Leu2 reporter activation, after a 4 h incubation at 30°C in a galactose containing medium according to a one step screening procedure (Finley and Brent, 1996). The variable moiety of the aptamer sequences was determined using an ABIPRISM automatic sequencer (Perkin-Elmer). Selected aptamers (Apt) were excised from pJM1 as *Eco*RI/*Not*I and *Eco*RI/*Xba*I fragments and cloned into the *Eco*RI/*Not*I sites of pGEX4T1 (GST-Apt) and into the *Eco*RI/*Xba*I sites of pCDNA3 (pCDNA3-Apt), respectively. pCMV-E2F1 and pCMV-DP1 expression vectors (Hayashida *et al.*, 1996) as well as T7-driven E2F1, E2F4, E2F5 and DP1 constructs (Sardet *et al.*, 1995) used for EMSAs have been described elsewhere. In pBSKHADP1, the WIGL amino acid sequence found in the third helix of DP proteins was mutated into GIRM (Helin *et al.*, 1993) using a mutagenesis kit (Promega) according to the manufacturer's instructions.

Recombinant protein production, cell extracts and peptide synthesis

For EMSAs, we transcribed and translated T7-driven full length cDNAs encoding various E2Fs and DP1 proteins (Sardet *et al.*, 1995) *in vitro* in reticulocyte lysates (Quick T7 TNT kit, Promega). We obtained whole cell extracts from CCL39 fibroblasts according to the following procedure. Cells were detached with PBS/EDTA 5 mM, packed in eppendorf tubes at 5000 *g* for 30 s, resuspended in two packed cell volumes of extraction buffer (20 mM HEPES pH 7.9, 0.58 M, NaCl 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 25% glycerol), frozen ($-70°C$)/thawed (in ice) three times and finally centrifuged at 40 000 r.p.m. for 20 min (2°C). We adjusted the protein concentration of supernatants to 5 mg/ml with extraction buffer and froze aliquots at $-70°C$. We expressed recombinant Gst-Apts and affinity purified them according to standard protocols. Purified Gst-Apts were then extensively dialysed against elution buffer without Glutathione (50 mM Tris-HCl pH 7.6, 0.25 M KCl, 1 mM DTT) and kept frozen at $-70°C$. Pept5, the free variable region of Apt5 and the unrelated control peptide (PeptCtl: RSQPAPPADPDGSPG-GAA) were synthesized and purified by Synt:em (Nimes, France) and their sequences were confirmed by mass spectrometry analysis.

Cell culture, transfection and reporter assays

CCL39 Chinese hamster lung fibroblasts and hs68 primary human fibroblasts were maintained in DMEM (GIBCO) + 10% FCS (Biomedia). To prepare the whole cell extracts used in EMSAs, we transfected 10^5 CCL39 cells/well in 6 well plates using lipofectamine (Gibco) in the presence of 3 μg of carrier DNA (pBluescript, Stratagene) and pept5 or control peptide at 200 nM final concentration. To measure E2F-mediated transactivation, we transfected the cells with 500 ng of a plasmid bearing the 3X-E2FLUC synthetic promoter (Krek *et al.*, 1993) in the presence of lipofectamine (Hayashida *et al.*, 1996), as described above, together with 200 ng of pCH110 β-galactosidase vector, and 20 ng of pCMV-E2F1 (Helin *et al.*, 1993) and/or pCMV-DP1 (Helin *et al.*, 1993) plasmids, and in the presence of various concentrations of pept5 (10, 50 and 200 nM). We monitored luciferase activity according to a standard protocol 24 h after transfection and normalized this figure to β-galactosidase activity. For colony formation assays, we transfected CCL39 cells using a standard calcium phosphate procedure (Sardet *et al.*, 1989; Counillon *et al.*, 1993).

Colony formation assay

Colony formation assay was performed essentially as described elsewhere (Pages *et al.*, 1993). Briefly, 150 ng of either pCDNA3-Apt4, pCDNA3-Apt5, or the control vectors pCDNA3-Trx and pCDNA3-p16 were transfected into CCL39 cells together with 500 ng of pECE-NHE3 vector expressing the selectable amiloride-resistant Na+/H+ exchanger NHE3. Transfectants were selected for the presence of NHE3 at 48 h and 72 h using the proton-suicide test in the presence of 100 μM of the amiloride-like NHE inhibitor (HOE694) as described elsewhere (Sardet *et al.*, 1989; Counillon *et al.*, 1993). Selected cells were then left in culture for four more days before plates were stained with crystal violet dye.

Electro-Mobility Shift Assay (EMSA)

We used the following double-stranded probes for EMSAs: E2wt (consensus E2F binding site derived from the Adenovirus E2 promoter) 5′-GCATAAGTTTCGCGCCC-TTTCTCAG-3′, and E2mut (E2 with the corresponding mutations) 5′-GCATAAGTTTCG<u>AT</u>CCCTTTCTCAG-3′. EMSAs were performed as follows: 10 μg of cell extracts were pre-incubated for 15 min at 22°C in 15 μL of EMSA buffer (2.5 mM MgCl$_2$, 20 mM HEPES pH 7.8, 0.1 mM DTT, 10% glycerol and 0.5 μg of calf thymus genomic DNA

(Pharmacia). 4×10^4 c.p.m. of ^{32}P-kinased E2wt probe was then added for 30 min at 22°C before samples were electrophoresed on 4% (Acrylamide:bis-Acrylamide, 29:1) non-denaturating gels in TBE 0.25× at 22°C and 10 V/cm. When *in vitro* translated E2Fs and DP1 were used, 2 μL of programmed TNT lysates were mixed for 15 min with GST-Apt (125 nM) or different concentrations of pept5 (5, 1 and 0.5 μM).

Microinjection and immunofluorescence

Microinjection was performed as described previously (Lamb *et al.*, 1990). Briefly, we rendered hs68 cells subcultured onto glass coverslips quiescent by 36 h of serum deprivation in 0.5% heat-inactivated serum. We microinjected cells with 200 μg/mL of pept5 and control peptide in the presence of BrdU and inert affinity-purified mouse anti-rabbit IgG (0.5 mg/mL), which we used as marker of microinjection 3 h after restimulation with 10% FCS. We monitored immunofluorescence 18 h after serum restimulation in a humidified chamber as previously described (Lamb *et al.*, 1990). Briefly, after fixation in 3.7% paraformaldehyde in PBS for 5 min at 25°C, cells were extracted for 30 s in acetone (−20°C) rinsed and rehydrated with PBS/BSA 1%. We treated the cells with 4N HCl, treatment was then performed for 10 min at 25°C and after two washes in PBS, cells were saturated in PBS-BSA 3% and incubated in the presence of immunodetection reagents. We detected cells with a Texas Red-conjugated goat anti rabbit IgG, and cells reentering S with a fluorescein-conjugated rabbit anti-BrdU antibody.

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