A small peptide inhibitor of DNA replication defines the site of interaction between the cyclin-dependent kinase inhibitor p21^{WAF1} and proliferating cell nuclear antigen

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Background: p21^{WAF1} is a potent inhibitor of the cell-cycle regulatory cyclin-dependent kinases (Cdks). It acts on Cdks in the G1 and S phases of the cell cycle, and also binds to proliferating cell nuclear antigen (PCNA), blocking DNA replication *in vitro*. Transcription of p21^{WAF1} can be induced by the human tumour suppressor protein p53, suggesting that the action of p21^{WAF1} may be important in cancer prevention. We have investigated the interaction between p21^{WAF1} and PCNA using a genetic two-hybrid screen and with arrays of synthetic peptides derived from the p21^{WAF1} protein sequence.

Results: We have established that the carboxy-terminal region of p21^{WAF1} interacts with PCNA in a yeast two-hybrid screen. Interaction with p21^{WAF1} involves the central loop of PCNA, which connects the two domains of the PCNA monomer. The interaction was finely mapped using peptides derived from the entire sequence of the p21^{WAF1} protein, and the critical residues were found to be QTSMTDFY (amino acids 144–151 of p21^{WAF1}). Remarkably, a 20-residue peptide containing this sequence inhibited replication of simian virus 40 (SV40) DNA *in vitro* and could capture PCNA from whole cell extracts, demonstrating that small molecules can retain the biological activity characteristic of the whole protein. Sequential alanine-scan mutations of the peptide demonstrated that its ability to block replication correlates with its affinity for binding PCNA.

Conclusions: We have shown that PCNA and the cell-cycle regulator p21^{WAF1} interact *in vivo*, and that this interaction requires the central loop of PCNA and an eight amino-acid motif from the carboxyl terminus of p21^{WAF1}. Peptides of p21^{WAF1} that interact with PCNA can inhibit DNA replication; such peptides or mimetics may thus prove useful in the treatment of hyperproliferative diseases, including cancer.

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Background

Cyclin-dependent kinases (Cdks) are required for certain key cell-cycle transitions in eukaryotic cells. A family of small protein inhibitors of Cdks — called CdkIs — , have been identified [1]. One of these inhibitors, p21^{WAF1} [2] (also known as p21^{Cip1} [3], p21^{pic1} [4], p20CAP [5] and Sdi [6]), seems to be a universal inhibitor of Cdks; p21^{WAF1} can be transcriptionally induced by the tumour suppressor protein p53, although its expression is not always dependent upon p53 [2,7,8]. Thus, p21^{WAF1} may act to regulate the cell cycle in response to activation of the p53 checkpoint pathway that responds to DNA damage. [2,7]. The p21^{WAF1} protein is extremely stable to heat, retaining inhibitory activity even after treatment at 100 °C for 10 minutes [3].

Kinase complexes containing p21^{WAF1} can exist in both catalytically active and inactive forms, suggesting a subtle regulation of kinase activity by p21^{WAF1} [9]. In normal human cells, p21^{WAF1} exists in a quaternary complex with a cyclin, a Cdk, and the proliferating cell nuclear antigen (PCNA), although the stoichiometry of these complexes is not clear [2,3,5,10]. In transformed cell lines, p21^{WAF1} expression is depressed and Cdks are found in Cdk–cyclin binary complexes rather than in Cdk–Cyclin–p21^{WAF1}–PCNA quaternary complexes [2,3,5,10].

PCNA is a processivity factor for DNA polymerase δ, which plays an essential role in DNA replication and repair [11,12]. PCNA may also be involved in recruiting replication factors to sites at which DNA replication is initiated, as PCNA localizes to pre-replicative sites considerably before the onset of DNA replication [13]. The interaction of p21^{WAF1} and PCNA blocks the ability of PCNA to support simian virus 40 (SV40) DNA replication *in vitro* without apparently interfering with PCNA's repair activity [14–17]. In cytoplasmic extracts of *Xenopus* eggs that undergo repeated cell cycles *in vitro*, p21^{WAF1} inhibits replication by acting on cyclin E–Cdk2 to arrest the cell cycle, rather than by binding directly to PCNA [18]. This contrast between experimental systems may highlight yet another mechanism of regulation by p21^{WAF1} that depends on post-translational modification (for example, phosphorylation) of p21^{WAF1} and its interacting partners. Various points of the cell cycle are therefore amenable to regulation by p21^{WAF1}, allowing the coordination of cell-cycle progression with DNA replication and repair.

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In this paper, we have used a yeast two-hybrid genetic screening technique to establish that there is an interaction *in vivo* between the carboxy-terminal half of $p21^{WAF1}$ and the central loop of PCNA that connects the two domains of the PCNA monomer. We have identified a small peptide from the carboxy-terminal region of $p21^{WAF1}$ that binds to PCNA with high affinity and selectivity, and we show that its interaction with PCNA accounts for the inhibition of SV40 DNA replication *in vitro* [14,15]. Variants of this peptide have allowed us to define the residues of $p21^{WAF1}$ that are critical for PCNA binding, and to show that inhibition of SV40 DNA replication by $p21^{WAF1}$ is a function of PCNA binding. A precise understanding of the interaction of $p21^{WAF1}$ with Cdks and PCNA should illuminate its role in coordinating cell proliferation and cell-cycle control. The high affinity of interaction between PCNA and the $p21^{WAF1}$ peptide, and the potent biological activity of the latter, suggest possible uses for the peptide and mimetics in tumour therapy.

Results and Discussion

The carboxy-terminal 89 amino acids of $p21^{WAF1}$ bind PCNA in an interaction trap

We have screened for proteins that physically interact with human PCNA using a two-hybrid screening method that detects polypeptide interactions by means of the reconstitution of a functional transcriptional activator in the yeast *Saccharomyces cerevisiae* [19,20]. A plasmid that expresses a hybrid protein comprising the DNA-binding domain of GAL4 ($GAL4^{AS}$) combined with human PCNA (pAS-PCNA-Hs) was used to screen plasmids expressing hybrid fusion constructs made up of DNA molecules from a human cDNA library and the DNA encoding the transcriptional activation domain of GAL4 ($GAL4^{ACT}$); this is known as the pACT-derived library (Fig. 1 and Materials and methods) [20].

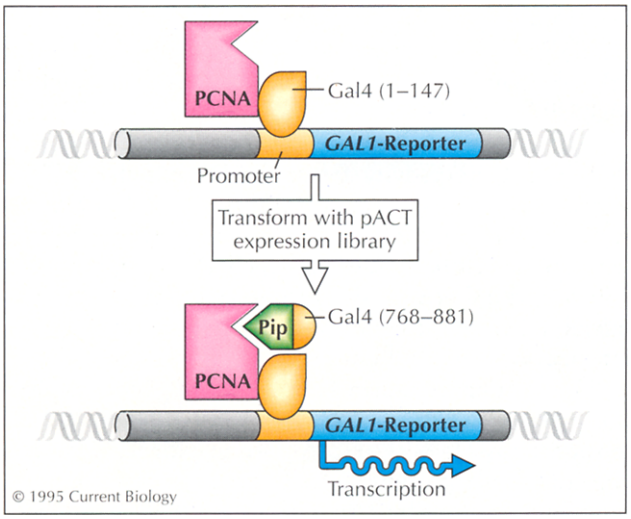

Fig. 1. Strategy for detecting PCNA-interacting proteins (Pip) using the two-hybrid system. The *S. cerevisiae* strain Y190, which expresses the reporter genes *lacZ* (*E. coli*) and *HIS3* (*S. cerevisiae*) under the control of the *GAL1* promoter was used to detect the reconstitution of GAL4 activity using the two-hybrid system . Y190 was co-transformed with pAS-PCNA-Hs (encoding the DNA-binding domain of GAL4 (amino acids 1–147, $GAL4^{AS}$) fused to human PCNA) and also with the pACT-derived human cDNA library which expresses human proteins as fusion constructs with the activation domain of GAL4 (amino acids 768–881) [20]. Tranformants containing library-encoded PCNA-interacting proteins result in the reconstitution of GAL4 activity, and thus in the expression of reporter constructs.

We picked 77 His^+ colonies from over 1 x 10^6 transformants, of which 14 expressed β galactosidase; pACT-derived library plasmids were isolated from 12 of these strains which retested positive upon co-transformation with pAS-PCNA-Hs. Each plasmid encoding a human PCNA-interacting protein (pHSpip) was tested for non-specific interactions with other $GAL4^{AS}$ fusions by co-transforming with various pAS plasmids (Table 1). The pAS-PCNA plasmids contain the entire open reading frame of PCNA from *Homo sapiens* (Hs), *Drosophila melanogaster* (Dm) and *Schizosaccharomyces pombe* (Sp), respectively, in the vector pAS2. The control plasmids pAS-SNF1, pAS-lamin, pAS-p53 and pAS-CDK2 are as described [3]. The plasmids were grouped according to cross-hybridization and DNA sequence analysis. Each pHSpip was also tested for interactions with both *D. melanogaster* and *S. pombe* PCNA.

The 12 positives fell into three classes. One class (9 plasmids) was shown to contain shared sequences by cross-hybridization, and will be described elsewhere. A second class (pHSpip19) showed non-specific interactions with the control pAS plasmids, and so was not investigated further. The third class (pHSpip9 and pHSpip31) showed a specific interaction with pAS-PCNA-Hs. Sequence analysis (data not shown) indicated that both these plasmids express the carboxy-terminal 89 amino acids of

Table 1. Summary of two-hybrid screening results.

	No plasmid	pHSpip9	pHSpip31	pHSpip35
pAS-SNF1	–	–	–	–
pAS-lamin	–	–	–	–
pAS-p53	–	–	–	–
pAS-CDK2	–	–	–	–
pAS-PCNA-Hs	–	+	+	+
pAS-PCNA-Dm	–	–	–	(+)
pAS-PCNA-Sp	–	–	–	+

The symbols + and – indicate the result of β galactosidase colony-lift assays and *His3* expression as judged by growth on SDA containing 3-aminotriazole. These assays were performed on single or co-transformant strains, as indicated by plasmids named on the horizontal and vertical axes. + indicates a strongly positive result from both tests, (+) a weakly positive and – a negative result. pHSpip9 and pHSpip31 encode Gal4-$p21^{WAF1}$ fusions as described in the text; pSPpip35 expresses a fusion protein made up of the $Gal4^{ACT}$ domain and *S. pombe* PCNA.

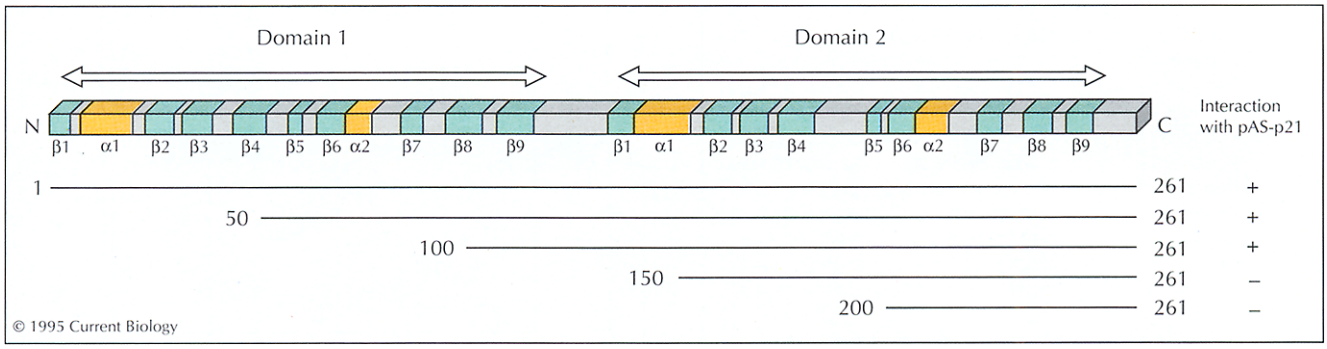

Fig. 2. Identification of the regions of PCNA that interact with p21^{WAF1}. The structural domains within the PCNA molecule are shown. Numbers indicate β-sheet domains and α helices based on the three-dimensional structure of *S. cerevisiae* PCNA [23]. Bars below indicate the regions of PCNA expressed as fusions with GAL4^{ACT}, and the column on the right (+/–) indicates a positive/negative result when tested for interaction with p21^{WAF1} in the two-hybrid system.

p21^{WAF1} [3,5,10] in frame with GAL4^{ACT}, although the vector sequences of one of the plasmids showed a slight rearrangement. A p21^{WAF1} construct encoding the full-length protein in frame with GAL4^{ACT} interacted with both Cdk2 and PCNA in the two-hybrid system, although the carboxy-terminal half of p21^{WAF1} did not interact with Cdk2. This is the first report of an interaction between p21^{WAF1} and PCNA *in vivo*, and shows that the carboxy-terminal half of p21^{WAF1} is sufficient for this interaction.

PCNA–PCNA interactions

We undertook a similar screen to search for *S. pombe* cDNAs encoding proteins that would interact with *S. pombe* PCNA (encoded by *pcn1*$^+$) [21]. Two of the clones identified expressed the entire open reading frame of *pcn1*$^+$ (data not shown), suggesting that PCNA is able to interact with itself. These results are consistent with the model proposed by Kong *et al.* [22], which is supported by evidence from Krishna et al. [23], in which PCNA forms a homotrimer that acts as a sliding clamp, tethering the replication complex to the DNA strand. Furthermore, the *pcn1*$^+$ product was found to interact with both human and *D. melanogaster* PCNA, suggesting that the interaction is evolutionarily conserved (Table 1). Human PCNA has sufficient DNA-binding capacity for a PCNA–GAL4^{ACT} hybrid to activate reporter gene expression in the two-hybrid system, so such a construct could not be used for two-hybrid interaction analysis (data not shown).

p21^{WAF1} interacts with the central region of PCNA

We next tested a series of carboxy-terminal deletion constructs of human PCNA fused to GAL4^{AS}, in order to identify the regions of PCNA that interact with p21^{WAF1} in the two-hybrid assay [20]. The results of these experiments (Fig. 2) suggest that p21^{WAF1} interacts with a central region of PCNA. Structural and sequence similarities between the β subunit DNA polymerase III of *E. coli* and PCNA have suggested a model in which three PCNA subunits, each consisting of two repeated domains, form a toroid structure that encircles the DNA strand [22]. The three-dimensional structure of *S. cerevisiae* PCNA has recently been described [23], and this molecule does indeed form a trimeric ring with close topological symmetry to the β subunit of DNA polymerase III, although the sequence alignment on which the previous model was based has been found to be largely incorrect. The data in Figure 2 are consistent with p21^{WAF1} interacting with the 'junctional loop' that connects the two domains.

Peptide mapping of sites on p21^{WAF1} important for its interaction with PCNA

In order to refine the map of the site(s) on p21^{WAF1} that interact with PCNA, we tested the binding capability of a series of 11 overlapping 20-residue peptides derived from the p21^{WAF1} sequence (Fig. 3a). This approach has been used successfully to fine-map protein–protein interactions (for example, the interaction between p53 and MDM2) and antibody epitopes [24–26]. The p21^{WAF1} peptides were linked to biotin, permitting attachment to streptavidin-coated ELISA plates (see Materials and methods). PCNA from various sources, including HeLa cell extract, *Xenopus* egg extract and lysate of *E. coli* BL21 overexpressing a human PCNA clone [27], were applied to the immobilized peptide array. Bound PCNA was detected using the polyclonal antibody 3009, which recognizes the carboxy-terminal 15 amino acids of native and denatured PCNA (L.S.C., S. Picksley, P.A. Hall, A.T.M. Rennie and D.P.L., unpublished data). PCNA from the three sources tested binds with great specificity to peptide 10 (KRRQTSMTDFYHSKRRLIFS in the single-letter amino-acid code; Fig. 3b), which we designate p21PBP (for p21^{WAF1} PCNA-binding peptide).

The interaction of PCNA with the adjacent peptide 11, which shares all but the initial four residues with peptide 10, is much weaker (13 % of the level of binding for bacterially expressed PCNA, and only 3.8 % for HeLa PCNA), suggesting that some or all of the residues KRRQ are important in determining the affinity of p21^{WAF1} for PCNA. Similar results were obtained when the monoclonal antibody PC10 was used to detect PCNA [28], demonstrating that the interaction between PCNA and p21PBP of p21^{WAF1} is specific to PCNA and

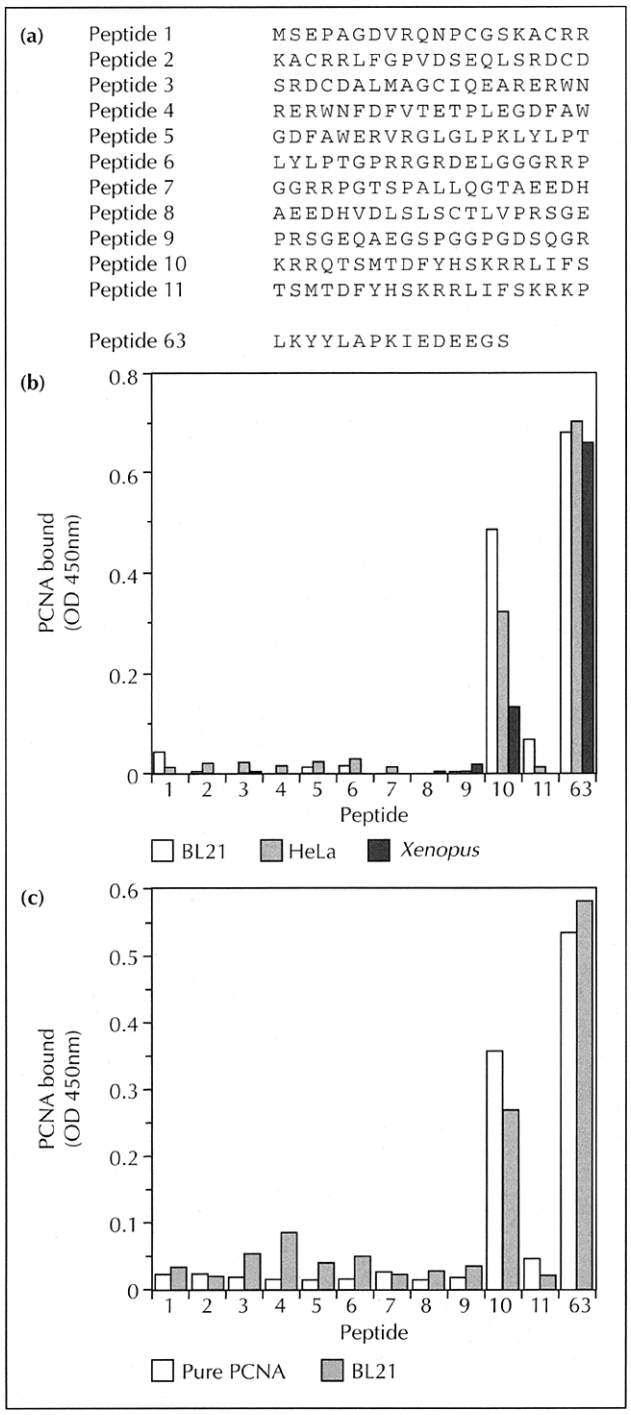

Fig. 3. Peptides of p21^{WAF1} and analysis of PCNA binding by ELISA. **(a)** Peptides 1–11 cover the entire protein sequence of p21^{WAF1} [2], with overlaps between adjacent peptides. Peptide 63 represents the carboxy-terminal 15 amino acids of PCNA. **(b)** Peptides were incubated with cell extracts from HeLa, *Xenopus* or *E.coli* BL21 cells overexpressing human PCNA, and binding was assessed by ELISA (OD 450 nm). **(c)** Purified human PCNA from BL21 cells was compared with crude lysates, as in (b) above. PCNA was detected using polyclonal anti-PCNA antibody 3009.

not due to antibody cross-reaction. This specificity was confirmed using purified PCNA which bound strongly and specifically to p21PBP (Fig. 3c). This result also confirms that the interaction detected in the two-hybrid screen was direct and not mediated by a bridging protein.

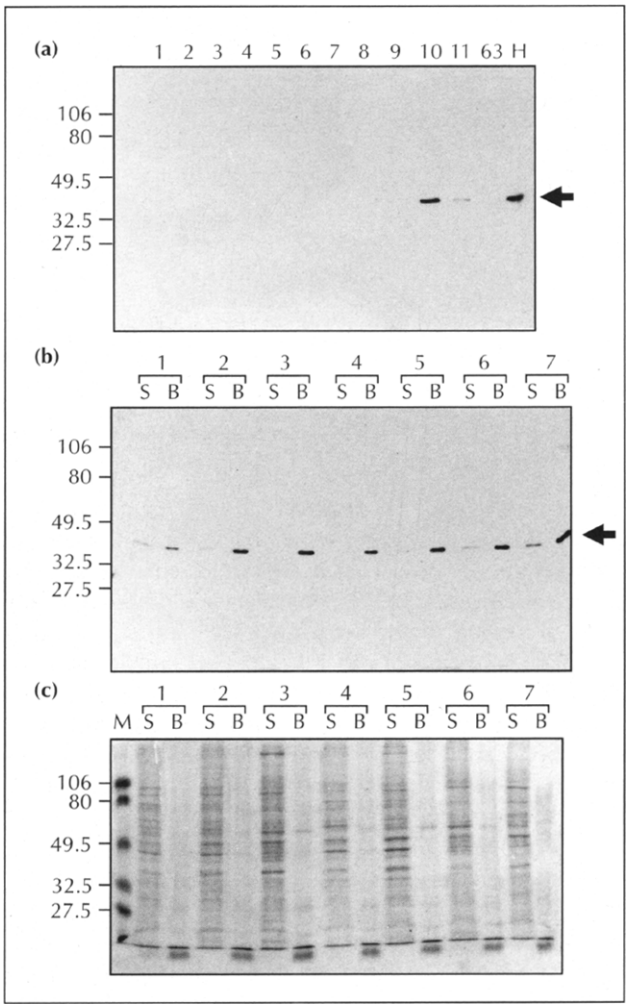

Fig. 4. Mapping the sites of p21^{WAF1} required for PCNA binding using precipitation analysis. **(a)** All 11 peptides of p21WAF1, separately attached to streptavidin–agarose beads, were incubated in HeLa cell extract; beads were then separated from the lysate by centrifugation. Bound proteins were analysed by SDS polyacrylamide gel electrophoresis, and then western immunoblotted with PC10 monoclonal anti-PCNA antibody followed by horseradish peroxidase–anti-mouse secondary antibody and enhanced chemiluminescent (ECL) detection. Peptides are numbered as in Fig. 3a. H indicates undepleted HeLa extract. Molecular weight marker sizes are shown in kD on the left. The position of PCNA is marked by an arrow. **(b)** Western blot probed with monoclonal antibody PC10, and **(c)** Coomassie-stained gel of p21PBP–streptavidin bead precipitation from extracts of various human tumour cell lines. The results from seven out of fourteen lines tested are shown; A431 (sample 1), HOS (sample 2), SKBR3 (sample 3), BT549 (sample 4), MDA231 (sample 5), T47D (sample 6) and DLD1 (sample 7). Similar results to those shown were found for the remaining seven lines (data not shown). S indicates supernatant after incubation with p21PBP beads; B contains proteins precipitated by the p21PBP-streptavidin agarose beads; M shows the position of molecular weight standards and sizes are given in kD.

p21^{WAF1} peptides can precipitate PCNA from cell extracts

To confirm the specificity of the interaction between native PCNA and p21^{WAF1} peptides, the peptides were attached to streptavidin–agarose beads and incubated in HeLa cell extract. The proteins that bound to the beads were separated electrophoretically, blotted onto

nitrocellulose, and probed with the anti-PCNA monoclonal antibody PC10 (Fig. 4a). PCNA was precipitated by beads carrying p21PBP, and also weakly by peptide 11. The p21PBP precipitate contained a similar amount of PCNA to the untreated control extract (HeLa), suggesting a highly efficient capture of soluble PCNA. By contrast, PCNA was not detected in the precipitates of any other p21^{WAF1} peptide.

PCNA could be quantitatively removed from extracts of 14 other human tumour cell lines by p21PBP (7 are shown in Fig. 4b). In some cases, such as SKBR3 and BT549 cells, there was almost complete transfer of PCNA from the cell lysate to the beads. This result suggests that PCNA from these tumour cell lines is not altered in its ability to bind p21^{WAF1}. Parallel gels stained with Coomassie brilliant blue show that the interaction with PCNA is highly specific, as the amount of total protein precipitating with the beads is negligible (Fig. 4c). The interaction of PCNA with the p21PBP peptide could not be disrupted using 100 mM glycine pH 2.5, 100 mM triethylamine pH 12.5 or 1.4 M NaCl under batch elution conditions (data not shown), demonstrating the strength of the binding. Strong binding could even be detected when only 500 pg of peptide was applied to the wells in the ELISA assay. These results suggest that p21PBP beads may be used to deplete cell extracts of PCNA, and should therefore prove to be a useful tool in further elucidating the action of PCNA in DNA replication and repair.

Inhibition of SV40 DNA replication by p21PBP

As p21^{WAF1} has recently been reported to bind PCNA and to inhibit the replication of SV40 DNA [14,15], we examined the 20mer peptides for their effect on the replication of SV40 DNA *in vitro* (Fig. 5a). Of all the peptides, only p21PBP (peptide 10) had a significant effect on DNA replication *in vitro*, reducing incorporation of [^{3}H]dTMP to 51 % of control levels at 20 μg ml^{-1} and to 31 % of control values at 40 μg ml^{-1}. The concentration-dependence of the inhibition of replication by p21PBP is shown in Figure 5b. The activity is remarkably high for a peptide: DNA replication is inhibited at 25 μg ml^{-1} (12.5 μM), which is only a ten-fold higher molar ratio than the full-length p21^{WAF1} protein used by Waga *et al.* [15]. It is remarkable that a small peptide shows any activity in this biological assay, and the finding that this peptide that can inhibit DNA replication, and can bind to PCNA in tumour cell extracts, raises important therapeutic possibilities.

The minimum PCNA binding site on p21^{WAF1}

The sequence of p21PBP was scanned to determine the minimum size of the recognition sequence by creating a set of 20mer peptides with 4 amino-acid overhangs (that is, with a 16 amino-acid overlap between adjacent peptides). These were based on the p21^{WAF1} sequence from amino acids 121–164, and included the initially identified reactive peptide (p21PBP) of sequence KRRQTSMTDFYHSKRRLIFS (Fig. 6a). These peptides were immobilized on streptavidin-coated ELISA dishes, and screened for binding to PCNA over-expressed in *E. coli* BL21. Of the eight peptides in this array, only peptides 68, 69, and 70 bound strongly to PCNA (Fig. 6b). Of these three, peptide 70 (identical to p21PBP) gave the highest signal. These peptides were also tested for their ability to inhibit the replication of SV40 DNA *in vitro*. Of all the peptides, only peptide 70 (p21PBP) showed any appreciable inhibitory activity, although a slight decrease in label incorporation was observed with peptide 71 (Fig. 6b).

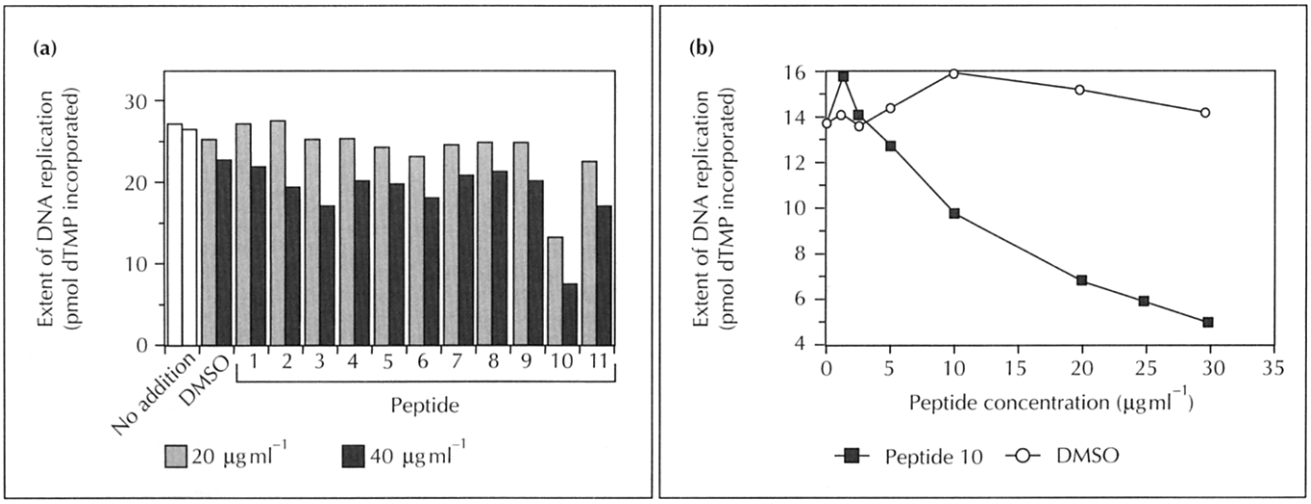

Fig. 5. Inhibition of DNA replication by p21PBP. **(a)** All peptides of p21^{WAF1} were added individually to an SV40 DNA replication reaction *in vitro*, to a final concentration of 20 μg ml^{-1} (grey columns) or 40 μg ml^{-1} (black columns), compared with DMSO at equivalent dilutions, or with no addition (duplicate result, white columns). After a 2 hour incubation at 37 °C, incorporation of [^{3}H]dTMP was measured by TCA precipitation and scintillation counting. **(b)** p21PBP (peptide 10) was added to the replication reaction at a range of concentrations up to 30 μg ml^{-1} and compared with equivalent dilutions of the solvent DMSO. The extent of label incorporation was analysed after 2 hours as above. The same concentrations of peptide 9 (data not shown) or the solvent alone had no appreciable effect on levels of DNA synthesis.

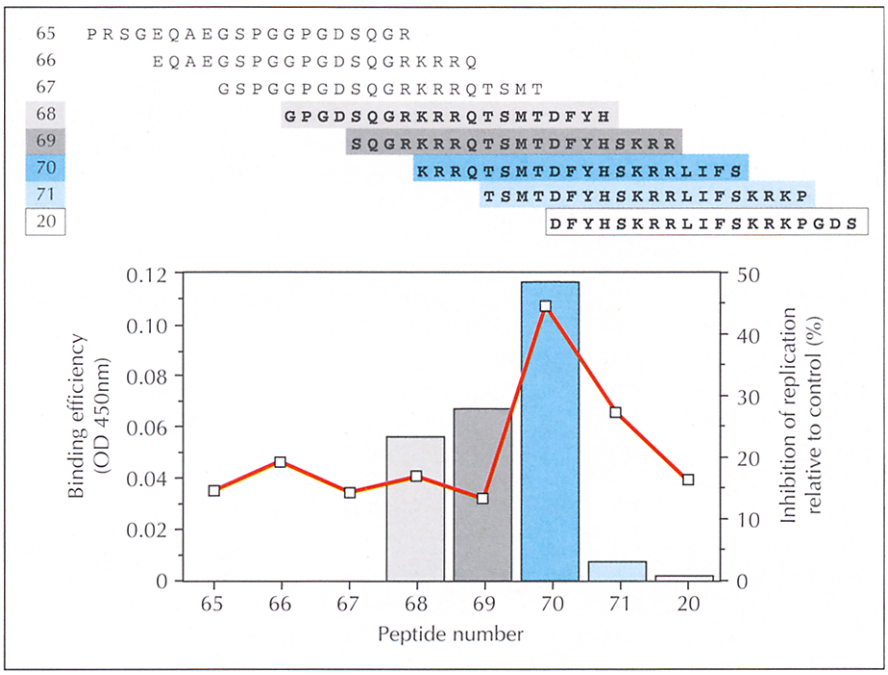

Fig. 6. Determination of the minimal binding site of PCNA on p21^{WAF1}. **(a)** A series of 20mer peptides covering p21PBP were synthesized with 4 amino-acid overhangs (16 amino-acid overlaps) **(b)** These peptides were used to coat ELISA dishes and the binding of bacterially expressed PCNA was detected by antibody 3009. Optical density at 450 nm after colour development is plotted as bars for each of the peptides. In a separate experiment, these peptides were added at 40 μg ml^{-1} to SV40 DNA replication reactions *in vitro*, and the percentage inhibition of label incorporation, calculated against the 'no addition' positive control, is plotted in red.

Interestingly, neither peptide 68 nor peptide 69 showed any capacity to block SV40 DNA replication, despite the ability of each to bind PCNA. Using a modified form of p21PBP with an additional four amino acids at the amino terminus and a concomitant loss of four carboxy-terminal amino acids, the relationship between PCNA binding and inhibition of replication was not so straightforward. These results confirm our earlier observations that the motif KRRQTSMTDFYH is required for binding to PCNA, but that the more carboxy-terminal residues SKRRLIFS also contribute to the biological activity of p21^{WAF1}.

Critical residues within p21PBP

In order to define the critical residues within the PCNA binding site of p21^{WAF1}, a series of peptides was generated in which each residue of p21PBP was altered sequentially to alanine; a double mutant with two arginines substituted by alanine was also made. In addition, a peptide was generated containing the same amino acids as p21PBP but in a different order ('jumbled p21PBP'). These peptides were tested in the ELISA for PCNA-binding capacity. It was found that residues M and F were absolutely required for recognition, whereas mutation of Q, D or Y considerably decreased the ability to bind PCNA (Fig. 7). Jumbled p21PBP did not bind PCNA, indicating that the order of amino acids as well as the overall composition is important for p21PBP activity. In general, of these altered peptides, those with the strongest PCNA-binding activity were most able to inhibit SV40 DNA replication *in vitro*, and conversely, loss of PCNA binding correlated with lack of inhibition of SV40 DNA replication (Fig. 7). These results allow us to define the region of p21^{WAF1} involved in PCNA binding as QTSMTDFY. The residues shown in bold are critical for PCNA binding, and those underlined are important. Phosphorylation of the serine and threonine residues within this motif could regulate PCNA binding to p21^{WAF1}, and it is also interesting to note that this motif lies within a consensus bipartite nuclear localization signal [29]. We are currently investigating the possibility that modification of these sites may alter the stoichiometry of 21^{WAF1}–cyclin–Cdk–PCNA complexes.

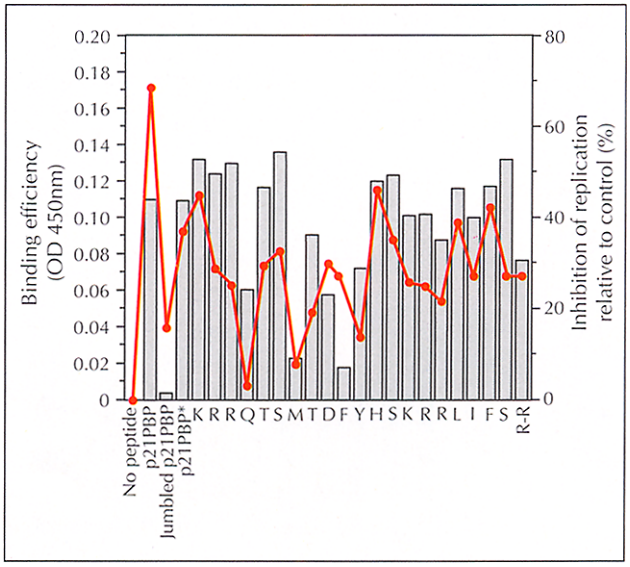

Fig. 7. Alanine scanning to determine the critical residues of p21PBP. Peptide variants of p21PBP were generated with sequential alanine changes, or with two arginines (residues 2 and 15 in the peptide) altered to alanine. The efficiency of binding of these peptides to bacterially expressed PCNA was tested by chromogenic ELISA, and the optical density at 450 nm is shown by the shaded bars. These peptides were also tested for their ability to inhibit SV40 DNA replication *in vitro*, calculated against the 'no peptide' control, and shown by the red line; 'jumbled p21PBP' contains the same amino acids as p21PBP, but in the order QDKTRYFHRTMSRSKSIRLF, and p21PBP* is the same sequence as p21PBP but from a different peptide synthesis batch.

Conclusions

We have demonstrated a strong interaction between the central loop of PCNA and the carboxy-terminal region of p21^{WAF1} using the yeast two-hybrid screening method. A 20 amino-acid peptide, p21PBP, that is derived from p21^{WAF1}, binds to PCNA strongly and specifically and is capable of inhibiting DNA replication *in vitro*. Knowledge of the critical sites for p21^{WAF1}–PCNA interaction and function should assist searches for mutations in the genes encoding these vital cell-cycle regulators in human tumours. Our results lead us to suggest that p21PBP itself, or its derivatives or mimetics, might be used therapeutically. Drugs based on p21PBP may be capable of functionally down-regulating PCNA replication activity in tumour cells. As such therapy would reinforce the endogenous cell-cycle regulatory pathway without impairing DNA repair, we anticipate that hyperproliferation could be checked whilst genomic integrity is maintained.

Materials and methods

Cloning and expression plasmid construction

Manipulation of *E. coli* and DNA was by standard methods [30]. All plasmid constructs containing fragments produced by the polymerase chain reaction (PCR) were initially subcloned into pBC-SK (Stratagene) and sequenced using oligonucleotide primers on one strand to check for PCR errors before a second step of subcloning into the required expression vector. DNA sequencing was performed using double-stranded plasmid template DNA with a modification of the Sequenase protocol (USB). Sequence analysis was carried out using the UWGCG package at the Daresbury Seqnet facility. The human PCNA open reading frame was adapted as *Nde*I–*Bam*HI fragments using PCR, to give either the full-length open reading frame, or the deletion constructs described in Figure 2. One full-length isolate was used to subclone the insert into pAS2 [3] to give pAS-PCNA-Hs, and also into the vector pREP1 which is used for expression in *S. pombe* under the control of the *nmt1* promoter [31]. This plasmid, pREP1-PCNA-Hs, was capable of complementing the lethality resulting from *pcn1* deletion, suggesting that the protein expressed from this construct is functional. The plasmid pAS-PCNA-Sp was derived from the plasmid pREP1*pcn1* [21]. The insert in this plasmid was found to contain a frameshift mutation compared to the genomic sequence (data not shown) and was modified before cloning into pAS2 to ensure that the entire protein was expressed. The plasmid pAS-PCNA-Dm was constructed in a similar fashion to that described above. The full-length open reading frame of p21^{WAF1} was cloned as a *Bam*HI fragment using PCR. This was subcloned into the *Bam*HI site in pACT to give plasmids pACT-p21 The *S. pombe* expression library was constructed in pACT and was a gift from Steve Elledge.

Yeast two-hybrid methods

Growth and maintenance of *S. cerevisiae* was according to Rose *et al.* [32]. Transformation was carried out by the method of Gietz *et al.* [33]. All growth was carried out at 30 °C. The *S. cerevisiae* strain Y190 (*MATa leu2-3,112, ura3-52, trp1-901, his3-D200, ade2-101, gal4D, gal80 D, cyh^R URA3::GAL1-lacZ, LYS2::GAL1-HIS3*) was used for all two-hybrid analysis. This strain expresses the reporter genes *lacZ* (*E. coli*) and *HIS3* (*S. cerevisiae*) under the control of the *GAL1* promoter. Expression of *lacZ* was assayed by a filter-lift assay for β galactosidase [34]. The *HIS3* reporter construct described here gives residual *HIS3* expression, although not sufficient to render the cells resistant to 3-aminotriazole, a chemical inhibitor of HIS3 (IPG dehydratase) at 50 mM. In order to test for *HIS3* expression from the *GAL1* promoter, cells were streaked out on SDA [32] plates containing 50mM 3-aminotriazole, incubated for 1 week, and plates examined for the formation of single colonies. Where growth occurred, a filter-lift was taken from these plates to assay β galactosidase activity. Only transformants which expressed both reporter genes were counted as true positives. Plasmid DNA was recovered from *S. cerevisiae* using a modified version of the method described by Hoffman and Winston [35] and further purified using GeneClean (Stratagene). The *E. coli* strain JA226 (*recBC leuB6 trpES hsdR$^-$ hsdM+ lacY600*) was used; ampicillin-resistant colonies were tested for leucine prototrophy to determine whether they contained a pACT or pAS2 derived plasmid, as the *LEU2* sequences in pACT complement the *leuB6* mutation in JA226.

Two-hybrid screening

Y190 transformed with pAS-PCNA-Hs was tested and found not to express either of the reporter constructs in this strain. This transformant strain was then itself transformed with a human cDNA library in the vector pACT, which has cDNAs expressed as fusion constructs with the activation domain of GAL4 (amino acids 768–881) [20]. Transformants containing library-encoded PCNA-interacting proteins result in the reconstitution of GAL4 activity, and thus in the expression of reporter constructs. These were selected for by plating on SDA containing 3-aminotriazole, while an aliquot was plated on SDA without 3-aminotriazole to determine transformation frequency. Putative positives were tested for *lacZ* expression, streaked out on SDA containing 3-aminotriazole, and a single colony isolated for further analysis; cDNA library plasmids were rescued into *E. coli* strain JA226.

Peptides and ELISA

Peptides were synthesized (Chiron Mimotopes, Australia) to cover the wild-type p21^{WAF1} protein sequence, with 5 or 16 amino-acid overlaps, linked at their amino termini to biotin by a 4-amino-acid linker of sequence SGSG. An alternative set of peptides was also generated, in which each residue from amino acids 144–164 was substituted in turn by alanine. Peptides were dissolved in DMSO at 5 or 10 mg ml^{-1} and stored at −80 °C. Plastic plates for ELISA (enzyme-linked immunoabsorption assay; Falcon) were coated overnight at 37 °C with 5 μg ml^{-1} streptavidin (Vector), washed with PBS plus 0.2 % Tween 20 (PBST) then blocked in 5 % non-fat milk powder in PBS (milk–PBS) for 2 h at room temperature (r.t.). Each well of the plates was incubated with the following, washing extensively with PBST between each incubation : (i) 0.5 μg peptide diluted in 0.1 % milk-PBS, 1 h, r.t.; (ii) ~6 μg total protein from cell extracts diluted in 0.1 % milk-PBS, 1 h, r.t.; (iii) primary antibody 3009 diluted 1:1000 in 2 % milk-PBST or undiluted hybridoma supernatant of PC10, 1 h, r.t.; (iv) secondary horseradish peroxidase conjugated anti-rabbit (for 3009) or anti-mouse (for PC10) antibody at 1:1000 in 2 % milk-PBST, 1 h, r.t.; then 50 μl of the chromogenic substrate TMB was added per well (prepared from 10 mg ml^{-1} stock in DMSO and diluted to 100 μg ml^{-1} final concentration in 0.1 M sodium acetate, pH 6.0, with 1:1000 diluted 30 % stock solution H_2O_2). Once a visible blue colour had developed, the reaction was stopped by addition of approximately 1 M H_2SO_4, and

the plate read using a Dynatech 5000 ELISA plate reader at 450 nm. Human PCNA was purified from lysates of BL21 transformed with the pT7.7hPCNA construct [27].

Peptide precipitation of PCNA

Peptide (1.5 μg) was bound to 10 μl packed streptavidin-agarose beads for 1 h at room temperature. The beads were washed extensively in PBS prior to incubation with 20 μg total cell protein for 1 h at 4 °C. After washing three times in 1.5 x PBS (220 mM NaCl), the beads were boiled in loading buffer (0.2 M dithiothreitol, 2% SDS, 1% bromophenol blue and 20% glycerol) and then proteins were separated by 10 % SDS polyacrylamide gel electrophoresis and either stained directly with Coomassie brilliant blue or electrophoretically transferred onto nitrocellulose. Blots were blocked in 5 % milk–PBST, washed in PBS and incubated with undiluted culture supernatant from PC10 hybridomas, followed by horseradish peroxidase-conjugated rabbit anti-mouse antibody (Dako) at 1:1000 dilution in 2 % milk–PBST. Proteins were visualised by the enhanced chemiluminescence (ECL) reaction using Hyperfilm-MP (Amersham, UK).

Replication reactions

SV40 DNA replication reactions were carried out essentially as described by Wang *et al.* [36] with the exception that all incubation volumes were 10 μl.

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