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Identification of DNA replication and cell cycle proteins that interact with PCNA

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ABSTRACT

The identity of DNA replication proteins and cell cycle regulatory proteins which can be found in complexes involving PCNA were investigated by the use of PCNA immobilized on Sepharose 4B. A column containing bovine serum albumin (BSA) bound to Sepharose was used as a control. Fetal calf thymus extracts were chromatographed on PCNA–Sepharose and BSA–Sepharose. The columns were washed and then eluted with 0.5 M KCl. The salt eluates were examined for the presence of both DNA replication proteins (Pol α, δ, ε, PCNA, RFC, RFA, DNA ligase I, NDH II, Topo I and Topo II) and cell cycle proteins (Cyclins A, B1, D1, D2, D3, E, CDK2, CDK4, CDK5 and p21) by western blotting with specific antibodies. The DNA replication proteins which bound to PCNA–Sepharose included DNA polymerase δ and ε, PCNA, the 37 and 40 kDa subunits of RFC, the 70 kDa subunit of RPA, NDH II and topoisomerase I. No evidence for the binding of DNA polymerase α, DNA ligase I or topoisomerase II was obtained. Of the cell cycle proteins investigated, CDK2, CDK4 and CDK5 were bound. This study presents strong evidence that PCNA is a component of protein complexes containing DNA replication, repair and cell cycle regulatory proteins.

INTRODUCTION

The discovery of a stimulating factor for DNA polymerase δ (1,2) that eventually led to its identification as proliferating cell nuclear antigen (PCNA) (3) stimulated major advances in our understanding of DNA synthesis at the replication fork. PCNA functions as a sliding clamp which endows pol δ with a high degree of processivity (4). Studies of *in vitro* SV40 DNA replication have now led to a fuller understanding of the protein machinery required for the formation of a functional mammalian DNA replication fork in which DNA polymerase δ (pol δ) and PCNA play a central role. The current model is one in which replication factor C (RFC, also known as activator-1), a complex of five subunits, first binds to the primer-template terminus and loads the PCNA onto the 3′ hydroxyl end of the primer strand of the DNA primer-template in an ATP-dependent process. Following the formation of a RFC/PCNA complex, pol δ is then recruited to assemble an elongation complex that catalyzes DNA synthesis in the presence of deoxynucleotide triphosphates (5–9). Replication protein A (RPA), a ssDNA binding protein, is involved in both initiation and elongation, as it stimulates pol δ activity in the presence of RFC and PCNA (6,10). A DNA helicase activity is essential to the replication machinery and serves mainly to unwind replication origins during the initiation phase of DNA replication and to separate parental DNA strands during the elongation phase. A helicase which is highly associated with pol δ has been isolated (11). Recently, six human helicases have been purified to near homogeneity (12). Furthermore, a nuclear DNA helicase II (NDH II) has also been purified (13). Like the large T antigen of SV40, it was found to unwind both DNA and RNA. Molecular cloning of NDH II revealed a high homology to human RNA helicase A (14). Pol α/primase is primarily involved in the synthesis of RNA primers plus short stretches of DNA primers on the lagging strand, while the actual elongation of the primers is performed by pol δ in a process requiring polymerase ‘switching’ (15). A topoisomerase activity is also required, and studies using the SV40 system showed that either topoisomerase I or topoisomerase II is capable of removing positive supercoils ahead of the replication fork (16,17). The model of the protein assembly at the replication fork now resembles that of the well defined prokaryotic systems, requiring the presence of two pol δ molecules (18).

A major area of research which is currently emerging is the exploration of the biochemical and genetic mechanisms by which cell cycle regulation of DNA synthesis is achieved. There have been rapid advances in delineating the existence of cell cycle proteins: these include the cyclins A and B, a family of G1 cyclins (E, D cyclins) and a family of cyclin dependent kinases (CDKs) (19,20). There is now evidence for the cell cycle control of mammalian DNA replication by the cyclin–CDK system (21). A number of studies point to the existence of protein–protein interactions of DNA synthesis proteins with cell cycle dependent protein kinases or cyclins, as well as the phosphorylation of DNA synthesis proteins by CDKs. DNA polymerase α is phosphorylated in a cell cycle specific manner and is a substrate for $p34^{cdc2}$ (22,23). The RPA complex purified from HeLa cells or Manca cells is also phosphorylated in a cell cycle dependent manner by one or more members of cyclin/CDK2 family, and its phosphorylation has been shown to stimulate the initiation of SV40 DNA synthesis *in vitro* (24,25). Recent studies by Pan *et al.* (26) showed that both CDK2/cyclin A and DNA-dependent protein

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kinase phosphorylate the 34 kDa subunit of RPA. However, phosphorylated and unphosphorylated forms of RPA were equally active in SV40 DNA replication and nucleotide excision repair (26). Using immunoprecipitation and western blot experiments, Xiong *et al*. (27,28) showed combinatorial interactions of D type cyclins, cyclin-dependent kinases with PCNA and with p21. p21, also known as WAF1, CIP1 or Sdi1, is an inhibitor of the CDKs that control the initiation of the S phase of the cell cycle and DNA replication. The N-terminal region of p21 contains the CDK inhibitory domain whereas the C-terminal region contains a PCNA binding domain that leads to the inhibition of DNA synthesis (29).

In addition, both pol δ and PCNA have been shown to be required for DNA repair (30). Thus, PCNA, through its interactions with elements of both the DNA replication apparatus and the cell cycle regulatory system, has emerged as an important locus for protein–protein interactions that may provide communication between DNA replication, DNA repair and cell cycle control. Definition of the number and nature of these protein–protein interactions will therefore be important. In this study, immobilized recombinant PCNA is used as a means for the isolation of proteins that bind to PCNA.

MATERIALS AND METHODS

Immunoblotting

After electrophoresis in 5–15% gradient gels, proteins were transferred to nitrocellulose membranes. Prestained protein standards (Sigma Chemical Co.) were used as molecular weight markers and provided visual confirmation of efficient transfer. The nitrocellulose blots were incubated with 3% BSA in phosphate-buffered saline as a blocking agent. The blot was then incubated with the primary monoclonal antibody at a final concentration of 5 µg/ml or with a polyclonal antibody at ~1:500 dilution for 12 h at 25°C. After washing, the blot was incubated with biotinylated sheep anti-mouse immunoglobulin, followed by streptavidin-biotinylated peroxidase preformed complex. When polyclonal antibodies were used, the second antibody was anti-rabbit IgG biotinylated species-specific whole antibody instead of anti-mouse IgG. Color development was performed by incubation with 4-chloro-1-napthol and hydrogen peroxide and was terminated with sodium azide.

Antibodies used were as follows: polyclonal antibodies against the p145, p40, p37 and p38 subunits of RFC (Dr J.Hurwitz, Memorial Sloan Kettering Cancer Center, NY); polyclonal antibodies against the p70 and p11 subunits of RPA and monoclonal antibody against the p34 subunit of RPA (Dr S.H.Lee, St. Jude's Children's Hospital, Memphis, TN); polyclonal antibodies to human topoisomerase I and II (ToPoGen Inc.); PCNA monoclonal antibody mAB19F4 (American Biotech. Inc., Plantation, FL); p21 monoclonal antibody (Santa Cruz); monoclonal antibody against DNA polymerase ε (Dr J.E.Syvaoja, University of Oulu, Finland); monoclonal antibody against polymerase α (American Type Culture Collection); DNA ligase I polyclonal antibody (Dr A.Tomkinson, University of Texas Health Science Center at San Antonio); rabbit antiserum to mouse cyclins D1, D2 and D3 (Dr C.J.Sherr, St. Jude's Children's Hospital, Memphis, TN); monoclonal antibodies to cyclins A, B1 and E and to both CDK2 and CDK5 (Dr E.Lee, Massachusetts General Hospital, Boston); polyclonal antibody to CDK4 (Dr S.Hanks, Vanderbilt University, TN); monoclonal antibody to NDH II (Dr F.Grosse, Heinrich-Pette Institute for Experimental Virology and Immunology, Germany).

Preparation of PCNA and BSA affinity columns

Recombinant PCNA was overexpressed in *E.coli* and purified to homogeneity as previously described (31). Activated CH–Sepharose, which allows coupling to a six carbon spacer arm, was obtained from Pharmacia LKB Biotech. Purified recombinant PCNA (20 mg in 50 ml) was dialyzed against 2 l of 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.0 (four changes at 6–8 h intervals). Activated CH–Sepharose 4B (2 g) was suspended in 30 ml of cold 1 mM HCl. The gel was washed in a column with 400 ml of cold 1 mM HCl followed by 200 ml of 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.0. The coupling reaction was performed by addition of PCNA (20 mg, 50 ml in 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.0) to the washed gel. The suspension was rotated end over end for 18 h at 4°C. The protein content of the supernatant was checked at intervals by absorbance at 280 nm to monitor the progress of the reaction. After an overnight reaction it was estimated that ~2 mg PCNA was bound/ml of gel. The suspension was centrifuged and the supernatant discarded. The gel was then suspended in 50 ml of 1 M ethanolamine, pH 9.0 for 18 h to block unreacted groups. The gel was washed with 200 ml of 1 M NaCl–0.1 M sodium acetate, pH 6.0 followed by 200 ml of 1 M NaCl–0.1 M Tris–HCl, pH 8.0 and 200 ml of 0.5 M NaCl–0.1 M Tris–HCl, pH 8.0. The gel was equilibrated with TGEED buffer (50 mM Tris–HCl, pH 7.8, 10% glycerol, 0.5 M EDTA, 0.1 mM EGTA and 1 mM dithiothreitol). A control column in which bovine serum albumin (BSA) was substituted for PCNA was prepared by the same procedure. In this case ~8.4 mg of BSA were coupled to 5 ml of activated CH–Sepharose 4B. All operations were performed at 4°C.

Preparation of calf thymus extracts

Frozen fetal calf thymus tissue (10 g) was used to prepare 50 ml of tissue extract. The tissue was homogenized in a blender with 50 ml of lysis buffer (50 mM Tris–HCl, pH 7.8, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM $MgCl_2$, 0.25 M sucrose, 10% glycerol, 10 mM KCl, 0.1 mg/ml soybean trypsin inhibitor, 1 mM benzamidine, 0.1 mg/ml bacitracin, 1 mg/ml pepstatin A, 1 mg/ml leupeptin, 0.2 mM phenylmethyl sulfonyl fluoride and 10 mM sodium bisulfite). The homogenate was centrifuged for 1 h at 15 000 *g*. The extract was then centrifuged at 100 000 *g* for 1 h. All operations were performed at 4°C.

Affinity chromatography

Affinity chromatography was performed by mixing 50 ml of calf thymus extract with the PCNA–Sepharose (5 ml) and rotating the suspension end over end for 2 h. The gel was then packed into a column and washed with 100 ml of 50 mM KCl in TGEED buffer and eluted with 0.5 M KCl in TGEED buffer. Fractions of 0.3 ml were collected. Control experiments in which immobilized BSA was used as the column support was performed in parallel.

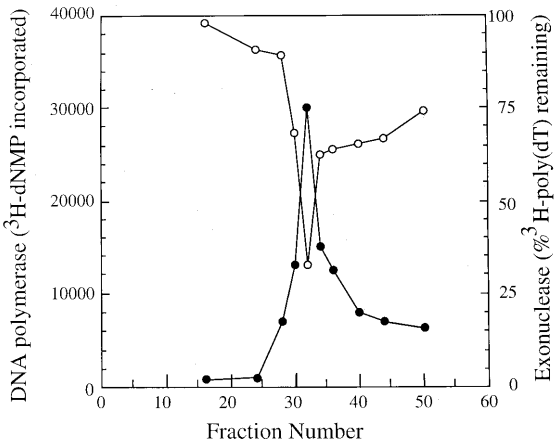

Figure 1. Affinity chromatography of calf thymus extract on PCNA–Sepharose. Crude calf thymus extract (50 ml) was rotated end over end with 5 ml of PCNA–Sepharose for 2 h. The gel was then packed onto a column, washed with 100 ml of 50 mM KCl TGEED buffer and stripped with 0.5 M KCl in TGEED (Materials and Methods). Fractions (0.3 ml) were collected and assayed for polymerase activity using poly dA/oligo dT as a template in the presence of PCNA (closed circles) and for exonuclease activity using [^{3}H]dT$_{50}$ (open circles) as previously described (1).

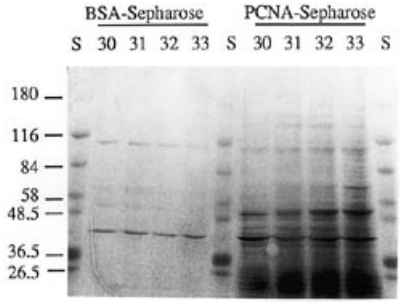

Figure 2. SDS–gel electrophoresis of proteins bound to PCNA and BSA affinity columns. Fractions 30–33 from the BSA-control column and the PCNA column were subjected to SDS–PAGE and stained for protein. From left to right are fractions 30–33 from the BSA control column, followed by fractions 30–33 from the PCNA column. The latter fractions correspond to the peak of pol δ activity. S: pre-stained protein standards (Sigma Chem. Co., α-2-macroglobulin, 180 kDa; β-galactosidase, 116 kDa; fructose 6-phosphate kinase, 84 kDa; pyruvate kinase, 58 kDa; fumarase, 48 kDa; lactate dehydrogenase, 36 kDa; triosephosphate isomerase, 26 kDa).

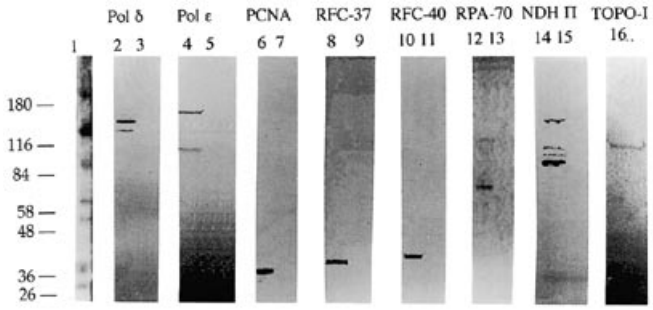

Figure 3. Immunoblots against DNA replication proteins bound to the PCNA affinity column. Lane 1: molecular weight markers with weights in kDa as indicated. Lanes 2, 4, 6, 8, 10, 12 ,14 and 16 each illustrate fraction 32 from the PCNA column western blotted against pol δ, pol ε, PCNA, RFC-37, RFC-40, RPA-70, NDH II and topoisomerase I antibodies, respectively. Lanes 3, 5, 7, 9, 11, 13 and 15 depict fraction 32 eluted from the BSA column and blotted against the same antibodies. Immunoblots were performed as described in Materials and Methods.

RESULTS

Affinity purification of DNA replication complex on PCNA–Sepharose

Calf thymus extracts were chromatographed on PCNA–Sepharose as described in Materials and Methods. The eluted fractions were assayed for DNA polymerase δ activity using poly dA/oligo dT as a template and for exonuclease activity using [^{3}H]dT$_{50}$. Preliminary experiments established that pol δ was bound to the column and was eluted at ~250 mM KCl when a KCl gradient was applied (not shown). A standard protocol was then used in which the bound material was eluted with 0.5 M KCl (Materials and Methods). No activity was detected in the flow through fractions, and both DNA polymerase and 3′→5′ exonuclease activities eluted together and were only detected in the eluate from the PCNA column (Fig. 1). (No activity was bound to a control BSA–Sepharose column when tested with a calf thymus extract.) The SDS–PAGE profile of polypeptides bound to PCNA–Sepharose and eluted with 0.5 M KCl is shown in Figure 2. A number of protein bands ranging from 18 to 210 kDa were present in the eluate from the PCNA–Sepharose column. The gels shown in Figure 2 were deliberately overloaded to show the presence of all bound polypeptides. Comparison with calf thymus extracts chromatographed on a control BSA column showed that there were several bands with two prominent polypeptides (110 and 43 kDa) that also adhered to the BSA column. Experiments were also performed in the presence of 1 mM ATP, since the interaction of PCNA with RFC is ATP dependent (6,7). However, the compositions of the polypeptides that were eluted were the same in the presence or absence of ATP (not shown).

The 0.5 M KCl eluate from the PCNA column was systematically tested for the presence of other replication proteins by western blotting. Representative blots are shown in Figure 3 from a number of individual experiments. The catalytic polypeptides of pol δ and ε were found to be present by western blot analysis using specific antibodies to pol δ (Fig. 3, lane 2) and pol ε (Fig. 3, lane 4). The pol δ antibody immunoblotted a band of 125 kDa, and the pol ε antibody detected a band of 145 kDa, in agreement with the previously reported molecular mass of pol ε isolated from calf thymus extracts (32,33). DNA polymerase α was not detected in the eluates by western blotting. PCNA itself was detected in the eluate as a 31 kDa band (Fig. 3, lane 6). This could be attributed either to stripping from the column, given that PCNA is trimeric, or due to an interaction of calf thymus PCNA subunits with immobilized PCNA. Antibodies against the individual 145, 40, 37 and 38 kDa subunits of RFC revealed positive results only for RFC-37 and RFC-40 (Fig. 3, lanes 8 and 10). Western blotting was also performed using antibodies to the 70, 34 and 11 kDa subunits of RPA. A positive blot was obtained for the 70 kDa subunit (Fig. 3, lane 12). These results indicate that both RFC and RPA are bound to the PCNA column.

The nuclear DNA helicase II (NDH II) enzyme was readily detected in the 0.5 M KCl eluate by immunoblotting as a 130 kDa band and three other bands of lower molecular weight ranging from 100 to 84 kDa (Fig. 3, lane 14). The lower molecular weight bands are likely to be proteolytic products. It has been reported that limited tryptic digestion of recombinant NDH II produced active helicases with molecular masses of 130 and 100 kDa (14). The presence of topoisomerase I and II, and ligase I was also tested for by immunoblotting. Only topoisomerase I was detected in the 0.5 M KCl eluate as a 100 kDa band (Table 1 and Fig. 3, lane 16). Examination of the fractions eluted from the BSA column by western blot yielded negative results for all of the above.

It is shown in this report that, in addition to pol δ, pol ε, RFC, RPA, PCNA, nuclear DNA helicase II (NDH II) and topoisomerase I are also present in the 0.5 M salt eluate from the PCNA column (Table 1). This collective elution of various constituents of the DNA replication machinery provides direct evidence for strong interactions between these proteins that directly or indirectly involve PCNA.

Table 1. Proteins which bind to immobilized PCNA as determined by western blotting of column eluates

Protein	PCNA column	BSA column	Protein	PCNA column	BSA column
Pol α	–	–	CDK2	+	–
Pol δ	+	–	CDK4	+	–
Pol ε	+	–	CDK5	+	–
PCNA	+	–	Cyclin A	–	–
RFC-37	+	–	Cyclin B1	–	–
RFC-38	–	–	Cyclin D1	–	–
RFC-40	+	–	Cyclin D2	–	–
RFC-145	–	–	Cyclin D3	–	–
RPA-11	–	–	Cyclin E	–	–
RPA-34	–	–	p21	–	–
RPA-70	+	–			
Ligase I	–	–			
NDH II	+	–			
Topo I	+	–			
Topo II	–	–			

Presence or absence in column eluates as determined by western blotting is shown as + or – respectively.

Binding of cell cycle regulatory proteins to PCNA–Sepharose

The PCNA–Sepharose column fractions containing peak pol δ activity were also tested for the presence of proteins involved in cell cycle regulation. Some principal components were detected through a series of western blots. Positive blots were obtained for cyclin dependent kinase 2 (CDK2), CDK4 and CDK5 (Fig. 4). Neither cyclins nor p21 were detected in the eluates (Table 1).

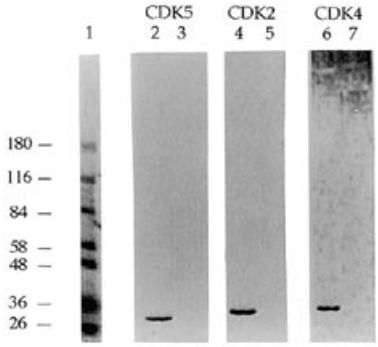

Figure 4. Immunoblots of cell cycle regulatory proteins eluting from the PCNA column. Lane 1: prestained protein markers with weights in kDa as shown. Lanes 2, 4 and 6: cell cycle regulatory proteins, CDK5 (31 kDa), CDK2 (33 kDa) and CDK4 (34 kDa), were bound by and eluted from the PCNA column. Shown also are the corresponding western blots of fraction 32 (lanes 3, 5 and 7) from the control BSA column where no CDK proteins were detected.

DISCUSSION

Recombinant PCNA was immobilized on Sepharose 4B and was systematically used to investigate the binding of replication and cell cycle proteins from fetal calf thymus extracts by affinity chromatography. As expected, tests for the binding of pol δ both by activity and by western blotting confirmed that it was bound. In addition, examination of the protein bands present in the peak of the bound fractions showed the presence of multiple polypeptide components. Some of these may represent adventitious binding although it may be noted that only a few bands with two prominent polypeptides of 110 and 43 kDa were observed in the eluates from the same fractions in the BSA control column. In addition to pol δ, several other replication proteins were bound to the affinity column. Specifically, the binding of PCNA, pol ε, RFC, RPA, NDH II and topoisomerase I was detected. The binding of pol δ and RFC was anticipated, since it is known that these proteins interact with PCNA (6). The collective elution of PCNA, pol δ, RFC and RPA was striking, in view of the fact that these are all components of the proposed replication complex involved in leading and lagging strand DNA synthesis (16,34).

The interaction of PCNA with pol ε is still controversial. Lee *et al.* (7) reported that RPA, RFC and PCNA could overcome the salt inhibition of DNA polymerase ε. Chui and Linn (35) observed strong inhibition of DNA polymerase ε by salt and found that this inhibition could not be completely overcome by RFC, RPA and PCNA which had little, if any, effect on the processivity of DNA polymerase ε. A major significance of these present findings is that they reveal a definite interaction, either direct or indirect, between pol ε and PCNA, thus linking pol ε to the replication fork. Navas *et al.* (36) have identified the DUN2 gene of *Saccharomyces cerevisiae* as DNA polymerase ε (Pol2). Mutations in the DUN2 gene displayed properties that suggest that pol ε has a role as a sensor of replication blocks and some forms of DNA damage, thus linking the DNA replication machinery to the S phase checkpoint (36). However, in *Schizosaccharomyces pombe* it was demonstrated that *cdc* 20^{+} encodes the catalytic subunit of pol ε and the gene product is required for chromosomal replication but not for the S phase checkpoint (G.D'Urso, personal communication). A pol ε holoenzyme

consisting of pol ε, PCNA, RPA and RFC may function on the lagging strand of the replication fork (37). This could provide a mechanism for proofreading in the lagging strand because pol ε, unlike pol α and similar to pol δ, has a 3′→5′ exonuclease activity (38,39). Zlotkin *et al.* (40), using UV crosslinking of nascent cellular DNA and immunoprecipitation, showed that DNA polymerase ε is essential in cellular nuclear DNA replication. Studies of *S.pombe cdc* 20^+ mutants showed that pol ε plays an important role in the elongation of nascent DNA chains, suggesting that pol ε participates in the switch from primer extension by pol α primase to leading strand synthesis (G.D'Urso, personal communication).

The presence of topoisomerase I in the eluates from PCNA–Sepharose is interesting, as it functions to relieve positive superhelicity during replication (41). The positive immunoblots for nuclear DNA helicase II (NDH II) (Fig. 3) in the peak eluates reveals the possibility of a complex involving the helicase enzyme. This is interesting in view of the fact that there may be differences between viral and host chromosomal DNA replication, so that there may be limitations of the *in vitro* SV40 replication system as a model system. Recently, a human nuclear protein that interacts with the constitutive transport element (CTE) of simian retrovirus was identified as RNA helicase A (42). The latter has a high degree of similarity to NDH II which also has RNA helicase activity (14). RNA helicase A was found to be concentrated in the nucleus in normal cells (42). It was also identified as an inherent shuttling protein that interacts with CTE *in vitro* and associates with CTE in its trafficking from the nucleus to the cytoplasm *in vivo* (42). Whether the presence of NDH II is physiologically relevant in the DNA replication complex purified from the PCNA–Sepharose affinity column is still an open question.

These findings are in concert with, and support other studies which have led to the partial purification of macromolecular complexes using conventional protein purification methods (43–45). Immobilized T4 bacteriophage gene 32 protein has been successfully used to characterize and isolate the interacting components of the T4 replication complex (46). The existence of a physical assembly of a mammalian replication complex, the 'replisome' has been inferred from studies of the prokaryotic system, and by consideration of the requirement for processive and uninterrupted DNA synthesis during replication. Evidence for the existence of such complexes is still fragmentary, and is based on the isolation of partially purified protein fractions by conventional methods that contain a number of replication proteins that can functionally replicate viral DNA (18,34). The advantage of an affinity chromatography approach over conventional methods is that it is rapid and is based on protein–protein interactions. This greatly lessens concerns that these complexes may be artifacts of the isolation methods. The current studies using affinity chromatography demonstrates the existence of a system of protein–protein interactions involving the replication proteins that could provide the molecular basis for the formation of a replication complex.

Since PCNA has been reported to bind to the cyclins (27,28), the binding of the cyclins and associated cyclin dependent kinases to the PCNA–Sepharose was also tested. Blots for the cyclin dependent kinases were positive in the case of CDK2, CDK4 and CDK5, while tests for associated cyclins were negative. This is surprising, since both CDKs and cyclins have been reported to associate in quaternary complexes with PCNA and p21 (27,28). It may be that tissue levels of the cell cycle proteins in calf thymus were too low for detection (28); also, expression of p21 occurs as a result of DNA damage (47). In general, a failure to observe any given protein in our experiments does not preclude its involvement in a replication complex, since the experiments are dependent on the sensitivity of the antibodies, the strength of the association and the stability of the given protein–protein interaction during purification. Recent studies have described several intermolecular interactions between cell cycle proteins and the replication proteins that may be of mechanistic significance in the cell cycle regulation of DNA replication. These include the demonstration that cyclin A or cyclin E–CDK complexes can trigger initiation of DNA synthesis (48), and that cyclin A is required for *in vitro* DNA replication (49). The phosphorylation of replication proteins by cyclin/CDKs has been demonstrated in the case of HSSB-p34 (50). In the latter case, cyclinA/CDK2 but not cyclinE/cdk2 was shown to phosphorylate HSSB. This critical observation indicates that targeting of the CDK2 to HSSB is necessary for phosphorylation to occur. Pol δ was reported to be phosphorylated *in vivo* (51). Recently, pol δ was found to be phosphorylated by cyclin/CDKs (Zeng and Lee, unpublished observations).

These studies which show the binding of both replication and cell cycle proteins to PCNA provides additional support for a central role of PCNA in the linkage of the processes of DNA replication and cell cycle regulation via protein–protein interactions. In addition, these findings demonstrate the existence of protein–protein interactions between DNA replication proteins and cell cycle regulatory proteins. This interaction of the cyclin–CDK cell cycle regulatory proteins with polymerases and elements of the DNA replication system could be important in understanding the cell cycle control of DNA replication. The findings that PCNA exhibits interaction with multiple protein partners suggest that it may have an important role in the formation of macromolecular complexes involved in DNA replication and its cell cycle control. For this reason, immobilized PCNA may be a particularly useful tool for the isolation of these complexes, a view that is supported by the studies reported. A molecular basis for the multiple protein partners with which PCNA interacts is now emerging, in the form of the recent identification of a short peptide consensus sequence which is found in several PCNA binding proteins including p21 (52), Fen1 (53) and cdc27 (54). There remain major questions as to the number and nature of the interacting protein partners of PCNA, and the mechanisms of how these interactions provide the necessary functional and regulatory outcomes in DNA replication and repair.

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REFERENCES

1 Lee, M.Y.W.T., Tan, C.K., Downey, K.M. and So, A.G. (1984) *Biochemistry* **23**, 1906–1913.
2 Tan, C.K., Castillo, C., So, A.G. and Downey, K.M. (1986) *J. Biol. Chem.* **2257**, 8386–8390.

3 Prelich, G., Tan, C.K., Mikostura, M., Mathews, M.B., So, A.G., Downey, K.M. and Stillman, B. (1987) *Nature (London)* **326**, 517–520.
4 Kuriyan, J. and O'Donnell, M. (1993) *J. Mol. Biol.* **234**, 915–925.
5 Tsurimoto, T. and Stillman, B. (1989) *Mol. Cell. Biol.* **9**, 609–612.
6 Lee, S.H. and Hurwitz, J. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5672–5676.
7 Lee, S.H., Pan, Z.Q., Kwong, A.D., Burgers, P.M.J. and Hurwitz, J. (1991) *J. Biol. Chem.* **266**, 22707–22717.
8 Pan, Z.Q., Chen, M. and Hurwitz, J. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6–10.
9 Chen, M., Pan, Z.Q. and Hurwitz, J. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5211–5215.
10 Kenney, M.K., Schlegell, U., Furneaux, H. and Hurwitz, J. (1990) *J. Biol. Chem.* **266**, 7693–7700.
11 Li, X., Tan, L., So, A.G. and Downey, K.M. (1992) *Biochemistry* **31**, 3507–3513.
12 Tuteja, N., Ochem, A., Taneja, P., Tuteja, R., Skopac, D. and Falaschi, A. (1995) *Nucleic Acids Res.* **23**, 2457–2463.
13 Zhang, S. and Grosse, F. (1994) *Biochemistry* **33**, 3906–3912.
14 Zhang, S. and Grosse, F. (1997) *J. Biol. Chem.* **272**, 11487–11494.
15 Tsurimoto, T., Melendy, T. and Stillman, B. (1990) *Nature (London)* **346**, 534–539.
16 Wang, J. (1988) *Annu. Rev. Biochem.* **54**, 665–697.
17 Yang, L., Wold, M.S., Li, L.L., Kelly, T.J. and Liu, L.F. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 950–954.
18 Waga, S. and Stillman, B. (1994) *Nature (London)* **369**, 207–212.
19 Norbury, C. and Nurse, P. (1992) *Annu. Rev. Biochem.* **61**, 441–470.
20 Sherr, C.J. (1993) *Cell* **73**, 10059–10065.
21 D'Urso, G., Marraccino, R.L., Marshak, D.R. and Roberts, J.M. (1990) *Science* **250**, 786–791.
22 Nasheuer, H.P., Moore, A., Wahl, A.F. and Wang, T.S.F. (1991) *J. Biol. Chem.* **266**, 7893–7903.
23 Cripps-Wolfman, J., Henshaw, E.C. and Bambara, R.A. (1989) *J. Biol. Chem.* **264**, 19475–19486.
24 Dutta, A. and Stillman, B. (1992) *EMBO J.* **11**, 2189–2199.
25 Fotedor, R. and Robert, J.M. (1992) *EMBO J.* **11**, 2177–2187.
26 Pan, Z.Q., Park, C.H., Hurwitz, J. and Sancar, A. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 4636–4640.
27 Xiong, Y., Zhang, H. and Beach, D. (1992) *Cell* **71**, 505–514.
28 Xiong, Y., Zhang, H. and Beach, D. (1993) *Genes Dev.* **7**, 1572–1583.
29 Pan, Z.Q., Reardon, J.T., Li, L., Flores-Rozas, H., Legerski, R., Sancar, A. and Hurwitz, J. (1995) *J. Biol. Chem.* **270**, 22008–22016.
30 Zeng, X.R., Jiang, Y., Zhang, S.J., Hao, H. and Lee, M.Y.W.T. (1994) *J. Biol. Chem.* **269**, 13748–13751.
31 Zhang, P., Zhang, S.J., Zhang, Z., Woessner, J.F. and Lee, M.Y.W.T. (1995) *Biochemistry* **34**, 10703–10712.
32 Siegal, G., Turchi, J.J., Myers, T.W. and Bambara, R.A. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 9377–9381.
33 Weiser, T., Gassmann, M., Thommes, P., Ferrari, E., Hafkemayer, P. and Hubscher, U. (1991) *J. Biol. Chem.* **266**, 10420–10428.
34 Hurwitz, J., Dean, F.B., Kwong, A.D. and Lee, S.K. (1990) *J. Biol. Chem.* **265**, 18043–18046.
35 Chui, G. and Linn, S. (1995) *J. Biol. Chem.* **270**, 7799–7808.
36 Navas, T.A., Zhou, Z. and Elledge, S.J. (1995) *Cell* **80**, 29–39.
37 Burgers, P.M.J. (1991) *J. Biol. Chem.* **266**, 22698–22706.
38 Lee, M.Y.W.T. and Toomey, N.L. (1987) *Biochemistry* **26**, 1076–1085.
39 Nishida, C., Reinhard, P. and Linn, S. (1988) *J. Biol. Chem.* **263**, 501–510.
40 Zlotkin, T., Kaufmann, G., Jiang, Y., Lee, M.Y.W.T., Uitto, L., Syvaoja, J., Dornreiter, I., Fanning, E. and Nethanel, T. (1996) *EMBO J.* **15**, 2298–2305.
41 Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1994) In *Molecular Biology of the Cell.* Garland Publishing, Inc., New York, ed. 3, pp. 260–262.
42 Tang, H., Gaietta, G.M., Fischer, W.H., Ellisman, M.H., Wong-Staal, F. (1997) *Science*, **276**, 1412–1415.
43 Wu, Y., Hickey, R., Lawlor, K., Wills, P., Yu, F., Ozer, H., Starr, R., Jiang, Y., Lee, M.Y.W.T. and Malkas, L. (1994) *J. Cell. Biochem.* **54**, 32–46.
44 Applegren, N., Hickey, R.J., Kleinschmidt, A.M., Zhou, Q., Coll, J., Wills, P., Swaby, R., Wei, Y., Jiang, Y., Lee, M.Y.W.T. and Malkkas, L.H. (1995) *J. Cell. Biochem.* **59**, 91–107.
45 Cao, Q.P., McGrath, C.A., Baril, E.F., Quesenberry, P.J. and Reddy, G.P.V. (1995) *Biochemistry* **34**, 3878–3883.
46 Formosa, T., Barry, J., Alberts, B.M. and Greenblatt, J. (1991) *Methods Enzymol.* **208**, 24–45.
47 Pines, J. (1994) *Nature* **369**, 520–521.
48 Krude, T., Jackman, M., Pines, J. and Laskey, R.A. (1997) *Cell* **88**, 109–119.
49 Fotedar, A., Cannella, D., Fitzgerald, P., Rousselle, T., Gupta, S., Doree, M. and Fotedar, R. (1996) *J. Biol. Chem.* **271**, 31627–31637.
50 Gibbs, E., Pan, Z.Q., Niu, H. and Hurwitz, J. (1996) *J. Biol. Chem.* **272**, 22847–22854.
51 Zeng, X.R., Hao, H., Jiang, Y. and Lee, M.Y.W.T. (1994) *J. Biol. Chem.* **269**, 24027–24033.
52 Gulbis, J.M., Kelman, Z., Hurwitz, J., O'Donnell, M. and Kuriyan, J. (1996) *Cell*, **87**, 297–306.
53 Warbrick, E., Lane, D.P., Glover, D.M. and Cox, L.S. (1997) *Oncogene* **14**, 2313–2321.
54 MacNeill, S.A., Reynolds, M.S., Nurse, P. and Fantes, P.A. (1996) *EMBO J.* **15**, 4613–4628.