Localisation of human Y-family DNA polymerase κ: relationship to PCNA foci

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Summary

DNA polymerases of the Y-family are involved in translesion DNA synthesis past different types of DNA damage. Previous work has shown that DNA polymerases η and ι are localised in replication factories during S phase, where they colocalise one-to-one with PCNA. Cells with factories containing these polymerases accumulate after treatment with DNA damaging agents because replication forks are stalled at sites of damage. We now show that DNA polymerase κ (polκ) has a different localisation pattern. Although, like the other Y-family polymerases, it is exclusively localised in the nucleus, polκ is found in replication foci in only a small proportion of S-phase cells. It does not colocalise in those foci with proliferating cell nuclear antigen (PCNA) in the majority of cells. This reduced number of cells with polκ foci, when compared with those containing polη foci, is observed both in untreated cells and in cells treated with hydroxyurea, UV irradiation or benzo[a]pyrene. The C-terminal 97 amino acids of polκ are sufficient for this limited localisation into nuclear foci, and include a C_2HC zinc finger, bipartite nuclear localisation signal and putative PCNA binding site.

Key words: DNA polymerase, PCNA, Replication foci, Translesion synthesis, UV irradiation

Introduction

All cells have evolved a variety of pathways for repairing different types of DNA damage. Despite the efficiency of these pathways, unrepaired lesions remain in the DNA during DNA replication, and most types of DNA damage block the progress of the replication machinery. The replicative DNA polymerases are very efficient and processive, and replicate DNA with high fidelity. However, they are unable to accommodate damaged DNA bases in their active sites and such lesions block their progress. A major way in which mammalian cells overcome this barrier is to use specialised translesion synthesis (TLS) polymerases. These polymerases have low efficiencies and fidelities, but are able to replicate DNA past different types of damage (reviewed by Lehmann, 2002; Prakash and Prakash, 2002). Four of these TLS polymerases belong to the recently discovered Y-family (Ohmori et al., 2001). DNA polymerase η (polη) is able to replicate DNA containing the major UV photoproduct, the cyclobutane pyrimidine dimer (CPD) with similar efficiency to undamaged DNA, and in the case of the T-T CPD, the 'correct' nucleotides (A-A) are usually inserted opposite the damage (Johnson et al., 2000b; Masutani et al., 2000). Deficiency in polη results in the variant form of xeroderma pigmentosum (Broughton et al., 2002; Johnson et al., 1999; Masutani et al., 1999). Polι is a paralog of polη (Tissier et al., 2000), but despite extensive studies on its activities in vitro, its function in vivo remains unknown.

Polκ is able to carry out TLS past benzo[a]pyrene (BaP) adducts in DNA (Rechkoblit et al., 2002; Suzuki et al., 2002; Zhang et al., 2002) and also past apurinic or apyrimidinic (AP) sites, acetylaminofluorene-DNA adducts (Ohashi et al., 2000b) and thymine glycols (Fischhaber et al., 2002). *Polk*$^{-/-}$ mouse embryonic stem cells are hypersensitive to both killing and mutagenesis by BaP (Ogi et al., 2002), suggesting that this polymerase might carry out TLS past polycyclic hydrocarbon adducts in vivo. However *Polk*$^{-/-}$ embryonic stem cells and fibroblasts are also sensitive to UV irradiation, implicating polκ in the response to UV photoproducts (Ogi et al., 2002; Schenten et al., 2002), even though it is unable to bypass either of the major UV photoproducts (Ohashi et al., 2000b; Zhang et al., 2002). Polζ is a heterodimer comprised of a catalytic subunit Rev3, which is a member of the B-family of polymerases, together with the Rev7 regulatory subunit. Current theories suggest that polζ is required for extension from nucleotides inserted by other polymerases opposite damaged bases (Guo et al., 2001; Johnson et al., 2000a). The fourth member of the Y-family is Rev1, which does not have DNA polymerase activity, but does have dCMP transferase activity (Nelson et al., 1996). Studies in yeast have shown that Rev1, 3 and 7 are required for UV mutagenesis, but the mutagenic function and dCMP transferase activity of Rev1 can be separated (Nelson et al., 2000).

The Y-family DNA polymerases have a conserved sequence of about 400 amino acids, which contain the catalytic site and C-terminal extensions that are not conserved between members. In previous work, we showed that polη is localised in the nucleus, and is found constitutively in nuclear foci, which contain PCNA and represent replication factories in S-phase cells (Kannouche et al., 2001). Following treatment with UV irradiation, stalling of replication forks at damaged sites results in an accumulation of cells in S phase, and the number

of cells with polη-containing foci increases substantially as a consequence. Treatment of cultures with hydroxyurea similarly results in an accumulation of cells with polη in replication foci (P.K. and A.R.L., unpublished). In all these cases, the polη foci colocalise with PCNA. The C-terminal 119 amino acids are sufficient for correct localisation of polη into nuclei and nuclear foci (Kannouche et al., 2001). This C-terminal fragment contains a C_2H_2 zinc finger, a nuclear localisation signal and a PCNA binding site, all of which are required for correct localisation (P.K. and A.R.L., unpublished). In subsequent work, we found that polι and Rev1 had identical localisation patterns to polη, and in the case of polι (but not Rev1), its localisation was dependent on the presence of polη (Kannouche et al., 2003; Tissier et al., 2004).

Polκ is an 870 amino acid protein, related to DNA polymerase IV (DinB) of *Escherichia coli*. Amino acids 100-376 contain polymerase domains conserved throughout the Y-family, whereas amino acids 376-500 are conserved only within the DinB sub-family. Truncated protein containing the first 560 residues has polymerase activity, although less than the full-length protein (Ohashi et al., 2000a). The C-terminal 270 amino acids of the protein contain two C_2HC zinc fingers, a bipartite NLS and a putative PCNA binding site at the extreme C terminus (Gerlach et al., 1999; Haracska et al., 2002). This region thus encompasses several motifs that resemble those in the C-terminal part of polη. We therefore anticipated that the localisation of polκ would be similar to that of the other Y-family polymerases. Here we describe an investigation of the localisation of polκ. Surprisingly we found that, although it was always located in the nucleus, the proportion of nuclei containing polκ in nuclear foci was much lower than for polη. We have identified the elements required for its localisation.

Materials and Methods

Plasmid construction

GFP-tagged human polκ, pEGFPC2-polκ, was provided by J. S. Hoffmann (Bergoglio et al., 2002). We generated a similar construct with a different GFP vector. We modified *POLK* cDNA by deleting the first ATG codon by PCR using plasmid pSHE2, which contains intact human *POLK* cDNA, as a template and 5′-gggctcgagctcGATAGCACAAAGGAGAAGTGTGACAG-3′ and 5′-ggggatccTTACTTAAAAAATATATCAAGGGTATGTTTGGG-3′ as primers. PCR products were digested with *Xho*I and *Bam*HI, and then cloned into *Xho*I-*Bam*HI sites of pEGFP-C3 (Clontech) to generate pEGFPC3-HsPOLK, which we abbreviate to pEGFPpolκ.

A series of deletion and point mutations of GFP-tagged human POLK were generated from pEGFPpolκ: Sa11 (dK870), deletion of final lysine residue K870; Sb31 (FF868/9AA), substitution of double phenylalanine residues F868F869 to alanines; Sc11 (dPCNA), deletion of C-terminal 9 amino acids K862 to K870; and Sd11 (dNLSdPCNA), deletion of C-terminal 29 amino acids K842 to K870 were obtained by fragment replacement of corresponding regions. PCR was performed using pSHE2 as a template, 5′-GACAGGAAACACCAACAAAGGAGCAT-3′ as a 5′ common primer, and 3′ specific primers: for Sa11, 5′-ggggatccTTAAAAAAATATATCAAGGGTATGTTTGGG-3′; Sb31, 5′-ggggatccTTACTTAGCAGCTATATCAAGGGTATGTTTGGG-3′; Sc11, 5′-ggggatccTTAGGGATTGTTTGGTTTTATTTTCTTTG-3′; Sd11, 5′-ggggatccTTATGTTCTTGTTACAGCCTTCTGTACTCC-3′. PCR fragments were digested with *Xba*I and *Bam*HI, and replaced the corresponding *Xba*I-*Bam*HI fragment of pEGFPpolκ.

N-terminal truncation mutants were generated by PCR amplification of the desired regions and cloning into pEGFP-C3. PCR was performed using pSHE2 as a template, 5′-ggggatccTTACTTAAAAAATATATCAAGGGTATGTTTG-3′ as a 5′ common primer, and 3′ specific primers; for TA (c510-870; C-terminal 510-870 amino acids), 5′-gggctcgagGGTGTTCGGATATCTAGTTTTC-3′; TB (c570-870), 5′-gggctcgagAAAAAACGATCAGAAAGGAAATGGAG-3′; TC (c547-870), 5′-gggctcgagTTAGAGAAAACTGACAAAGATAAGTTTG-3′; TD (c603-870), 5′-gggctcgagAAGAAGAAGATGAATGAGAATTTGG-3′; TE (c824-870), 5′-gggctcgagAGCTCCAGAAGTACTGGTAGC-3′; TF (c842-870), 5′-gggctcgagAAAAGGCCAGGATTGATGACAAAG-3′; TH (c710-870), 5′-gggctcgagTTAAATAAAAGTTTTATCCAAGAATTAAG-3′; TI (c774-870), 5′-gggctcgagGGCCAAGCTCTAGTTTGTCCTGTTTG-3′; TJ (c774-870:C779C782AA), 5′-gggctcgagGGCCAAGCTCTAGTTGCTCCTGTTGCTAACGTAG-3′; TK (c710-870), 5′-ggctcgagTCATCTAAAGCAGAAAGCATAGATGC-3′. PCR products were digested with *Xho*I and *Bam*HI, and then cloned into the *Xho*I-*Bam*HI sites of pEGFP-C3.

Cells and transfection of plasmid DNA

SV40-transformed wild-type MRC5 and polη-deficient XP30RO human fibroblasts were used in all experiments. Cells were grown in DMEM supplemented with 10% fetal calf serum, and antibiotics. Plasmid transfections were carried out by lipofection with lipofectamine (Gibco) or FuGENE 6 (Roche).

UV irradiation, gamma irradiation and drug treatments

254 nm UVC irradiation was performed with a germicidal lamp at a fluence rate of 0.4 J/m^2/second. Cells cultivated on coverslips were washed once with PBS and UV irradiated followed by further incubation. For γ irradiation, cells were trypsinised, suspended in PBS, and irradiated with a ^{60}Co irradiator at a dose rate of 1 Gy/minute. For hydroxyurea (HU) treatment, cells were incubated in complete medium with 10 mM HU for indicated times. For BaP treatment, the drug was activated with S-9 fraction of rat liver homogenates (S9, Sigma) just before treatment. Cells were treated for the indicated times in complete medium containing 20 μM BaP, 0.1% S9 and 0.1% DMSO.

Sub-nuclear fractionation and western blotting

2×10^6 MRC5 cells were transfected with pEGFPpolκ or pEGFPpolη plasmids and cultured for 20 hours. They were then UV irradiated and incubated for 6 hours, prior to washing twice with PBS and scraping off into PBS. Cell pellets were collected by centrifugation (200 ***g***) and resuspended in 500 μl hypotonic buffer [HB; 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 m DTT, 0.5% NP-40, 1 mM PMSF, ×1 Complete protease inhibitor mix (Roche)]. Cell suspensions were kept on ice for 30 minutes and then centrifuged. The supernatant was collected for cytoplasm and unbound fraction (UB). Pellets were washed with HB twice, and resuspended in 100 μl extraction buffer (EB; 20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.1 mM DTT, 0.2 mM EDTA, 25% Glycerol v/v, 500 mM NaCl, 1 mM PMSF, ×1 Complete protease inhibitor mix). Nuclear extraction was performed with gentle agitation for 30 minutes at 4°C, and then centrifuged. The supernatant is the nuclear binding fraction (NcB). Pellets were then washed twice with HB, resuspended in HB containing 5 U/ml Benzonase (Novagen) for 2 hours at 16°C, and then centrifuged. The supernatant was used as the chromatin-binding fraction (ChrB). Fractionated proteins were desalted and concentrated. Protein extracts (10 μg) were separated in 8% SDS-PAGE, transferred to PVDF membranes, and probed with rabbit anti-GFP primary antibody (Roche) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (DAKO). GFP-tagged proteins were detected by the ECL detection system.

Microscopic observation

To visualise the eGFP proteins, cells were grown on coverslips, transfected and then treated with DNA damaging agents. At the end of the experiment, cells were washed once with PBS, fixed in 3.7% paraformaldehyde for 20 minutes, rinsed twice with PBS and mounted with Glycergel (Dako). To detect the colocalisation of eGFPpolκ and PCNA, cells were fixed in cold methanol for 20 minutes at –20°C and then incubated for 30 seconds with cold acetone to extract the soluble PCNA fraction. Cells were washed with PBS twice, and then incubated with anti-PCNA antibody (PC-10, SantaCruz) diluted 1:100 in 3% BSA containing PBS for 1 hour. Then, cells were washed twice with PBS and incubated with rhodamine-conjugated anti-mouse antibody (Jackson Immunoresearch Laboratories) diluted 1:250 in PBS containing 3% BSA. After washing three times with PBS, cells were mounted with Glycergel. Polκ was visualised by autofluorescence of the eGFP.

Photographs of the cells were captured with a Zeiss Axiophot2 microscope equipped with CCD camera, and captured images were analysed with MetaMorph and Photoshop software. A minimum of 300 nuclei were captured and analysed for colocalisation.

Results

Limited localisation of polκ in nuclear foci

In our previous work, we showed that polη, polι and Rev1 were constitutively localised in replication factories during S phase. S-phase cells with replication foci containing polη, polι and Rev1 accumulated following UV irradiation, because replication forks stalled at damaged sites (Kannouche et al., 2001; Kannouche et al., 2003; Tissier et al., 2004). We therefore anticipated a similar localisation pattern for polκ. In all our experiments, we compared the localisation of eGFPpolκ with that of eGFPpolη used in previous experiments. Consistent with previous reports, eGFPpolη localised in nuclei and up to 80% of the cell population formed eGFPpolη foci 16 hours after UV irradiation with 10 J/m^2 (Kannouche et al., 2001; Kannouche et al., 2003). We obtained a similar result 16 hours after treatment with HU (Fig. 1A). We found that the number of cells with eGFPpolκ foci increased following treatment with UV or HU, but the proportion of the cell population that formed eGFPpolκ foci (~25%) was much lower than for eGFPpolη foci, irrespective of whether the cells were untreated or treated with HU or UV (Fig. 1A). Without any DNA damaging treatments, we found that eGFPpolη accumulated in foci in approximately 20% of the cell population, corresponding to cells in S phase, whereas only around 5% of the cell population formed eGFPpolκ foci (Fig. 1A, compare open black bars with open red bars). After UV or HU treatment, polκ foci were observed in 15-20 and 20-25% of the population respectively, whereas polη foci were found in 60-80%. Typical images of UV irradiated MRC5 cells expressing eGFPpolκ and eGFPpolη are shown (Fig. 1B,C).

The cellular localisation of polκ protein and its behaviour after DNA damaging treatments has been reported (Bergoglio et al., 2002). Using a similar N-terminal eGFP-tagged polκ construct, this group reported a substantially greater DNA damage-dependent localisation of polκ into nuclear foci than we found. In order to determine the reason for this apparent discrepancy, even though we had designed and used a very similar plasmid and the same SV40-transformed MRC5 cells that were used in their report, we obtained the exact plasmid and cell line used by these authors. We checked whether the different plasmid and cells affected polκ nuclear foci formation (Fig. 1A). However, neither the plasmid nor the cell line affected the results. The blue bars in Fig. 1A represent results obtained with plasmid from Bergoglio and colleagues (GFPK-Tou), and the solid bars are data using the cell line obtained from them (Bergoglio et al., 2002). It is clear that neither the plasmid nor the cells could account for the discrepancies between the two sets of findings.

There is evidence from both in vitro and in vivo studies that polκ might participate in translesion synthesis across BaP-adducted bases (Ogi et al., 2002; Zhang et al., 2002), so we checked whether polκ accumulated in nuclear foci following treatment with 20 μM BaP treatment. As with UV irradiation and HU treatment, polη accumulated in nuclear foci in a high proportion of cells. The number of cells containing polκ foci also increased, but again we found foci containing polκ in a much lower proportion (20%) of the population (Fig. 1A, right). Similar results were obtained with other doses of BaP and incubation times (results not shown). With γ irradiation, neither polη nor polκ foci accumulated.

We next examined the correlation between foci formation and accumulation of protein in the chromatin fraction (Fig. 1D). The accumulation of eGFPpolη protein into nuclear foci after UV irradiation is accompanied by an increased amount of eGFPpolη protein in the chromatin fraction after UV irradiation (lane 12, compare lane 9). In contrast, we could not detect any significant increase of eGFPpolκ protein in the chromatin fraction after UV irradiation (compare lanes 6 and 3), consistent with the low number of cells in which polκ was present in nuclear foci. We obtained similar results with HU-treated cells (data not shown).

Localisation of polκ and PCNA

We previously showed that polη and polι colocalised with PCNA in nuclear foci (Kannouche et al., 2001; Kannouche et al., 2003). This suggests that polη is tightly associated with the replication machinery. In contrast, the poor accumulation of polκ into nuclear foci after UV irradiation and the low fraction of the cell population that formed polκ foci in untreated cells and in cells treated with the replication inhibitor HU, suggest that the association of polκ protein with the replication fork or replication machinery is far weaker than for polη. To assess the colocalisation of polκ and PCNA foci, pEGFPpolκ-transfected cells were UV irradiated and stained with anti-PCNA antibody. First, eGFPpolκ-expressing cells were analysed and classified for the presence or absence of polκ and PCNA foci following UV irradiation (Fig. 2A). Consistent with previous reports, PCNA foci were observed in 79% of the cell population that expressed eGFPpolκ. Of these cells with PCNA foci, however, only 23% (18% of the whole population) also contained eGFPpolκ foci. Cells with PCNA foci were then further analysed as to whether these foci colocalised with polκ foci (Fig. 2A inner columns, top left). We observed four different types of localisation pattern: complete colocalisation of PCNA and eGFPpolκ (Fig. 2B); partial colocalisation (Fig. 2C); no eGFPpolκ foci in PCNA foci forming cells (Fig. 2D); no colocalisation, although both eGFPpolκ and PCNA formed foci (Fig. 2E). Both completely and partially colocalised cases were classified as colocalisation positive, and the others were classified as colocalisation negative. Our data show that the

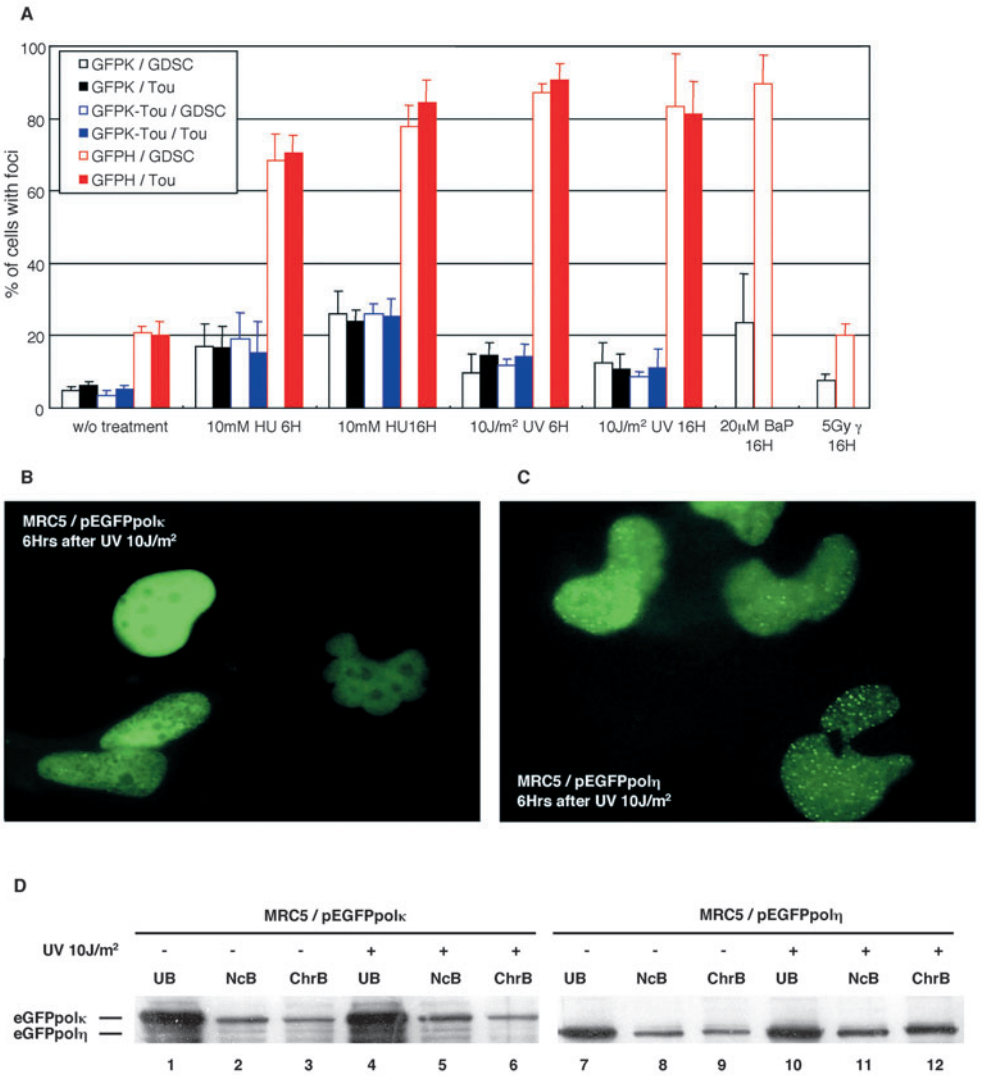

Fig. 1. Localisation of polκ following DNA damage. (A) Foci formation frequencies of polκ after DNA damaging (UV irradiation, BaP and γ-irradiation) or replication inhibitory (HU) treatments. MRC5 cells from our laboratory (GDSC, open bars) or from the laboratory of Bergoglio and colleagues (Tou, shaded bars) (Bergoglio et al., 2002) were transfected with eGFPpolκ constructed in our laboratory (GFPK, black), eGFP-C2-*HsPOLK* constructed in the laboratory of Bergoglio (GFPK-Tou, blue), or eGFPpolη (GFPH, red) and incubated for 20 hours. Cells were then treated with the indicated doses of damaging agents and further incubated for the indicated times. The proportion of eGFPpolκ (or eGFPpolη)-expressing cells in which the protein was localised in nuclear foci was determined. All experiments were carried out in triplicate and each data point is the mean of three independent scorings. Benzo[a]pyrene (BaP) and γ-ray experiments were carried out only with cells and plasmid from our laboratory. Error bars indicate standard error. (B,C) Typical images of cells expressing eGFPpolκ (B), or eGFPpolη (C) 6 hours after 10 J/m^2 UV irradiation. (D) Sub-nuclear polκ protein localisation after UV irradiation. MRC5 cells were transfected with pEGFPpolκ, or pEGFPpolη and incubated for 20 hours. Cells were then UV irradiated with 10 J/m^2 and incubated for 6 hours. Cellular proteins were fractionated into detergent extractable (unbound, UB), salt extractable nuclear matrix binding fraction (NcB) and a fraction resistant to salt extraction (chromatin binding, ChrB) and analysed by immunoblotting.

colocalisation of polκ with PCNA is quite different from that of polη. Similar results were obtained after HU treatment.

The C-terminal region of polκ is essential for nuclear localisation and localisation of protein into nuclear foci after UV irradiation and HU treatment

All the mammalian Y-family polymerases consist of N-terminal TLS polymerase domains and C-terminal domains of an extra 200-300 amino acids, the latter being dispensable for DNA synthesis and translesion synthesis in vitro (Masutani et al., 2000; Ohashi et al., 2000a). It has also been reported that truncation of the C-terminal 310 amino acids of polκ protein reduced the processivity of the enzyme (Ohashi et al., 2000a). The C-terminal domain of polκ contains two C_2HC zinc fingers, a bipartite NLS and, at the extreme C-terminus, a putative PCNA binding sequence. To identify the sequences that are involved in nuclear localisation and foci formation of polκ, a series of eGFP-tagged deletion mutants were generated (C-terminal truncations and amino acid substitution mutants are summarized in Fig. 3A; N-terminal truncation mutants are shown in Fig. 3B). Fluorescence microscopy showed that all the eGFP fusion proteins were expressed, and we did not detect any protein aggregation in cytoplasmic particles. The predicted NLS is located in polκ at position 842-859. The eGFPpolκ construct deleting C-terminal amino acids 842-870 (dNLSdPCNA) was excluded from nuclei (Fig. 3A, bottom row; Fig. 3C) and no nuclear foci were detected with this construct in cells treated with UV or HU. Constructs c547-870, c570-870 and c603-870, which completely lack the polymerase domain, displayed 100% nuclear localisation and formed foci in undamaged, UV-irradiated or HU-treated cells with similar frequencies to wild-type constructs (Fig. 3B, top four rows; Fig. 3D). These results show that the polymerase catalytic domain is not required for protein localisation, as also found in our previous work with polη and polι (Kannouche et al., 2001; Kannouche et al., 2003). We next tested if the C_2HC type Zn finger domains were essential for nuclear localisation and foci formation. Removal of the N-terminal zinc finger (construct c710-870) did not affect localisation. eGFP-tagged

A

	eGFPpolκ foci positive 18.2 ± 3.2		eGFPpolκ foci negative 81.8 ± 3.2
PCNA foci positive 79.1 ± 7.4	Co-localization positive 13.9 ± 2.1	Co-localization negative 4.3 ± 1.3	60.9 ± 4.5
PCNA foci negative 20.9 ± 7.4	0		20.9 ± 7.4

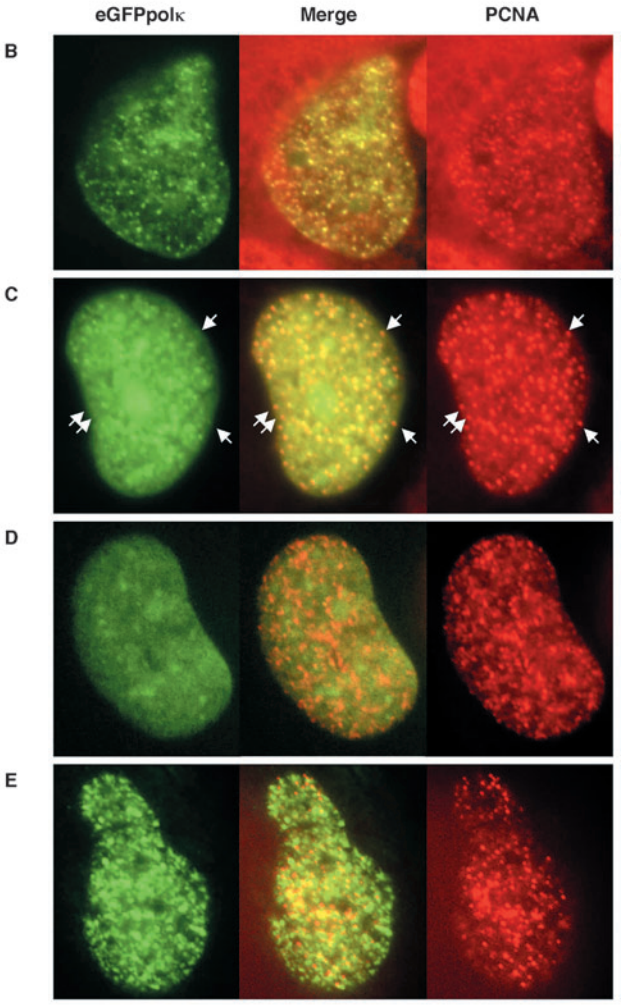

Fig. 2. Localisation of polκ and PCNA after UV irradiation. MRC5 cells were transfected with pEGFPpolκ and incubated for 20 hours. Cells were UV irradiated with 10 J/m^2 and incubated for 6 hours. Cells were then fixed and stained with anti-PCNA mouse monoclonal antibody and rhodamine-conjugated secondary antibody. Colocalisation of eGFPpolκ and PCNA foci was analysed. (A) eGFPpolκ-expressing cells were selected and sorted by the foci formation property of polκ and PCNA. To check the colocalisation of foci, images of the cells that form both eGFPpolκ and PCNA foci were captured and then further analysed. Experiments were carried out in triplicate, and indicated numbers are the averages and the standard deviations of three independent experiments. More than 100 cells were analysed in each experiment. (B-E) Typical staining patterns of the auto-fluorescent signal of eGFPpolκ (green) and PCNA (red) in the same cell are shown. (B) Complete colocalisation of eGFPpolκ foci and PCNA foci was observed as shown by yellow staining. (C) Partial colocalisation of eGFPpolκ foci and PCNA foci. Most of the PCNA foci colocalised with eGFPpolκ foci, but significant numbers of eGFPpolκ foci were missing (white arrows). (D) eGFPpolκ foci were completely absent in cells with PCNA foci. (E) eGFPpolκ foci and PCNA foci were not colocalised at all.

constructs c802-870, c824-870 and c842-870, lacking both C_2HC domains were still mainly localised in nuclei, although there was some leakage of the protein into the cytoplasm (Fig. 3B, last three rows; Fig. 3E,F). However, no nuclear foci were observed even after UV or HU treatment, suggesting that one of the Zn finger motifs is important for polκ localisation into nuclear foci. We made two further deletion constructs, c774-870 and c774-870C779C782AA. The N-terminus of these constructs is just five amino acids upstream of the first cysteine of the C-terminal zinc finger and both constructs were localised in the nucleus. In the former however, foci formation was significantly reduced (Fig. 3G). Most surprisingly however, in the latter construct, in which two of the three cysteines in the zinc finger were converted into alanines, foci formation was actually improved and was similar to that with full-length polκ (Fig. 3H). Thus although the domain containing this zinc finger is required for foci formation, the zinc finger motif itself is not required. Indeed, it appears to be counterproductive in this context.

We also tested whether the conserved PCNA binding motif was involved in foci formation. Human polκ has a postulated PCNA binding domain at position 862-870 that is conserved in vertebrate polκ. We made three different mutations in this domain: eGFP-tagged dK870, in which the final lysine residue located at 870 was deleted; FF868/9AA, substitutions of tandem phenylalanine residues to alanines; dPCNA, deletion of amino acids 862-870. All these mutants were localised in the nucleus, but none of them formed foci even after UV irradiation or HU treatment (Fig. 3A top four panels; Fig. 3I).

Polη is not necessary for polκ foci formation

We previously reported that polη and polι interacted physically and colocalised in nuclear foci (Kannouche et al., 2003). Furthermore, the localisation of polι in foci was largely dependent on polη, as polι foci formation was much reduced in the XP variant cell line XP30RO, which is defective in polη. In contrast, in similar experiments using polκ, we found no difference in the localisation patterns in nuclei and nuclear foci in XP30RO and MRC5 cells, with or without UV irradiation (Fig. 4). Similar results were obtained after HU treatment (not shown). Thus, the limited localisation of polκ into nuclear foci is not dependent on polη.

Discussion

We have shown that, like the other Y-family polymerases, polκ is localised in the nucleus in human cells. Interestingly, however, the localisation of polκ in replication foci and the accumulation of nuclei with foci containing polκ following UV or HU treatment are much more limited than with the other polymerases. In the cases of polη and polι, there is a one-to-one correspondence of foci containing PCNA and those containing polymerase (Kannouche et al., 2001; Kannouche et al., 2003). In other words, each replication factory contains polη and polι molecules. As Rev1 colocalises with polη, we

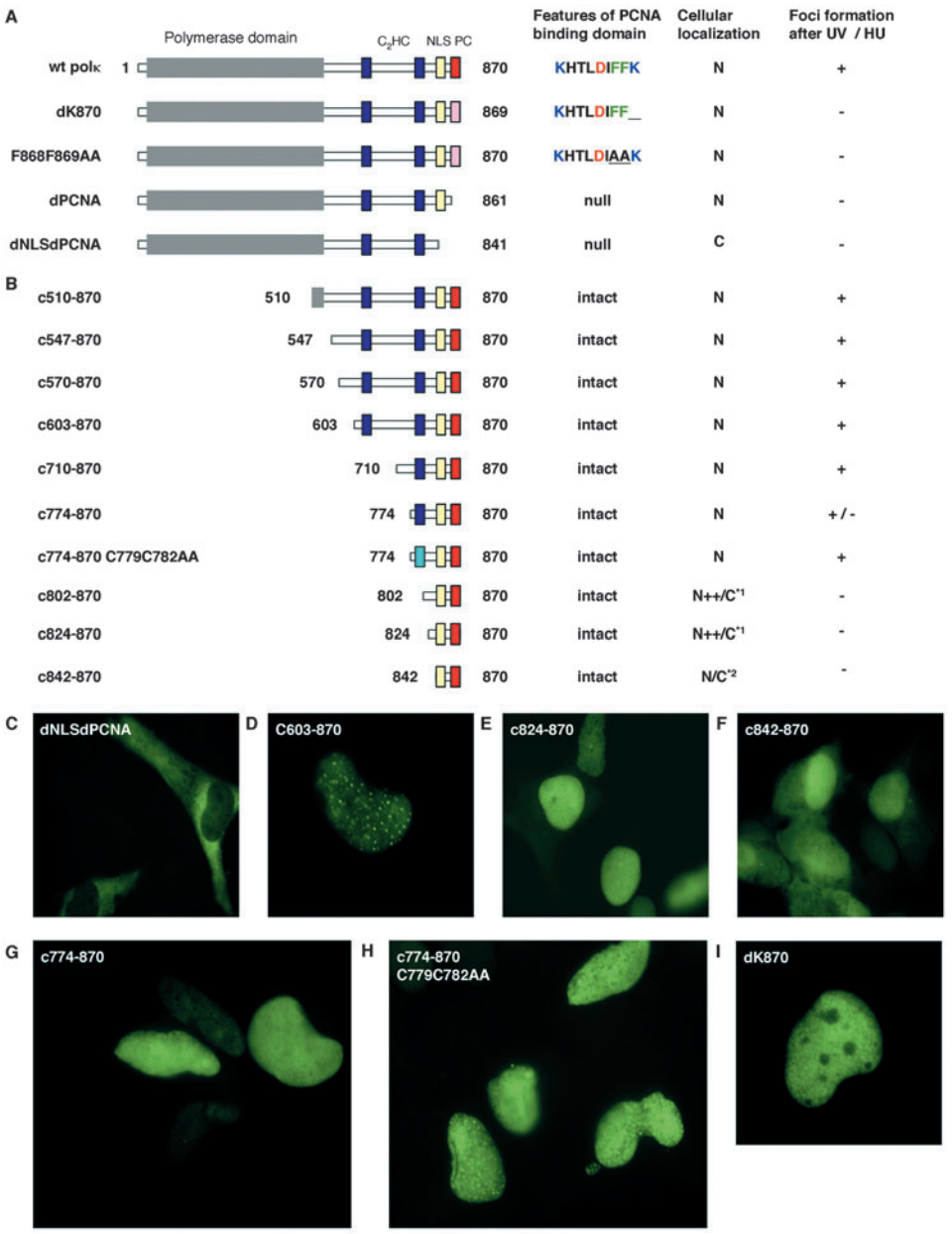

Fig. 3. The C-terminal region of polκ protein is required for foci formation. MRC5 cells were transfected with plasmids expressing various eGFP-tagged polκ deletion proteins and incubated for 20 hours. Cells were then UV irradiated with 10 J/m^2 or 10 mM HU and further incubated for 6 hours. (A) Summary of foci formation and cellular localisation properties of C-terminal truncation mutants. (B) Summary of foci formation and cellular localisation properties of N-terminal truncation mutants. C, cytoplasmic; C_2HC, C_2HC type Zinc finger domain; N, nuclear; NLS, nuclear localisation signal like domain; PC, similar to PCNA binding domain consensus; *1, both nuclear and cytoplasmic localisation, but majority was nuclear; *2, both nuclear and cytoplasmic localisation. (C-I) Typical images of UV-irradiated cells expressing eGFP-tagged polκ deletion proteins.

can assume that it is also present in replication factories (Tissier et al., 2004). This was not the case with polκ: only a small proportion of cells with PCNA foci also contained colocalising polκ. Our findings appeared to be different from previously published data (Bergoglio et al., 2002). The results of these authors suggested a localisation pattern for polκ similar to that reported in our previous work for polη and polι. By exchanging materials, we eliminated the possibility that this discrepancy was caused by the use of different plasmids and cell lines. A visit by T.O. to the laboratory of Bergoglio and co-workers clarified the discrepancy. In the experiments carried out in our laboratory, all experiments were done as a comparison between localisation of polη and polκ, and the differences were immediately apparent. A nucleus was only scored as positive for foci formation if there were many bright foci, as seen in our previous work with polη and polι and exemplified for polκ in Fig. 2C-E. Bergoglio and colleagues did not carry out a comparison with polη and included as positives nuclei with only a very small number of 'foci'. The origin of these foci is not clear, but they would not have been included as positives in our analyses. Irrespective of the precise definition of cells containing foci, our laboratories agree that the pattern of foci formation for polκ is completely different from that for polη.

We have considered the possibility that the eGFP protein linked to the N-terminus of polκ might impede its correct localisation. Although this cannot be ruled out absolutely, we consider this to be unlikely as: (1) we obtained the same results using two different constructs, in which the linker joining GFP to polκ was respectively 4 and 12 amino acids in length; (2) the nature of our constructs was identical to those we used previously for polη and polι; and (3) in preliminary experiments we have shown that an adenovirus vector containing our eGFPpolκ construct is able to restore substantial

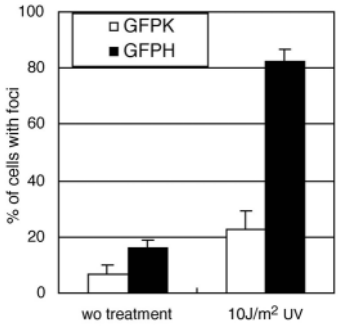

Fig. 4. polκ foci formation is independent of polη. Polη-deficient XP30RO cells were transfected with pEGFPpolκ (GFPK, open bars), pEGFPpolη (GFPH, filled bars). 20 hours later, cells were UV irradiated with 10 J/m^2 and then incubated for a further 6 hours. The proportion of cells expressing eGFPpolκ or eGFPpolη in which the protein was localised in nuclear foci was determined. All the experiments were carried out in triplicate and each data point is the mean of three independent scorings. Error bars indicate standard error.

UV resistance to mouse *Polk*$^{-/-}$ cells, confirming that it is biologically active.

The precise function of polκ is not clear. However, the substantial sensitivity of *Polk*$^{-/-}$ embryonic stem cells to BaP (Ogi et al., 2002), the inducibility of polκ by treatment of mice with the polycyclic hydrocarbon, 3-methylcholanthrene (Ogi et al., 2001) and the ability of polκ to bypass BaP adducts in vitro (in general inserting C opposite adducted G) (Rechkoblit et al., 2002; Suzuki et al., 2002; Zhang et al., 2002), are all consistent with polκ playing a role in TLS past BaP adducts. Our previous work showed that polη, polι and Rev1 are constitutively localised in replication factories in S-phase cells. Thus, we expect that any DNA damaging treatment that blocks the replication fork and causes an accumulation of cells in S phase will result in an increase in the number of cells with foci containing PCNA, polη, polι and Rev1. Therefore, even though the ability of polη to bypass BaP adducts is weak and in general mutagenic (e.g. Chiapperino et al., 2002), the replication factories that accumulate when replication forks are blocked by BaP adducts, all contain polη, as with UV and HU treatment, whereas only a small proportion appear to contain polκ, at least as visualised by fluorescent microscopy.

Given the likely function of polκ in TLS, why might the localisation pattern of polκ and polη be different, despite the rather similar structural features of the proteins? In order to be visible by fluorescence microscopy, a 'focus' must contain 50-100 fluorescently tagged molecules. Recent studies using real-time imaging on living cells have shown that many nuclear proteins involved in responses to DNA damage are highly dynamic within the nucleus (Houtsmuller et al., 1999). Assuming this is also true for TLS polymerases, the number of molecules in a focus will be dependent on the concentration of the tagged molecules, the rates at which molecules enter and leave the focus and the residence time in the focus. Alterations in any of these parameters could affect the observed proportion of cells with foci. Thus, it may indeed be that polκ resides in replication foci, but the time of residence is short, so that there are rarely enough polκ molecules in a replication factory to be visible as foci. Our results raise the intriguing question as to how the appropriate polymerase is selected for TLS past different adducts. In the case of UV-induced cyclobutane pyrimidine dimers, polη is present in factories, and can carry out TLS efficiently and accurately. With HU, the issue does not arise, as the fork blockage is caused by depletion of deoxyribonucleotides and this cannot be alleviated by any of the polymerases. With BaP adducts, we may speculate that the apparently high concentrations of polη in the vicinity of the blocked forks enable polη to be the first polymerase to attempt TLS, but as it is inefficient with this adduct, it is often out-competed by polκ, which may be present at lower levels but is able to effect TLS more efficiently. These ideas are entirely speculative and await further experimentation to clarify the way in which TLS polymerases are regulated.

Although the localisation of polκ in replication foci is much less than that of polη, the elements required for localisation in the nucleus and in nuclear foci are quite similar. The C-terminal domains of both proteins contain the zinc finger motif, bipartite NLS and PCNA binding motif in the same order (although the types of zinc finger differ between the two polymerases, C_2H_2 in polη and C_2HC in polκ). In both polymerases, the bipartite NLS is required for localisation in the nucleus, and the C-terminal PCNA binding sites, which are conserved in higher eukaryotes, are required for foci formation in both polκ (this paper) and polη (P.K., J. Wing and A.R.L., unpublished). Whereas we have shown that the zinc finger motif is required for localisation of polη in foci (our unpublished observations), the domain encompassing one of the zinc fingers is required for polκ foci formation, but missense mutations in the zinc finger surprisingly increased foci formation. Although the reason for this is not clear, our results would be consistent with the idea that the zinc finger was involved in turnover of the protein near the replication forks. Our current work is directed towards testing this hypothesis.

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