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The large subunit of replication factor C is a substrate for caspase-3 *in vitro* and is cleaved by a caspase-3-like protease during Fas-mediated apoptosis

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Caspase-3 is an ICE-like protease activated during apoptosis induced by different stimuli. Poly(ADP-ribose) polymerase (PARP), the first characterized substrate of caspase-3, shares a region of homology with the large subunit of Replication Factor C (RF-C), a five-subunit complex that is part of the processive eukaryotic DNA polymerase holoenzymes. Caspase-3 cleaves PARP at a DEVD-G motif present in the 140 kDa subunit of RF-C (RFC140) and evolutionarily conserved. We show that cleavage of RFC140 during Fas-mediated apoptosis in Jurkat cells and lymphocytes results in generation of multiple fragments. Cleavage is inhibited by the caspase-3-like protease inhibitor Ac-DEVD-CHO but not the caspase-1/ICE-type protease inhibitor Ac-YVAD-CHO. In addition, recombinant caspase-3 cleaves RFC140 *in vitro* at least at three different sites in the C-terminal half of the protein. Using amino-terminal microsequencing of radioactive fragments, we identified three sites: DEVD^{723}G, DLVD^{922}S and IETD^{1117}A. We did not detect cleavage of small subunits of RF-C of 36, 37, 38 and 40 kDa by recombinant caspase-3 or by apoptotic Jurkat cell lysates. Cleavage of RFC140 during apoptosis inactivates its function in DNA replication and generates truncated forms that further inhibit DNA replication. These results identify RFC140 as a critical target for caspase-3-like proteases and suggest that caspases could mediate cell cycle arrest.

Keywords: apoptosis/CPP32/PARP/programmed cell death/RF-C

Introduction

Human caspase-3 (CPP32/apopain/Yama), a member of the ICE/CED-3 family of aspartate-specific cysteine proteases (Fernandes-Alnemri *et al.*, 1994), is involved in apoptosis mediated by different stimuli, including Fas/Fas ligand interaction (Enari *et al.*, 1996). The first caspase-3 substrate that was identified is the nuclear DNA repair enzyme poly(ADP-ribose) polymerase (PARP) which is cleaved by caspase-3 at a DEVD↓G motif (Nicholson *et al.*, 1995). Other caspase-3 substrates identified include sterol regulatory element binding proteins (SREBP-1, SREBP-2) (Wang *et al.*, 1996), DNA-dependent protein kinase (Song *et al.*, 1996), the 70 kDa protein component of the U1 small nuclear ribonucleoprotein (U1-70 kD) (Casciola-Rosen *et al.*, 1996), huntingtin (Goldberg *et al.*, 1996), D4 GDP dissociation inhibitor (D4-GDI) (Na *et al.*, 1996), p21-activated kinase 2 (PAK2) (Rudel and Bokoch, 1997), Protein Kinase C δ (Ghayur *et al.*, 1996), the Cdc2-like PITSLRE kinases (Beyaert *et al.*, 1997) and DNA fragmentation factor (DFF) (Liu *et al.*, 1997). From these substrates, the consensus cleavage site for caspase-3, DXXD↓ followed by a small amino acid can be derived, although the presence of the P4 Asp does not appear to be an absolute requirement (Wang *et al.*, 1995a; Srinivasula *et al.*, 1996; Rudel and Bokoch, 1997).

The exact role of the cleavage of most caspase-3 substrates during apoptosis is still not clear. For instance, it was suggested that PARP proteolysis during apoptosis was probably not critical for the cell-death mechanism (Chinnaiyan and Dixit, 1996), given that PARP knock-out mice do not show severe pathologies related to apoptosis anomalies (Wang *et al.*, 1995b). However, a recent study showed that PARP knock-out mice are more susceptible to DNA fragmentation (de Murcia *et al.*, 1997). Several caspase-3 substrates are nuclear proteins and their sites of cleavage are often located between functional domains. The 140 kDa large subunit of human DNA replication factor C (RFC140) is a nuclear protein that possesses a domain of homology to prokaryotic DNA ligases and PARP, known as homology box I (Bunz *et al.*, 1993; Burbelo *et al.*, 1993; Cullmann *et al.*, 1995). This domain was recently described as a distinct version of the BRCT domain found in the superfamily of DNA damage-responsive cell cycle checkpoint proteins (Bork *et al.*, 1997). Such a domain is also present in the retinoblastoma (Rb) protein. Interestingly, RFC140 contains a putative caspase-3 cleavage site (DEVD↓G) located within a conserved region called replication factor C (RF-C) box V (Bunz *et al.*, 1993; Burbelo *et al.*, 1993; Cullmann *et al.*, 1995). This site is located precisely at the carboxy-terminal end of the PCNA-binding domain (Fotedar *et al.*, 1996) and cleavage would result in separation of the DNA- and PCNA-binding domains from the RF-C heteropentamerization domain (Uhlmann *et al.*, 1997).

DNA replication in eukaryotes requires three distinct DNA polymerases (pol), α, δ and ε. DNA pol α, complexed with DNA primase, initiates DNA synthesis and the production of pre-Okazaki fragments. The functions of pol δ and pol ε remain to be elucidated, but it is thought that they are involved in the maturation of pre-Okazaki

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fragments on the lagging strand and synthesis on the leading strand DNA. In contrast to pol α, pol δ and pol ε require two accessory protein factors, a sliding clamp, proliferating cell nuclear antigen (PCNA) that tethers the pols to the DNA, and a clamp loader, RF-C, that loads the sliding clamp onto DNA. RF-C recognizes primed DNA at the 3′-OH end of the primer and assemble PCNA onto DNA in the presence of ATP. Subsequent ATP hydrolysis, catalyzed by RF-C is required for pol δ or ε to join the complex and initiate chain elongation (Stillman, 1994). Human RF-C is a complex composed of five proteins of molecular weights of 36, 37, 38, 40 and 140 kDa (Cullmann *et al.*, 1995; Uhlmann *et al.*, 1996). The different subunits of RF-C share significant homology among themselves (Bunz *et al.*, 1993; Burbelo *et al.*, 1993) although each subunit is essential for RF-C function (Cullmann *et al.*, 1995; Uhlmann *et al.*, 1996). The small RF-C subunits, with the exception of RFC38, all have either DEAD-A or DEAD-S sequences located within the homology V region and these motifs could be potential caspase-3 cleavage sites.

The RFC140 subunit may carry out functions other than its role in DNA replication and repair (Aboussekhra *et al.*, 1995). RFC140 is highly homologous to the yeast CDC44 protein (Rfc1) which is required for cell cycle progression as its mutation causes G_2/M cell cycle arrest in yeast (Amatruda Howell *et al.*, 1994). By analogy to CDC44, a possible function of RFC140 in the G_2/M checkpoint might be related to its role in DNA repair (McAlear *et al.*, 1996). Moreover, RFC140 could be involved in telomere stability or turnover (Adams and Holm, 1996), DNA recombination (Halligan *et al.*, 1995) and adipoblast differentiation (Lyle *et al.*, 1996).

Three recently identified substrates of caspase-3 or caspase-3-like proteases, namely the Rb protein (Jänicke *et al.*, 1996), MDM2 (Erhardt *et al.*, 1997) and PITSLRE kinases (Beyaert *et al.*, 1997) are involved in the cell cycle process. Rb is critical for the regulation of S-phase entry (reviewed in Sherr, 1996), PITSLRE kinases are Cdc2 kinase-related proteins (Lahti *et al.*, 1995), and MDM2 is involved in the regulation of p53 and Rb functions (Xiao *et al.*, 1995). Thus, several cell cycle-related proteins are targets for caspases. As RF-C or RF-C subunits are also involved directly in cell cycle or in the S-phase checkpoint (Amatruda Howell *et al.*, 1994; McAlear *et al.*, 1996; Sugimoto *et al.*, 1996) and contain putative caspase-3 cleavage motifs, we investigated whether they could be cleaved during the apoptotic process. In this report, we show that RFC140 is cleaved to several fragments during Fas-induced apoptosis and we demonstrate that recombinant caspase-3 cleaves RFC140 *in vitro*.

Results

Cleavage of RFC140 in Jurkat cells during Fas-mediated apoptosis

We treated Jurkat cells with an anti-Fas antibody and levels of apoptosis and necrosis were assessed by FACS analysis using Annexin V/propidium iodide staining (Martin *et al.*, 1995). Figure 1A shows that phosphatidylserine externalization of Jurkat cells started after 1 h of anti-Fas treatment. Cell viability (lower left quadrant)

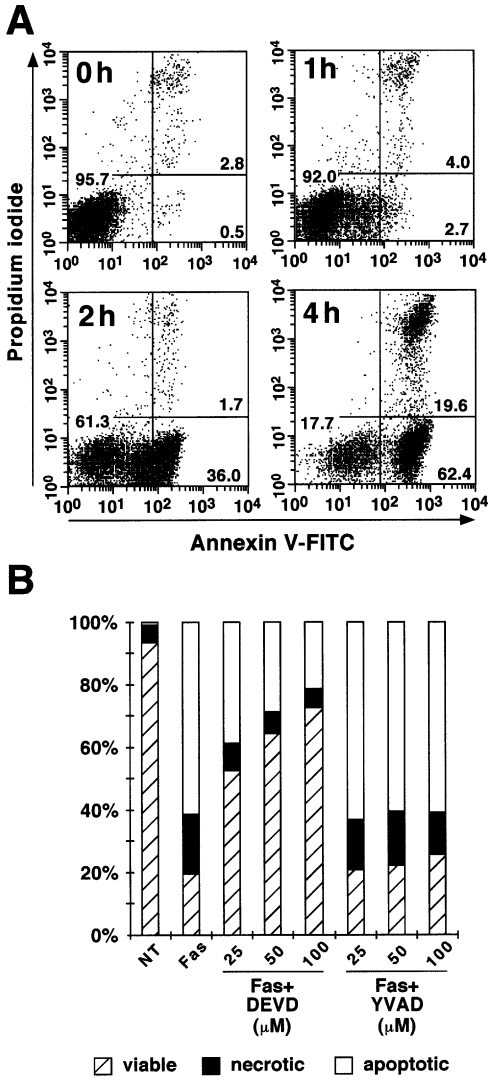

Fig. 1. Flow cytometric analysis of apoptosis in Jurkat cells induced by Fas cross-linking. (**A**) Annexin V binding and propidium iodide uptake of Fas-ligated Jurkat cells. Untreated Jurkat cells (0 h) and Jurkat cells that were transferred into anti-Fas coated plates for 1–4 h, at 37°C. (**B**) Jurkat cells were preincubated with indicated concentrations of Ac-DEVD-CHO or Ac-YVAD-CHO for 30 min, at 37°C prior to transfer to anti-Fas coated plates for 4 h, at 37°C. NT, not treated. Jurkat cells that were transferred to uncoated plates.

decreased to 18% after 4 h whereas the percentage of apoptotic cells (lower right) reached 62%. At this time-point, 20% of the cells incorporated propidium iodide (upper right quadrant) and were scored as necrotic cells.

Lysates from treated cells were fractionated on 10 or 12% SDS–PAGE and immunoblotted with anti-RFC140, anti-caspase-3 and anti-PARP antibodies (Figure 2). A band of 148 kDa corresponding to the full-length RFC140 was detected in all samples. Additional bands appeared at 97, 73 and 65 kDa after 2 h of anti-Fas treatment. After 4 h, two weakly detected bands of 50 and 45 kDa appeared with a concomitant decrease in the intensity of the 148 kDa band. PARP was processed 1 h earlier than RFC140. Preincubation of the cells with the caspase-3-like protease inhibitor Ac-DEVD-CHO resulted in a 60% inhibition of apoptosis and of PARP cleavage and in a complete block of RFC140 cleavage (Figures 1B and 2). This effect was

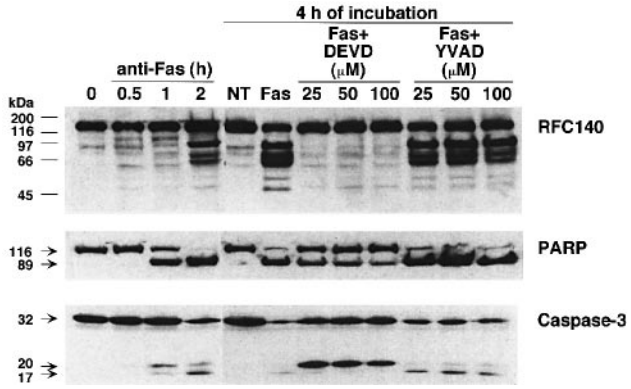

Fig. 2. Proteolysis of RFC140, PARP, and caspase-3 in Jurkat cells stimulated with anti-Fas. Cell lysates were prepared from cells treated as described in the experiment from Figure 1 and analyzed by Western blotting using anti-RFC140 (1:2000), anti-PARP (1:10 000) and anti-caspase-3 (1:1500) antibodies.

associated with a lack of caspase-3 processing, resulting in a block of p20 to p17 conversion. In contrast, the ICE-like protease inhibitor Ac-YVAD-CHO did not block PARP cleavage, exerted a partial and limited inhibition of apoptosis (10%) and had no significant effect on RFC140 processing. Thus, cleavage of RFC140 in anti-Fas-treated Jurkat cells might be mediated by an Ac-DEVD-CHO-inhibitable protease such as caspase-3, caspase-6/Mch2 or caspase-8/FLICE.

Processing of RFC140 in Fas-treated peripheral blood lymphocytes (PBLs)

We performed a similar set of experiments with activated human peripheral blood lymphocytes (PBLs). Untreated PBLs contained 7.5% necrotic cells and 1.8% apoptotic cells (Figure 3A). RFC140 and PARP were present as full-length proteins of 148 and 116 kDa, respectively (Figure 3B). The percentage of necrotic and apoptotic cells in anti-Fas-treated PBLs reached 39% and 35%, respectively (Figure 3A), and RFC140 was cleaved predominantly into fragments of 73, 65, 53 and 48 kDa. Two minor fragments, of 40 and 36 kDa, were also observed. The amount of the 97 kDa band formed was lower in PBLs than in Jurkat cells, probably due to more extensive proteolysis. Processing of caspase-3 (data not shown) and PARP (Figure 3B) was also induced by Fas treatment.

Cell necrosis and apoptosis of PBLs were only slightly inhibited by Ac-DEVD-CHO (Figure 3A), as their percentages decreased only from 35 to 32% and from 39 to 33%, respectively, in the presence of the caspase-3-like inhibitor. Although PARP cleavage was only slightly decreased, RFC140 fragmentation was almost completely blocked by Ac-DEVD-CHO exposure (Figure 3B). On the other hand, the ICE-like protease inhibitor Ac-YVAD-CHO had no protective effect on induction of apoptosis and RFC140 or PARP cleavage. The cell-permeable ICE-like protease inhibitor zVAD-fluoromethylketone (zVAD-fmk) partially protected PBLs against apoptosis as only 7% of the cells were scored as apoptotic. However, PARP and RFC140 cleavage were both totally blocked by this inhibitor (Figure 3B). The serine-protease inhibitor TLCK had no effect on apoptosis or on the processing of PARP and RFC140.

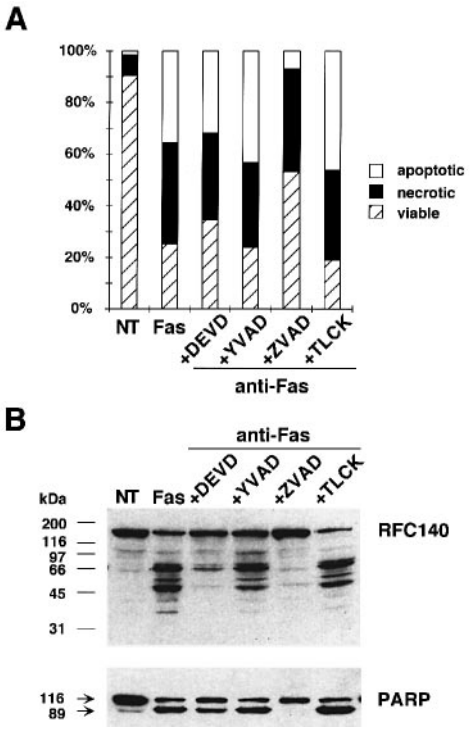

Fig. 3. Proteolysis of RFC140 and PARP in Fas-ligated peripheral blood lymphocytes (PBLs). (**A**) Activated PBLs were transferred to anti-Fas coated plates in the presence or absence of 100 μM of Ac-DEVD-CHO, Ac-YVAD-CHO, zVAD-fmk or 25 mM of TLCK. After 30 min of incubation at 37°C, cells were transferred to anti-Fas coated plates for 6 h at 37°C, followed by FACS analysis using Annexin V binding and propidium iodide incorporation. NT, not treated; PBLs that were transferred to uncoated plates. (**B**) Lysates were prepared from the cells treated in the experiment shown in (A) and analyzed by Western blotting with anti-RFC140 and anti-PARP antibodies. NT, not treated.

In vitro proteolysis of RF-C subunits by caspase-3

To address whether caspase-3 can cleave RFC140, the cDNA for RFC140 was used to direct *in vitro* transcription/translation in reticulocyte lysates and the resulting [^{35}S]-methionine-labeled protein was incubated with crude lysates of bacteria expressing GST alone (G) or a GST–caspase-3 fusion protein (C). Expression of pro-caspase-3 in bacteria yields a processed active caspase-3 that is indistinguishable from the native enzyme (Mittl *et al.*, 1997). As shown in Figure 4A, in the presence of lysates from GST-expressing bacteria, the major ^{35}S-labeled protein band corresponded to the full-length RFC140. However, after incubation in the presence of the lysate containing caspase-3, additional bands of 97, 50, 45, 32, 26 and 24 kDa appeared. We obtained ~90% of cleavage of [^{35}S]Met-labeled RFC140 in 1 h, whereas it took only 2.5 min for similar proteolysis of [^{35}S]Met-labeled PARP using identical amounts of bacterial lysates containing caspase-3 (data not shown). These results may explain the slower rate of cleavage of RFC140 as compared with PARP cleavage that we observed *in vivo* in Figure 2. RFC140 cleavage was completely inhibited by the addition of 0.1 μM Ac-DEVD-CHO (Figure 4A, lane 'CD'). Cleavage of the small subunits of RF-C by recombinant caspase-3 was examined and, as shown in Figure 4A, no significant cleavage of p40, p38, p37 or p36 RF-C subunits was detected. While a band of ~25 kDa in lane p40/C

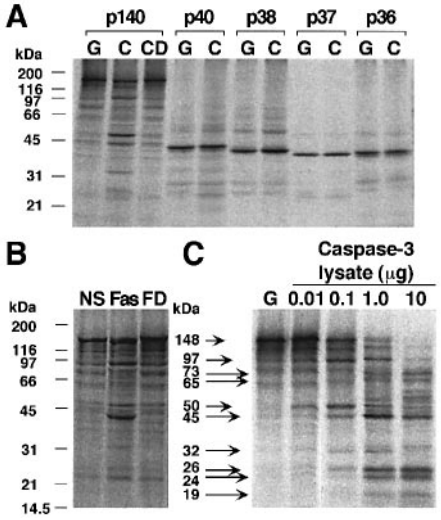

Fig. 4. *In vitro* cleavage of [^{35}S]methionine-labeled RF-C subunits by recombinant caspase-3 (**A** and **C**) or by Jurkat cell lysates (**B**). Incubations were performed at 30°C for 1 h and the reactions were analyzed by 8–20% SDS–PAGE and subjected to exposure on a PhosphorImager. 'G' represents lysate of *E.coli* expressing GST only and was used as a negative control. 'C' represents caspase-3 bacteria lysate. 'CD' and 'FD', caspase-3 bacterial lysate and Fas-stimulated Jurkat cell lysate, respectively, incubated with 0.1 μM Ac-DEVD-CHO prior to addition of [^{35}S]methionine-labeled RFC140. Jurkat cell lysates [Fas and FD lanes in (B)] were prepared after a 3-h anti-Fas treatment that resulted in 59% of apoptotic cells as determined by FACS analysis using Annexin V binding and propidium iodide incorporation. NS, non-stimulated Jurkat cell lysate. Reactions were carried out with 1 μg (A), 50 μg (B) or indicated amounts (C) of crude lysate proteins.

seems to be absent from the control p40/G lane, this is probably due to uneven loading between those two lanes. Radiolabeled small subunits of RF-C were not cleaved either when incubated with apoptotic Jurkat cells lysates (data not shown), suggesting that they are not cleaved *in vivo* during apoptosis. Thus, even though all small subunits of RF-C contain a potential caspase-3 cleavage site in their RF-C homology V domain, no cleavage of these subunits was detected under our experimental conditions.

Recombinant caspase-3 and apoptotic Jurkat cell extracts process RFC140 to identical fragments

To compare the proteolytic activity present in apoptotic Jurkat cells with that of recombinant caspase-3, [^{35}S]Met-labeled RFC140 was added to Fas-treated Jurkat cell extracts or to various amounts of bacterial lysates containing caspase-3. After incubation for 1 h at 30°C, both preparations yielded the same fragments of 97, 73, 65, 50, 45 and 32 kDa (Figure 4B and C). When increasing amounts of the caspase-3 lysates were used, the protein fragments generated were first the 97 and 50 kDa bands followed by the 45, 32 and 26 kDa bands and finally the 73, 65, 24 and 19 kDa bands (Figure 4C). In parallel, full-length RFC140, the 97 and the 50 kDa bands almost disappeared. Therefore the low level of 97 kDa band observed in PBLs (Figure 3B) could be due to more extensive processing of RFC140 than seen in Jurkat cells (Figure 2).

Microsequencing and mapping of the cleavage sites in RFC140

The proposed consensus sequence cleavage site of caspase-3 is DXXD↓X and this protease appears to undergo autocatalysis at the IETD↓S motif located between the p20 and p12 subunits of the proenzyme. For these reasons, the sequences DEED^{114}D, DEVD^{723}G, DKSD^{872}L, DLVD^{922}S, DEKD^{1110}S and IETD^{1117}A were considered as putative cleavage sites in RFC140. To identify which sites were recognized by caspase-3, RFC140 proteins were synthesized in the presence of different ^{3}H-labeled amino acids and mixed with bacterial lysates containing recombinant caspase-3. Figure 5A shows the results of an Edman degradation of some of the fragments isolated after cleavage. Analysis of the [^{3}H]leucine-labeled 26 kDa fragment revealed the presence of a leucine at position 14 and 17 of the amino-terminal part of the fragment in addition to an isoleucine at position 11 from the [^{3}H]isoleucine-labeled fragment. These results confirmed the DEVD^{723}G sequence as one cleavage site. Cleavage at the DEVD^{723}G site probably accounts for the 97 and 50 kDa fragments observed *in vivo* and *in vitro* (Figures 2 and 4C). The 24 kDa bands obtained from [^{3}H]leucine- and [^{3}H]isoleucine-labeled RFC140 proteins gave signals at position 11–12 and 3, respectively, identifying the sequence DLVD^{922}S as another cleavage site for caspase-3. The DLVD^{922}S site also generated the 32 kDa fragment since this fragment contained leucine residues at positions 11 and 12 (data not shown). Thus, the 32 kDa fragment must be cleaved further at a carboxy-terminal site that could either be the DEKD^{1110}S or the IETD^{1117}A site to generate the 24 kDa band and a small fragment of 5 kDa that was detected after electrophoresis through an 8–20% gradient SDS–PAGE using 16 cm gels followed by prolonged autoradiographic exposure (data not shown). For this reason, the [^{35}S]Met-labeled 5 kDa fragment was microsequenced and yielded a strong signal at position 2, suggesting that IETD^{1117}A is a third site of cleavage. Confirmation of this cleavage site with the [^{3}H]Ile-labeled 5 kDa fragment was not possible. However, we cannot rule out cleavage at the putative DEKD^{1110}S site.

Mutational analysis of RFC140 cleavage sites

Since we could not identify all the sites of cleavage of RFC140 by microsequencing, we further analyzed the pattern of cleavage of RFC140 by caspase-3 by carrying out *in vitro* cleavage assays with point mutants and truncated forms of RFC140. As shown in Figure 6A, the 555 N-terminal amino acids derivative of RFC140 (NTD) was not cleaved by the caspase-3 bacterial lysate; thus, the putative DEED^{114}D site was not used. Incubation of the C-terminal construct (CTD) consisting of amino acids 555–1148 of RFC140 with caspase-3 resulted in the formation of fragments of 50, 45, 32, 26 and 24 kDa products, similar to those formed with the full-length RFC140. An additional 21 kDa band was generated with CTD that could not be observed with the other RFC constructs which most likely contains amino acids 555–723. Incubation of the 776–1148 C-terminal part of RFC140 (776, Figure 6A) with caspase-3 yielded the 32 and 24 kDa fragments. However, the 26 kDa fragment was absent, consistent with the fact that this fragment is generated from the DEVD^{723}G site. Incubation of a

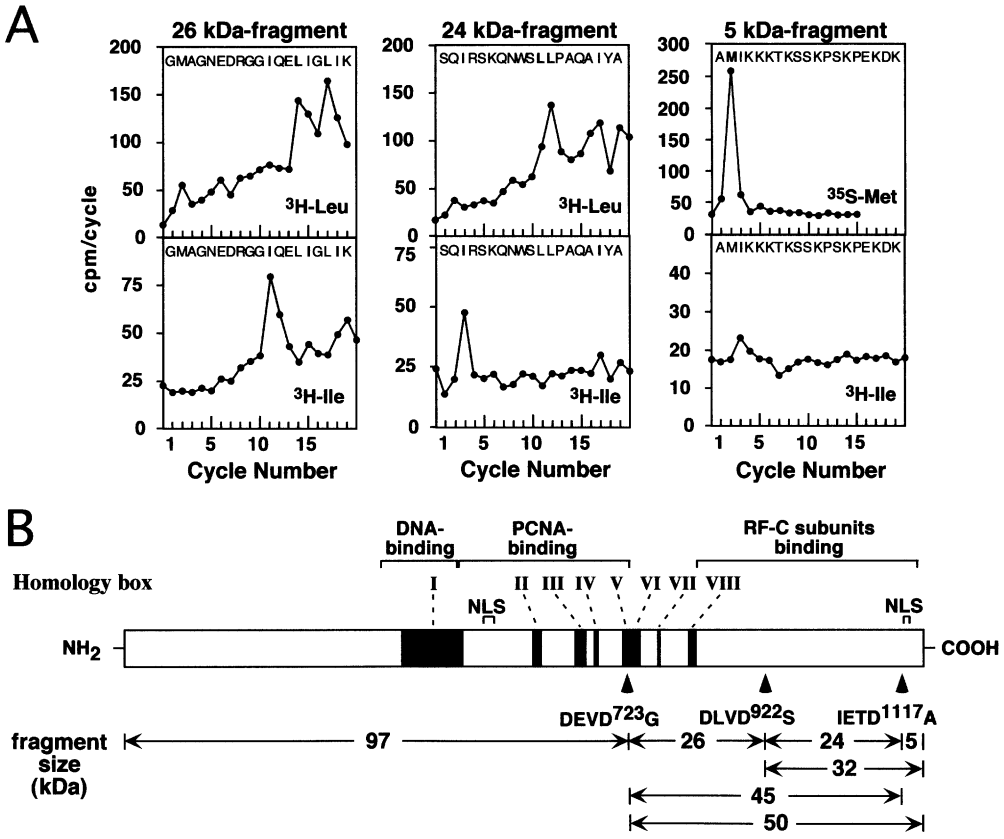

Fig. 5. Amino-terminal radiosequencing of some of the products formed by recombinant caspase-3 cleavage of RFC140 and localization of RFC140 functional domains and determined cleavage sites. (**A**) RFC140 was synthesized in a coupled transcription–translation T7 RNA polymerase–rabbit reticulocyte lysate system in the presence of the indicated ^{3}H- or ^{35}S-labeled amino acids and cleaved by recombinant caspase-3 lysates. The cleaved products were separated on an 8–20% SDS–PAGE and transferred to PVDF membranes. Bands corresponding to the indicated size fragments were excised and sequenced. The radioactivity recovered at each cycle of Edman degradation is plotted. Cycle 0 corresponds to the recovered radioactivity after a complete degradation cycle in absence of phenylisothiocyanate. (**B**) The size of the various fragments are calculated based on their electrophoretic migration from a 16-cm 8–20% SDS–polyacrylamide gel (data not shown). NLS, putative nuclear localization signals.

truncated form containing amino acids 877–1148 of RFC140 with caspase-3 led to formation of the 32 kDa fragment and possibly the 24 kDa fragment.

Site-directed mutagenesis of DEVD^{723}G to EEVE^{723}G (723E) was performed to abolish this site of cleavage. This alteration abrogated the cleavage, leading to generation of the 26 kDa fragment from the DEVD^{723}G site in wild-type RFC140 (Figure 5A and B and Figure 6B). The largest fragment formed from the 723E mutated RFC140 was a 114 kDa band, possibly arising from cleavage at DLVD^{922}S. A 91 kDa band observed in the 723E lane in the presence of caspase-3 (Figure 6B), probably resulted from cleavage of the contaminating band that appears at 116 kDa in the 723E control lane. The presence of extra bands at 41 and 44 kDa may have resulted from cleavage at sites between amino acid 555 and 723 and cleavage at 922. This could explain the disappearance of the 26, 19 and 15 kDa bands present in the wild-type RFC140/caspase-3 that were absent in mutant 723E/caspase-3 (Figure 6B). The deletion and point mutants confirm the results obtained from amino-terminal sequencing reported in Figure 5 and allow to assign amino acids 555–723 of RFC140 as a region containing two other cleavage sites that might explain the 65 and 73 kDa bands observed *in vivo* and *in vitro*.

An N-terminal fragment of RFC140 inhibits DNA replication

RF-C complexes formed with the RFC140 subunit deleted from the N-terminus to either amino acid 687 or 766 are devoid of RF-C activity (Uhlmann *et al.*, 1997). Thus, an RF-C complex containing RFC140 cleaved at amino acid position 723 should also be inactive. Fotedar and collaborators described a domain consisting of amino acids 481–728 of RFC140, which corresponds to the PCNA-binding domain, that can act as a dominant-negative inhibitor of DNA replication (Fotedar *et al.*, 1996). Fragment 1 to 723 of RFC140 generated by cleavage at DEVD^{723}G is also likely to act as an inhibitor of DNA replication. Since it appeared that cleavage sites between amino acids 555 and 723 existed, we decided to test whether a fragment (amino acids 1–555) without the complete PCNA-binding domain could also inhibit DNA replication. For this purpose, a highly purified N-terminal fragment of RFC140 spanning amino acids 1–555 was added to an elongation reaction (using singly primed M13 single-stranded DNA as the template) catalyzed by polymerase δ that is dependent on PCNA, RF-C and the multisubunit DNA binding protein RPA. Figure 7 shows that the fragment inhibited this replication reaction in a concentration-dependent manner. The inhibition observed

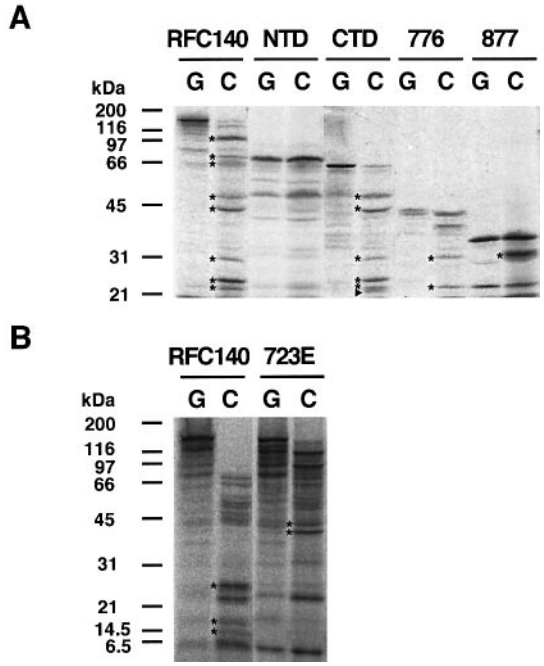

Fig. 6. Analysis of RFC140 truncated forms (**A**) and point mutant (**B**) cleavage by recombinant caspase-3. Various ^{35}S-labeled forms of RFC140 generated *in vitro* were added to bacterial lysates for 1 h at 30°C. Reactions were separated by 10% (A) or 8–20% (B) SDS–PAGE and analyzed after exposure in a PhosphorImager. 'G' represents lysate of *E.coli* expressing GST only which was used as a negative control. 'C' represents lysate from bacteria expressing caspase-3. 'NTD', amino acids 1–555; 'CTD', amino acids 555–1148; '776', amino acids 776–1148; '877', amino acids 877–1148. Asterisks indicate major fragments. A triangle indicates the position of a fragment that could correspond to amino acids 555–723 in the CTD/C lane. Reactions were carried out using 1 μg (A) or 10 μg (B) of crude lysate proteins.

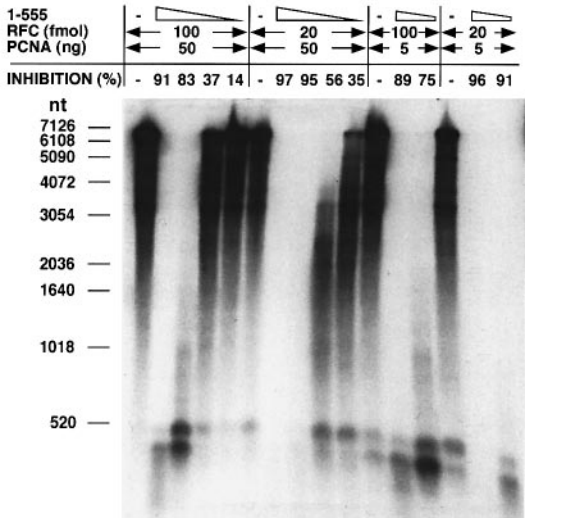

Fig. 7. A fragment of RFC140 spanning amino acids 1–555 inhibits RF-C-dependent DNA replication. The elongation of singly primed circular M13 DNA was carried out as described in Materials and methods. Shown is the autoradiograph of the replication products after alkaline agarose gel electrophoresis. The positions and sizes in nucleotides (nt) of markers are shown on the left. Reactions were performed in a high (100 fmol) or low (20 fmol) level of RF-C and in a high (50 ng) or low (5 ng) amount of PCNA. Where indicated, decreasing amounts of the purified RFC140 N-terminal fragment (1–555) were: 540, 270, 135 and 67.5 fmol. Only 270 and 135 fmol were used with low amounts of PCNA. The inhibition was calculated as percentage of total deoxynucleotide incorporated into replication products relative to reactions without fragment 1–555.

at low levels of both RF-C and PCNA was partly alleviated by increased levels of either component. At high levels of both RF-C and PCNA, the addition of the RFC140 N-terminal fragment, stoichiometric in amount to RF-C, inhibited the replication reaction 37%. These results suggest that this fragment containing the N-terminal DNA binding domain of RFC140 competes with intact RF-C for the binding to DNA as well as PCNA. Since the 1–555 fragment contains only part of the PCNA binding domain, the partial reversal of inhibition by high levels of PCNA may be indirect, reflecting an increased regeneration of the PCNA–pol δ complex at primer ends. Further experiments are needed to explain the PCNA effect described in Figure 7.

Discussion

Among the different substrates cleaved by caspase-3, PKCδ, DFF and PAK2 have been shown to be directly involved in mediating cellular responses characteristic of apoptosis, namely chromatin condensation (Ghayur *et al.*, 1996), DNA fragmentation (Ghayur *et al.*, 1996; Liu *et al.*, 1997), and membrane and morphological changes (Rudel and Bokoch, 1997). No direct role in cell death mechanisms have been determined for the other substrates. Therefore, the identification of caspase-3 and other caspase substrates may provide important clues about the coordinate execution phase of the apoptotic process.

Results presented here demonstrate that RFC140, a critical component of the DNA replication machinery, was cleaved at multiple positions by a caspase-3-like protease *in vivo* during anti-Fas-induced apoptosis of the Jurkat T cell line and activated primary PBLs. Moreover, we have shown that the proteolytic activity present in apoptotic Jurkat cells and recombinant caspase-3 cleave [^{35}S]-methionine-labeled RFC140 and generate the same fragments, confirming that caspase-3 is sufficient to mediate this processing. Inhibition of RFC140 and PARP cleavage by Ac-DEVD-CHO was more efficient in Jurkat cells than in PBLs. Moreover, this inhibitor blocked apoptosis in Jurkat cells but not in activated PBLs. This could be due to a less efficient passage of Ac-DEVD-CHO into PBLs as compared with Jurkat cells. As a confirmation, the general ICE-like protease inhibitor zVAD-fmk, that is more permeable, partially inhibited Fas-induced apoptosis and completely blocked PARP and RFC140 cleavage in PBLs. Ac-DEVD-CHO inhibited RFC140 cleavage more efficiently than PARP, which is consistent with our observation that caspase-3 cleaved PARP more efficiently than RFC140 *in vitro*. Interestingly, Ac-DEVD-CHO affected caspase-3 processing in Jurkat cells by blocking the propeptide removal but not cleavage between p20 and the p12.

Cleavage of RFC140 to a single 87 kDa fragment which could be inhibited by Ac-DEVD-CHO was shown to occur in irradiated or etoposide-treated cell lines undergoing apoptosis (Song *et al.*, 1997). This 87 kDa band probably corresponds to the 97 kDa fragment that we observed. Our results confirm the cleavage of RFC140 in Fas-mediated cell death, but we also found that RFC140 is cleaved *in vivo* to at least six fragments. The additional bands we detected probably result from usage of a different

anti-RFC140 antibody than that used by Song *et al*. By mutagenesis and microsequencing, we identified three cleavage sites: the DEVD^{723}G, DLVD^{922}S and the IETD^{1117}A sequences. The importance of the DEVD^{723}G site is underscored by the fact that this sequence is present in large RF-C subunit sequences of several species. Our demonstration that the small subunits of RF-C were not cleaved by recombinant caspase-3 is surprising, since the DEAD↓A/S sequence fits with the proposed consensus sequence recognized by caspase-3. Possible conformational differences between the RFC140 subunit and the small RF-C subunits might explain their relative resistance to caspase-3.

The putative caspase-3 DEED^{114}D sequence was not used by caspase-3 for cleavage of RFC140 since the N-terminal region (up to amino acid 555) was not cleaved by caspase-3 *in vitro*. It is possible that this site is not accessible to the protease; alternatively, the presence of an Asp residue at the P1′ position could prevent its recognition by caspase-3. The fact that the 65 and 73 kDa fragments appeared concomitantly with the decrease and eventual disappearance of the 97 kDa band suggest that they most likely arose from further processing of the 97 kDa fragment. This could explain the lack of 97 kDa band that we observed in PBLs. Abrogation of the DEVD^{723} site in the 723E mutant modified the 15/19 kDa doublet in a 41/44 kDa doublet resulting from the addition of the 26 kDa fragment downstream of site 723. Thus, it is likely that there are two caspase-3 sites located between amino acids 555 and 723, although these sites would not fit the proposed DXXD consensus.

The demonstration that there was no cleavage site in the first 555 amino acids of RFC140 may be important since a protein containing the N-terminal 555 amino acids of RFC140 inhibited DNA replication *in vitro* (Figure 7). The 97 kDa fragment cleavage product (containing amino acids 1–723 of RFC140) is likely to inhibit DNA replication. This suggests that during apoptosis, cleavage of RFC140 by caspase-3 and/or other members of the protease family could produce an inhibitor of DNA replication. This hypothesis is consistent with a recent finding that a fragment of RFC140 consisting of amino acids 481–728 can bind PCNA and also act as a dominant-negative inhibitor of DNA replication (Fotedar *et al*., 1996). It appears likely that the C-terminal 26, 45 and 50 kDa fragments generated after caspase-3 cleavage would interfere in RF-C complex formation (Uhlmann *et al.*, 1997). As RFC140, DNA-dependent protein kinase and PARP are proteins involved in DNA repair, their cleavage may reflect the commitment of the cell rapidly to abort unwanted DNA synthesis in order to proceed efficiently to the irreversible degradation of its DNA, which is part of its suicide program. Thus, a fully cleaved RFC140 molecule has the potential to inhibit the action of several RF-C complexes, which could rapidly lead to cell cycle arrest during the apoptotic process. Further functional studies, however, will be necessary to confirm this hypothesis.

Materials and methods

Reagents

The caspase inhibitors Ac-DEVD-CHO and Ac-YVAD-CHO were from Peptides International, Inc. zVAD-fluoromethylketone was from Enzyme Systems Products (Dublin, CA). Annexin V was from Biodesign and the phytohemagglutinin (PHA) from Murex Diagnostics (Guelph, ON, Canada). Recombinant IL-2 was obtained from the NIH AIDS Research and Reference Reagent Program. Propidium iodide and the protease inhibitors pepstatin, leupeptin, PMSF, TLCK and iodoacetamide were from Sigma (St Louis, USA). Aprotinin was purchased from Boehringer-Mannheim. The anti-Fas antibody M3 was a kind gift from Dr David Lynch (Immunex). The anti-RFC140 mAb 19 (Bunz *et al.*, 1993) antibody and plasmid pRFC140 were provided by Dr Bruce Stillman (Cold Spring Harbor Laboratory, NY, USA). The anti-PARP C-2-10 antibody was provided by Dr Guy Poirier (CHUL, Québec, Canada). The polyclonal anti-caspase-3 antibody was prepared by purification of a human GST-p20 subunit fusion protein followed by rabbit immunization (Alam *et al*., 1997).

Cell culture and treatments

Human peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque (Pharmacia, Baie d'Urfe, Qc, Canada) and resuspended in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum, 50 μM β-mercaptoethanol, 10 mM HEPES, 1 μg/ml of PHA, 20 U/ml of recombinant human IL-2 and cultured in 24-well plates (Falcon, Becton-Dickinson Labware, Toronto, ON, Canada) for 4 days at 37°C. The same amount of IL-2 was added to the wells after 48 h of culture. Non-adherent cells were harvested and incubated with or without 100 μM of caspase inhibitors for 30 min at 37°C. Cells were placed in 24-well plates (1.6×10^6 cells/ml) coated with the M3 anti-Fas antibody (20 μg/ml) or in control uncoated plates. After a 6 h incubation at 37°C, the cells were analyzed by flow cytometry for apoptosis and by Western blotting for cleavage of RFC140. The human T-cell leukemia cell line Jurkat was obtained from the American Type Culture Collection. Jurkat cells were maintained in the same medium as PBMCs, and treated with anti-Fas as the PBMCs.

Cell death assays

Treated cells were harvested and 2×10^5 cells were washed with PBS and resuspended in binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.4). Propidium iodide (2 μg/ml final) and FITC-conjugated Annexin V (1 μg/ml) were added and incubated for 10 min on ice. Cells were analyzed using a FACScan flow cytometer (Becton-Dickinson) with the CellQuest software and 10^4 events were accumulated per sample.

Preparation of cell lysates and immunoblots

All steps were performed on ice in presence of pepstatin A (1 μg/ml), aprotinin (2 μg/ml), leupeptin (0.5 μg/ml), PMSF (1 mM) and iodoacetamide (5 mM). 5×10^6 cells were resuspended in 50 μl of buffer A (20 mM HEPES, pH 7.8, 10 mM KCl, 0.15 mM EDTA, 0.15 mM EGTA) followed by lysis with 3 μl of 0.53% NP-40 and brief vortexing. Immediately, 15 μl of sucrose restore buffer (50 mM HEPES, pH 7.0, 0.25 mM EDTA, 10 mM KCl, 70% sucrose) was added and samples centrifuged at 5000 r.p.m. in a microfuge. The cytoplasmic fraction was kept and nuclei were washed in 50 μl buffer B (10 mM HEPES, pH 8.0, 0.1 M NaCl, 0.1 mM EDTA, 25% glycerol), lysed by resuspension in 40 μl buffer C (10 mM HEPES, pH 8.0, 0.4 M NaCl, 25% glycerol, 0.1 mM EDTA) and incubated for 30 min on ice with frequent shaking. Samples were centrifuged for 10 min at 10 000 r.p.m. and nuclear proteins were mixed with cytoplasmic proteins and one volume of 2× sample buffer (4% SDS, 125 mM Tris–HCl, pH 6.8, 1.4 M β-mercaptoethanol, 20% glycerol) was added and samples were boiled for 5 min. The equivalent of 8×10^5 cells was loaded into each well. SDS–PAGE was carried out in 10, 12 or 8–20% gels. Proteins were transferred to PVDF membranes (Boehringer-Mannheim, Laval, Qc, Canada) and immunoblotting was performed with the indicated antibodies followed by HRP-conjugated anti-rabbit (Amersham) or anti-mouse (Amersham) antibodies. The complexes were revealed using the Renaissance Western blot luminescence reagent (NEN).

Caspase-3 construct and lysates

The human caspase-3 cDNA was amplified by RT–PCR with the following primers: 5′-GGGATCCATGGAGAACACTGAAAAACTC-3′ and 5′-ATGAATTCCCAACCAACCATTTCTTTAGTG-3′ from Jurkat total RNA and subcloned into the *Bam*HI and *Eco*RI sites of pBSKS+ (Stratagene). The cDNA was sequenced and subcloned in pGEX2TK (Pharmacia). DH5α bacteria were transformed with the caspase-3–pGEX plasmid and grown overnight at 37°C, followed by induction with 1 mM IPTG for 4 h at 30°C. Bacteria were lysed by sonication in 1/50 of the culture volume in caspase-3 buffer (25 mM MES, pH 6.5, 5 mM EDTA,

2 mM DTT, 0.1% CHAPS, 10% sucrose) containing protease inhibitors (pepstatin A, 1 μg/ml; aprotinin, 2 μg/ml; leupeptin, 0.5 μg/ml; PMSF, 1 mM). The lysates were centrifuged at 10 000 *g* for 10 min, the supernatant was incubated at 30°C for 1 h and aliquots kept frozen at –80°C. Aliquots were thawed at 4°C and kept for less than 1 month.

Constructs for expression of RF-C subunits

Constructs for expression of the five human RF-C subunits and the various deleted variants of the RFC140 subunit were described previously (Uhlmann *et al.*, 1996, 1997). Mutant 723E of RFC140 was generated by the PCR by overlap extension method using primers 5′-CTGATCATG-GAGGAAGTAGAGGGCATGGCAG-3′ and 5′-GCCCTCTACTTCCT-CCATGATCAGAGCATGTT-3′. The mutation was first created by PCR amplification of plasmid pRFC140 and inserted in the same vector and a *Bst*EII fragment was cloned using the same sites of pET16ap140 (Uhlmann *et al.*, 1996).

Coupled in vitro transcription/translation of RF-C subunits and in vitro cleavage assay

The *in vitro* transcription and translation of the cDNAs encoding the five subunits of RF-C and the RFC140 deletion mutants were carried out with the TNT Coupled Reticulocyte Lysate System (Promega, Madison, WI, USA) according to the manufacturer's protocols. Reactions (25 μl) containing 0.5 μg of DNA, reaction buffer, T7 RNA polymerase, 20 μM of each amino acid except methionine, 20 μCi of L-[^{35}S]methionine (1200 Ci/mmol, NEN, *in vivo* labeling grade), 20 units of RNAsin (Promega) and 12.5 μl of rabbit reticulocyte lysate were incubated at 30°C for 90 min. For full-length RFC140, 1 mM magnesium acetate was added to the reaction mixture.

For *in vitro* cleavage by recombinant caspase-3, 2 μl of ^{35}S-labeled RFCs were incubated with 1 μl caspase-3 bacteria lysate and 9 μl caspase-3 buffer with 2% sucrose at 30°C for 1 h. Cleavage products were fractionated on 10 or 8–20% PAGE and exposed to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA, USA). Alternatively, ^{35}S-labeled subunits of RF-C were incubated with 50 μg of proteins obtained from apoptotic Jurkat cells for 1 h at 30°C. Jurkat lysates used for *in vitro* cleavage assays were prepared by four freeze–thaw cycles of 10×10^6 cells in 100 μl of caspase-3 buffer with 2% sucrose and protease inhibitors.

Amino-terminal sequence analysis of radioactively labeled RFC140

The coupled *in vitro* transcription and translation reactions were supplemented with the corresponding radioactive amino acids: 800 μCi/ml L-[^{35}S]methionine (1200 Ci/mmol, NEN), 500 μCi/ml L-[3,4,5-^{3}H(N)]leucine (180 Ci/mmol, NEN), or 500 μCi/ml L-[4,5-^{3}H]isoleucine (94 Ci/mmol, Amersham Life Science, Oakville, ON, Canada). 20 μl aliquots of each labeled RFC140 were incubated at 30°C for 1 h with 200 μg of protein from lysates of *Escherichia coli* expressing caspase-3, in 100 μl caspase-3 buffer with protease inhibitors. The reaction mixtures were fractionated by electrophoresis on 8–20% SDS–PAGE and transferred to PVDF membranes. After drying, the membranes were autoradiographed, the bands, excised and subjected to Edman degradation on an Applied Biosystems model 470A sequencer. Fractions from each cycle (100 μl) were collected and counted by scintillation.

DNA replication assay

The N-terminal fragment of RFC140 spanning amino acids 1–555 was expressed in the *E.coli* strain BL21 (DE3) from the plasmid pET16p140C555 (Uhlmann *et al.*, 1997) and was purified from the soluble fraction of the cell extracts to homogeneity by four chromatographic steps on SP Sepharose, Q Sepharose (Fast Flow, Pharmacia), Hydroxylapatite (HT Gel, BioRad) and double-stranded DNA cellulose (Sigma) columns, followed by glycerol gradient centrifugation.

The elongation of singly primed circular M13 DNA was carried out as follows. Reaction mixtures (10 μl) contained 40 mM Tris–HCl, pH 7.8, 0.5 mM DTT, 1 μg BSA, 7 mM $MgCl_2$, 2 mM ATP, 100 μM each of dATP, dGTP and dTTP, 20 μM [α-^{32}P]dCTP (15 400 c.p.m./pmol), 5.4 fmol of singly primed M13 mp7 DNA, 240 ng of human single-stranded DNA binding protein (RPA), 0.1 unit of DNA polymerase δ and PCNA, RFC and RFC140 spanning amino acids 1–555 as indicated the legend of Figure 7. Reactions were assembled on ice and incubated for 30 min at 37°C prior to being terminated by the addition of EDTA (10 mM). An aliquot was used to measure DNA synthesis and the remainder subjected to alkaline agarose gel electrophoresis. Gels were dried and autoradiographed for 4 h at –80°C.

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