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A conserved domain of the large subunit of replication factor C binds PCNA and acts like a dominant negative inhibitor of DNA replication in mammalian cells

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Replication factor C (RF-C), a complex of five polypeptides, is essential for cell-free SV40 origin-dependent DNA replication and viability in yeast. The cDNA encoding the large subunit of human RF-C (RF-Cp145) was cloned in a Southwestern screen. Using deletion mutants of RF-Cp145 we have mapped the DNA binding domain of RF-Cp145 to amino acid residues 369–480. This domain is conserved among both prokaryotic DNA ligases and eukaryotic poly(ADP-ribose) polymerases and is absent in other subunits of RF-C. The PCNA binding domain maps to amino acid residues 481–728 and is conserved in all five subunits of RF-C. The PCNA binding domain of RF-Cp145 inhibits several functions of RF-C, such as: (i) *in vitro* DNA replication of SV40 origin-containing DNA; (ii) RF-C-dependent loading of PCNA onto DNA; and (iii) RF-C-dependent DNA elongation. The PCNA binding domain of RF-Cp145 localizes to the nucleus and inhibits DNA synthesis in transfected mammalian cells. In contrast, the DNA binding domain of RF-Cp145 does not inhibit DNA synthesis *in vitro* or *in vivo*. We therefore conclude that amino acid residues 481–728 of human RF-Cp145 are critical and act as a dominant negative mutant of RF-C function in DNA replication *in vivo*.

Keywords: DNA elongation/DNA replication/PCNA/replication factor C

Introduction

Replication of DNA is a highly regulated process which occurs during the S phase of the cell cycle. Our current understanding of eukaryotic DNA replication emerges primarily from studies of cell-free assays of DNA replication using DNA templates containing SV40 origin of replication (Li and Kelly, 1984; Stillman and Gluzman, 1985; Wobbe *et al.*, 1985). The recognition of the replication origin by the origin binding protein is one of the first steps in initiation of DNA replication. Subsequent unwinding and stabilization of the unwound DNA requires the single-stranded DNA binding protein, replication protein A (RP-A) which is a complex of three tightly associated subunits, RP-A 70, RP-A 34 and RP-A 11 (Fairman and Stillman, 1988; Kenny *et al.*, 1989). The subsequent recruitment of the DNA polymerase α-primase complex to the origin leads to the generation of RNA–DNA primers for initiation of leading strand synthesis and for the synthesis of each Okazaki fragment during lagging strand replication (Tsurimoto and Stillman, 1989b). This is followed by a polymerase switch from DNA polymerase α (pol α) to DNA polymerase δ (pol δ) (Tsurimoto and Stillman, 1989a; Tsurimoto *et al.*, 1990). DNA pol δ then catalyses the replication of the leading strand and is utilized for the completion of replication of the viral lagging strand (Tsurimoto and Stillman, 1989b; Lee and Hurwitz, 1990; Lee *et al.*, 1991a,b).

Two accessory proteins, proliferating cell nuclear antigen (PCNA) and a complex of five polypeptides termed replication factor C (RF-C), are essential for the polymerase switch during SV40 DNA elongation (reviewed in Hübscher *et al.*, 1995). PCNA is a 36 kDa protein originally discovered as a cell cycle-regulated protein (Miyachi *et al.*, 1978; Bravo and Celis 1980). RF-C is comprised of polypeptides with masses of 145, 40, 38, 37 and 36 kDa in humans (Lee *et al.*, 1989; Tsurimoto and Stillman, 1989b; Weinberg *et al.*, 1990; Cullmann *et al.*, 1995). RF-C binds preferentially to 3′ end of a DNA primer bound to template DNA and thus has been suggested to be a structure-specific DNA binding protein. The binding of RF-C to primers synthesized by pol α–primase, blocks primers from further elongation by this polymerase (Lee *et al.*, 1991b; Tsurimoto and Stillman, 1991), and increases the affinity of pol δ or pol ε for these primers. PCNA recognizes and binds to the RF-C:DNA complex in an ATP-dependent manner (Tsurimoto and Stillman, 1990). RF-C loads the PCNA sliding clamp onto DNA (Podust *et al.*, 1995a), thereby increasing the processivity of pol δ (Podust *et al.*, 1992; Stillman, 1994). Subsequently, DNA pol δ or pol ε recognizes the RF-C–PCNA–DNA template/primer complex, thus allowing DNA elongation to be completed.

In this paper we describe the functional analysis of the large subunit of RF-C (RF-Cp145). We have mapped the binding of DNA and PCNA to adjacent but non-overlapping regions of RF-Cp145. The PCNA binding domain of RF-Cp145 behaves like a dominant negative mutant and inhibits DNA replication in mammalian cells. The dominant negative mutant phenotype is consistent with the ability of this domain to inhibit RF-C-dependent loading of the sliding PCNA clamp and to inhibit DNA elongation *in vitro*. We have thus identified a critical region comprising 335 amino acids of RF-Cp145 which inhibits RF-C function in a cell-free system and behaves as a dominant negative mutant in mammalian cells *in vivo*.

Fig. 1. Structural characteristics of the RF-Cp145 protein. A schematic representation of the RF-Cp145 protein is shown in the top panel. The two conserved domains A and B are located in the middle of the open reading frame. The middle panel shows a comparison between the RF-Cp145 domain A and homologous sequences in eukaryotic poly(ADP-ribose) polymerases and prokaryotic ligases. Identical amino acid residues in the homologous regions are highlighted. The bottom panel shows a comparison between the RF-Cp145 domain B and homologous sequences in other subunits of the RF-C complex.

Results

Identification and tissue distribution of the RF-Cp145 gene

Southwestern screens of human T-cell (Jurkat) λgt11 cDNA libraries with two different double-stranded DNA (dsDNA) motifs led to the identification of 30 clones which bind non-specifically to DNA. These independent clones all represent the same gene that encodes for a protein which binds DNA in a sequence-independent manner (Figure 1). This gene encodes a 1171 amino acid protein with a molecular mass of ~145 kDa. Our sequence is identical to the sequence reported for the human large subunit of RF-C complex (Bunz *et al.*, 1993) which is highly homologous to the murine (Luckow *et al.*, 1994) and yeast gene (Howell *et al.*, 1994; Cullmann *et al.*, 1995). Database searches reveal two tandem conserved domains in the middle of the open reading frame (Figure 1). One of these domains (domain A) is conserved among prokaryotic ligases (Ishino *et al.*, 1986) and eukaryotic poly(ADP-ribose) polymerases (PARP) (Cherney *et al.*, 1987; Kurosaki *et al.*, 1987; Huppi *et al.*, 1989) (Figure 1). It is still unclear if the homology reflects divergence of a primordial gene or conservation of function. The second domain (domain B) is conserved among other RF-C subunits (Chen *et al.*, 1992a,b; O'Donnell *et al.*, 1993; Li and Burgers, 1994a,b; Cullmann *et al.*, 1995) (Figure 1). The tissue distribution of the RF-Cp145 transcripts revealed that RF-Cp145 is a ubiquitously expressed gene, although transcript levels vary among different tissues (data not shown).

Non-overlapping domains of RF-Cp145 bind to DNA and PCNA respectively

In order to determine if the two conserved domains of RF-Cp145 had distinct functional characteristics, we generated GST fusion proteins of RF-Cp145 domain A (containing amino acid residues 369–480), RF-Cp145 domain B (residues 481–728) and RF-Cp145 domains A+B (residues 369–734) (Figure 2A). We then determined which of these recombinant RF-Cp145 proteins bind end-labelled dsDNA in a gel-shift assay. Domains A+B (369–734) and domain A (369–480) bind dsDNA, whereas domain B (481–728) did not (Figure 2C). We also found that recombinant RF-Cp145 domain A does not bind

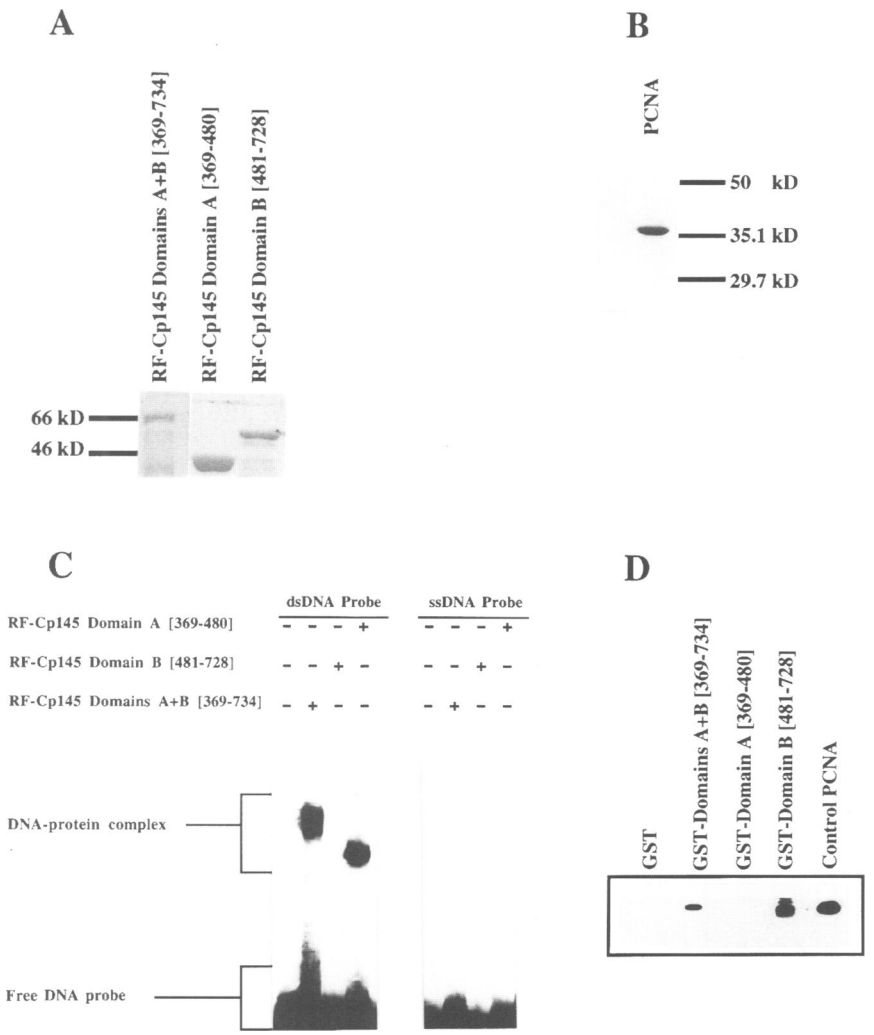

Fig. 2. Distinct non-overlapping domains of RF-Cp145 bind PCNA and DNA: RF-Cp145 domain A [369–480] binds DNA whereas RF-Cp145 domain B [481–728] binds PCNA. (**A**) Analysis of GST–RF-Cp145 domain fusion proteins on SDS–polyacrylamide gels. Shown are GST–RF-Cp145 domain A [369–480], GST–RF-Cp145 domain B [481–728] and GST–RF-Cp145 domains A+B [369–734]. Proteins resolved on 10% SDS–polyacrylamide gels were stained with Coomassie brilliant blue. (**B**) Analysis of purified bacterially expressed recombinant PCNA on SDS–polyacrylamide gels is shown. (**C**) RF-Cp145 domain A [369–480] binds dsDNA but not ssDNA. The ability of control GST–protein or recombinant GST–RF-Cp145 domain A, -domain B, -domains A+B to bind to dsDNA or to ssDNA was determined in a gel-shift assay. The dsDNA (octamer motif) and ssDNA (octamer sense strand only) were end-labelled with ^{32}P and the DNA–protein complexes run on non-denaturing gels. Equal amounts of radioactivity were loaded in each lane. Similar results have been obtained in three separate experiments. (**D**) RF-Cp145 domain B [481–728] binds PCNA. GST–RF-Cp145 fusion proteins and control GST–protein were incubated with PCNA in the presence of Mg^{2+} and ATP. The GST fusion proteins were isolated on glutathione–agarose beads and the bound proteins solubilized in SDS sample buffer and run on SDS–polyacrylamide gels. The ability of the respective recombinant proteins to bind PCNA was determined by immunoblotting with PCNA-specific antibodies. Identical results have been obtained in three separate experiments.

single-stranded DNA (ssDNA) (Figure 2C). The ability to bind dsDNA therefore maps to within residues 369–480 in RF-Cp145.

We next determined which domain of RF-Cp145 binds recombinant purified PCNA (Figure 2B). The GST fusion proteins of RF-Cp145 domains A, B or A+B were incubated with PCNA and isolated by immobilization on glutathione–agarose. The ability of GST–RF-Cp145 proteins to bind PCNA was then determined by immunoblotting using PCNA-specific monoclonal antibody. Domain B (481–728) and domains A+B (369–734) bind PCNA, whereas domain A (369–480) does not (Figure 2D). We conclude that while residues 369–480 of RF-Cp145 bind DNA, residues 481–728 bind PCNA. Domain B binds PCNA more effectively than domains A+B; this probably reflects that domains A+B GST fusion protein is less soluble as compared with domain A or domain B (see Materials and methods). The ability of RF-Cp145 domain B to bind PCNA was also confirmed in an independent assay, in which ^{35}S-labelled PCNA generated by *in vitro* transcription/translation (Figure 3A) was tested for its ability to bind GST–RF-Cp145 fusion proteins (Figure 3B). In this assay domain B but not domain A binds PCNA.

RF-Cp145 and p21 bind PCNA at distinct sites

$p21^{Waf1/Cip1}$ is an inhibitor of cdk–cyclin kinase activity, and of PCNA function in DNA replication (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994). The N-terminal region of p21 has been shown to bind cdk2–cyclin complexes and inhibit kinase activity while the C-terminal region of p21 has been demonstrated to bind PCNA and to inhibit *in vitro* DNA replication (Chen *et al.*, 1995; Goubin and

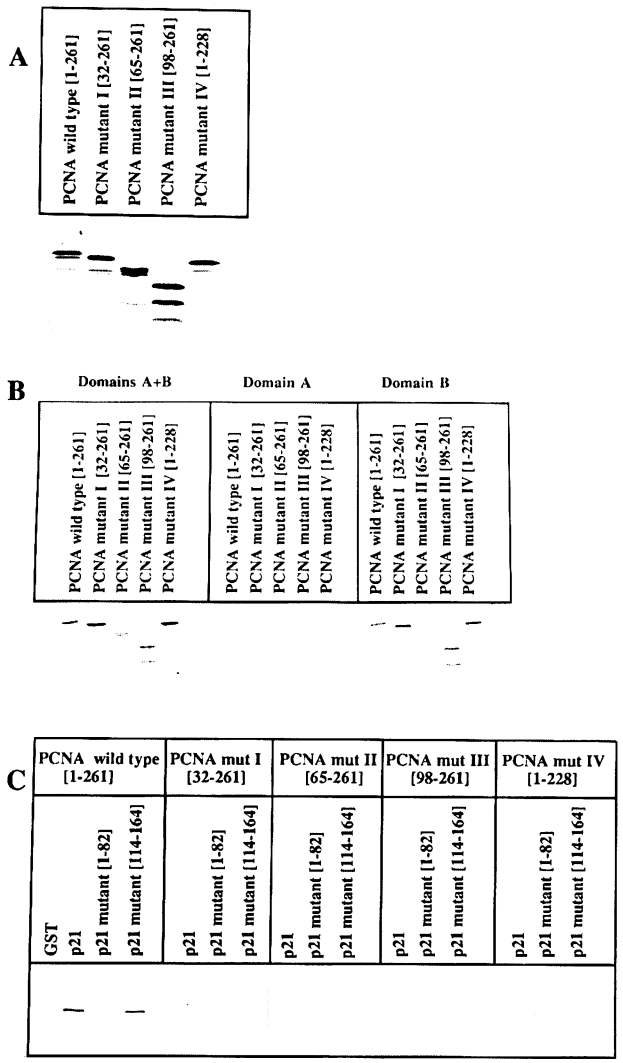

Fig. 3. p21 and RF-Cp145 bind differentially to a panel of PCNA deletion mutants. (**A**) *In vitro* transcribed and translated ^{35}S-labelled full-length and deletion mutants of PCNA. The amino acids which comprise the PCNA deletion mutants I to IV are indicated in parentheses. The products were run on SDS–polyacrylamide gels and visualized by autoradiography. (**B**) The ability of RF-Cp145 GST fusion proteins immobilized on glutathione–agarose to bind ^{35}S-labelled PCNA or its mutants is shown. (**C**) The ability of GST–p21 to bind ^{35}S-labelled PCNA or its mutants is shown. p21 mutant containing residues 1–82 represents the cdk–cyclin binding domain whereas p21 mutant containing residues 114–164 represents the PCNA binding domain.

Ducommun, 1995; Harper *et al.*, 1995; Luo *et al.*, 1995; Nakanishi *et al.*, 1995; Warbrick *et al.*, 1995; Fotedar *et al.*, 1996). As p21 inhibits DNA elongation via binding to PCNA, we wanted to determine if p21 and RF-Cp145 bind to the same region of PCNA. To test this, ^{35}S-labelled deletion mutants of PCNA (Matsuoka *et al.*, 1994) were generated by *in vitro* transcription/translation. The ^{35}S-labelled PCNA mutants were then tested for their ability to bind immobilized GST–RF-Cp145 (domains A, B or A+B). In parallel, the ability of PCNA mutants to bind GST fusion proteins comprising full-length GST–p21, cdk–cyclin domain of p21 (residues 1–82) and PCNA binding domain of p21 (residues 114–164) was also examined (Fotedar *et al.*, 1996). Distinct deletion mutants of PCNA bind RF-Cp145 (Figure 3B) whereas none of the deletion mutants of PCNA bind p21 (Figure 3C). Only full-length PCNA binds p21 (Figure 3C). We interpret the data as indicating that p21 and RF-Cp145 interact with PCNA via distinct sites. However, as PCNA has a complex three-dimensional structure (Krishna *et al.*, 1994) deletion mutants may not be adequate to map the RF-C binding region in PCNA.

RF-Cp145 domain B (481–728) inhibits DNA replication

To clarify if either of the two RF-Cp145 domains is functionally involved in DNA replication, we determined the ability of RF-Cp145 domains to inhibit DNA replication. For this assay, we used extracts from S-phase Manca cells to drive *in vitro* DNA replication on SV40 origin-containing plasmids. The effect of adding either GST–RF-Cp145 domain A, domain B or domains A+B on DNA replication was then determined. RF-Cp145 domains A+B (369–734) and RF-Cp145 domain B (481–728) which contain the PCNA binding domain inhibit DNA replication, whereas domain A (369–480), which lacks the PCNA binding domain, had no significant effect on DNA replication (Figure 4A).

We also determined the ability of the RF-Cp145 mutant proteins to inhibit DNA elongation using the two-step SV40 *in vitro* replication assay described in Materials and methods. In the first step, plasmid DNA containing the SV40 origin of replication is incubated for 30 min under conditions that allow only initiation to occur. Under these conditions no measurable DNA synthesis occurs. In the second step, DNA synthesis is initiated by the addition of deoxyribo- and ribonucleoside triphosphates to preincubated reactions. RF-Cp145 domain A, domain B or domains A+B were then added to reactions in which elongation had proceeded for 10 min. Domain B (481–728) was very effective in inhibiting DNA elongation, whereas domain A (369–480) had no such effect (Figure 4B). Domain B thus inhibits DNA elongation, a result that is consistent with the predicted function of the RF-C complex in DNA elongation.

RF-Cp145 domain B inhibits RF-C-dependent loading of the PCNA clamp

RF-C is required for loading of the sliding PCNA clamp, and for the recruitment of DNA polymerase δ to the RF-C–PCNA–DNA complex. We therefore decided to test if our recombinant RF-Cp145 proteins inhibit RF-C-dependent loading of PCNA onto DNA. The assay for measuring RF-C-catalysed loading of PCNA utilized a ^{32}P-labelled recombinant PCNA ([^{32}P]ph-PCNA) (Podust *et al.*, 1995a). The ability of RF-C to load [^{32}P]ph-PCNA onto gapped circular DNA was determined after fixing the complex with glutaraldehyde followed by agarose gel electrophoresis. The appearance of labelled [^{32}P]ph-PCNA co-migrating with DNA was absolutely dependent on RF-C and ATP. In this assay, RF-Cp145 domain B efficiently inhibited RF-C-dependent loading of PCNA onto a gapped circular plasmid, whereas domain A had no effect (Figure 5A and B). These results reinforce our finding that domain B inhibits cell-free DNA replication by virtue of its ability to inhibit RF-C function. Domains A+B also inhibited loading but its effect was variable. In some experiments (Figure 5A) domains A+B was equally effective as

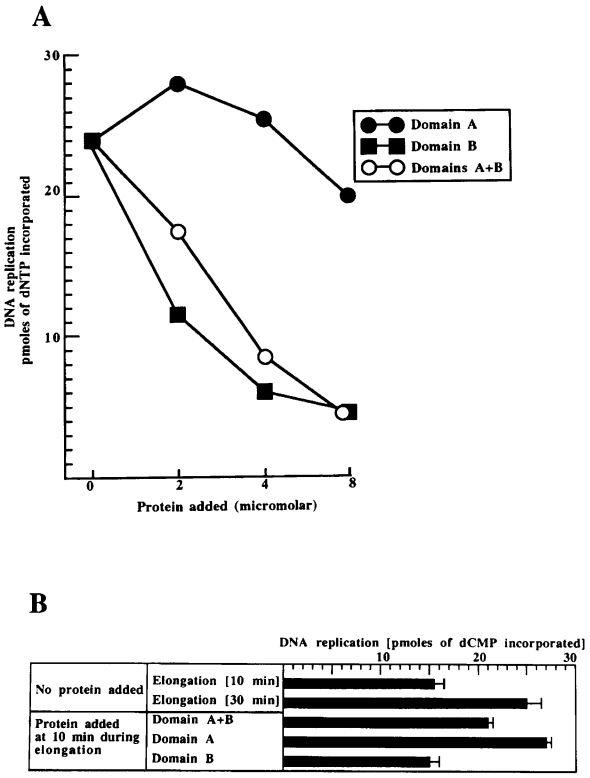

Fig. 4. RF-Cp145 domain B (481–728) inhibits DNA replication in cell free assays of SV40 replication. (**A**) Effect of addition of RF-Cp145 mutants on SV40 origin-dependent *in vitro* DNA replication. Varying amounts of GST fusion proteins of domain A (369–480), domain B (481–728), domains A+B (369–734) were added during initiation to SV40 replication reactions. DNA synthesis (pmol dCMP incorporated) was measured at 90 min. The values are plotted as % DNA replication relative to values obtained in control assays without added protein. DNA synthesis in control assays without added proteins was 23.9 ± 1.7 pmol dCMP incorporated/90 min/50 µl reaction. (**B**) RF-Cp145 domain B (481–728) which binds PCNA inhibits elongation phase of SV40 origin-dependent DNA replication. Replication reactions were performed in two steps as described in Materials and methods. Proteins were added at a final concentration of 4 µM to a replication reaction in which elongation had proceeded for 10 min. The effect on DNA elongation was determined when reactions with added proteins had been incubated for a total of 30 min. DNA synthesis measured at 10 min in control reactions indicates the level of replication at the time the proteins were added. Data are plotted as mean ± standard deviation of three separate experiments.

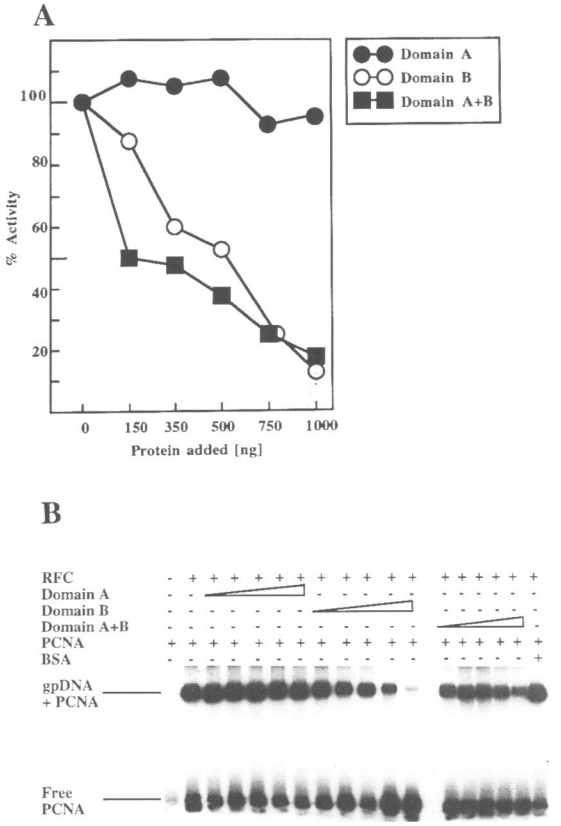

Fig. 5. RF-Cp145 domain B inhibits RF-C-dependent loading of PCNA. (**A**) Inhibition of RF-C-catalysed loading of ^{32}P-labelled PCNA onto gapped circular DNA by RF-Cp145 domain B. ^{32}P-labelled PCNA, RF-C, gapped circular DNA and indicated amounts of RF-Cp145 were incubated followed by glutaraldehyde fixation and analysis on 0.1% SDS, neutral 0.8% agarose gels. The radioactivity co-migrating with DNA was quantified with a phosphorimager. (**B**) Autoradiography of the agarose gel analysis of RF-C-dependent loading of ^{32}P-labelled PCNA onto gapped circular DNA (gpDNA). The effect of various concentrations (150, 350, 500, 750 and 1000 ng) of RF-Cp145 domain A (369–480), domain B (481–728) or domains A+B (369–734) on PCNA loading is shown. No RF-C complex was added in the first lane on the left. On the last lane on the right, adding BSA (1 µg) had no effect on PCNA loading. Equal amounts of radioactive PCNA were loaded in each lane with the exception of the first lane on the left, where free PCNA is loaded only as a marker. Free PCNA is loaded onto gpDNA in a RF-C-dependent manner. This is a different experiment from that shown in Figure 5A. Quantitation of the experiment in Figure 5B revealed PCNA loading of 99%, 91% and 95% with domain A; 73%, 54% and 30% with domains A+B; and 61%, 38% and 13% with domain B when 350, 750 and 1000 ng of recombinant protein was added to the reaction. Loading in the presence of BSA (1 µg) was designated as 100%.

domain B in inhibiting loading, but less effective in others (Figure 5B).

Since the loading of the PCNA sliding clamp leads to recruitment of pol δ, we then directly tested the effect of the two domains of RF-Cp145 on pol δ-dependent DNA synthesis using a primed ssDNA substrate. The ability of pol δ to replicate DNA correlates directly with the ability of RF-C to load PCNA onto DNA in the same assay system (Podust *et al.*, 1992). As expected, the same domain (domain B) which inhibits RF-C-dependent loading of PCNA also inhibits pol δ holoenzyme activity (Figure 6A). Analysis of the replication products on gels confirmed that domain B interfered with RF-C function (Figure 6B). Consistent with these conclusions, we have observed that RF-Cp145 domain B inhibits RF-C-dependent pol δ activity but not RF-C-independent pol δ activity (data not shown). We conclude, therefore, that domain B of RF-Cp145 (PCNA binding domain) inhibits RF-C function during DNA elongation.

Domain B of RF-Cp145 behaves like a dominant negative mutant and inhibits DNA replication in mammalian cells

We determined the *in vivo* effect on DNA replication of RF-Cp145 domain A (369–480), domain B (481–728), and domains A+B (369–734), in transient transfection experiments. These vectors were designed to express RF-Cp145 mutant proteins with an N-terminal haemagglutinin (HA) nonapeptide tag. RF-Cp145 expression vectors were transfected into U20S cells. The effect of expressing such RF-Cp145 mutants on DNA replication

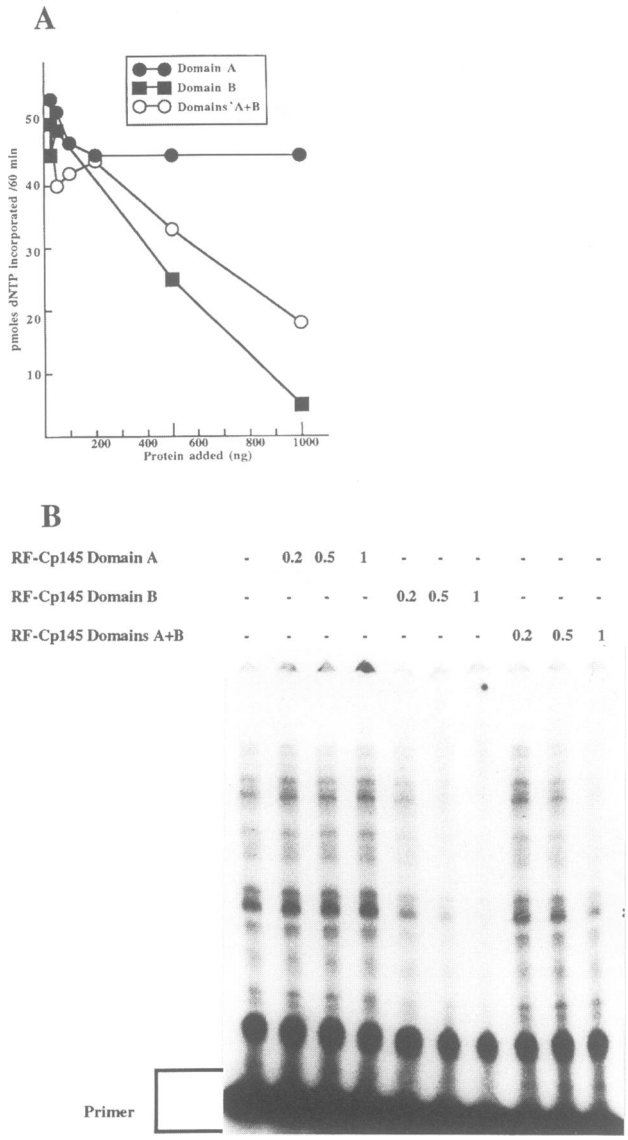

Fig. 6. RF-Cp145 domain B inhibits RF-C-dependent pol δ enzyme activity. (**A**) RF-Cp145 domain B inhibits RF-C-dependent pol δ activity. Primed ssDNA substrate was incubated with dNTPs, RF-C, pol δ, ATP and indicated amounts of RF-Cp145 proteins and products analysed on neutral agarose gels. The pol δ enzymatic activity was quantified by TCA precipitation. (**B**) Generation of RF-C-dependent pol δ replication products is inhibited by RF-Cp145 domain B. Primed ssDNA was a used as a substrate. The primer was end-labelled before annealing to the ssDNA substrate. Autoradiographic analysis of the effect of adding 0.2, 0.5 or 1 μg of RF-Cp145 domain A, domain B or domains A+B on pol δ-catalysed replication products is shown. The position of the unused primer is indicated. Pausing with this template under these conditions results in discrete products not a smear (Podust *et al.*, 1995b).

was determined by examination of individual cells expressing the HA tag by double staining with antibodies for HA and bromodeoxyuridine, as a marker of DNA replication. Epitope-tagged RF-Cp145 domain B and domains A+B localized exclusively to the nucleus whereas the RF-Cp145 domain A was present in both cytoplasm and the nucleus (Figure 7). RF-Cp145 domains A+B inhibited DNA replication, since none of the cells expressing the epitope tag stained for bromodeoxyuridine (Table I). Cells transfected with vector alone incorporate bromodeoxyuridine. RF-Cp145 domain B was an effective inhibitor of DNA replication. In contrast, consistent with our observations in cell-free assays described above, RF-Cp145 domain A did not inhibit DNA replication. These data demonstrate that the RF-Cp145 domain B acts like a dominant negative mutant in mammalian cells by inhibiting DNA synthesis.

Discussion

We have ascribed unique functional characteristics to each of two tandem domains in the middle of the large subunit of replication factor C (RF-Cp145). One of the domains (domain A) binds DNA, is conserved among prokaryotic DNA ligases (Ishino *et al.*, 1986) and eukaryotic PARPs (Cherney *et al.*, 1987; Kurosaki *et al.*, 1987; Huppi *et al.*, 1989), but is absent in other subunits of RF-C. Such absence suggests that the DNA binding ability of the RF-C complex might reside in the large subunit of RF-C. The other domain (domain B), which is conserved in all five subunits of RF-C (Chen *et al.*, 1992a,b; Bunz *et al.*, 1993; Burbelo *et al.*, 1993; O'Donnell *et al.*, 1993; Howell *et al.*, 1994; Li and Burgers, 1994a,b; Luckow *et al.*, 1994), binds PCNA. We show that the PCNA binding domain of RF-Cp145 inhibits SV40 DNA replication *in vitro*, RF-C-dependent loading of PCNA onto DNA, and behaves like a dominant negative mutant when expressed in mammalian cells.

We have demonstrated that human RF-Cp145 domain A (amino acid residues 369–480) binds to dsDNA which corresponds to residues 366–477 in murine RF-Cp145 and residues 117–231 in the large subunit of RFC in *Saccharomyces cerevisiae*. PARP also binds DNA and abrogation of PARP activity sensitizes cells to γ radiation (Küpper *et al.*, 1990; Ding *et al.*, 1992). It is of interest to note that the DNA binding domain A in RF-Cp145 is not homologous to the DNA binding domain of PARP. The DNA binding domain of PARP maps instead to the N-terminal zinc finger region (Küpper *et al.*, 1990). The automodification region of PARP is, however, homologous to domain A of RF-Cp145. Domain A of RF-Cp145 when expressed transiently in mammalian cells, localizes throughout the cell, suggesting that other regions of RF-C are required to ensure localization of RF-C to the nucleus. The inability of domain A to inhibit DNA replication *in vivo* is consistent with its inability to inhibit either DNA replication *in vitro* or RF-C-dependent loading of PCNA.

The flanking but non-overlapping PCNA binding domain of RF-Cp145 (domain B) is conserved in all subunits of RF-C. The conservation of this domain has been reported earlier (Bunz *et al.*, 1993; Burbelo *et al.*, 1993; Howell *et al.*, 1994; Luckow *et al.*, 1994). Although other reports have suggested that the RFC complex associates with PCNA (Burgers, 1991; Tsurimoto and Stillman, 1991), we show for the first time that RF-Cp145 can bind PCNA in the absence of other RF-C subunits. We have mapped the PCNA binding domain in human RF-Cp145 to amino acids 481–728 which correspond to amino acid residues 478–712 in murine RF-Cp145 and 232–431 in the large subunit of RF-C in *S.cerevisiae*. Transfection of domain B in mammalian cells reveals that this domain localizes to the nucleus. This might reflect the presence of an internal nuclear localization signal in this region of

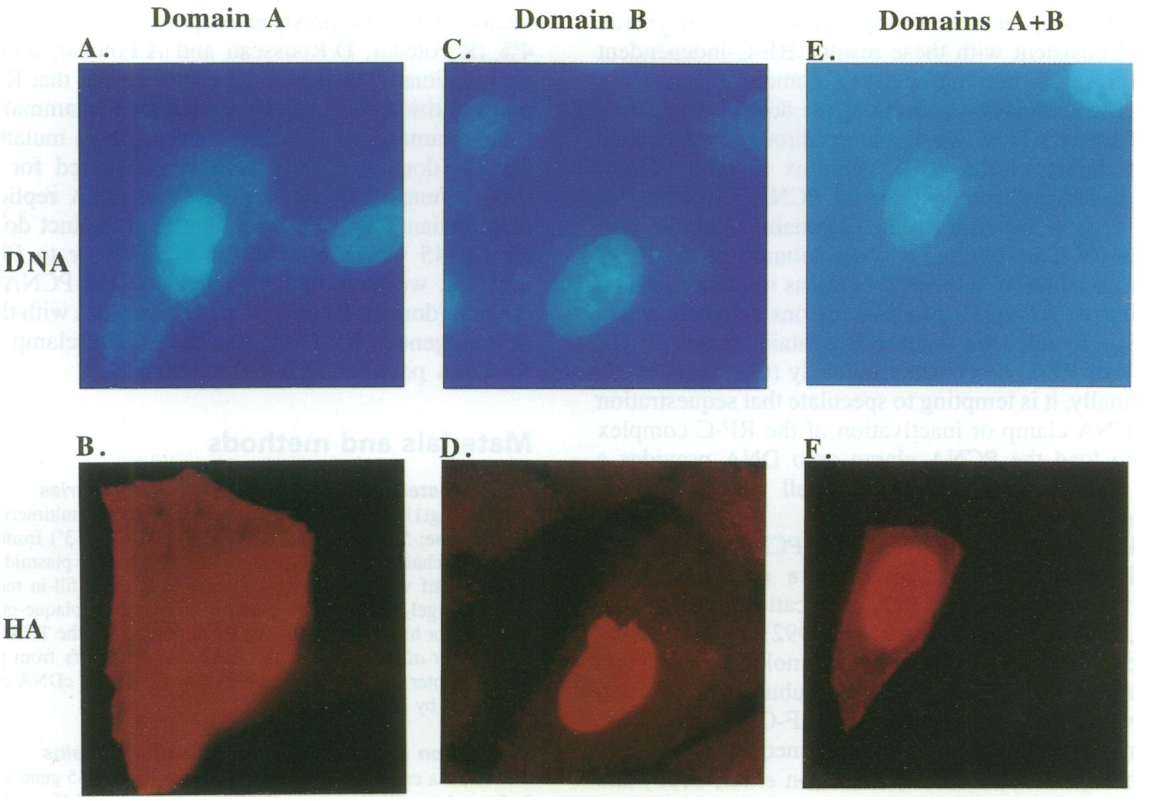

Fig. 7. Subcellular localization of RF-Cp145 domains. U20S cells were transiently transfected with vectors expressing either RF-Cp145 domain A, domain B, or domains A+B. All the expressed proteins were tagged with the HA epitope. The cells were double-stained with anti-HA antibody and with Hoechst 33258, a chromatin dye. Domain A and domains A+B localized exclusively to the nucleus whereas domain A was present throughout the cell.

Table I. RF-Cp145 domain B inhibits DNA replication in mammalian cells

Treatment	% of HA^+ $BrdU^+$ cells[a]	Cellular localization
Mock transfection	No HA+ cells detected	
Vector	34	99% whole cell
RF-Cp145 domains A+B (369–734)	<1	96% only nuclear
RF-Cp145 domain A (369–480)	54	99% whole cell
RF-Cp145 domain B (481–728)	<1	97% only nuclear

[a]The % of HA-positive cells which are HA^+BrdU^+ double-positive was used to score cells in S phase. U20S cells were transiently transfected with either control vector, or vectors expressing domain A, domain B, or domains A+B of RF-Cp145. All the expressed proteins were tagged with the HA epitope. The cells were double-stained for HA and bromodeoxyuridine (BrdU). The percentage of HA^+ cells which are also $BrdU^+$ was used to score cells in S phase. The subcellular localization of the different HA epitope-tagged proteins was scored by immunofluorescence. The data shown are for a representative experiment, and 400 cells were counted in each set. None of the mock-transfected cells was stained with HA antibody. The transfection efficiency was ~25%. These experiments have been repeated four times and similar results were obtained each time.

RF-Cp145 or binding to a nuclear protein such as PCNA might ensure nuclear localization. The functional importance of domain B is clear from its ability to inhibit DNA replication in mammalian cells, and SV40 origin-dependent DNA replication *in vitro*.

Mammalian PCNA (Miyachi *et al.*, 1978) is the processivity factor or sliding clamp required for pol δ-dependent leading and lagging strand synthesis (Muller *et al.*, 1994; Waga and Stillman, 1994). The sliding clamp tracking on DNA while holding the polymerase onto the template is consistent with the biochemical analysis of the interactions with DNA of PCNA (Stillman, 1994; Stukenberg *et al.*, 1994; Podust *et al.*, 1995a). Using radioisotope labelling or cross-linking it has been shown that PCNA clamps cannot be loaded onto closed DNA without the assistance of the RF-C ATPase subunits. Once loaded onto DNA they track along DNA unless prevented by steric hindrances. Complexes bound stably to circular DNA can be disassociated by linearizing the DNA, thus allowing the clamp to slide off the DNA (Burgers and Yoder, 1993; Stukenberg *et al.*, 1994; Podust *et al.*, 1995a). Analysis of such experiments has led to the suggestion that PCNA forms a torodial structure that encircles DNA. This has been confirmed by the analysis of the crystal structure of PCNA (Krishna *et al.*, 1994) and its *Escherichia coli* homologue (Kong *et al.*, 1992). PCNA forms a multimeric closed circle with a hole in the middle large enough for the passage of DNA without steric hindrance (Krishna *et al.*, 1994).

Domain B of RF-Cp145 inhibits DNA replication by inhibition of RF-C function. This is supported by the ability of domain B to inhibit RF-C-dependent loading of

the PCNA clamp and RF-C-dependent pol δ enzymatic activity. Consistent with these results, RF-C-independent pol δ activity is not inhibited by domain B (data not shown). The mechanism underlying the dominant negative effect of domain B *in vivo* could be through interference with the ability of the RF-C complex to bind PCNA, thereby decreasing the amount of PCNA available for loading onto a DNA template. Domain B could also inactivate RF-C by binding to other subunits of the RF-C complex. In addition, domain B contains sequences which are similar to ATP/GTP binding regions (Howell *et al.*, 1994). Specifically, the domain B contains boxes II, III, IV and V of RF-C described previously (Cullmann *et al.*, 1995). Finally, it is tempting to speculate that sequestration of the PCNA clamp or inactivation of the RF-C complex needed to load the PCNA clamp onto DNA provides a critical nodal point at which the cell regulates DNA replication.

The POL30 gene which encodes PCNA in yeast is essential for cell growth, revealing a requirement for PCNA in chromosomal DNA replication (Bauer and Burgers, 1988, 1990; Waseem *et al.*, 1992). A *S.cerevisiae* protein complex that is functionally homologous to human RF-C has been identified, and has a subunit composition and activity similar to that of human RF-C. All five RF-C genes in *S.cerevisiae* have been cloned (Chen *et al.*, 1992a,b; Howell *et al.*, 1994; Cullmann *et al.*, 1995) and all genes are essential for survival (Howell *et al.*, 1994; Cullmann *et al.*, 1995). Genetic analysis of the *S.cerevisiae cdc44* gene (large subunit of RF-C) and pol30 (PCNA) has led to the identification of pol30 mutations which suppress the phenotype of *cdc44* mutations (McAlear *et al.*, 1994). Since the suppression observed was not allele-specific it provided limited information regarding a potential physical interaction (McAlear *et al.*, 1994) and thus concluded that it could be the result of an indirect functional interaction. An independent study concluded that suppression of *cdc44* cold sensitivity by pol30 mutants seemed to occur by several different mechanisms which still need to be clarified (Ayyagari *et al.*, 1995). We show here for the first time a direct interaction between PCNA and RF-Cp145 (in the absence of other RF-C subunits). We now know that the *cdc44* mutations previously used to screen for suppressor pol30 mutants (McAlear *et al.*, 1994) correspond to residues 809 and 810 of human RF-Cp145 which lie outside the PCNA binding domain in RF-Cp145 that we have mapped (481–728). In the context of the results presented here it will now be interesting to determine if the previously identified *cdc44* mutants are still capable of binding to wild-type or mutant pol30. It should be noted that *cdc44* mutants complete S phase and exit the cell cycle with a DNA content of G_2/M (Howell *et al.*, 1994; McAlear *et al.*, 1994, 1996). Using a transient transfection approach we have shown for the first time that a dominant negative mutant of the large subunit of RF-C, which suppresses RF-C activity *in vitro*, also inhibits DNA replication in intact mammalian cells. We have extended these results by determining if domain B inhibits cell growth of transfected cells in soft agar. Transfected U20S cells were selected in the presence of neomycin and the number of colonies scored after 12 days. Expression of domain B leads to a 40% growth suppression as compared with growth in the presence of vector alone. Suppression of growth by domain A was 4% (R.Fotedar, D.Rousseau and A.Fotedar, unpublished observations). We have thus demonstrated that RF-Cp145 plays a critical role in DNA replication in mammalian cells.

In summary, using a dominant negative mutant of RF-Cp145 (domain B) we have demonstrated for the first time a functional role for RF-C in DNA replication in mammalian cells. We have identified distinct domains in RF-Cp145 which bind either to DNA or to PCNA. In addition, we have demonstrated that the PCNA binding domain (domain B) of RF-Cp145 competes with the ability of endogenous RF-C to load the PCNA clamp essential for DNA polymerase δ and ε activity.

Materials and methods

Southwestern screening of λgt11 cDNA libraries

A Jurkat λgt11 cDNA library was screened with a multimerized dsE4A motif (sense: 5′-TCTGGGTGTTTATCTGTAAGTA-3′) from the T-cell receptor β chain enhancer (Messier *et al.*, 1993b). The plasmid containing the E4 motif was linearized, labelled by a Klenow fill-in reaction, and the insert gel-purified. The positive clones were plaque-purified and analysed for binding the E3A and E4A motifs from the TCR b enhancer (Messier *et al.*, 1993a) and the AP-1 and ets motifs from the murine Vβ2 promoter (Messier *et al.*, 1992). The RF-Cp145 cDNA clones were sequenced by standard methods.

Generation of recombinant GST fusion proteins

The cDNAs encoding the fragments of the RF-Cp145 gene were cloned in-frame into pGEX vectors which expressed RF-Cp145 as a GST fusion protein. Recombinant protein was produced in *E.coli* (DH5α), as described earlier (Fotedar *et al.*, 1996). Briefly, bacteria were grown to an OD_{270} of 0.7 at 37°C and induced with IPTG at 20°C overnight. The bacterial pellet (500 ml bacterial culture) was resuspended in 20 ml of 20 mM Tris, pH 7.5, 1 mM EDTA, 100 mM KCl, 0.5% NP-40, 1 mM DTT, 0.1 mM PMSF, 1.5% sarcosyl, 2 μg/ml benzamidine, 2 μg/ml aprotinin, 2 μg/ml pepstatin A and 2 μg/ml trypsin inhibitor. The bacterial suspension was sonicated, freeze–thawed and the solubilized proteins mixed with 0.6 ml glutathione–Sepharose beads at 4°C for 1 h. The beads were washed and GST fusion proteins eluted with 50 mM Tris, pH 9.5, 20 mM glutathione, 120 mM NaCl and dialysed extensively against 20 mM Tris, pH 8, 1 mM EDTA, 10 mM NaCl, 1 mM DTT and 10% glycerol. The deletion fragments of RF-Cp145 were generated by PCR and completely sequenced to ensure that no errors were introduced by PCR. GST–RF-Cp145 domain proteins were produced as described above. We have consistently observed that recombinant domains A+B protein is less soluble compared with either domain A or domain B alone. Further, the yields of recombinant domains A+B protein is much lower than that of domain A or domain B and a significant amount of the domains A+B protein is retained in the inclusion bodies in *E.coli*. For Figure 3B, p21 fusion proteins were generated as described earlier (Fotedar *et al.*, 1996).

Gel-shift assays

End-labelled (10 000 c.p.m.) octamer motif (Messier *et al.*, 1993b), was incubated with 1 μg of purified recombinant RF-Cp145 domain proteins in the presence of 10 mM HEPES, pH 7.8, 4 mM $MgCl_2$, 0.1 mM EDTA, 4 mM spermidine, 100 mg/ml BSA, 2 mM DTT, 15% glycerol and 2 μg poly(dI:dC). The DNA–protein complexes were then resolved on a 4% non-denaturing polyacrylamide (0.25× TBE) gel.

Binding assays

For *in vitro* binding with RF-Cp145, 20 μl of glutathione–agarose beads were incubated with 5 μM GST–RF-Cp145 domain proteins and 0.5 μM PCNA for 20 min at 25°C in the presence of 40 mM HEPES, pH 7.5 and 8 mM $MgCl_2$. The binding of RF-Cp145 to PCNA was significantly lower if the assay was performed at 4°C using identical buffer conditions. The glutathione–agarose beads were washed four times with binding buffer [20 mM Tris, pH 8.0, 1 mM EDTA, 100 mM KCl, 0.5% (v/v) NP-40 and 2 μg/μl leupeptin and aprotinin] at 4°C and bound proteins eluted with SDS sample buffer. The purified recombinant PCNA used in this assay resolved as a single band on SDS–polyacrylamide gels after Coomassie blue staining (Figure 2).

In vitro transcription/translation

The *in vitro* transcription/translation vectors of PCNA and its deletion mutants (Matsuoka *et al.*, 1994) and conditions for *in vitro* transcription/translation have been described (Messier *et al.*, 1993b). [^{35}S]methionine-labelled proteins were used in binding assays with GST–RF-Cp145 and GST–p21 fusion proteins as described above. Binding of GST–p21 to PCNA was identical whether the binding assay was performed at 4°C or at 25°C. In contrast, the binding of RF-Cp145 to PCNA was only significant at 25°C.

Immunoblotting and antibodies

Proteins were resolved on 12% SDS–PAGE, transferred onto nitrocellulose (Fotedar and Roberts, 1991) and immunoblotted with modifications as described (Brenot-Bosc *et al.*, 1995). Anti-human PCNA antibody (Clone 19A2) was purchased from Coulter. The immunoblots were processed for ECL (Amersham) as described.

DNA replication assays

S-phase Manca cells were obtained by synchronizing exponentially growing cells with 2 mM hydroxyurea as described earlier (Brenot-Bosc *et al.*, 1995). S100 supernatants containing 100 mM NaCl were then prepared from hypotonic lysates of S-phase cells as described (Fotedar and Roberts, 1992). Replication reactions (Fotedar and Roberts, 1989) containing 150 ng of SV40 origin-containing plasmid DNA, with 1 μg of SV40 T antigen and 100 μg of S100 extract from S-phase Manca cells were performed as described (Brenot-Bosc *et al.*, 1995). The amount of DNA synthesis was quantitated by measuring the incorporation of [α-^{32}P]dCTP into tricholoroacetic acid-precipitable counts. The effect of RF-Cp145 proteins on elongation of DNA during replication was determined by performing replication in two steps. SV40 T antigen was pre-incubated for 30 min at 37°C with S-phase cell extracts and SV40 origin-containing DNA in the presence of 3 mM ATP to allow the formation of initiation complexes on DNA (Fotedar and Roberts, 1992). The start of DNA synthesis is prevented by the omission of deoxyribo- and ribonucleotide triphosphates. Elongation was then initiated by the addition of ribonucleoside triphosphates (except ATP) and deoxyribonucleoside triphosphates. After 10 min of elongation, GST–RF-Cp145 domains were added and the reactions allowed to continue for an additional 20 min.

DNA polymerase δ activity on primed ssDNA substrate

The ssDNA substrate was generated as described (Podust *et al.*, 1995a). A reaction mixture in a final volume of 25 μl contained 40 mM Tris–HCl, pH 7.5, 0.2 mg/ml BSA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP, 50 μM each of dGTP, dCTP, dTTP and dATP, 50 ng of primed ssDNA (5′-[α-^{32}P]phosphorylated primer), 15 ng PCNA, 45 ng RF-C, 0.3 U pol δ, 175 ng *E.coli* SSB and the designated RF-Cp145 domain. After 60 min at 37°C, DNA synthesis was stopped by the addition of 10% TCA. Acid-precipitable radioactivity was then counted. For product analysis, reactions were stopped by 1% SDS and 20 mM EDTA, pH 8. The DNA was precipitated with ethanol and analysed on an alkaline 1.5% agarose gel as described (Podust *et al.*, 1995a). Pausing with this template under these conditions (Podust *et al.*, 1995b) leads to the generation of discrete products and not a smear when products are run on a gel.

RF-C-dependent loading of PCNA

A recombinant PCNA engineered to be phosphorylated at the N-terminus with a cAMP-dependent protein kinase *in vitro* was used (Podust *et al.*, 1995a). Phosphorylated PCNA (ph-PCNA) retained complete biological activity (Podust *et al.*, 1995a). Briefly, ph-PCNA was expressed in *E.coli*, purified and phosphorylated *in vitro* with cAMP-dependent protein kinase using [γ-^{32}P]ATP as described. A reaction mixture in a final volume of 25 μl contained triethanolamine–HCl, pH 7.5, 0.2 mg/ml BSA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP, 50 ng of [^{32}P]ph-PCNA, 45 ng of RF-C, 100 ng of gapped circular DNA, and the designated RF-Cp145 domain. The samples were incubated at 37°C for 3 min, followed by addition of glutaraldehyde to a final concentration of 0.1% (w/v), incubated for 10 min at 37°C and samples run on a neutral 0.8% agarose gel in the presence of 0.1% SDS (Podust *et al.* 1995a). Finally, the gel was fixed in 10% acetic acid, 12% methanol, dried and exposed to X-ray film.

Transient transfection assays

The RF-Cp145 domains A, B or A+B were expressed as HA epitope-tagged (at the N-terminus) proteins in U20S cells under the control of the SRα promoter. Transfections were performed on U20S cells grown overnight on poly D-lysine-coated coverslips (Fotedar *et al.*, 1995). Cells were pulsed with bromodeoxyuridine (BrdU) for 30 min before harvesting to monitor DNA synthesis during S phase (Fotedar *et al.*, 1995). Under these conditions, DNA repair is not detectable (Pagano *et al.*, 1994). To detect DNA repair requires BrdU pulsing for 3–4 h (Pagano *et al.*, 1994). The effect of expressing RF-Cp145 mutants on cell cycle progression was monitored by double staining for HA and BrdU. For immunofluorescence, cells were fixed with 2% paraformaldehyde at 37°C followed by 70% ethanol at 4°C. Cells permeabilized with 0.2% (v/v) Triton X-100 were stained for HA epitope using the 12CA5 antibody and goat anti-mouse IgG (H+L)-Cy3 fluorochrome (Caltag). DNA was denatured for anti-BrdU staining by treatment with 4 M HCl–0.2% Triton X-100 followed by staining with FITC-labelled BrdU-specific antibody (Becton-Dickinson) and finally with Hoechst 33258, to visualize chromatin.

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