A Novel PCNA-Binding Motif Identified by the Panning of a Random Peptide Display Library†

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ABSTRACT: Proliferating cell nuclear antigen (PCNA) has recently been identified as a target for the binding of proteins involved in DNA replication, DNA repair, and cell cycle control. The interactions between PCNA and a number of these proteins are known to be mediated by a conserved peptide motif. In this study, a random peptide library in which peptide sequences are displayed on the *E. coli* bacterial flagellin protein was screened for PCNA-binding sequences. Analysis of the retrieved peptide sequences verified the presence of the known PCNA-binding motif. In addition, a second, larger group of peptides containing a different consensus sequence for PCNA binding was discovered. This sequence was found to be present on DNA polymerase δ, and a peptide conforming to this sequence was demonstrated to bind to PCNA. Database search and analysis show that many proteins contain the second consensus sequence. These include proteins that are involved in DNA replication, repair, and cell cycle control. The demonstration of this second PCNA-binding motif may provide a basis for identifying and experimentally testing specific proteins for the structural basis for PCNA binding.

Proliferating cell nuclear antigen (PCNA) is a highly conserved eukaryotic protein that functions in DNA replication as a molecular sliding clamp that permits highly processive synthesis by DNA polymerase δ (pol δ) (*1*). PCNA, originally identified as a processivity factor for pol δ, has been intensively investigated both in terms of its structure and in terms of its role in cellular processes. Expression of recombinant human PCNA and its physicochemical characterization established that it is a trimeric protein (*2*), and the crystal structures of both yeast PCNA (*3*) and human PCNA (*4*) have shown that they are structurally and functionally homologous to the T4 gene 45 protein and the β subunit of *E. coli* DNA polymerase III holoenzyme DNA sliding clamps (*5, 6*). PCNA interacts with the clamp loader replication factor C (RFC) (*7*−*9*), DNA polymerase δ (*10, 11*), replication endonuclease FEN-1 (*12, 13*), and DNA ligase I (*14*) and plays a role in both leading- and lagging-strand DNA synthesis at the replication fork. During the past several years, it has been shown that PCNA also interacts with proteins involved in cell cycle progression and DNA repair. The DNA repair endonuclease XPG (*15*), the major nuclear uracil DNA−glycosylase (UNG2) (*16*), and the mismatch repair protein MSH2−MSH3 heterodimer (*17*) interact with PCNA. PCNA also binds to DNA (cytosine-5) methyltransferase (MCMT) (*18*), cyclin D (*19*), the cell cycle regulated nuclear protein Gadd45 (*20, 21*), and the cell cycle regulatory protein p21 (*22, 23*). Thus, PCNA, by virtue of its functions as a sliding clamp, may have multiple cellular functions associated with processes involving DNA modification or synthesis. Moreover, it may be a target for cell cycle regulation, as evidenced by its interactions with cyclin D, p21, p57 (*24*), and GADD45 (*20, 21*).

The ability of PCNA to interact with multiple protein partners having disparate structures is explicable at least in part through the existence of a PCNA-binding motif that is present on a number of its binding proteins (*25*). This conserved PCNA-binding motif was termed the PCNA interaction protein box (PIP-box) (*26*). An alignment of these binding motifs shows that it consists of the sequence Q-x-x-(h)-x-x-(a)-(a) (where "h" represents residues with moderately hydrophobic side chains, e.g., L, I, M; "a" represents residues with highly hydrophobic, aromatic side chains, e.g., F, Y; and "x" is any residue) (*25, 26*). Proteins which exhibit such a PCNA-binding PIP-box include p21, Fen1, XPG, and Dacapo protein, which is a cyclin-dependent kinase inhibitor (*27, 28*), and the *Pogo* transposon (*29*). In the case of p21, the structural basis for the interaction of this motif with PCNA has been determined at the atomic level by crystallographic analysis of a p21 peptide−PCNA complex (*4*). The peptide consisted of residues 139-GRKRRQTSMTDFYHS-KRRLIFS-160, and harbors the sequence QTSMTDFY which conforms to the PIP-box. The interaction of the peptide with PCNA involves three general features: interaction with a large hydrophobic pocket, a small hydrophobic pocket, and an extended interaction with the interdomain connector loop of PCNA (*4*). The residues in the PIP-box interact with the large hydrophobic pocket, and involve the two aromatic residues of the PIP-box. Thus, in the case of p21, the PIP-box represents only part of the protein−protein interface with PCNA. A 20 amino acid sequence that exists in both the N-terminal region of DNA ligase I and the large subunit of

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RFC has been proposed to function as a replication factory targeting sequence (RFTS). This sequence also binds to PCNA (*30*).

Because PCNA appears to be involved in the binding of a number of protein partners, the question of whether it contains sites for recognizing other peptide motifs can be raised. In addition, the short sequence of the PIP-box, and the fact that only three of eight residues in this sequence are conserved, suggests that its presence in a given protein does not necessarily provide strong evidence that the protein binds PCNA. Conversely, not all the known PCNA-binding proteins contain sequences which might conform to the PIP-box motif, e.g., cyclin D and pol ϵ. Mutational analyses of PCNA have shown that mutations in different regions of PCNA can result in differential effects with a given partner [reviewed by Tsurimoto (*31*)]. To obtain more information on the peptide motifs that are recognized by PCNA, a random peptide library was screened against PCNA. The results provide evidence that PCNA may bind to a second class of peptides in addition to those conforming to the PIP-box, and further verify the existence of the conserved motif (PIP-box).

EXPERIMENTAL PROCEDURES

Materials. The FliTrx random peptide library was obtained from Invitrogen (San Diego, CA). The Sephacryl S-200 column and Mono P column for PCNA purification, the activated CH-Sepharose, the T7 Sequenase version 2.0 DNA sequencing kit, the protein biotinylation system, and the ECL chemiluminescence detection reagents were purchased from Amersham−Pharmacia Biotech Inc. The QIA prep miniprep kit for plasmid isolation was obtained from QIAGEN. Pol δ synthetic peptides, C1 (1047-LEERFSRLWTQCQRCQGSL-HED-1068), C2 (1069-VICTSRDCPIFYMRKKVRKDLED-1090), C3 (1091-DLEDQEQLLRRFGPPGPEAW-1107), N2 (129-GVTDEGFSVCCHIHGFAPYFY-149), N2a (129-GVTDEGFSVC-138), N2b (139-CHIHGFAPYFY-149), N2−1 (mutant of N2, 129-GVTDEG**A**SVCCHIHGFAPYFY-149, F → A), N2−2 (mutant of N2, 129-GVTDEGF**R**VC-CHIHGFAPYFY-149, S → R), N2−3 (mutant of N2, 129-GVTDEGFSV**A**CHIHGFAPYFY-149, C → A), N2−4 (mutant of N2, 129-GVTDEGFSVCC**A**IHGFAPYFY-149, H → A), N2−5 (mutant of N2, 129-GVTDEGFSVCCHI-**A**GFAPYFY-149, H → A), N2−6 (mutant of N2, 129-GVTDEGFSV**AA**HIHGFAPYFY-149, CC → AA), N2AAA (mutant of N2, 129-GVTDEGFSVCCHIHGFAP**AAA**-149, YFY → AAA), and N4 (276-RLKEKATQCQLEADVL-WSDV-295), were synthesized by the Protein Chemistry Core Laboratories (Miami, FL). The IMC medium used for growth of the *E. coli* peptide library was 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, pH 7.4, 0.2% casamino acids, 0.5% glucose, and 1 mM $MgCl_2$. TGD buffer consists of 50 mM Tris-HCl, 5% glycerol, 1 mM DTT, pH 7.8. The blocking solution consisted of 1% nonfat dry milk, 150 mM NaCl, 1% α-methyl mannoside, and 100 μg/mL ampicillin in IMC medium. The 1 L of RM medium contained 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, pH 7.4, 20 g of casamino acids, 10 mL of 50% glycerol, 100 μg/mL ampicillin, and 1 mM $MgCl_2$. The plating medium for RMG plates contained 15 g of agar in 1 L of RM medium containing 100 μg/mL ampicillin. TGEED buffer consists of 50 mM Tris-HCl (pH 7.8), 5% glycerol 0.5 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol.

Preparation of PCNA. Five milliliter overnight cell cultures of *E. coli* DHα containing the PCNA expression plasmid previously described (*2*) were used to inoculate 1 L cultures (Terrific media) and grown at 37 °C until the A_{600} reached 0.3. After addition of isopropyl-1-thio-β-D-galacto-pyranoside to a concentration of 0.3 mM, the cultures were grown for another 16 h at 28 °C. The cells were harvested, and the PCNA were purified as described by Zhang et al. with minor modifications (*2*). Before loading on a Sephacryl S-200 column for purification, the preparation was first purified by chromatography on a Mono P column.

Growth of the E. coli Peptide Library. The FliTrx random peptide library is based on the system described by Lu et al. (*32*). The *E. coli* strain GI826 containing pFliTrx with inserts was grown with shaking for 18 h at 25 °C in IMC medium containing 100 μg/mL ampicillin. The cultures (1×10^{10} cells) in 50 mL of IMC medium were induced to express the thioredoxin−flagellin fusion proteins containing the peptide inserts by adding ampicillin (100 μg/mL) and tryptophan (100 μg/mL) and grown for another 6−8 h at 25 °C.

Immobilization of PCNA on Culture Plates. The purified PCNA was dialyzed overnight with TGD buffer. PCNA was immobilized on 60 mm plastic Petri dishes by adsorption from 1 mL of PCNA solution (100−120 μg of PCNA/plate) with gentle agitation for 1 h. After washing the plate with 10 mL of sterile water, the immobilized plate was agitated for 1 h with 10 mL of blocking solution.

Panning of the Random Peptide Display Library. The panning technique was performed as described in the manufacturer's procedure with minor modifications. After the 6 h induction, the following were added to the 50 mL of induced cells: 0.5 g of nonfat dry milk, 1.5 mL of 5 M NaCl, and 2.5 mL of 20% α-methyl mannoside. The induced cells (10 mL) were added to the plate containing immobilized PCNA. The plate was rotated gently for 1 min at 50 rpm and allowed to incubate for 60 min at room temperature. The bacterial culture was decanted. The plate was washed by gentle agitation for 5 min with 10 mL of IMC medium containing 100 μg/mL ampicillin and 1% α-methyl manno-side, and the wash was repeated 4 times. The fifth wash was fully decanted, and the PCNA-bound bacteria were detached into a small volume of IMC medium by vortexing the plate for 30 s. The detached bacteria were grown as described above. The culture was then induced by growth with tryptophan-containing medium, and the cycle of panning was repeated. Nine rounds of panning were performed.

Biotin-Labeled PCNA. Purified recombinant PCNA (1 mg/mL) was dialyzed overnight in 40 mM sodium bicarbonate buffer, and concentrated on a Centricon-30 filter. PCNA was labeled with biotin by reaction with biotinamidocaproate *N*-hydroxysuccinamide ester. One milliliter of PCNA solution (0.5 mg/mL) was incubated with 25 μL of biotinamido-caproate *N*-hydroxysuccinamide ester (5 mg/mL) at room temperature for 1 h with constant agitation. The reaction mixture was passed through a Sephadex G-25 column equilibrated with 5 mL of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). The conjugated PCNA was diluted 1000-fold in PBS containing 1% BSA and 0.2% sodium azide, and stored at 4 °C.

Preparation of Positive Clones for Analysis. After the ninth panning, the cultures were streaked onto RMG-Amp plates,

and single colonies were selected. Each colony was inoculated into 4 mL of RM medium, and grown at 30 °C to saturation with shaking (245 rpm) for 20 h until the A_{600} reached 2.0−3.0. The cultures (100 μL) were then inoculated with 3 mL of IMC medium containing 100 μg/mL ampicillin and 100 μg/mL tryptophan, and grown at 30 °C for 8 h until the cells reached mid-log phase (0.6 OD_{600}). The remainder of the noninduced cultures was saved at 4 °C for DNA isolations.

Overlay Blotting with Biotinylated PCNA. After induction, the cells were harvested and resuspended in 150 μL of SDS−PAGE sample buffer and heated at 100 °C for 5 min. Ten microliters of sample was loaded and subjected to electrophoresis on SDS−PAGE (10% acrylamide), and transferred to a nitrocellulose membrane. The blot was blocked with 5% nonfat dry milk in PBST (1 × PBS, plus 0.05% Tween 20) for 2 h at room temperature with shaking followed by four washes of PBST for 10 min each. The blot was then incubated with biotinylated PCNA (0.5 μg/mL) at 4 °C overnight or for 2 h at room temperature with shaking. The blot was washed 5 times with PBST for 15 min each. It was subsequently incubated with streptavidin−horseradish peroxidase conjugate diluted in PBST (1:10 000) for 1 h at room temperature with shaking. After washing 5 times with PBST for 15 min each, the blot was developed using a chemiluminescence method (ECL detection system, Amersham).

DNA Sequencing. The remaining noninduced cultures from above were collected, and the pFliTrx plasmids were isolated. DNA sequencing was performed by the dideoxy chain termination method using the T7 Sequenase version 2.0 DNA sequencing system (Amersham). The primer used for DNA sequencing was the FliTrx forward sequencing primer, 5′-ATTCACCTGACTGACGAC-3′.

Sequence Analysis. The amino acid sequences of inserted peptides were analyzed by the Motif program at the GenomeNet Database (Kyoto University and the University of Tokyo, Japan). The protein sequence database used for search was SWISSPROT. The query pattern entered in the search was written in PROSITE format.

Dot Blot Analysis for the Interaction between Pol δ Synthetic Peptides and PCNA. About 5 μg of each peptide was dotted onto a nitrocellulose membrane and air-dried for 45 min at room temperature. After blocking the membrane with 5% nonfat dry milk in TBST (1 × TBS, plus 0.05% Tween 20) for 2 h at room temperature with shaking, the membrane was visualized by following the same procedures as for the PCNA overlay described above.

Preparation of Immobilized Peptides and Adsorption of PCNA on N4- or N2−6-Sepharose. Activated CH-Sepharose (0.25 g) was suspended in cold 2 mM HCl for 15 min and then washed 6 times with ice-cold 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 (separated by centrifugation, 3000 rpm, 8 s). The gel was then mixed with the peptide solution (N4 and N2−6, 2.5 mg in 1 mL of 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3), and the suspension was rotated end-over-end overnight at 4 °C. The gel was blocked by incubation with 1 mL of 0.2 M glycine, pH 8, for 20 h at 4 °C and then washed 6 times with 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 (separated by centrifugation, 3000 rpm, 8 s). Purified recombinant human PCNA (40 μL, 0.1 μg/μL) was mixed with 40 μL of N4 or N2−6 coupled to CH-Sepharose. The suspension was mixed at 37 °C for 1 h. The beads were then centrifuged (3000 rpm, 8 s) and washed 7 times with 0.1 M $NaHCO_3$, 0.6 M NaCl, pH 8.3. After the final wash, the beads were resuspended in 40 μL of SDS−PAGE sample buffer and boiled for 5 min. The supernatant was then taken directly for SDS−PAGE and Western blot analysis using anti-PCNA antibody (*33*). The same procedure was used for coupling of bovine serum albumin, which was used as a control.

Enzyme Activity Assay. DNA polymerase δ activity was assayed with poly(dA)/oligo(dT) as a template-primer and [^{3}H]dTTP as the nucleotide donor. First, increasing amounts (1 mg/mL) of various synthetic peptides were added to 5 μL of PCNA (0.1 mg/mL) in each tube. The reactions consisted of either 0, 1, 2, 4, or 8 μL of the peptide(s). Each reaction was brought to the same volume by adding TGEED buffer. Each volume was mixed and allowed to react at room temperature for 1 h. Then, 5 μL of DNA polymerase and 45 μL of poly(dA)/oligo(dT) were added to each reaction. The reaction mixtures were incubated at 37 °C for 30 min. The samples were spotted onto DE81 filter paper circles and placed under a lamp for 10 min. Filters were washed 3 times in 0.3 M ammonium formate for 10 min each time and once with 95% ethanol for 5 min. Pol δ activity was determined by DNA binding to the DE81 filters as described by Lee et al. (*34*).

RESULTS

Panning of the FliTrx Random Peptide Display Library Using PCNA. The FliTrx random peptide display library used is based on the display of peptides on the flagella of *E. coli*. The FliTrx library has a diversity of 1.77×10^8 individual dodecapeptides; each is flanked by the sequences CPG and GPC at its N and C termini, respectively. These peptides are inserted into the active-site loop of thioredoxin, which is itself fused into the major flagellin protein of *E. coli.* After induction of the flagellin fusion protein in the cells, the peptides are thus displayed on the flagella. The screening consisted of consecutive rounds of panning on Petri dishes to which PCNA was immobilized. After nine rounds of panning (see Experimental Procedures), the plasmids from individual *E. coli* isolates were isolated and analyzed. To confirm that these isolates were indeed binding to PCNA, a PCNA overlay method was employed to detect thioredoxin−flagellin fusion proteins that bind to PCNA. This was done by SDS−PAGE of the *E. coli* proteins, followed by overlay blotting with biotin-conjugated PCNA. Figure 1 shows an example of this analysis in which eight isolates were tested. It can be seen that there are five isolates which show a positive overlay for PCNA binding to a protein of 68 kDa. The latter size is consistent with an expected size of the fusion protein since *E. coli* thioredoxin has a molecular mass of 12 kDa (*35*) and *E. coli* flagellin has a molecular mass of 60 kDa (*36*).

Analysis of the Peptide Sequences Isolated by Panning of the FliTrx Library. Eighty-five peptide inserts were obtained from the panning of the FliTrx library, and 49 of these were positive clones by the PCNA overlay method. Inspection of the insert peptide sequences of these positive clones shows that they did not fall into a single family. In Table 1, two groups of peptide sequences which clearly form two separate families are shown. The first of these (Group I, Table 1) contained those sequences that matched or were similar to

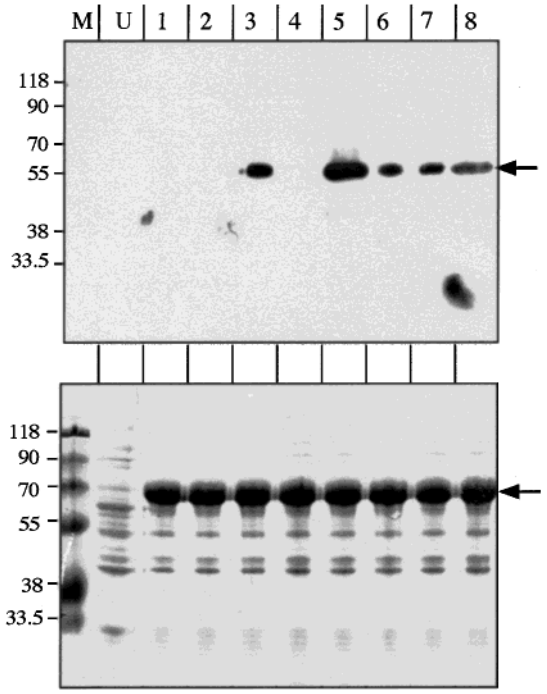

FIGURE 1: Binding of PCNA to thioredoxin−flagellin fusion proteins. The binding of PCNA to the thioredoxin−flagellin fusion proteins of the isolates obtained by panning of the random peptide library was tested by PCNA overlay of bacterial extracts (see Experimental Procedures). Induced cultures of isolated colonies were collected and resuspended in 150 μL of SDS−PAGE loading buffer. The samples were boiled for 5 min and then loaded onto SDS−PAGE (5 μL). After transfer to the nitrocellulose membrane, the blot was exposed to biotinylated PCNA and detected using streptavidin−horseradish peroxidase conjugate (see Experimental Procedures). The upper panel shows a representative overlay blot for eight individual isolates (lanes 1−8). The lane marked "M" contained the protein standards, and the lane marked "U" is that of a culture containing the FliTrx plasmid without induction. The lower panel shows the Coomassie blue stain for protein. The position of the 68 kDa thioredoxin−flagellin fusion protein is indicated by arrows.

Table 1: Peptide Sequences from Screening of the FliTrx Random Peptide Library with PCNA as the Bait

Group I (PIP-Box motif)	
8-6	K**Q**GR**L**AG**FF**KLG
9-15	F**Q**PA**V**GD**FY**ASK
7-1a	ATQHSKRA**Y**AVG
7-12	S**Q**RRSGP**Y**AATL
9-47	ACVNR**Q**SREDE**F**
9-19	TTGSGHG**FY**KPG
9-21	MWRSE**FY**TPPDE
7-3	EF**Q**CPREALKAH
7-2	DF**Q**CPREALKAH
Group II.	
8-2	**Y**EGE**Y**IRSC**W**PG
8-9b	**Y**EGE**Y**IRSC**W**PG
8-9a	**Y**EGST IRSC**W**PG
8-9	**Y**EGST IPSC**W**PG

the known PCNA PIP-box motif. Only the first five of the sequences in Group I can be obviously matched to the PIP-box. Sequences 8−6 and 9−15 conformed closely to the specifications of the PIP-box, Qxx(h)xx(a)(a) (h, residues with moderately hydrophobic side chains, e.g., L, I, M; a, residues with highly hydrophobic, aromatic side chains, e.g., F, Y; x, any residues). The next three possessed only a single aromatic residue, as has been found for the PIP-box for *Drosophila* p21 (*26*). The next two peptides (9−19 and 9−21) contained a pair of aromatic residues but without the conserved glutamine. PCNA overlay analysis confirmed that 9−19 reacts with PCNA. Members of the PIP-box motif which also do not contain the conserved glutamine are those present in p57 (*24*), and the PIP-box sequence that has been proposed to reside in the N2 region of the 125 kDa catalytic subunit of pol δ (*37*). Two additional sequences (7−3 and 7−2) are shown in Group I, but these can only be considered as marginal members of the PIP-box as they lack the aromatic residues.

The second group (Group II) only contained four members which are strongly related and are characterized by the presence of two or three aromatic residues. The search sequence Y-x(3)-[YT]-x(4)-W was used to search for related sequences in the SWISSPROT database using the PROSITE search engine. Nearly 1500 hits were obtained, and among the hits were a number of enzymes/proteins related to the replication of human viruses (not shown).

A New PCNA-Binding Motif Found from the Isolates of the FliTrx Random Peptide Library. The third group of peptides that were aligned is shown in Table 2, and is subdivided into Groups IIIA, IIIB, and IIIC. This group consists of 29 members, about one-third of the total sequences that were isolated. Group IIIB contains 13 members, and is the largest of the 3 subgroups. Groups IIIA and IIIB are clearly related, while Group IIIC is more closely related to Group IIIB. The distinguishing feature of Groups IIIA and IIIB is the presence of a highly conserved KA pair, followed by two to four aliphatic hydrophobic residues that are mainly leucines. Group IIIA is distinguished from Groups IIIB and IIIC by the presence of a conserved pair of residues that consists of a basic residue often followed by leucine that is N-terminal to the KA pair that is absent in Groups IIIB and IIIC. Group IIIC is lacking the conserved basic residue of the KA pair that is present in Group IIIA and Group IIIB. The overall motif was termed the KA-box.

Attempts were made to search for related sequences using query patterns for the KA-box motifs in Groups IIIA and IIIB in the SWISSPROT database using the PROSITE search engine. However, because of the shortness of the sequence and a lack of very strong conservation, a large number of "hits" were obtained. For example, using a query pattern based on Group IIIA, over 600 hits were obtained using the PROSITE program. Using a shorter query pattern, based on Group IIIB, nearly 3000 hits were obtained. It is noted parenthetically that use of the PIP-box motif provides even larger numbers of hits. Included among the hits were a number of proteins that are involved with DNA replication, DNA repair, and cell cycle control. Among these were sequences present in the N-terminus of the catalytic subunit

Table 2: The Major Group of PCNA-Binding Peptides Isolated by Peptide Library Screening[a]

Group IIIA	9-44	HG**L**G**A**FQGRE**VI**
	9-36	K**I**R**HA**GSG**V**GS**L**
	9-47b	R**L**C**KAL**NGPNES
	9-47a	R**L**C**K**P**A**NPPNES
	9-47c	H**L**C**K**P**L**NG**A**NES
	9-26	H**L**C**KA**HQWP**L**RE
	9-47d	H**L**C**KAL**MARTR**V**
Group IIIB	9-13	PGS**GRAIL**HPW**A**
	9-13a	PGS**VRAI**RHPW**A**
	9-11	**LKALIA**KGNFPS
	9-59	**AGKA**S**LI**T**L**RGR
	9-43	**KA**R**L**GS**LAI**RCG
	9-22	**KA**G**L**GS**LAI**KCG
	9-51	MC**GKAVL**D**L**K**L**H
	9-61	**RAL**D**A**R**L**G**A**GGR
	9-18	A**I**R**KA**GQVT**LI**M
	9-4	A**IIRVL**R**L**Q**L**GR
	9-62	**LK**PG**L**G**VTAI**TL
	9-56	TE**GKA**TNSRT**LI**
	9-52	**LKAV**EM**L**MG**V**LR
Group IIIC	8-16:	T**A**DS**LL**H**I**QNGK
	8-7	**LAL**R**R**V**KAI**STV
	7-1	**A**H**AAL**K**R**A**Y**A**V**G
	7-15	**AALA**VS**L**MQCLR
	9-28	R**GAV**S**V**GM**LA**GS
	8-21	VS**G**CS**L**RQ**V**C**LL**
	8-8	V**AG**G**A**GGRGA**L**R
	9-48	**A**D**V**I**V**G**L**C**V**H**A**L
	9-3	**V**TWR**I**D**V**Q**LA**RP

[a] Residues that are conserved over more than one group are shown in boldface and are shaded. Where a second residue in the same column is conserved, it is shown in boldface alone.

of pol δ. The region of similarity of the pol δ sequences falls in what is termed the N4 region by Yang et al. (*38*), and is conserved among pol δ enzymes from eukaryotes as well as in several viral polymerases including those of HSV, EBV, and CMV. An alignment of the N4 regions of pol δ of different species with members of the Group IIIA and IIIB peptides is shown in Table 3.

The Peptide, N4, Containing the New PCNA-Binding Motif Interacts with PCNA. In previous studies, we had examined the binding of PCNA to peptides conforming to conserved regions in the N-terminus of human pol δ, and have found that the N2 region, but not the N4 region, bound to PCNA by a dot blot procedure using a PCNA overlay method followed by detection with a monoclonal antibody against PCNA and a chemical staining procedure (*39*). For this reason, the issue of whether a peptide containing the N4 region would bind to PCNA was reinvestigated with the use of more sensitive detection methods. Dot blot experiments were performed in which samples of peptides were placed on nitrocellulose membranes and overlayed with biotinylated PCNA. The blot was visualized using a chemiluminescence method (see Experimental Procedures). A peptide corresponding to the N4 region of human pol δ p125 (276-RLKEKATQCQLEADVLWSDV-295) was tested together with peptides derived from other portions of the pol δ sequence (Figure 2). The peptides tested included three from the C-terminus (C1, C2, C3), and peptides based on the conserved N2 regions of pol δ. These were the N2 peptide (129-GVTDEGFSVCCHIHGFAPYFY-149), N2−6 (129-GVTDEGFSV**AA**HIHGFAPYFY-149), in which cysteines 138 and 139 were mutated to alanines and which has previously been shown to bind PCNA (*37*, *39*), the two half-peptides of the N2 sequence, N2a (129-GVTDEGFSVC-138) and N2b (139-CHIHGFAPYFY-149), and N2AAA (129-GVTDEGFSVCCHIHGFAP**AAA**-149). Also included in these experiments were PCNA, purified calf thymus pol δ, and bovine serum albumin (BSA). The results showed that only peptides N4, N2, N2−6, and N2b gave positive signals (Figure 2). In the same experiment, positive signals were obtained with PCNA itself and with the pol δ enzyme but not with BSA. The difference between this experiment and our previous studies (*39*) is the use of a more sensitive chemiluminescence assay for binding, and suggests that the N4 peptide, while it does bind to PCNA, may not bind as strongly as the N2 peptide. It was also found that addition of the N4 peptide could successfully block the binding of biotinylated PCNA to the p125 catalytic subunit of pol δ in overlay experiments (data not shown).

A second experimental approach to confirm if these peptides could bind to PCNA was to test for their ability to inhibit the PCNA stimulation of pol δ activity. The results are shown in Figure 3. The N2 peptide, as a positive control, inhibits the PCNA stimulation of pol δ as we have previously shown (*39*). The N4 peptide also inhibited the PCNA stimulation of pol δ, as did the half-peptide N2b, although both of these were clearly less potent than the full-length N2 peptide. None of the other peptides tested (C1, C3, N2a, N2AAA) inhibited pol δ activity. The potency of inhibition of pol δ by N4 was roughly 5-fold less effective than the N2 peptide. These results demonstrate the ability of the N4 peptide to inhibit the PCNA stimulation of pol δ, consistent with its having the ability to bind to pol δ.

The demonstration that the C-terminal half, but not the N-terminal half of the N2 peptide, showed positive interactions with PCNA by inhibition assays provides additional evidence that the N2 peptide contains a variant of the PIP-box (*37*, *39*). This is also consistent with the demonstration that the N2 peptide, but not the N2AAA peptide in which the aromatic resides of the PIP-box (*25*, *26*) are mutated to alanine, is able to inhibit the PCNA stimulation of pol δ. Experiments in which other mutations of N2 (Figure 4) were tested showed that all these were able to inhibit pol δ activity, consistent with our previous data showing that these same peptides were able to bind biotinylated PCNA in overlay experiments (*37*).

A more direct test of the ability of the N4 peptide to bind PCNA was performed. The N4 and N2−6 peptides were immobilized on CH-Sepharose (see Experimental Procedures) and tested for their abilities to bind PCNA. Recombinant human PCNA was incubated with the peptide−Sepharose beads to which either N4 or N2−6 had been

Table 3: Alignment of the N4 Regions of the 125 kDa Subunit of Pol δ with the Group III Peptides[a]

N4 regions of pol δ:					
soy-bean		237	**K**TAKS**LS**YC**QLE**FDCL	252	AF020193
S. pombe		260	**R**YQN**RVSN**C**QIEA**WIN	275	L07734
EBV		259	**RL**QH**R**D**S**Y**AELE**YDCE	274	V01555
hamster		272	**R**TEK**KATQ**C**QLEV**DVL	287	AJ222691
rat		272	**RA**EK**KATL**C**QLEV**DVL	287	O54747
bovine		275	**R**PEG**KATL**C**QLEA**DVL	290	M80395
human		276	**RL**KE**KATQ**C**QLEA**DVL	291	M01735
Peptides: IIIA	9-47b		**RL**C-**KALN**GPN**E**S		
	9-47a		**RL**C-**K**P**AN**PPN**E**S		
	9-26		**HL**C-**KA**H**Q**WP**L**RE		
	9-47d		**HL**C-**KAL**M**A**RTR**V**		
Peptides: IIIB	9-59		AG**KASLI**T**L**RGR		
	9-18		**A**IR**KA**G**QV**T**L**IM		
	9-43		**KA**R**L**GS**L**A**I**RCG		
	9-61		**RALDA**R**L**G**A**GGR		
	9-52		L**KAVE**ML**V**LR		
	9-56		TEG**KATN**SRTL**I**		
	9-4		**A**II**RVL**R**L**Q**L**GR		

[a] Sequences from the N4 regions of pol δ are aligned with representative peptides from Groups IIIA and IIIB. Conserved residues are shown in boldface and are shaded. Accession numbers are given on the right.

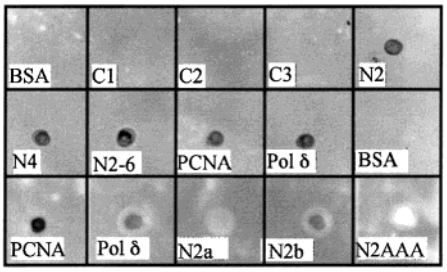

FIGURE 2: Dot blot analysis of the binding of PCNA to synthetic peptides. The synthetic peptides or proteins were dot blotted onto nitrocellulose and tested for PCNA binding by the use of biotinylated PCNA (Experimental Procedures). The blots were visualized using a chemiluminescence method. The synthetic peptides used were as follows: C1 (1047-LEERFSRLWTQCQRCQGSLHED-1068), C2 (1069-VICTSRDCPIFYMRKKVRKDLED-1090), C3 (1091-DLEDQEQLLRRFGPPGPEAW-1107), N2 (129-GVTDEGFSVCCHIHGFAPYFY-149), N2a (129-GVTDEGFSVC-138), N2b (139-CHIHGFAPYFY-149), N2−6 (mutant of N2, 129-GVTDEGFSV**AA**HIHGFAPYFY-149, CC → AA), N2AAA (mutant of N2, 129-GVTDEGFSVCCHIHGFAP**AAA**-149, YFY → AAA), and N4 (276-RLKEKATQCQLEADVLWSDV-295). BSA, purified calf thymus pol δ, and PCNA were also tested.

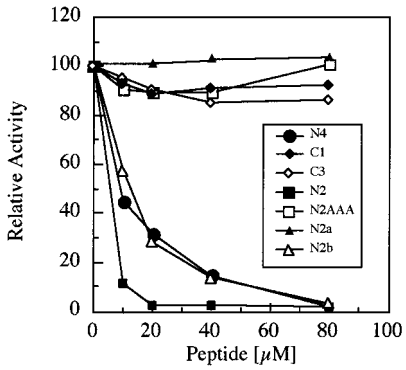

FIGURE 3: Inhibition of pol δ activity by the synthetic peptides N4 and N2. Human pol δ activity was assayed as described under Experimental Procedures in the presence of PCNA and varying concentrations (μM) of the peptides. Results are shown as relative activities. Pol δ synthetic peptides used in the assay include: C1 (1047-LEERFSRLWTQCQRCQGSLHED-1068), C3 (1091-DLEDQEQLLRRFGPPGPEAW-1107), N2 (129-GVTDEGFSVCCHIHGFAPYFY-149), N2a (129-GVTDEGFSVC-138), N2b (139-CHIHGFAPYFY-149), N4 (276-RLKEKATQCQLEADVLWSDV-295), and N2AAA (mutant of N2, 129-GVTDEGFSVCCHIHGFAP**AAA**-149, YFY → AAA).

attached; the beads were washed, extracted with SDS buffer, and then Western blotted with antibody against PCNA (see Experimental Procedures). The results show that both the N4-Sepharose and the N2−6-Sepharose beads were capable of binding PCNA as shown by SDS−PAGE and silver staining (Figure 5). Similar tests of bovine serum albumin coupled to Sepharose were negative.

Both experiments, dot blot and adsorption of PCNA on N4- or N2−6-Sepharose, show that peptides N4 and N2−6 are able to bind PCNA. Zhang et al. (*37*) recently found that the N2 region of DNA pol δ could interact with PCNA. The peptide N2−6 is similar to its parent peptide, N2, in the ability of PCNA binding. The ability of N2−6 to bind to PCNA further verifies that the C-terminal portion of the conserved region in the N-terminus of DNA polymerase δ is involved in PCNA binding.

DISCUSSION

The results of this study show not only that sequences corresponding to the known PIP-box can be selected using a random peptide display library, but also that there is a novel family of peptides that bind to PCNA. This novel group comprised approximately one-third of the sequences isolated. While the alignment of these peptides did not provide a singularly tight consensus, the results reveal a novel peptide

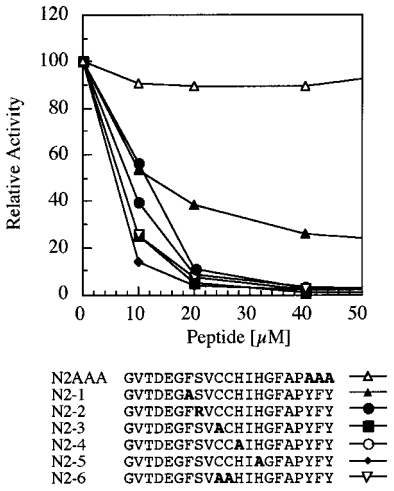

FIGURE 4: Inhibition of pol δ activity by the mutant peptides of N2. Human pol δ activity was assayed as described under Experimental Procedures in the presence of PCNA and varying concentrations (μM) of the mutant peptides of N2. Results are shown as relative activities. Pol δ synthetic peptides used in the assay are shown below the figure (mutated amino acids are marked in boldface letters).

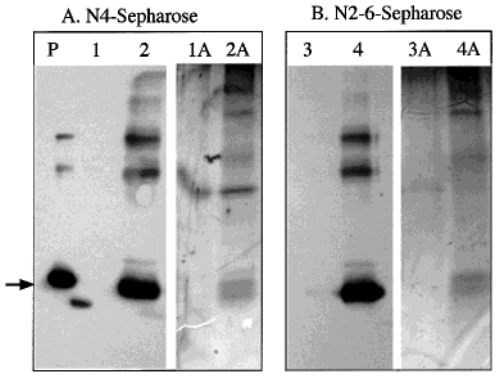

FIGURE 5: Binding of PCNA to immobilized N4 and N2−6 peptides. N4 and N2−6 peptides were covalently coupled to CH-Sepharose. The Sepharose beads (40 μL) were then used to bind purified recombinant human PCNA (40 μL, 0.1 μg/μL). After stringent washing to remove nonspecifically bound material, the beads were extracted with SDS-buffer and subjected to SDS−PAGE and Western blotting using an antibody to PCNA (see Experimental Procedures). Panel A: binding of PCNA to N4-Sepharose. Lane P, purified PCNA; lane 1, binding of PCNA to BSA−Sepharose; lane 2, binding of PCNA to N4-Sepharose; lanes 1A and 2B, silver stain of eluates corresponding to lanes 1 and 2. Panel B: binding to N2−6-Sepharose. Lane 3, binding of PCNA to BSA−Sepharose; lane 4, binding of PCNA to N2−6-Sepharose; lanes 3A and 4B, silver stain of eluates corresponding to lanes 3 and 4. The arrow marks the position of PCNA.

motif (KA-box) that is able to bind PCNA. This was confirmed by a positive overlay of the flagellin−thioredoxin fusion proteins with biotinylated PCNA. As a result of database searches, it was noted that the N4 region of pol δ contains a sequence that conforms to the KA-box. Experimental tests by the use of synthetic peptides in overlay and affinity chromatography experiments showed that the N4 peptide could bind to PCNA. This provides strong evidence that the N4 region of pol δ participates in PCNA binding, and also the first evidence for a functional KA-box in a PCNA-binding protein.

The identification of a KA-box in pol δ is of interest, since we have demonstrated by a number of experimental approaches that the p125 subunit of pol δ interacts with PCNA through a region identified as the conserved N2 region (*37, 39*) which contains a variant of the PIP-box. Thus, the implications of this study are that pol δ interacts with PCNA through the possession of at least two PCNA-binding motifs. A multisite interaction would favor formation of the pol δ−PCNA complex, since it would strengthen the association of the two proteins. Moreover, there is the additional possibility that these interactions could involve different PCNA molecules, since PCNA is a homotrimeric protein. This type of interaction could provide additional strength to the stability of a trimeric PCNA−pol δ complex. Indeed, there is some experimental evidence based on cross-linking experiments which indicates that p125 preferentially forms a complex with trimeric PCNA (*37*).

As already noted under Results, both the PIP-box and the KA-box motifs involve relatively short peptide sequences which do not have very strong signatures, so that database searching provides a large number of hits. This emphasizes the need for experimental verification of the functional ability of any proposed sequence in a given protein to participate in PCNA binding. The identification of a second motif that may be involved in PCNA binding is of particular significance, as it may be of utility in identifying PCNA-binding domains in candidate proteins that bind to PCNA. Furthermore, not all known PCNA-binding proteins contain the PIP-box sequence, and the existence of a second motif may provide an explanation.

The identification of PCNA-binding motifs is of significance since it would provide insights to the structural basis for the versatility of PCNA, given the current information that it is the nexus for multiple protein−protein interactions that underlie its participation in DNA replication, repair, and cell cycle control processes. A role for PCNA in cell cycle control has been indicated by the finding that cyclin/cdks formed quaternary complexes with PCNA and p21 (*19, 40, 41*). The interaction of PCNA with different cyclin/cdks during the cell cycle suggested that the regulated distribution of PCNA could be an important link to cell cycle control of DNA. p21 has been intensively studied since it was discovered to be an inhibitor of the of cyclin/cdks (*42*) and to be transcriptionally regulated by p53 (*43*). p21 inhibits pol δ in vitro and provides an attractive potential molecular mechanism whereby p53 induction could arrest DNA synthesis (*23, 44*) by competing for binding to PCNA (*45, 46*).

Recently, a number of additional proteins that bind to PCNA have been identified (*7*−*21, 24, 47*−*52*). These findings have major implications for understanding the role of PCNA. These PCNA-binding proteins fall into three major groups: DNA replication proteins—the catalytic and third subunits of pol δ (*10, 11, 37, 47*−*49*), RFC (*7*−*9*), pol ϵ (*50*), FEN1 (*12, 13*), and DNA ligase I (*14*); DNA repair proteins—XPG (*15*), uracil−DNA glycosylase (UNG2, *16*), mismatch repair proteins MSH2, MLH1, and PMS2 (*51*); cell cycle regulatory proteins—p21 (*22, 23*), p57 (*24*), and the cyclins (*19*). The use of PCNA affinity chromatography has also identified an association of a number of replication and repair proteins with PCNA (*47, 52*). A number of these have been shown to possess functional PIP-boxes, and sequence alignments have provided additional candidates for PCNA binding (*25, 26, 47*−*49*).

Table 4: Selected Proteins Containing Regions of Similarity to the N4 Regions of Pol δ and the Group III PCNA-Binding Peptides

Pol δ (N4 region)	276	**RL**KE**KA**T**Q**CQ**L**EA**D**V**L**	291	M01735
Pol ε	1533	**K**T**I**C**RAIQ**RF**LLA**YKE	1548	Q07864
RFC-140	682	**K**SSL**KAIV**AES**L**NNTS	697	P35251
RFC 38	221	**R**N**LRKALL**MCE**A**CR**V**Q	236	P40938
RFC 37	244	GD**LRKAI**TFLQS**A**TRL	259	P35249
XPC	712	**RA**-**RKA**R**L**AEPQ**L**REE	726	Q01831
XPC (Mouse)	676	**RA**-**RKA**RHLG**A**Q**L**HDH	690	P51612
XPD	224	E**LARKAVV**VFDE**A**HNI	239	P18074
	612	**H**HYG**RAVI**MFG**V**PY**V**Y	627	P18074
	630	SR**I**L**KA**R**L**EY**L**RDQFQ	645	P18074
XPG	116	**R**Q**A**I**KTA**FRSKRDE**A**L	131	P28715
XPG (xenopus)	116	**R**Q**A**I**KAAL**SGNKQSNE	131	P14629
UNG2	74	IQRN**KAAAL**LR**L**AARN	89	X15653
UNG2 (mouse)	56	IQRN**KAAAL**LR**L**AARN	71	P97931
MCM3	311	DY**VKKAIL**CL**LL**GG**V**E	326	P25205
MCM3 (S. cere)	374	DH**IKKAIL**LM**L**MGG**V**E	390	P24279
MCM5	706	**HAI**H**KVLQ**LM**L**RRGEI	721	P33992
MCM5 (S. cere)	746	L**AL**D**KAL**YALEKHETI	766	P29496
MCM7	348	ED**VKKALL**LL**LV**GG**V**D	363	P33993
MCM7 (xenopus)	347	ED**VKKALL**LL**LV**GG**V**D	362	Q91876
MSH6	724	A**I**FT**KA**Y**Q**RM**VL**DA**V**T	739	P52701
MSH6 (mouse)	721	A**V**FT**KA**S**Q**RM**VL**DA**V**T	736	P54276
Cyclin D3	92	VPT**RKA**Q**L**QL**L**G**A**VCM	107	P30281
Cyclin D3 (mouse)	92	VPT**RKA**Q**L**QL**L**GTVCL	107	P30282
BRCA 1	516	DF**IKKA**D**L**A-**V**QKTPE	530	P38398
BRCA 1 (mouse)	509	DF**IKKA**DSAG**V**QRTPD	524	P48754
BRCA 2	2725	WY**A**V**KA**Q**L**DPP**LL**A**V**L	2740	P51587
BRCA 2 (mouse)	2646	WY**A**V**KA**Q**L**DPP**LL**A**V**L	2661	P97929

[a] Conserved residues are shown in boldface and are shaded. Accession numbers are shown on the right, and proteins are those of human unless otherwise noted.

As already noted, database searches using the limited sequences of the PIP-box and the KA-box need to be treated with caution. Nevertheless, an intriguing number of proteins that contain the KA-box could be identified. Some of these are shown in Table 4. This list includes members of the MCM (mini chromosome maintenance protein) family, pol ϵ, three XP proteins (XPC, XPG, XPD), mismatch repair protein MSH6, cyclin D3, and BRCA1 and BRCA2. While the possibility that the KA-boxes in these proteins could serve as interaction sites for PCNA binding is purely speculative, evidence that these in fact interact with PCNA and the potential functional significance of such interactions need to be considered.

The nuclear uracil−DNA glycosylase (UNG2) that is involved in base excision repair has been shown to bind both PCNA and RPA (*16*). UNG2 contains a PIP-box at the N-terminus, and two binding sites for RPA (*16*). One of the RPA-binding sites is located between residues 7 and 18, and overlaps the PCNA-binding site. The other site is located between residues 73 and 90 and has the sequence RIQRNKAAALLRLAARNV. Curiously, this site consists of a KA-box (Table 4). Thus, UNG2 contains two sites for RPA, the first of which overlaps the PIP-box, and the second of which conforms to the PCNA-binding KA motif that is described here. Tests by Otterlei et al. (*16*) of the ability of N-terminally truncated UNG2 mutants to bind to PCNA by an ELISA method revealed that the PIP-box was the major determinant for PCNA binding by UNG2; nevertheless, a weak ELISA reaction was observed with a truncated UNG2 mutant in which the PIP-box had been deleted but which still contained the KA-box, leaving open the possibility that this might be due to PCNA interaction with the KA-box.

In summary, we have provided the first evidence for novel peptide motifs for PCNA binding. These findings point to potential relationships that may be important to explore, and also provide a starting point for site-directed mutational studies that could provide evidence for the identities of the PCNA-binding regions. Clearly, much further work needs to be done to establish the function of these motifs in PCNA binding.

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