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Suppression of pea nuclear topoisomerase I enzyme activity by pea PCNA

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Summary

Proliferating cell nuclear antigen (PCNA), a highly conserved DNA polymerase accessory protein of eukaryotic kingdom, has not been studied thoroughly in biochemical terms in plants. We describe the isolation of the cDNA encoding PCNA from the pea cDNA library using the PCR approach. The cDNA was used for expression of pea PCNA in bacteria as a fusion protein (GST.PCNA) with the GST tag at the amino terminal end. The GST.PCNA stimulated the partially purified pea DNA polymerases approximately 30-fold. The stimulation was due to the oligomeric form of GST.PCNA. The pea PCNA interacted with the recombinant type I pea topoisomerase as well as the native pea nuclear topoisomerase I and repressed the DNA relaxation activities. However, the DNA binding activity of Topo I remained undisturbed in the presence of high amounts of PCNA, thereby signifying that the catalysis of Topo I was probably affected by PCNA.

Introduction

Both *in vivo* and *in vitro* studies have demonstrated that PCNA is a cell-cycle regulated DNA replication and repair protein (Amin and Holm, 1996; Jonsson and Hubscher, 1997; Solov'eva *et al.*, 1996). The molecular mechanism behind its accessory function for the DNA polymerase δ (Pol. δ) is fairly well known (Uhlmann *et al.*, 1997; Zhou *et al.*, 1997). The key event for its accessory function appears to be the formation of a stable trimeric ring even at a very low concentration (Yao *et al.*, 1996). It also interacts with a variety of other proteins involved in, for example, cell-cycle control, post-replication mismatch repair, apoptosis, cytosine methylation, etc. (Kelman, 1997). Since PCNA, a moderately small protein, is required for such a wide array of cellular functions, it is evolutionarily conserved across the eukaryotic kingdom in terms of its primary and tertiary structure (Kelman, 1997; Krishna *et al.*, 1994). Although the overall functional properties of PCNA are conserved, interesting species-specific variations of its biochemical interactions have also been reported (Gibbs *et al.*, 1997; Lopez *et al.*, 1997).

PCNA of yeast, *Drosophila* and mammalian sources have been rigorously studied. However, not much is known about plant PCNAs, although the *pcna* genes have been cloned from a variety of plant sources such as rice, maize, carrot, brassica and soyabean. Only the rice PCNA gene is characterized to a great extent in terms of transcription control (Kosugi and Ohashi, 1997). The biochemical features of plant PCNAs, their contributions towards the plant cell cycle, and the regulatory events occurring at their promoters have not been reported. Consequently, we have made an attempt to study the biochemical features of pea PCNA. Here we report on the isolation and characterization of the full-length *pcna* from pea. The recombinant PCNA protein was produced in the form of N-terminal GST protein fusion (GST.PCNA). The trimeric form of GST.PCNA activated the partially purified pea DNA polymerases. The fusion protein also interacted directly with pea Topo I, an enzyme required at the flanking ends of the DNA replication fork. GST.PCNA did not disrupt the DNA binding activity of Topo I but affected its DNA relaxation activity.

Results

Isolation of cDNA encoding PCNA of pea

Using the degenerate oligonucleotides representing two conserved sites, a DNA fragment of 450 bp was amplified from the pea cDNA plasmid library. Using this fragment, the plasmid library was screened and four independent positive spots were detected out of 4×10^4 screened clones. Two positive clones with insert sizes of 1.1 kb were selected and sequenced using the chain termination method.

Both clones had identical nucleotide sequences of 1031 bases. The 5′ and 3′ untranslated regions are 57 and 176 bp long, respectively. The open reading frame (ORF) consists of 266 amino acids and the deduced amino acid sequences of pea PCNA show high homology with other eukaryotic PCNA proteins, especially the plant PCNA. The predicted molecular size of the encoded protein (approximately 30 kDa) was in good agreement with the observed size of the *in vitro* translated (lanes 1–2, Figure 5a) and bacterially expressed protein (lane 4, Figure 1). In order to understand the biochemical features of pea PCNA, the recombinant protein was produced in the bacterial host.

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Expression and purification of the recombinant GST.PCNA fusion protein

Figure 1 shows that the recombinant clone containing pea *pcna* produced the fusion protein of 60 kDa in IPTG-induced *E. coli* cells (lane 2). Employing the GST affinity chromatography, the fusion protein was purified to apparent homogeneity (lane 3). This soluble fusion protein was used for biochemical characterisation (described below) except in the glycerol gradient sedimentation analysis where the insoluble but later refolded form was used. The GST and recombinant pea PCNA were liberated from the fusion protein following proteolysis with thrombin (lane 4). The identity of the cleaved individual proteins was established in a separate Western blot experiment using the anti-GST antibodies. Lane 5 shows the purified GST protein obtained from the induced *E. coli* cells harbouring the vector pGEX 4T1.

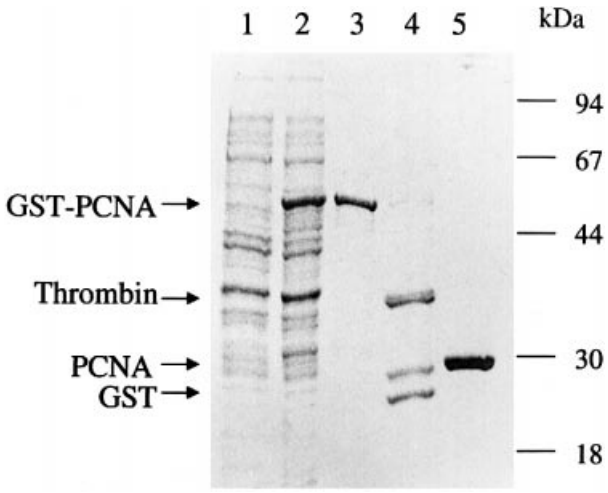

Figure 1. Purification of GST.PCNA fusion protein.
An SDS PAGE analysis of various protein fractions including 100 μg of lysate proteins of *E. coli* DH5α cells that were either induced (lane 2) or uninduced (lane 1) with 0.5 mM IPTG. Lane 3, about 2 μg purified GST.PCNA; lane 4, about 5 μg fusion protein digested with 0.6 U of thrombin; lane 5, about 8 μg of purified GST. The relative mobilities of Thrombin, PCNA and GST are shown by arrows in the left panel.

GST.PCNA enhances the homologous DNA polymerase activity

A nuclease free pea nuclear extract (NE) containing the partially purified DNA polymerase δ, along with other DNA polymerases and accessory factors was prepared. Figure 2a shows that one unit of NE preparation alone synthesised a residual amount of DNA (3.5×10^3 cpm, i.e. about one picomole of TMP) with 100 ng template. The addition of 10 μg fusion protein stimulated the synthesis up to 28-fold over the background value whereas the GST alone enhanced DNA synthesis only up to twofold. Hence the major stimulatory contribution was from the PCNA component only and GST.PCNA had little effect on the T7 DNA polymerase- (Pharmacia) mediated DNA synthesis.

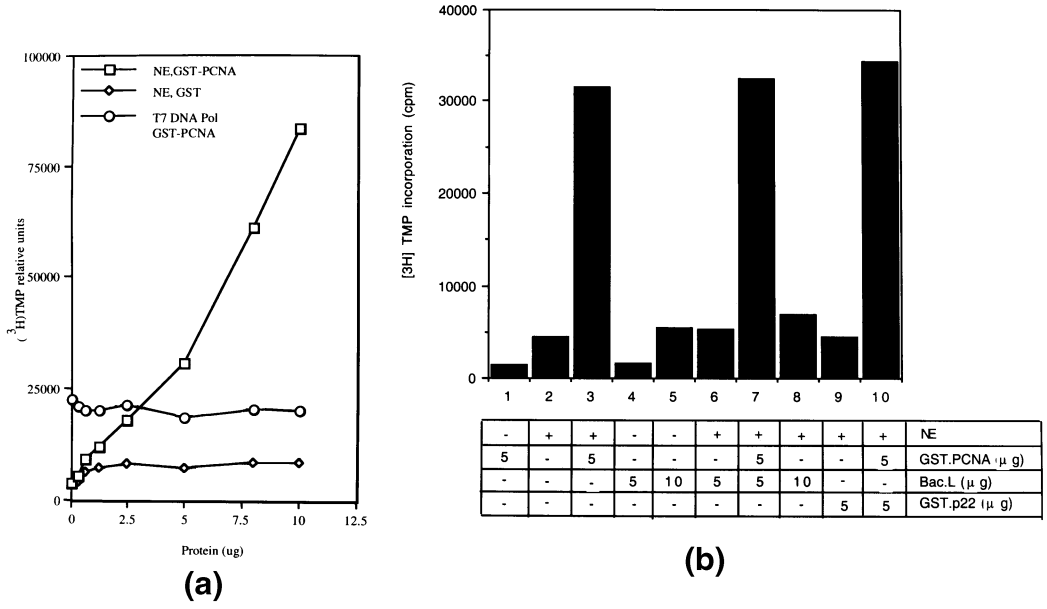

Figure 2. PCNA activates specific DNA-polymerase.
(a) Stimulation of the pea nuclear DNA polymerase activity. The graph shows [^{3}H] TMP incorporation in cpm as an indicator of DNA polymerase activity of NE assayed either in the presence of GST/PCNA (□) or GST alone (◇). The DNA polymerase activity of 1 unit of T7 DNA polymerase is also shown (○). (b) DNA synthesis in the presence of bacterial proteins. Five or 10 μg of lysate proteins of DH5α cells (Bac.L.) and 5 μg of GST.p22 were tested for DNA synthesis in various combinations with NE DNA polymerases as well as GST.PCNA. The p22 is a C-terminal fragment of a chloroplast protein p43 and is tagged with GST.

Dnase-1 nicked calf thymus DNA (i.e. ACTD or activated calf thymus DNA), consisting of only short gaps, has previously been shown to be a template valid for DNA polymerase α (Wang, 1991) and was therefore used in this study. Approximately 2.5-fold stimulation of DNA polymerase activity of one unit of NE preparation was observed with 12 μg of fusion protein.

The possibility of stimulation caused by trace amounts of bacterial contaminants that might remain associated with GST.PCNA following purification was also examined. Accordingly, the *E. coli* sonic lysate was prepared, mixed with NE, and the DNA synthesis of the resulting mixture was assayed. A comparison among columns 2, 5 and 8 of Figure 2(b) reveals that the activities of DNA polymerase present both in *E. coli* as well as pea nuclear extract could at best be synergistic. It is also apparent from Figure 2(b) that, unlike GST.PCNA, bacterial components could not stimulate pea nuclear DNA polymerase activities.

The trimeric form of fusion protein is involved in the stimulation of the DNA polymerase activity

Since the recombinant pea PCNA contained an upstream GST tag along with other 11 amino acids, it was necessary to examine the oligomeric status of the fusion protein and, therefore, glycerol gradient sedimentation of 100 μg GST.PCNA was carried out. Because a large amount of protein was required, only the refolded fusion protein was used. Prior to this the functionality of the refolded GST.PCNA was tested. About two- to fourfold excess protein was required for DNA polymerase stimulation activity.

A visual inspection of Figure 3 reveals that about 25% of the refolded protein existed as trimer. The dimer was the most abundant of the prevailing three forms, although the trimer was most efficient in stimulating pea DNA polymerases.

Pea PCNA interacts with pea Topo I enzyme

Apart from the DNA polymerase apparatus, no other protein related to DNA replication is known that interacts with PCNA. Although there is indirect evidence of interaction between recombinant human PCNA and Topo I enzyme of fetal calf thymus extract (Loor *et al.*, 1997), the direct interaction between these two proteins was not established. Pea cDNA encoding the nuclear type I DNA topoisomerase has been cloned in our laboratory previously (Reddy *et al.*, 1998). Consequently, the recombinant type I pea topoisomerase was prepared and

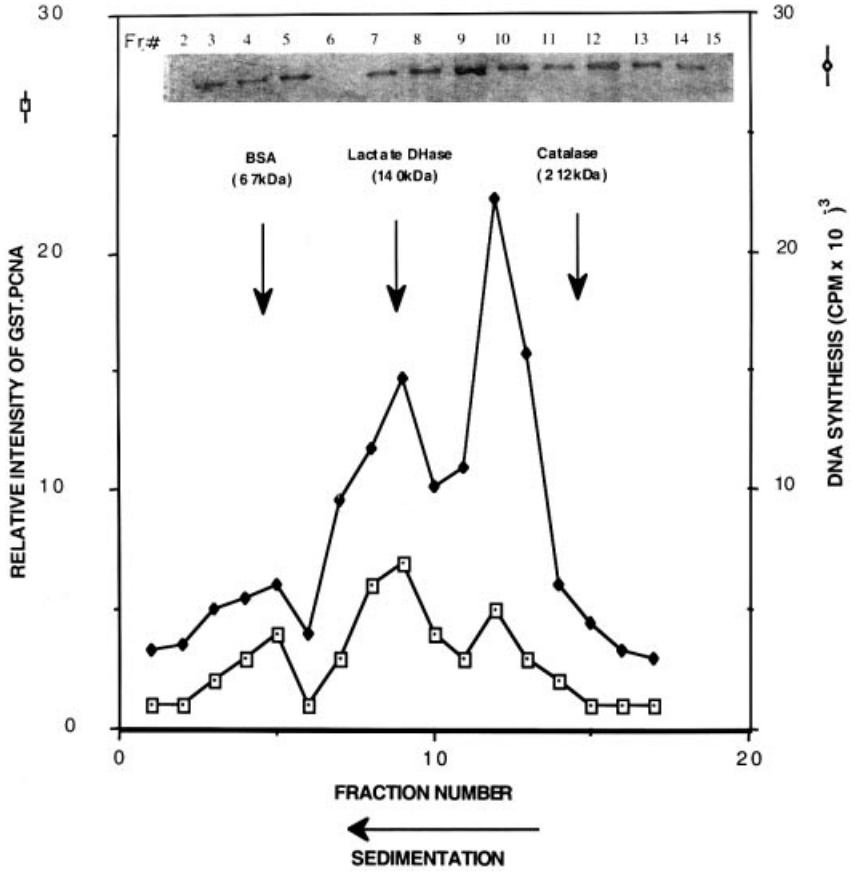

Figure 3. Fractionation of GST.PCNA fusion protein on glycerol gradient.
All the fractions were processed on a 10% SDS gel. The arrow at the bottom shows the direction of the sedimentation. The left hand *Y*-axis shows the densitometric scan of the Coomassie blue stained gel. The appropriate portion of the gel has been shown as an inset. The right hand *Y*-axis corresponds to stimulation of the DNA polymerase activity of various glycerol gradient fractions. Positions of the standard protein M.W. markers such as BSA (67 kDa), lactate dehydrogenase (140 kDa) and catalase (212 kDa) are shown with vertical arrows pointing downwards.

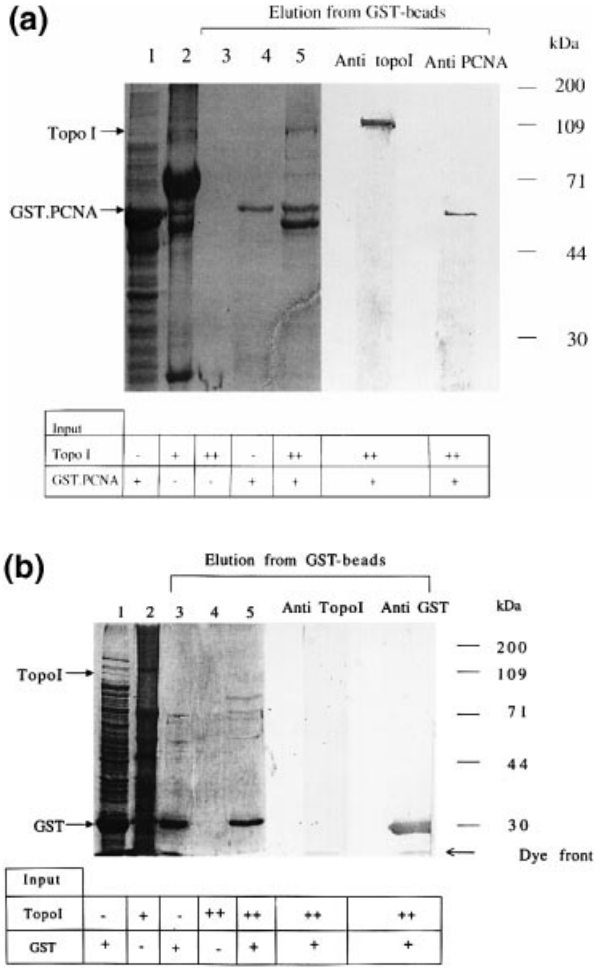

Figure 4. Profile of the proteins eluted from glutathione-beads.
(a) Lanes 1–5 show a Coomassie stained gel containing various proteins. Two separate immunoblots of the proteins present in lane 5 are shown on the right side. Lane 1, about 50 μg *E. coli* proteins including overexpressed GST.PCNA. Lane 2, about 35 μg proteins containing recombinant pea Topo I. The darkest band represents 20 μg BSA. Lane 3, proteins eluted when 70 μg of partially pure pea Topo I were input. Lane 4, proteins eluted when the input was 50 μg of partially pure GST.PCNA. Lane 5, proteins eluted when the complex of 50 μg partially pure GST.PCNA and 70 μg of partially pure pea Topo I was applied to the beads. (b) Same as in (a) except that GST was used in place of GST.PCNA. A different batch of recombinant pea Topo I was also used.

examined for its ability to interact with pea PCNA using three independent approaches.

(i) Interaction trapped by beads

In this approach, the glutathione sepharose beads were allowed to bind to *E. coli* proteins containing the recombinant pea Topo I, GST.PCNA or the recombinant pea Topo I along with GST.PCNA. Bound proteins were eluted with reduced glutathione. The partially pure recombinant pea Topo I failed to bind to the beads (lane 3, Figure 4a). The most abundant protein in the input lane of Topo I (lane 2) was BSA which was used as a stabilising carrier protein for pea Topo I. As expected, however, GST.PCNA was specifically retained from the partially pure proteins by the beads, as shown in lane 4. However, when the mixture of partially pure GST.PCNA and pea Topo I were allowed to bind, pea Topo I was retained and co-eluted with GST.PCNA (lane 5). Some unknown *E. coli* proteins also co-eluted in this process which were not detected in the immunoblots probed either with anti-Topo I or anti-GST.PCNA antibodies. In contrast, when the partially pure GST protein was pre-incubated with the partially pure pea Topo I and the affinity chromatography was carried out, only the GST protein and no Topo I remained bound to the beads (lane 5, Figure 4b). The molecular sizes of some of the minor eluted proteins (e.g. 57, 71 and 84 kDa) co-purifying with either the GST.PCNA/Topo I complex or GST were similar (compare lane 5 of Figure 4(a) with lane 5 of Figure 4(b). Such an experiment revealed that the recombinant pea Topo I interacted with pea PCNA.

Although the usage of partially pure proteins reflected the high specificity of interaction between the partners, the possibility of having a third protein mediating the interaction could not be ruled out, and hence direct interaction was examined using another approach.

(ii) Interaction on the polystyrene surface

In vitro translated and [^{35}S] labelled proteins were allowed to bind to the recombinant Topo I that was immunoimmobilised on the well of an ELISA plate. The bound proteins were eluted with 0.5 M NaCl. Figure 5(a) shows that the labelled pea PCNA eluted (lane 2) but luciferase did not (lane 4) since it failed to bind to Topo I. Various amounts of labelled proteins were allowed to bind to the fixed amount of immunoimmobilised pea Topo I and the eluted proteins were autoradiographed and quantitated as shown in Figure 5(b,c). This study clearly demonstrates a strong and direct interaction between PCNA and pea Topo I.

(iii) Interaction within NE

Since the *in vitro* synthesised pea PCNA interacted directly with the recombinant pea Topo I, we wanted to explore whether the pea PCNA and pea Topo I present in NE could form a complex. Identification of any such complex would reflect the (quasi) *in vivo* nature of the interaction. The co-immunoprecipitation method was employed to detect such an interaction.

The PCNA-interacting proteins of NE were immunoprecipitated either in the presence or absence of exogenous 1 μg GST.PCNA using the anti-GST.PCNA antibodies. The immunoprecipitated proteins were analysed in the

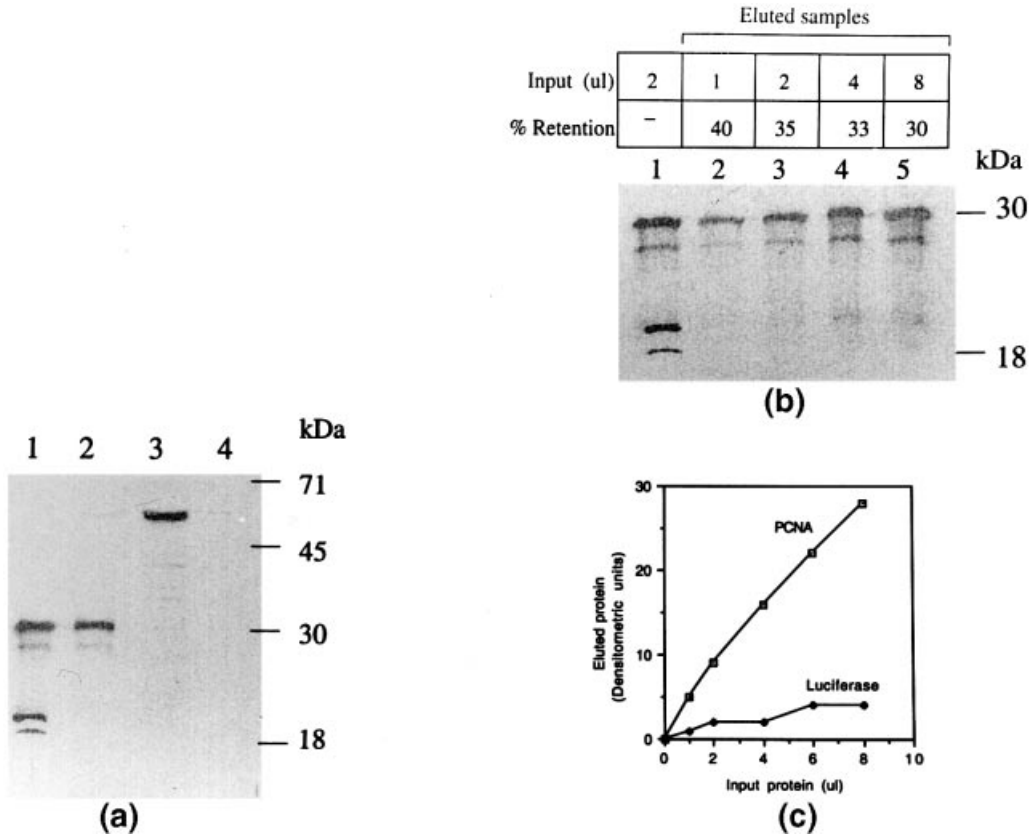

Figure 5. Interaction of PCNA with recombinant pea topoisomerase.
The figure shows autoradiographic analysis of the solid phase interaction assay. (a) Lane 1 corresponds to the input radiolabeled PCNA (2 μl). Lane 2 shows eluted PCNA when 6 μl of *in vitro* translated PCNA was applied on the well. Lane 3 shows 2 μl of *in vitro* translated luciferase. Lane 4, eluted luciferase when the input was 6 μl of *in vitro* translated luciferase. (b) Autoradiograph of eluted PCNA proteins when various amounts of labelled PCNA was used as the input. (c) Plot of intensity of autoradiographic bands of the eluted proteins in arbitrary densitometric units versus the amount of input PCNA.

immunoblots employing either the anti-pea Topo I or anti-GST.PCNA antibodies. A comparison between lanes 3 and 9 in Figure 6 reveals that both the Topo I and PCNA of NE co-precipitated, signifying the complex formation. Purified GST.PCNA was added exogenously to NE with the hope of aiding the process of complex formation. However, a comparison between lanes 2 and 3 does not reveal any additional complex formation upon exogenous addition of GST.PCNA. Figure 6 shows that GST.PCNA and the heavy chains of anti-GST.PCNA antibodies co-migrated almost together under the gel electrophoretic conditions used for this experiment. The polypeptides present in 20 μl NE were also resolved and visualised by Coomassie-stain (lane NE).

Pea PCNA suppresses the recombinant pea Topo I activity

In order to understand the functional significance of interaction, DNA relaxation activity of the recombinant Topo I was examined either in the absence or presence of soluble fusion protein. Lane 4 in Figure 7(a) shows the normal DNA ladder formation by two units of recombinant Topo I. This activity was suppressed when Topo I was pre-complexed either with 200 ng (lane 2) or 1 μg (lane 6) of GST.PCNA. Control proteins (GST) or control reaction conditions did not significantly affect the DNA relaxation activity of Topo I. Thus the association of PCNA with Topo I seems to be detrimental for the DNA relaxation activity. In order to rule out the contribution of the trace amounts of *E. coli* proteins contaminating the GST.PCNA preparation towards the repression of topoisomerisation, GST.PCNA was immunodepleted from the preparation. However, the resulting supernatant which only contained the contaminants hardly affected the ladder forming activity (data not shown).

The glycerol gradient fractions of refolded GST.PCNA proteins were used to explore the oligomeric state of PCNA affecting the DNA relaxation activity. Fractions 5, 8 and 12 (Figure 3), representing the monomeric (M), dimeric (D) and trimeric (T) forms of GST.PCNA, respectively, were used. Care was taken to uniform the effect of salt concentration and glycerol while assaying DNA relaxation with various oligomeric forms of PCNA. The

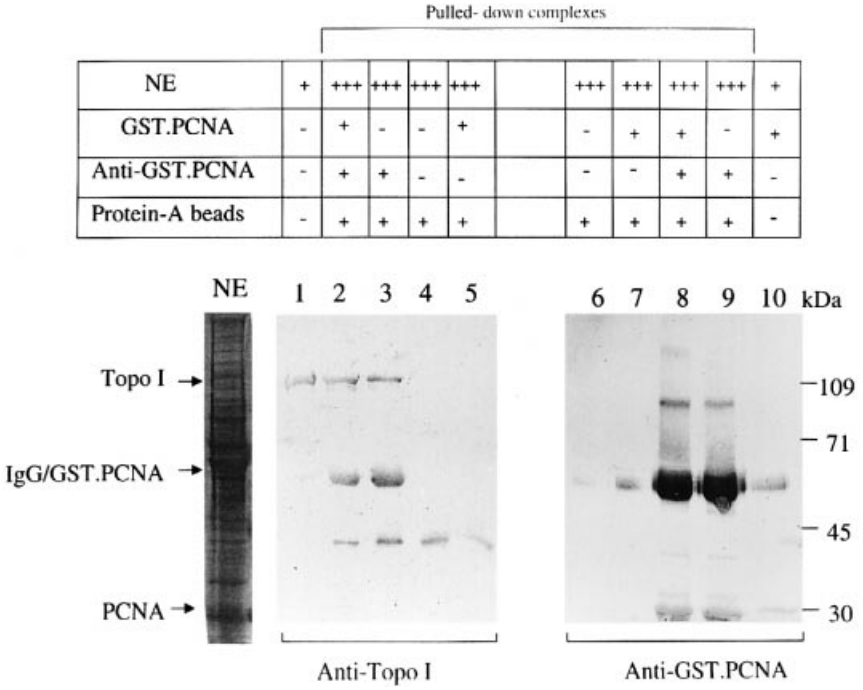

Figure 6. Co-immunoprecipitation of pea nuclear Topo I and PCNA.
Approximately 10 μg of anti-GST.PCNA antibodies were used to pull down the complex of Topo I and PCNA present in 150 μg of NE (+++). The components used in co-immunoprecipitation are indicated at the top of the figure. Precipitated complexes were immunoblotted using anti-Topo I and anti-GST.PCNA antibodies, respectively. Lanes 4, 5, 9 and 10 are the control lanes showing proteins brought down by the protein A Sepharose beads only. The positions of the relevant proteins and molecular weight standards are shown on the left and right of the figure, respectively.

usual DNA relaxation pattern of pea Topo I has been shown in lane 2 of Figure 7(b). Approximately 1 μg of monomeric GST.PCNA partially suppressed the pea Topo I activity (lane 4) while the same amount of either dimeric or trimeric fusion PCNA blocked the topo-activity almost totally (lanes 5,6). Thus it appeared that the oligomeric forms of PCNA were more efficient in suppressing the DNA relaxation activity.

GST.PCNA cannot influence DNA binding ability of recombinant pea Topo I

The inability of the complex of PCNA and Topo I to relax DNA superhelicity could be attributed to the loss of either DNA binding activity or nicking/closing, i.e. catalytic activity of Topo I. Here we have analysed the DNA binding of Topo I with or without PCNA using the protein-adsorbent filters as described by Roca and Wang (1992). About 0.5 μg of pBSK plasmid DNA was allowed to bind separately to GST.PCNA, pea Topo I or GST.PCNA plus Topo I in the binding buffer for 10 min at 30°C and the reaction mixture was passed through the filter. The unbound flow through DNA and the bound DNA that was eluted from the filter following proteolysis of the DNA binding proteins in the presence of 0.3% SDS at 42°C were analysed by agarose gel electrophoresis in the presence of ethidium bromide (Figure 8a). The amount of bound DNA in the filter measured the DNA binding ability of Topo I or any other DNA-binding proteins. Comparison between lanes 1 and 5 shows that the filter retained a very small amount of free DNA. Lanes 2 and 6 show that GST.PCNA also bound to an insignificant amount of plasmid DNA. Lanes 3 and 7 reveal that about 0.5 μg of plasmid DNA was bound completely by 1.2 μg of Topo I (approximately 3 U). Lanes 4 and 8 clearly show that 3 μg GST.PCNA could not disturb the DNA-binding activity of Topo I at all since the amount of bound DNA was not reduced in the presence of PCNA. Thus, the blockage of ladder formation shown in Figure 7 was not due to the loss of the DNA binding ability of Topo I in the presence of PCNA.

In order to probe the nature of DNA binding of a wide range of recombinant pea Topo I either in the presence or absence of GST.PCNA, we used the ^{32}P-labelled circular DNA substrates for binding. Protein-bound DNA complexes were studied, employing the method described by Pommier *et al.* (1989). The DNA bound to the filters was quantitated and expressed as the bound fraction of input DNA, as shown in Figure 8(b). Neither GST nor GST.PCNA could bind the labelled DNA effectively, but 30 ng (600 pg μl^{-1}) of pea Topo I was sufficient to bind 10 ng of labelled DNA (c.p.m. = 4×10^5) completely. Under similar binding conditions, 10 and 25 ng of Topo I was effective to bind 33% and 70% input DNA, respectively. When the Topo I amount was held fixed (at either 10 or 25 ng) with the varying amount of GST.PCNA (Figure 8b), the amount of bound DNA did not fall below the threshold level, i.e. the level observed with Topo I alone. These experiments clearly show that the complex of GST.PCNA and pea Topo I retain the DNA binding ability as efficiently as the Topo I itself.

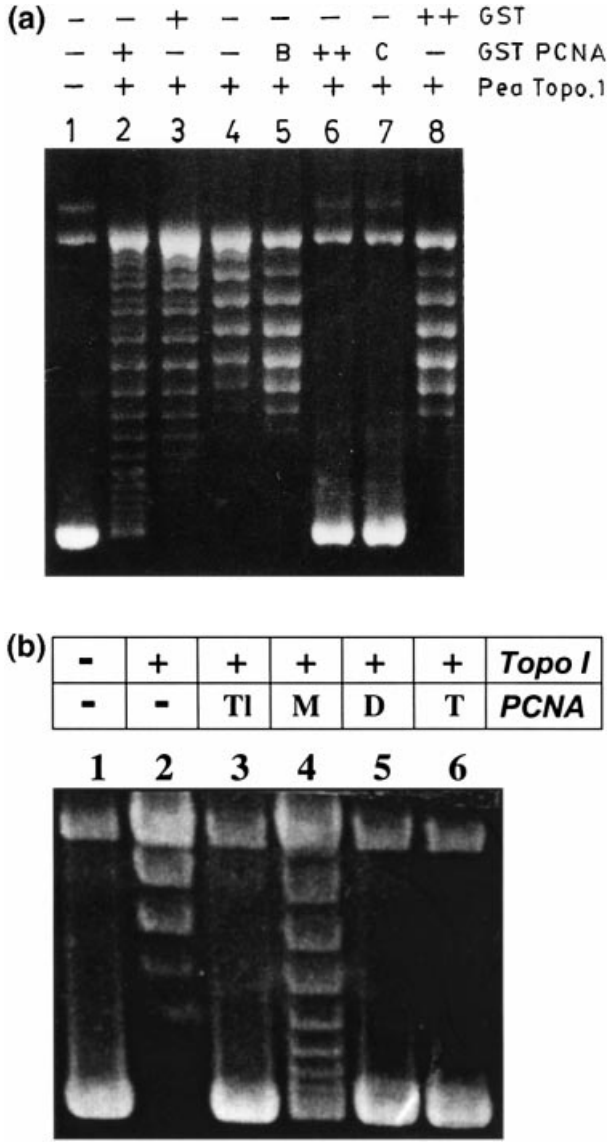

Figure 7. PCNA inhibits topoisomerisation.
(a) Suppression of DNA relaxation by PCNA. Lane 1 shows 2 μg of control pBR322 supercoiled DNA. In lanes 2–8, about two units of purified recombinant topoisomerase enzyme was used for ladder formation. Lane 2, Topo I pre-complexed with 200 ng of soluble GST.PCNA. Lane 3, Topo I with 200 ng of GST. Lane 4, Topo I alone. Lane 5, about 1 μg heat-inactivated GST.PCNA (B) added to the pea topoisomerase. Lane 6, 1 μg of the GST.PCNA with Topo I. Lane 7, 1 μg cleaved (C) GST.PCNA with Topo I. Lane 8, 1 μg purified GST precomplexed with Topo I. (b) Inhibition of pea Topo I activity with various oligomeric forms of PCNA. Lane 1, about 2 μg of pBKS supercoiled DNA. About three units of pea Topo I enzyme was used for lanes 2–6. About 1.5 μg of refolded GST.PCNA containing all the forms (Tl), 1 μg each of the monomeric (M), dimeric (D) and trimeric (T) form of GST.PCNA were used in lanes 3, 4, 5 and 6, respectively.

Discussion

In this paper we report on cloning of the cDNA encoding pea PCNA and its expression. The recombinant pea PCNA significantly stimulated the pea DNA polymerases. Since PCNA from other sources are known to stimulate δ and ε types of DNA polymerases, and the template choice for the reported DNA synthesis experiments was specific for the above-mentioned polymerases, it is quite likely that the recombinant PCNA probably activated similar polymerases of pea. The recombinant PCNA would probably also not have undergone any post-translational modifications and hence these may not be necessary for the activation of DNA polymerases.

The stretches of N-terminal 8 amino acids and C-terminal 12 amino acids are crucial for maintenance of the native trimeric structure of PCNA (Fukuda *et al.*, 1995). As the recombinant pea PCNA contained the upstream GST and additional 11 amino acids, it was essential to determine the folding status of the recombinant GST.PCNA. The glycerol gradient sedimentation analysis revealed that only a fraction (0.25) of the refolded population formed trimers. This finding implicates that the native or non-fusion form of pea PCNA would probably be more efficient than the fusion form in stimulating pea DNA polymerases as the native protein is expected to be totally trimeric in structure.

The dimeric GST.PCNA was most abundant in the mixed population and also stimulated the pea DNA polymerases. Bacterial GST proteins are known to form homodimers (Nishida *et al.*, 1998) and the abundance of GST.PCNA dimers could thus be explained in view of the homodimerisation property of bacterial GST. Some of the dimers might also convert to trimers in the presence of template DNA and the DNA polymerase apparatus, and thus GST.PCNA dimers might also partially stimulate DNA polymerases.

Using three independent approaches, we observed that pea PCNA was competent to form a complex with pea Topo I. The *in vivo* significance of this interaction remains to be ascertained, however, particularly in view of the promiscuity of PCNA in forming complexes with a host of other cellular factors. It is apparent from Figure 7 that the complex of PCNA and Topo I would keep Topo I in an inactive state. As the *in vivo* concentration of Topo I is not usually high, the PCNA.Topo I complex might be useful for the cell. The complexed form might offer a storage mechanism for the Topo I activity which is utilized whenever necessary by allowing dissociation of the complex. For example, following the complex formation at the pre-replicative stage, PCNA may be taken up from the complex as one of the fork-associated proteins during ongoing DNA replication because the free energy of interaction between PCNA, RFC and polymerase δ is expected to be higher than that between PCNA and Topo I (Bovayadi *et al.*, 1997; Yao *et al.*, 1996). In this event, the decoupled Topo I would be functional at the flanking ends of DNA replication forks to facilitate DNA replication. As soon as the DNA replication fork reaches the terminus, however, Topo I might pull

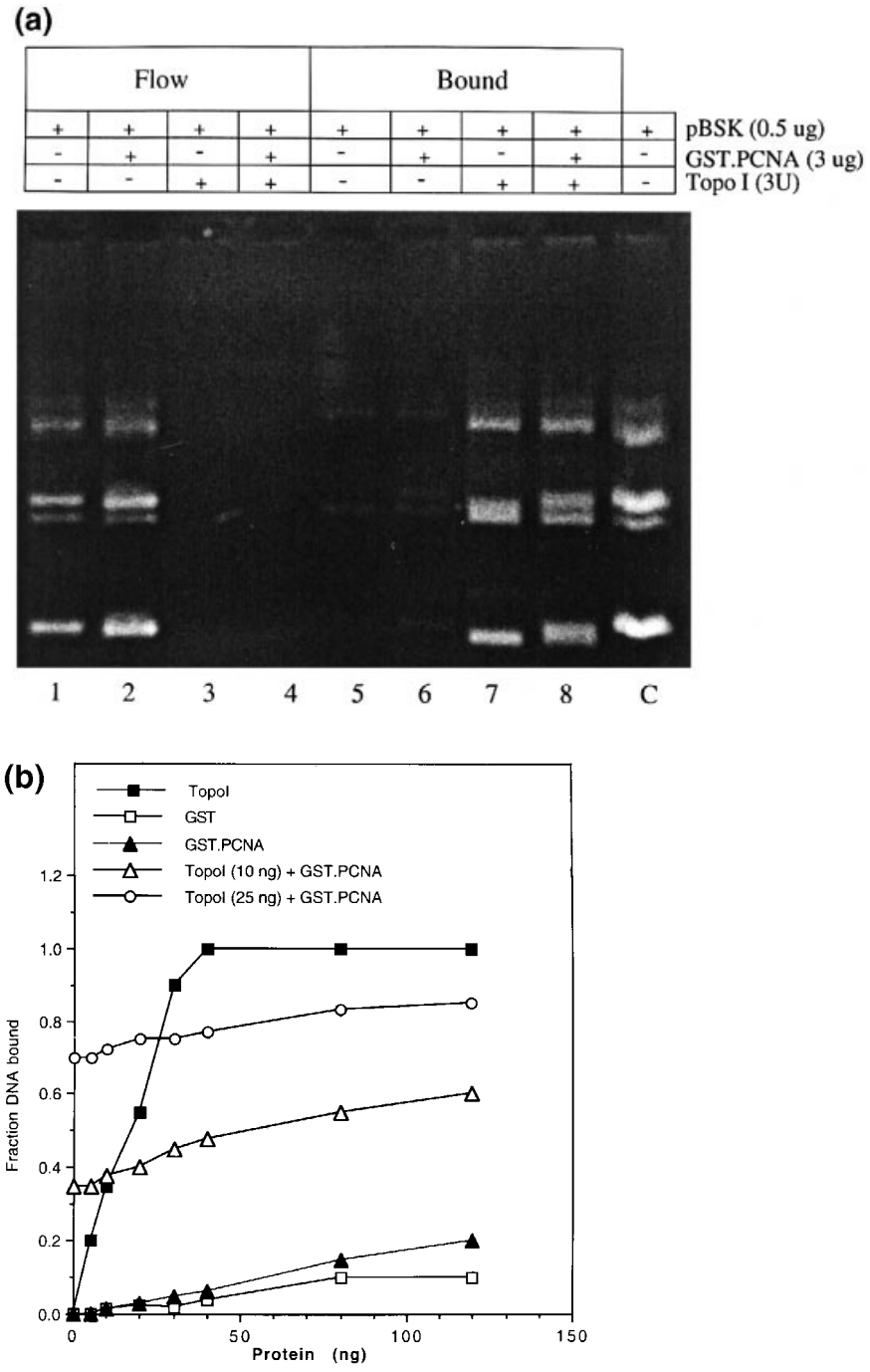

Figure 8. Binding of PCNA and Topo 1 complex to DNA.
(a) Agarose gel electrophoresis of DNA processed through filters. The contents of the various lanes have been shown at the top of the figure. C, control DNA which was not processed through the filter. The minor variations in the amount of DNA of pairs of lanes such as 1,2 and 7,8 could be traced to DNA purification steps. (b) Formation of protein–DNA complexes. The radioactivity of DNA was quantitated by Cerenkov counting. The symbols at the top of the graph show the various proteins that were used for DNA binding. When mixtures of Topo I and PCNA were used, the amount of Topo I was kept constant and the concomitant amount of GST. PCNA was spread over a wide range.

PCNA out of the stalled replisome and thereby facilitate recycling of the process of further fork formation and DNA synthesis (Yao *et al.*, 1996).

Many cellular proteins interact with PCNA using a PCNA-binding motif (Warbrick *et al.*, 1998). Since pea Topo I does not possess such a motif, the mechanism of Topo I binding to PCNA may be different from proteins such as p21, Fen1, XPG, etc. Topo I binding to PCNA resulted in the loss of DNA relaxation activity (Figure 7) without affecting the DNA binding of pea Topo I (Figure 8). As it appears that only the catalytic site of Topo I may be affected as a result of this binding, it would be interesting to explore whether the PCNA binding site of Topo I is located near its catalytic site.

Experimental procedures

Screening and isolation of cDNA

The conserved peptide sequences chosen were FEMKLMDID and APKIEEDE, respectively. The sequences of two oligonucleotides

for PCR were 5′TTYGARATGAARYTIATGGAYATHGA3′ for forward primer and 5′YTCRTCYTCYTCDATYTTNGGWGC3′ for the reverse primer, respectively. PCR and DNA sequencing were carried out using standard procedures.

The purified 450 bp amplified fragment was used to screen the plasmid-based pea cDNA library by colony hybridization using nylon membranes (Hybond N, Amersham). Four independent clones with a strong signal were obtained and purified.

Overexpression of the recombinant pea PCNA protein in E. coli *and purification of the fusion protein*

The purified pea *pcna* was excised as *Sma*I/*Xho*I fragment and recloned in pGEX4T1 vector (Pharmacia). Besides the glutathione S-transferase (GST) protein, another 11 amino acids were added at the N-terminal region of pea PCNA by virtue of the construction of the expression clone.

The clear sonic lysate of IPTG-induced *E. coli* cells was used to purify GST or GST tagged proteins using Glutathione Sepharose 4B (Pharmacia, Sweden) beads following the manufacturer's protocol. Inclusion bodies isolated from the sonic pellet were resolubilised and the fusion proteins were allowed to refold using conventional procedures. DNA synthesis assay was carried out to test the functionality of refolded GST.PCNA.

Raising antibodies against GST.PCNA

Purified GST.PCNA was used to generate antiserum in rabbits following standard protocols. Anti-GST.PCNA antiserum was adsorbed with overexpressed GST protein along with other *E. coli* proteins. The antiserum was then used to purify antibodies using protein A Sepharose column (Pharmacia).

Preparation of pea nuclear extract (NE)

Pea nuclear extract was made essentially as described by Dignam *et al.* (1983). The extract was subjected to a one step DE-52 column chromatographic step to remove excess nucleases.

DNA synthesis

Incorporation of [^{3}H] TMP [specific activity of [^{3}H] TTP = 5×10^{12} cpm mmole^{-1} (Amersham)] was carried out (Chen *et al.*, 1996). PolydA_{400} and oligodT_{15} (Pharmacia) were annealed at a molar ratio of 1 : 1 to prepare the template.

Determination of oligomeric forms of the fusion protein

Approximately 100 μg of refolded PCNA.GST fusion protein was applied on a pre-equilibrated 15–45% glycerol gradient in 50 mM Tris–HCl pH 8.0, 500 mM KCl, 1 mM EDTA, 2 mM β mercaptoethanol, and a set of protease inhibitors consisting 1 mM PMSF, 1 mM sodium metabisulfite, 1 mg ml^{-1} benzamidine and 1 mM pepstatin alongwith standard protein markers. Centrifugation was carried out at 200 000 ***g*** for 24 h in an SW41 rotor using Beckman L8–70 M centrifuge. About 0.3 ml fractions were collected from the bottom of the tube and about a 60 μl sample was resolved on a 10% SDS-PAGE and the gel was visualized by Coomassie staining. Intensity of the stained band was quantitated by laser densitometric scanning. Aliquots of 10 μl of each fraction were also taken for DNA synthesis assay.

In vitro translation of proteins

The full-length cDNA of *pcna* was cloned into pSGI vector (modified from, Stratagene). For *in vitro* transcription and translation of these genes, commercially available TnT coupled Wheat germ lysate from Promega along with [^{35}S] Methionine (specific activity 1000 Ci mmol^{-1}, DuPont NEN) was used, following the instructions from the manufacturer (Promega).

Solid phase assay for Topo I–PCNA interaction

Approximately 500 μg antibodies of recombinant pea Topo I was allowed to adsorb onto the polystyrene surface of the wells of an ELISA plate in 50 μl B1 buffer (50 mM Tris, pH 8.0, 10 mM β mercaptoethanol, 10% glycerol and protease inhibitors) with 150 mM NaCl for 2 h at 37°C. The wells were then blocked with 1% BSA. About 20 units of purified recombinant pea Topo I in 50 μl of B1 buffer was allowed to bind to the antibodies for 1 h at room temperature. Indicated amounts of *in vitro* translated labelled protein in 50 μl of B1 buffer were added on the wells. Unbound labelled protein was washed three times with 200 μl of B1 buffer containing 300 mM NaCl. The labelled protein was eluted with 50 μl of B1 buffer containing 500 mM NaCl, TCA precipitated, resolved on SDS-PAGE and subjected to autoradiography. Various lanes of the autoradiograph were also subjected to densitometric scanning.

DNA relaxation assay of Topoisomerase I

Purification and assay of the recombinant pea topoisomerase I was performed (Reddy *et al.*, 1998). One unit of enzyme activity was defined as the amount required to relax 50% of 2 μg pBR322 supercoiled DNA in 15 min at 30°C.

Protein mediated DNA binding to the adsorbent filters

Plasmid DNA-Topo I complexes were applied to a protein-adsorbent filter (filter type: DA, pore size 0.65 μM, Millipore) and experiments were carried out as described previously (Roca and Wang, 1992).

Protein titration experiments were performed with ^{32}P-labelled circular DNA (Figure 8b). The pBSK plasmid DNA was digested with *Xma*1 enzyme and the ends were repaired by Klenow enzyme in the presence of [α^{32}P] dCTP. Blunt-end ligation was carried out by T4 DNA ligase to obtain the labelled circular DNA (sp. activity approximately 4×10^7 c.p.m. μg^{-1}). About 10 ng of this DNA was mixed with various amounts of protein for 15 min at 30°C in the presence of the 50 μl reaction buffer. The reaction mixture was applied to DA (Millipore) protein-adsorbent filters and processed as mentioned previously (Pommier *et al.*, 1989).

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GenBank accession number Y16796 (*pcna* from *Pisum sativum*).