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Human and plant proliferating-cell nuclear antigen have a highly conserved binding site for the p53-inducible gene product p21^{WAF1}

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The mechanism(s) whereby higher plants respond to environmental agents that damage their DNA, which leads to the arrest of cell division, is poorly understood. In mammalian cells, the tumour-suppressor protein p53 plays a central role in a DNA-damage-induced cell-cycle-checkpoint pathway by induction of transcription of a set of gene products that have a direct role in a DNA-damage-induced cell-cycle growth arrest. One such protein, p21^{WAF1}, has been shown to be essential for radiation-induced growth arrest. There appear to be at least two cellular targets of p21^{WAF1} during checkpoint control, the G$_1$-cyclin-dependent kinases (CDK) and proliferating-cell nuclear antigen (PCNA). The aim of the research reported here was to determine whether the interactions between the human growth inhibitor p21^{WAF1} and PCNA from plants and humans are conserved. If so, this would suggest that modulation of PCNA activity may play an important role in plant responses to DNA damage and would imply that functional homologue(s) of p21^{WAF1} exist in plants. We show that the p21^{WAF1}-interaction domain of PCNA is conserved between humans and plants. A peptide that contains the site of human p21^{WAF1} that binds human PCNA has been used to precipitate PCNA from crude pea (Pisum savitum) extracts. We used the p21^{WAF1} peptide as an affinity matrix and showed that pea PCNA bound in a specific high-affinity manner. This finding was used to develop a purification protocol that allowed PCNA from plant tissue to be purified to homogeneity. Pure pea PCNA forms a stable complex with full-length human p21^{WAF1} and the specific amino acids of p21^{WAF1} required for the interaction have been identified. The critical residues were identical to those required for binding to human PCNA, which indicates that the interaction of human p21^{WAF1} with PCNA is highly conserved at each amino acid position between pea and human.

Keywords: proliferating-cell nuclear antigen; Pisum savitum; cell-cycle inhibitors; p21^{WAF1}; peptide-affinity chromatography.

When plants are exposed to ultraviolet or ionising radiation, a broad spectrum of physical and chemical modifications of their DNA occur, which eventually leads to arrest of cell division (Mateos et al., 1992; Chagvardieff et al., 1989; Inoue and van Huystee, 1984). Studies have shown that plants possess mechanisms for repair of damaged DNA which include photoreactivation, excision repair (McLennan, 1988) and recombinational repair (Cerutti et al., 1992). Recently, information about some of the genes and proteins involved in these processes has emerged (Cerutti et al., 1992, 1993; Pang et al., 1993; Batschauer, 1993). For example, poly(ADP-ribose) polymerase has been identified in maize, wheat and pea nuclei (Chen et al., 1994). In mammalian systems, this enzyme functions when DNA is cleaved and rejoined during processes such as DNA repair, DNA recombination and gene rearrangement, and a role in detection of DNA-strand breaks has also been proposed (Althaus et al., 1992; de Murcia and Menissier-de Murcia, 1994). In addition, the discovery of ultraviolet-sensitive and X-ray-sensitive mutants should provide a valuable tool to identify other enzymes and proteins involved in signalling and the response to genomic damage (Harlow et al., 1994; Britt et al., 1993; Davies et al., 1994). However, nothing is known about the signalling pathways that detect DNA damage and halt plant-cell division in response to environmental agents that affect genome integrity.

The response of mammalian cells to DNA-damaging agents such as ultraviolet and ionising radiation has been subject to much more intense investigation and is therefore better understood. The primary objective of a cell that has been subjected to DNA damage is to ensure that mutated DNA is not passed on to the next generation of cells as this could ultimately be disasterous for the organism. The mechanisms by which this is achieved are either to arrest the cell cycle at certain checkpoints, which allows repair to take place prior to DNA replication, or, if the level of chromosomal DNA damage exceeds the ability of the repair machinery to correct the lesions, the cell might undergo apoptosis or irreversible growth arrest. The tumour-suppressor protein p53 is central to the response of mammalian cells to radiation-induced DNA damage, as it is a key component in the pathways of cell-cycle arrest and apoptosis (reviewed by Lane, 1993). Although the events that lead from induction of p53 to apoptosis are not known, the role of p53 during cell-cycle-checkpoint control is better understood. After exposure of cells to DNA-damaging radiation, the sequence-specific transcriptional activity of p53 increases, which leads to the induction

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Abbreviations. PCNA, proliferating-cell nuclear antigen; CDK, cyclin-dependent kinase.

of a number of gene products implicated in growth control, including GADD45 (Kastan et al., 1992), mdm2 (Momand et al., 1992) and p21^{WAF1} (El-Deiry et al., 1993). The biochemical activity of p21^{WAF1} is well defined and this protein is thought to coordinate cell-cycle arrest and DNA replication because it has been shown to be an inhibitor of cyclin-dependent kinases (CDK; Harper et al., 1993; Xiong et al., 1993; Strausfeld et al., 1994), a group of kinases that regulate cell-cycle progression (reviewed by Nigg, 1995), and of proliferating-cell nuclear antigen (PCNA; Waga et al., 1994), a polymerase δ processivity factor, which is an essential DNA-replication protein (Tan et al., 1986; Prelich et al., 1987a,b; Wold et al., 1989; Waga et al., 1994). The model of p21^{WAF1} function is supported by the discovery that, although p21^{WAF1}-null mice are viable, p21^{WAF1}-null mouse-embryo fibroblasts are impaired in their ability to undergo cell-cycle arrest in G$_1$ after exposure to ionising radiation (Brugarolas et al., 1995; Deng et al., 1995).

Little is known about the signalling pathways that lead to cell-cycle arrest after DNA damage in higher plants. Because we are interested in the conservation of checkpoint mechanisms between plants and mammals, the possible conservation of one key radiation-responsive pathway, the p53-dependent p21^{WAF1} signalling network, requires consideration. As one of the main targets of p21^{WAF1} is PCNA, biochemical characterisation of purified plant PCNA could lead to the identification of conserved domains implicated in the regulation of replication and cell-cycle checkpoints in plants. Such information would provide valuable information about the possible physiological functions of PCNA and could reveal the existence of factors that negatively regulate the plant cell cycle.

Although little is known about the structure/function relationships of plant PCNA, cDNAs that encode PCNA from several higher plants including rice and soybean (Suzuka et al., 1991) have been cloned and sequenced. Comparison of the coding regions of PCNA from rice and rat reveal 64% similarity at the nucleotide level and 62% similarity at the amino acid level. Furthermore, comparison of the predicted amino acid sequence of PCNA from soybean and rice indicate a high degree of similarity (88%; Suzuka et al., 1991). In addition to the presumed structural similarity between mammalian and plant PCNA there is also evidence for functional conservation. A recombinant fusion protein of rice PCNA and maltose-binding protein stimulated DNA synthesis catalysed by human polymerase δ (although less effectively than human PCNA), while it had no effect on polymerase α activity (Matsumoto et al., 1994). Similarly, calf thymus PCNA stimulates the activity and processivity of two DNA polymerases from wheat that appear to be polymerase-δ-like enzymes (Laquel et al., 1993). Thus, the functional interaction of plant PCNA and a key replicative DNA polymerase appears to be conserved between plants and animals. It is not known, however, whether the interaction of PCNA interaction with the cell-cycle-checkpoint-control machinery is conserved.

In this report, we develop an affinity matrix to purify PCNA from plants to homogeneity, and we show that the p21^{WAF1}-interaction site of PCNA is highly conserved between human and pea. The results are discussed in terms of the implication that a functional homologue of p21^{WAF1} exists in plants. The conservation of such a high-affinity interaction between PCNA and p21^{WAF1} in plants and animals, despite their distant evolutionary relationship, indicates that there has been a strong selection pressure to conserve this binding domain. It also provides biochemical evidence to suggest that higher plants may regulate radiation-induced cell-cycle checkpoints through modulation of PCNA activity by proteins that inhibit cell-cycle progression.

EXPERIMENTAL PROCEDURES

Reagents. Phenyl-Sepharose and the Mono-Q 5/5 HR column were obtained from Pharmacia and streptavidin-agarose was from Sigma. All peptides were synthesised by Chiron Mimotopes Peptide Systems by means of a multipin peptide-synthesis system. Each peptide had a biotin-SGSG spacer at the C-terminus and a free NH$_2$ at the N-terminus. Peptides were dissolved in dimethyl sulfoxide to approximately 5 mg/ml and stored at $-70\,°C$. The concentration of each peptide was accurately determined by amino acid analysis; each peptide was hydrolysed and the amino acids were derivatised with phenylisothiocyanate, then separated by HPLC. All other reagents were of the highest available commercial grade.

Plant material. Peas (*Pisum savitum* v. Onward) were grown in a greenhouse under natural-light conditions, and the seedlings were harvested by removal just below the cotyledons 10 d after germination and placed directly into extraction buffer at 4°C.

Peptide precipitation of PCNA. Peptide (1.5 µg) was diluted in 100 µl 146 mM NaCl, 14 mM sodium phosphate, pH 7.4, NaCl/P$_i$ and incubated with 10 µl packed streptavidin-agarose beads for 1 h at room temperature. Unbound peptide was removed by extensive washing (four times with NaCl/P$_i$) and the beads with bound peptide were incubated for 1 h at 4°C with 20 µl crude pea seedling extract that contained approximately 40 µg total protein. The beads were washed three times with 1.5×NaCl/P$_i$ and heated at 95°C for 4 min in the presence of 125 mM Tris/HCl, pH 6.8, 4% (mass/vol.) SDS, 20% (by vol.) glycerol and 200 mM dithiothreitol. Proteins were analysed by SDS/PAGE and Western blotting.

SDS/PAGE and Western blots. PCNA protein was detected after SDS/PAGE (Laemmli, 1970) by means of staining with Coomassie Brilliant Blue or silver stain, or after electrophoretic transfer onto nitrocellulose. Blots were blocked in 5% dried milk in NaCl/P$_i$ containing 0.1% Tween 20 (NaCl/P$_i$/Tween 20) for 1 h, washed in NaCl/P$_i$/Tween 20 and incubated with culture supernatant from PC10 (or other mAb)-secreting hybridomas for 1 h, washed with NaCl/P$_i$/Tween 20 and incubated for 1 h with horseradish-peroxidase-conjugated rabbit anti-mouse Ig (Dako) diluted 1:1000 in NaCl/P$_i$/Tween 20 containing 5% dried milk. In addition, a rabbit polyclonal antibody (3009, raised against a synthetic peptide that corresponded to the C-terminus of human PCNA) was used on Western blots of plant material. The 3009 serum was diluted 1:500 in NaCl/P$_i$/Tween 20 containing 5% dried milk, and horseradish-peroxidase-congugated swine anti-rabbit Ig, diluted 1:1000 in NaCl/P$_i$/Tween 20 containing 5% dried milk was used as secondary antibody. After removal of excess secondary antibody by washing with NaCl/P$_i$/Tween 20, bound protein was detected by enhanced-chemiluminescence (ECL) reaction and Hyperfilm-MP (Amersham).

Purification of PCNA from peas. All steps were carried out at 4°C. Extracts were prepared in a Waring blender by homogenisation of 200 g pea seedlings in 200 ml 50 mM Hepes, pH 8.0, 0.1 mM EDTA, 0.15 M NaCl, 1 mM benzamidine, 2 mM dithiothreitol and 0.1 mM phenylmethanesulfonyl fluoride. After addition of Triton X-100 to 1% (by vol.), the homogenate was centrifuged at 24000×g for 20 min (the supernatant is referred to as the whole tissue extract). Ammonium sulfate was added to the supernatant to 40% saturation. The suspension was stirred for 20 min and the precipitate was collected by centrifugation at 24000×g for 15 min. The pellet was discarded and ammonium sulfate was added to the supernatant to 60% saturation. This suspension was stirred for 20 min and the precipitate was collected as described above. The precipitate was dissolved in 50 mM Hepes, pH 7.4, 0.1 mM EDTA, 1 mM benz-

amidine, 2 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride (buffer A) containing 1 M ammonium sulfate and applied to a 50-ml phenyl-Sepharose column equilibrated in buffer A plus 1 M ammonium sulfate. The column was washed with 200 ml buffer A plus 1 M ammonium sulfate, and proteins were eluted with a linear gradient from of 1 M to 0 M ammonium sulfate in buffer A over 500 ml. Fractions that were determined by Western blot analysis to contain PCNA were pooled and dialysed twice against 1 l 25 mM Hepes, pH 7.4, 0.1 mM EDTA, 0.1 M NaCl, 1 mM benzaminine, 2 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, 0.1% (by vol.) Triton X-100, 15% (by vol.) glycerol (buffer B). The dialysed protein was applied to a 2-ml peptide-10 affinity column equilibrated in buffer B, the column was washed with 12 ml buffer B containing 0.2 M NaCl and proteins were eluted with 12 ml buffer B containing 0.75 M NaCl. The peptide resin was made by incubation of 2 ml packed streptavidin-agarose beads with 300 µg peptide 10 overnight at 4°C with stirring. The column was poured and any unbound peptide was removed by extensive washing with NaCl/P$_i$. A 1-ml peptide column was used to determine binding of proteins from whole tissue extracts as described in the legend to Fig. 4. Fractions that contained PCNA were pooled, dialysed twice against 1 l buffer B, and the dialysed protein was applied to a MonoQ 5/5 HR column equilibrated in buffer B. The protein was eluted with a linear gradient from 0.1 M to 1.0 M NaCl in buffer B over 30 ml. Fractions that contained PCNA were pooled, concentrated to 250 µg/ml, frozen and stored at −70°C.

Immunoprecipitation. Extracts of *Escherichia coli* that expressed recombinant human p21^{WAF1} were prepared by lysis of cells in 50 mM Hepes, pH 8.0, 20% sucrose, 0.2 M KCl, 2 mM dithiothreitol, 1 mM benzamidine and 0.35 mg/ml lysozyme for 20 min at 4°C. The lysate was centrifuged at 14000×g for 20 min and the supernatant, which contained soluble p21^{WAF1}, was used in immunoprecipitation assays. Pure pea PCNA (1 µg) was incubated with 20 µl *E. coli* extract and 100 µl of NaCl/P$_i$/Tween 20 containing 0.1 mM phenylmethyane sulfonyl fluoride for 1 h at 4°C. A mAb (2 µg) that specifically recognises and immunoprecipitates p21^{WAF1} (WAF1-Ab1; Oncogene Sciences) was added and the incubation was continued overnight. The protein · antibody complex was precipitated by addition of 10 µl (packed volume) protein-G beads, washed three times with NaCl/P$_i$/Tween 20 and incubated for 1 h. The beads were washed with 4×NaCl/P$_i$/Tween 20 and the bound proteins were removed by addition of sample buffer and incubation at 95°C for 5 min. The absence or presence of pea PCNA in the p21^{WAF1} · antibody immune complex was determined by SDS/PAGE followed by Western blot analysis with PC10 as described above.

RESULTS

Monoclonal and polyclonal antibodies against mammalian PCNA can be used to detect denatured plant PCNA. Determination of conserved structural motifs in PCNA may provide valuable information on its regulation. We determined whether mAbs (PC2, PC5, PC8 and PC10) generated to rat PCNA by Waseem and Lane (1990) could recognise a protein from whole plant extracts by Western blot analysis. These antibodies have previously been shown to recognise HeLa, yeast (*Schizosaccharomyces pombe*) and insect (*Spodoptera frugiperda*) PCNA by denaturing immunoblot analysis (Waseem and Lane, 1990). However, the PC antibodies do not recognise denatured PCNA derived from *Saccharomyces cerevisiae* by Western blot analysis, which indicates that not all species retain the PC-type epitopes. Mapping of this antibody series by means of peptides derived from PCNA has established that the antibodies recognise
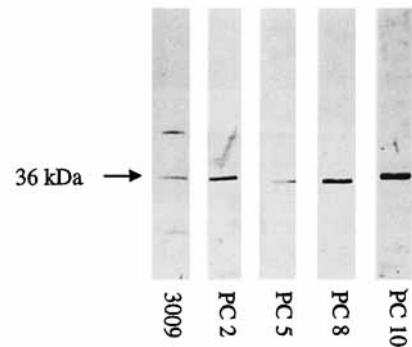

Fig. 1. Antibodies raised to mammalian PCNA recognise plant PCNA. Whole tissue extracts of pea were separated by SDS/PAGE, transferred to nitrocellulose and probed with mAbs PC2, PC5, PC8 and PC10 and the polyclonal antibody 3009. The position of PCNA (36 kDa) is indicated.

a region of PCNA between amino acids 111 and 120 (Cox, L. S. and Lane, D. P., unpublished results) described as the immunodominant region.

Since the PC antibodies recognise a highly conserved domain of mammalian PCNA, they were used to determine whether PCNA from plant lysates also contained this domain. mAbs PC2, PC5, PC8 and PC10 recognised a single polypeptide from denatured pea extracts with an apparent molecular mass of approximately 36 kDa (Fig. 1). This is in agreement with the apparent molecular mass of rice and soybean PCNA determined by SDS/PAGE (Suzuka et al., 1989; Daidoji et al., 1992). Although the amino acid sequence of pea PCNA is not known, the amino acid sequence of PCNA from several other higher plants has been deduced. Fig. 2A compares the sequences of plant PCNA with that of rat PCNA within the domain recognised by the PC series of antibodies. There is a high degree of cross-species conservation and the amino acid substitutions are highly conservative. These results are consistent with the proposal that the 36-kDa protein from plant lysates detected with the PC antibodies is plant PCNA.

Because PC10 gave the strongest signal upon denaturing immunoblots of crude pea extracts, it was also tested to determine whether it could recognise native pea PCNA from crude lysates by means of an immunoprecipitation assay. PC10 did not immunoprecipitate PCNA from HeLa cell extracts or crude extracts of pea (data not shown). These results suggest that this epitope is not exposed in native human or pea PCNA. Strong support for this hypothesis has been supplied from the X-ray-crystallographic analysis of *E. coli* pol III subunit (Kong et al., 1992) and yeast PCNA (Krishna et al., 1994), where it appears that the S, D and L residues of the PC10 epitope are folded into a β sheet buried within the protein and are therefore not solvent exposed in the native protein.

In addition to the PC mAbs, a polyclonal antibody (3009), generated to a 15-amino-acid peptide (Cox, L. S. and Lane, D. P., unpublished result) that represents the C-terminus of human PCNA, could be used to detect pea PCNA by denaturing-immunoblot analysis (Fig. 1). This finding supports the conclusion that the 36-kDa protein detected immunochemically from pea lysates is PCNA. If the deduced amino acid sequences of plant PCNA are compared with the human C-terminal 15 residues, we find that the central region shows a high degree of identity, but there is some divergence in the C-terminus (Fig. 2). The ability to detect immunochemically a 36-kDa protein from pea lysates by means of mAbs and polyclonal antibodies specific for human PCNA has provided a biochemical assay to identify plant PCNA during purification and biochemical characterisation.

A

```
111 SDYEMKLMDL 120    rat

    ADFEMKLMDI          rice

    SDFEMKLMDI          soybean

    ADFEMKLMDI          carrot (small PCNA)

    SDFEMKLMDI          carrot (large PCNA)
```

B

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    LKYYLAPKIEDEEGS              human

    IRFYLAPKIEEDEEMKS           rice

    VRFYLAPKIEEDEEDTKPQV        soybean

    IRFYLAPKIEEEEDES            carrot (small PCNA)

    IRYYLAPKIEEEDEAAN.....      carrot (small PCNA)
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Fig. 2. Alignment of the sequences of the PC10 and 3009 epitopes from rat with plant PCNA sequences. (A), the region of rat PCNA that contains the PC10 epitope; (B), the sequence of the peptide, which corresponds to the C-terminus of human PCNA, that was used to raise the polyclonal antibody 3009. These sequences are compared with the deduced amino acid sequences of rice PCNA, soybean PCNA (Suzuka et al., 1991) and the small and large PCNA from carrot (Hata et al., 1992).

A p21^WAF1 peptide can precipitate plant PCNA from whole tissue extracts.

It has been shown that p21^WAF1 can inhibit DNA through interaction with PCNA (Waga et al., 1994; Shivji et al., 1994) and evidence suggests that the physical binding of p21^WAF1 to PCNA (Flores-Rozas et al., 1994) prevents PCNA functioning as a processivity factor for polymerase δ. The interaction site has been mapped by Warbrick et al. (1995), who found that a 20-amino-acid peptide (that corresponded to peptide 10 in our study; Fig. 3) bound to PCNA from HeLa cell extracts and lysates of *E. coli* that overexpressed human PCNA protein.

We used a set of overlapping peptides that span the entire sequence of p21^WAF1 (Fig. 3) to identify peptides that can interact with PCNA from whole tissue extracts of pea seedlings. The p21^WAF1 peptides were linked to biotin which allowed them to be attached to streptavidin-agarose beads. The peptide-linked beads were incubated with pea extracts and the protein bound to the beads was analysed by SDS/PAGE, blotted onto nitrocellulose, and probed with mAb PC10 (Fig. 3). Plant PCNA was precipitated by beads linked to peptide 10 (KRRQTSMTDFYHSKRR-LIFS). As PCNA from pea was not precipitated with peptide 11, which lacks the four N-terminal amino acids (KRRQ) of peptide 10, it appears that these residues are important for the interaction with pea PCNA.

PCNA could be quantitatively removed form pea extracts such that no PCNA remained in the supernatant as determined by Western blot analysis with PC10 (data not shown). These results indicate that there is only one biochemical form of PCNA in pea seedlings (i.e., non-p21^WAF1-binding fractions of pea PCNA are not detected) and that the interaction between PCNA and p21^WAF1 peptide 10 is highly conserved between human and pea.

Binding of plant PCNA to a peptide-affinity column.

To further define the avidity and specificity of the interaction between peptide 10 and PCNA from peas, we made a peptide-affinity column by biotinylated peptide 10 to streptavidin-agarose. When crude extracts of pea were applied to a 1-ml column in the presence of 0.1 M NaCl the majority of the protein (14 mg protein were applied to the column) flowed through (12.88 mg protein),
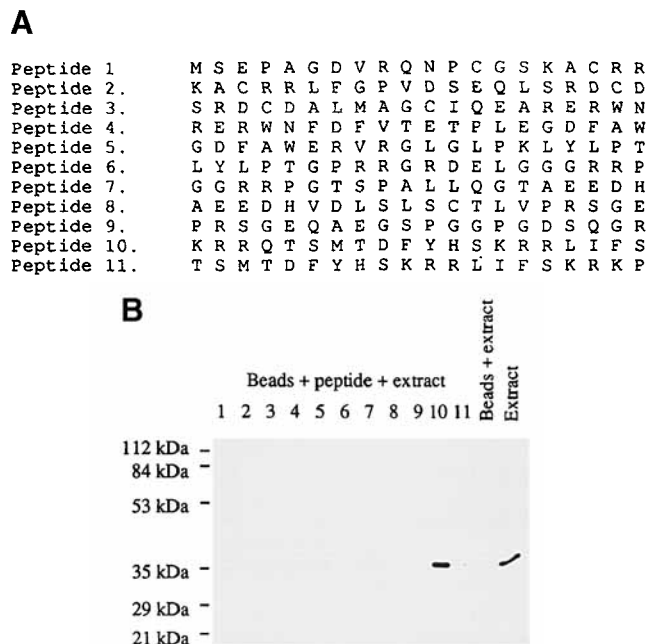
A

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Peptide 1      M S E P A G D V R Q N P C G S K A C R R
Peptide 2.     K A C R R L F G P V D S E Q L S R D C D
Peptide 3.     S R D C D A L M A G C I Q E A R E R W N
Peptide 4.     R E R W N F D F V T E T P L E G D F A W
Peptide 5.     G D F A W E R V R G L G L P K L Y L P T
Peptide 6.     L Y L P T G P R R G R D E L G G G R R P
Peptide 7.     G G R R P G T S P A L L Q G T A E E D H
Peptide 8.     A E E D H V D L S L S C T L V P R S G E
Peptide 9.     P R S G E Q A E G S P G G P G D S Q G R
Peptide 10.    K R R Q T S M T D F Y H S K R R L I F S
Peptide 11.    T S M T D F Y H S K R R L I F S K R K P
```

B

Fig. 3. Analysis of PCNA binding to p21^WAF1 peptides. (A), peptides 1−11, which overlap, cover the entire protein sequence of p21^WAF1. (B), each of the p21^WAF1 peptides were attached to streptavidin-agarose beads and incubated with whole tissue extracts of pea. The proteins that remained on the beads after extensive washing were analysed by SDS/PAGE, transferred to nitrocellulose and probed with mAb PC10, followed by horseradish-peroxidase-anti-mouse secondary antibody and enhanced-chemiluminescent (ECL) detection. The positions of molecular-mass standards are indicated.
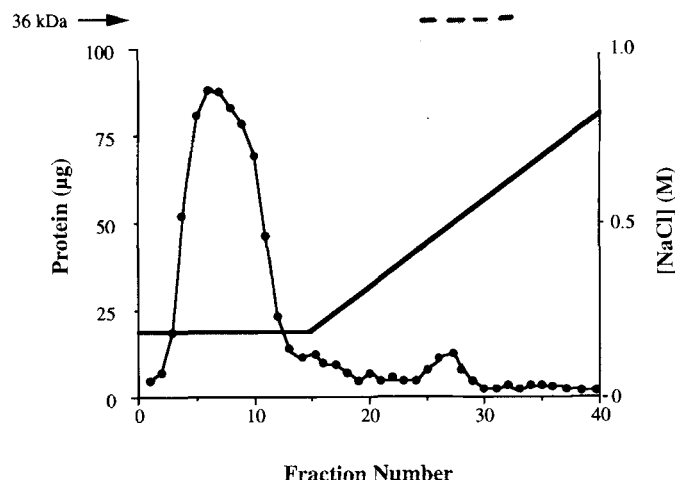

Fig. 4. Binding of plant PCNA to a peptide-affinity column. Whole tissue extracts of pea were applied to a 1-ml p21^WAF1 peptide-10−affinity column and proteins were eluted with an NaCl gradient. (●), amount of protein. The elution of PCNA, detected by Western blots probed with PC10, is shown. The position of PCNA (36 kDa) is indicated.

but no PCNA was detected in this fraction. Fig. 4 shows that when the column was developed with a NaCl gradient the vast majority of protein, which bound non-specifically to the column, eluted at 0.2 M NaCl whereas PCNA was not eluted from the column until the concentration of NaCl was greater than 0.4 M. No PCNA bound to streptavidin-agarose beads that were not linked to peptide 10. This chromatographic method shows that PCNA binds specifically to p21^WAF1 peptide 10 and elutes in one
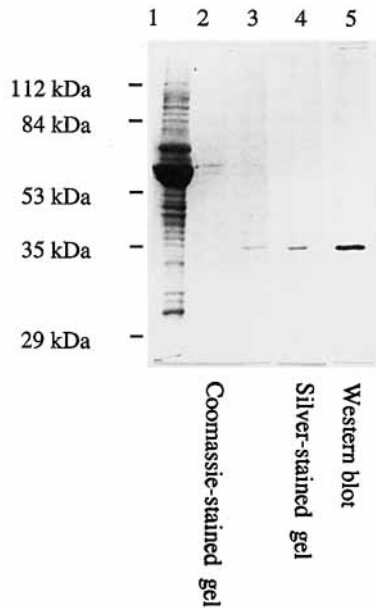
Ball and Lane (*Eur. J. Biochem. 237*)

Fig. 5. SDS/PAGE and Western blot analysis of PCNA purified from pea. Lane 1, pooled protein fractions after phenyl-Sepharose chromatography; lane 2, peptide-affinity-purified protein; lanes 3−5, purified protein after anion-exchange chromatography on MonoQ. The Western blot was probed with mAb PC10. The positions of molecular-mass standards are indicated.
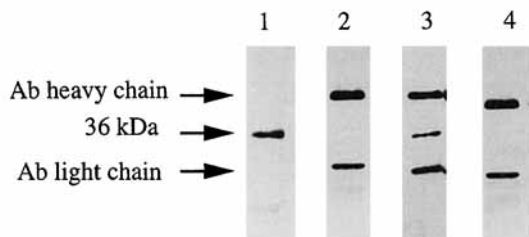

Fig. 6. Immunoprecipitation of human p21^WAF1 and pea PCNA with p21^WAF1-specific antibody. Lane 1, Western blot of purified pea PCNA; lanes 2−4, immunoprecipitations followed by Western blots. Lane 2, control mAb specific for human p53; lane 3, p21^WAF1-specific mAb WAF1-Ab1 (Oncogene Sciences); lane 4, as lane 3, except that the pea PCNA was pre-incubated with peptide 10 and incubation with antibody was performed in the presence of peptide 10 (100 µg/ml). The Western blot was probed with the PCNA-specific mAb PC10.

Fig. 7. Identification of the critical amino acid residues required for binding of plant PCNA to p21^WAF1. The p21^WAF1 residues that are critical for its interaction with pea PCNA were determined by generation of variants of peptide 10 in which each residue was sequentially changed to alanine. The ability of these peptides to interact with pea PCNA was determined by attachment of the peptides to streptavidine-agarose beads and incubation with whole tissue extracts of pea. Bound protein was analyzed by SDS/PAGE and Western blot analysis with PC10 to detect PCNA, as described in the legend for Fig. 3. (A), Western blot probed with PC10; (B), data expressed as relative binding affinity, where the binding of PCNA to each peptide has been standardised against the binding of PCNA to peptide 10, which is assumed to be 100%. These data were obtained by determination of the density of each band using a densitometer. The RR peptide had arginine residues 2 and 15 changed to alanine and the random peptide contained the same amino acids as peptide 10 but in a different order.

peak, which provides evidence for only one biochemical form of PCNA in pea extracts, and that the interaction is of high affinity but can be disrupted by an increase of the ionic-strength of the buffer, which indicates that the interaction of PCNA with peptide 10 is reversible.

Purification of PCNA from whole pea extracts. The high-affinity interaction of PCNA with the peptide-affinity column was exploited when a purification protocol for pea PCNA was designed. Crude pea tissue extract was fractionated by means of ammonium sulfate precipitation, hydrophobic-interaction chromatography, peptide-affinity chromatography and anion-exchange chromatography on a MonoQ column. Recovery of PCNA from the peptide column was extremely high (approximately 95%), as judged by densitometry after Western blot analysis. 40 mg total protein were applied to the column and approximately 600 µg was eluted in the 0.75-M-salt wash, and therefore we estimate that PCNA was purified approximately 200-fold during this step. Peptide-affinity purification is a rarely
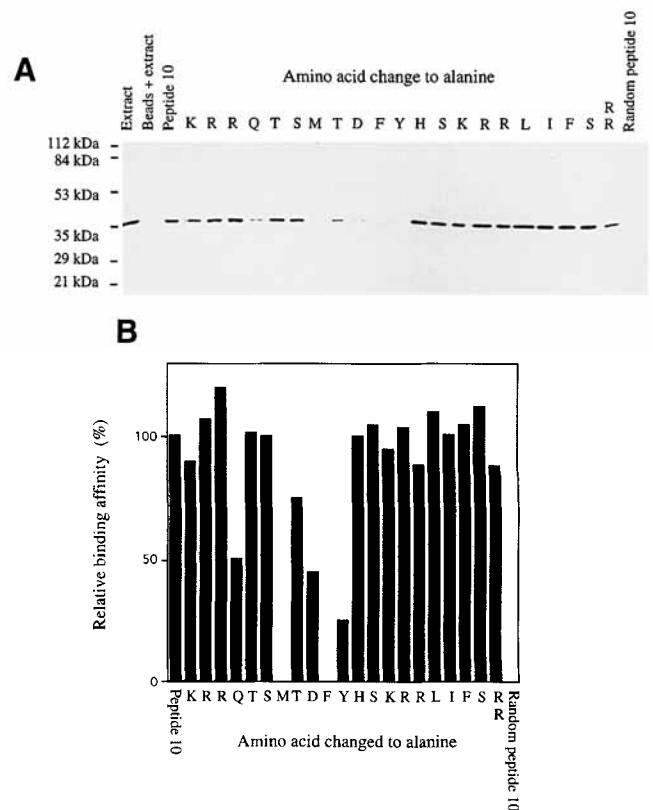
used method of purification, but has been very successful in the purification of protein kinases (Woodgett, 1991).

The final preparation of pea PCNA, which eluted from the MonoQ column in approximately 0.4 M NaCl, was estimated to be greater than 98% pure by SDS/PAGE detected with Coomassie blue or silver stain (Fig. 5). The identity of the purified polypeptide was confirmed by transfer to nitrocellulose and immunochemical detection with PC10. PCNA from rice and soybean have previously been partially purified (Suzuka et al., 1989; Daidoji et al., 1992) but several major contaminating polypeptides were present in both those preparations. The method described here was also used to purify human PCNA (Ball, K. L. and Lane, D. P., unpublished results), which shows that the method is broadly applicable.

Interaction of plant PCNA with full-length human P21^WAF1. After we showed that pea PCNA could form a stable complex with p21^WAF1 peptide 10, we needed to determine whether the pure pea PCNA could form a complex with full-length human p21^WAF1. When extracts from *E. coli* that expressed human

p21^{WAF1} were mixed with pure pea PCNA in the presence of a p21^{WAF1}-specific mAb, the isolated immune complex contained pea PCNA (Fig. 6). Formation of the complex was blocked if the PCNA was incubated with peptide 10. This finding indicates that the full-length human p21^{WAF1} and the peptide can both form stable complexes with plant PCNA and that they compete for binding at the same site.

Critical residues for the interaction between plant PCNA and human p21^{WAF1}.

Are residues that are critical for binding of p21^{WAF1} to human PCNA also important for binding to pea PCNA? To define the critical residues involved in the binding of p21^{WAF1} to pea PCNA we used a series of biotinylated peptides based on the sequence of peptide 10 in which each residue was changed sequentially to alanine (Fig. 7). Two other peptides were included in the analysis, a double substitution were two arginine residues were changed to alanine in the same peptide and a control peptide composed of the same residues as peptide 10 but in random order. We investigated the ability of the peptides linked to streptavidin-agarose to precipitate PCNA from pea extracts (as described above). Residues M and F were essential for binding as no PCNA was detected on Western blots if they were replaced with alanine (Fig. 7). Residue Y was also very important for binding, as only trace amounts of PCNA were detected when it was substituted, and mutations of Q and D considerably reduced the amount of PCNA precipitated. The random peptide did not precipitate any PCNA which suggests that it is the sequence and not just the presence of these amino acids that is of importance. Therefore, we are able to define the region of p21^{WAF1} involved in binding to pea PCNA as *Q*TS*MTDF*Y (residues underlined in bold are essential for binding, the Y in bold is extremely important for binding, and residues in italics contribute significantly to binding). These results are essentially identical to those found by Warbrick et al. (1995) for human PCNA. Thus, the binding site for p21^{WAF1} on PCNA is highly conserved at each amino acid position between human and pea.

DISCUSSION

Although some key components of the cell-cycle regulatory machinery have been conserved during evolution, there is no generic cell cycle, and the relative importance of individual regulatory mechanisms depends on the organism under study. Our current knowledge of the cell cycle and checkpoint control is based mainly on information from yeast and mammalian tissue-culture cells. It has become clear that the basic machinery for control of the cell cycle, namely the cyclin-dependent kinases and their regulatory subunits, the cyclins, are conserved between plants and other eukaryotes (reviewed by Staiger and Doonan, 1993). Several proteins involved in cell-cycle progression, including Cdc2 homologues (Feiler and Jacobs, 1990; Ferreira et al., 1991; Hirt et al., 1991) and G$_1$ and G$_2$ cyclins (Hata et al., 1991; Hemerly et al., 1992; Soni et al., 1995) have been identified in plants, and there is evidence for conservation of other regulators (Soni et al., 1995; Xie et al., 1995). Nothing, however, is known about the mechanism(s) of negative cell-cycle regulation and the possible existence of CDK-inhibitory or PCNA-inhibitory factors in plants. The first cell-cycle inhibitors were identified in yeast (Mendenhall, 1993; Schwob et al., 1994; Moreno and Nurse, 1994) and subsequently two groups (p21/KIP and INK4) of cell-cycle inhibitors were identified in mammalian cells (Sherr and Roberts, 1995), which are structurally unrelated to their yeast counterparts. The mammalian cell-cycle inhibitors respond to a variety of negative growth signals

but, as described above, p21^{WAF1} is induced by the p53-mediated response generated by radiation-induced DNA damage. Our results suggest that a p21^{WAF1}-like protein exists in plants and that such a protein could modulate PCNA and, perhaps, cyclin-CDK activity during cell-cycle arrest.

PCNA is a component of the polymerase δ holoenzyme complex which acts as a processivity factor for polymerase δ, and as such is involved in leading-strand and lagging-strand synthesis during DNA replication (reviewed by Waga and Stillman, 1994). PCNA is also essential for nucleotide-excision repair (Shivji et al., 1992) which requires synthesis of new DNA after excision of the damaged stretch of DNA. Intense interest in PCNA and its function(s) was generated when PCNA was shown to be a component of cyclin-dependent kinase quaternary complexes (Xiong et al., 1992), which also contained cyclin-CDK and p21^{WAF1}. Although the functional relevance of this association is not known, it led to the discovery that p21^{WAF1} inhibited DNA synthesis via a direct effect on PCNA (Waga et al., 1994; Flores-Rozas et al., 1994). Thus, PCNA not only interacts with components of the DNA-replication apparatus, it also interacts directly with a component of a cell-cycle-checkpoint pathway. While p21^{WAF1} seems to inhibit DNA synthesis during replication it does not prevent the DNA synthesis that is required for repair (Flores-Rozas et al., 1994; Li et al., 1994; Strausfeld et al., 1994). The interaction between p21^{WAF1} and PCNA has been mapped by Warbrick et al. (1995), who found that a 20-amino-acid peptide from the C-terminus of p21 bound to human PCNA and that this peptide was sufficient to inhibit simian virus 40 DNA replication *in vitro*. These results have been confirmed by deletion/mutation analysis (Chen et al., 1995; Goubin and Ducommun, 1995)

We have presented data that suggests that the p21^{WAF1}-responsive regulatory domain of PCNA is conserved between plants and animals. We used overlapping peptides that cover the entire sequence of human p21^{WAF1} to probe for a site on plant PCNA that binds to p21^{WAF1} or a p21^{WAF1}-like regulatory protein. One of the peptides (peptide 10) was able to interact with pea PCNA, and although the same peptide interacted with PCNA from HeLa cell extracts and lysates of *E. coli* that over-expressed human PCNA (Warbrick et al., 1995), the affinity and specificity were not determined. The interaction of p21^{WAF1} peptide 10 with pea PCNA is highly specific because greater than 99.5% of the total protein applied to a peptide-affinity column either does not interact with the column or interacts non-specifically with the resin and elutes with a low-salt wash. In contrast, all PCNA bound to the column and was only dissociated from it by NaCl concentrations in excess of 0.4 M. This finding indicates that the peptide · protein interaction is reversible and of very-high affinity. The peptide-affinity column gave a 200-fold purification of PCNA and allowed us to purify plant PCNA to homogeneity. Purification of intact native PCNA is essential to understand the biochemical functions of plant PCNA and studies are underway to characterise the activity of the pure pea protein in *in vitro* replication and repair assays.

We showed formation of a stable complex of pure pea PCNA with full-length human p21^{WAF1}. That the formation of this complex was prevented if PCNA was incubated with peptide 10 implies that they compete for the same site on PCNA. Therefore pea PCNA can form a complex with full-length human p21^{WAF1} and the p21^{WAF1} mimetic, peptide 10. The amino acid residues that were critical for the interaction of plant PCNA with p21^{WAF1} were determined and were essentially identical to those important for binding to human PCNA (Warbrick et al., 1995). Thus, the interaction of p21^{WAF1} and PCNA is highly conserved at each amino acid position between plants and humans.

The results raise the possibility that there is a functional homologue of p21^{WAF1} in higher plants, or that there is another protein, highly conserved during evolution, that interacts with PCNA from plant and animal sources with an identical specificity. Recent evidence also points to the existence in plants of a protein similar to retinoblastoma-protein in plants as its binding motif has been identified in a cyclin-D homologue from *Arabidopsis thaliana* (Soni et al., 1995) and in a protein from wheat geminivirus (Xie et al., 1995). The retinoblastoma protein is implicated in cell-cycle control as it is a substrate for the G$_1$-cyclin-CDK complexes and must be hyperphosphorylated for the cell cycle to proceed form G$_1$ into S phase (Weinberg, 1995). It seems probable that inhibition of PCNA and cyclin-CDK activity, which prevents replication and phosphorylation of key substrates such as retinoblastoma protein, is part of a checkpoint pathway, conserved between plants and animals, that leads to an arrest of the cell cycle. The factors that lead to activation of such a checkpoint pathway in plants are unknown. In mammalian cells, p53 is involved in detection of DNA damage. However, the p53 gene has only been found in the animal kingdom and more specifically in vertebrates (Soussi et al., 1990). Although p21^{WAF1} can be induced by other transcription factors (Jiang et al., 1994; Parker et al., 1995), p53-independent induction seems to be involved in senescence and differentiation rather than the damage response. It will therefore be of great interest to find out what induces a p21^{WAF1}-like protein in plants, as it may give us new information about signal-transduction pathways that can lead to cell-cycle arrest after cellular damage. In addition, it would provide information about novel mechanisms that plants may have evolved to deal with DNA-damaging environmental stress, such as ultraviolet irradiation.

In conclusion, the identification of a p21^{WAF1}-binding domain in PCNA derived from higher plants suggests a specific mechanism for modulation of PCNA activity. In addition, it suggests that cell-cycle inhibitors that can interact with PCNA and CDK during checkpoint control after DNA damage exist in higher plants. Future experiments will be aimed at the discovery of the protein(s) from plants that binds with high affinity to the p21^{WAF1}-binding domain of pure pea PCNA, and the determination of the ability of such a protein to regulate PCNA and CDK activity.

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