An oxidized nucleotide affects DNA replication through activation of protein kinases in *Xenopus* egg lysates

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ABSTRACT

To elucidate the response to oxidative stress in eukaryotic cells, the effect of an oxidized nucleotide, 8-oxo-2'-deoxyguanosine 5'-triphosphate (8-oxo-dGTP), generated from dGTP with an active oxygen, on DNA synthesis was studied using a cell-free DNA replication system derived from *Xenopus* egg lysates with a single-stranded DNA template. Amounts of newly synthesized DNA were reduced according to the increasing concentration of 8-oxo-dGTP. Pulse labeling analysis revealed that 8-oxo-dGTP could delay DNA synthesis by reducing the rate of chain elongation. This delay was recovered by addition of a protein kinase inhibitor, staurosporine or bisindolylmaleimide I. These results indicate that a staurosporine- or bisindolylmaleimide I-sensitive protein kinase, such as a protein kinase C family member, may contribute to the delay of DNA synthesis by 8-oxo-dGTP. UV-irradiated single-stranded DNA also caused a delay of DNA synthesis on the undamaged template in the lysates. However, this delay was not recovered by staurosporine or bisindolylmaleimide I. Therefore, the mechanism of delay of DNA synthesis by 8-oxo-dGTP may be different from that by UV lesions. This is the first report that demonstrates an effect of an oxidized nucleotide on DNA replication in eukaryotes.

INTRODUCTION

Reactive oxygen, a main by-product from mitochondria in eukaryotic cells, causes damage to many cellular components, one of which is 8-oxo-2'-deoxyguanosine 5'-triphosphate (8-oxo-dGTP), produced from dGTP (1). Several prokaryotic and eukaryotic DNA polymerases can incorporate this mutagenic nucleotide opposite either cytosine or adenine in a template (2–4). Studies with a human cell-free replication system dependent on an SV40 origin show that 8-oxo-dGTP

causes A:T→C:G transversion by being incorporated opposite adenine (5). To avoid this mutation, eukaryotic cells have an enzyme called 8-oxo-dGTPase, a homolog of *Escherichia coli* MutT protein, which hydrolyzes 8-oxo-dGTP to a non-mutagenic compound, 8-oxo-dGMP (6). 8-Oxo-dGMP is further metabolized to its nucleoside and excreted in urine. A high concentration of 8-oxo-dG nucleoside in urine suggests that a large amount of 8-oxo-dGTP is generated in eukaryotic cells. On the other hand, it has been demonstrated that DNA lesions trigger cell cycle arrest (7). Despite the fact that oxidative stress, as well as other factors causing DNA lesions, such as UV irradiation, X-ray irradiation and chemical reagents, causes cell cycle arrest (8), few studies have considered the effect of an oxidized nucleotide on cell cycle progression because of the difficulty of studying the effects in living cells.

Lysates prepared from *Xenopus* eggs have been frequently used to study cell cycle control, including the checkpoint mechanisms (9–11). Recently, Tatiana and Hanspeter (12) have reported that UV-irradiated single-stranded DNA inhibits DNA synthesis on an undamaged single-stranded DNA template in *Xenopus* egg lysates. This indicates that the egg lysate system with a single-stranded DNA template may be useful to elucidate the effects of several DNA-damaging agents on DNA replication. We attempted to study the effect of 8-oxo-dGTP on DNA replication using this cell-free DNA replication system in *Xenopus* egg lysates. The results demonstrate that 8-oxo-dGTP may inhibit DNA replication through activation of protein kinases. Furthermore, the mechanism of inhibition by 8-oxo-dGTP may be different from that by UV-irradiated single-stranded DNA, which also causes inhibition of DNA synthesis in *Xenopus* extracts.

MATERIALS AND METHODS

Materials

Xenopus egg lysates were prepared according to the method of Blow and Laskey (13). 8-Oxo-dGTP was chemically synthesized as described (14). Staurosporine and caffeine were purchased from Sigma Chemical Co. (St Louis, MO) and

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bisindolylmaleimide I (GF 109203X) was from Calbiochem-Novabiochem International (CA). Protein kinase C, containing the α , β I, β II, γ , δ and ϵ isoforms, was purchased from Promega (Madison, WI).

DNA synthesis reaction

DNA synthesis in *Xenopus* egg lysates was performed with a reaction mixture (25 μ l) containing 50 ng M13mp2 single-stranded DNA, 2 mM ATP, 50 μ M each dATP, dGTP, dTTP and [α - 32 P]dCTP (370 kBq), 20 mM creatine phosphate, 100 μ g/ml creatine kinase and an aliquot of egg lysate. The mixture was incubated at 23°C for 0–60 min. The reaction was terminated by adding 10 μ l of lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 500 mM NaCl and 2% SDS). The mixture was treated with 5 μ g RNase A at 37°C for 30 min, then with 5 μ g proteinase K at 37°C for 30 min and precipitated with ethanol. The precipitate was collected by centrifugation, dissolved in 50 μ l of TE buffer and extracted with phenol/chloroform. DNA was precipitated with ethanol and dissolved in 15 μ l of TE and subjected to 0.8% agarose gel electrophoresis. The 32 P-labeled product was detected and analyzed with a Fuji BAS-1500 phosphorimager. When testing the effect of 8-oxo-dGTP or UV-irradiated (360 J/m² at 254 nm) single-stranded M13 DNA, the indicated amounts of these were added to the reaction mixture.

RESULTS

Effect of 8-oxo-dGTP on DNA synthesis in *Xenopus* egg extracts

DNA synthesis in *Xenopus* egg extracts was performed in the presence or absence of 8-oxo-dGTP using M13 single-stranded DNA as a template. DNA chain elongation was monitored as incorporation of [α - 32 P]dCTP into single-stranded DNA. Products were analyzed by agarose gel electrophoresis and detected by autoradiography with a phosphorimager. The main products of the reaction without 8-oxo-dGTP appeared as either form I (a covalently closed supercoiled molecule) or II (an open circle molecule) on agarose gel electrophoresis (Fig. 1A). On the other hand, the total amounts of products were reduced according to the increasing concentration of 8-oxo-dGTP; for example, those with 60 μ M 8-oxo-dGTP were ~4% of those without 8-oxo-dGTP. This result indicates that the 8-oxo-dGTP may inhibit DNA synthesis in the lysates. To confirm this result, the products were again analyzed using a pulse labeling method. The results are shown in Figure 2A. Based on these results, the amounts of products synthesized in each 5 min period, indicating the rate of DNA synthesis, were determined and are shown in Figure 2B. Without 8-oxo-dGTP, the rate of DNA synthesis was rapidly increased after 20 min incubation, whereas that with 50 μ M 8-oxo-dGTP was very low in each incubation period. This indicates that 8-oxo-dGTP may delay DNA synthesis in the extract by reducing the rate of DNA chain elongation.

Effects of protein kinase inhibitors on the delay of DNA synthesis by 8-oxo-dGTP

Several protein kinases are reported to be activated in response to DNA lesions and act as a trigger for delay of replication (15,16). We examined whether the activation of a protein

Figure 1. Effects of 8-oxo-dGTP and UV-irradiated single-stranded DNA on DNA synthesis in *Xenopus* egg lysates. DNA synthesis on an M13 single-stranded DNA template in *Xenopus* egg lysates was carried out in the presence of the indicated amount of 8-oxo-dGTP (A) or UV-irradiated (360 J/m² at 254 nm) single-stranded DNA (B) for 30 min at 23°C. Products were purified and subjected to 0.7% agarose gel electrophoresis. The incorporated 32 P radioactivities were detected with a Fuji BAS-1500 phosphorimager. Amounts of 8-oxo-dGTP and UV-irradiated single-stranded DNA are shown at the top of (A) and (B), respectively. The total amounts of products were determined and are shown at the bottom of the panel as the relative amounts. I and II on the left indicate the positions of form I and II M13 double-stranded DNA, respectively, and ss indicates the position of single-stranded M13 DNA.

kinase was required for the delay of DNA synthesis with 8-oxo-dGTP using a protein kinase inhibitor, staurosporine. This compound is also known as a checkpoint abrogator (17). The inhibitor had few effects on DNA synthesis without 8-oxo-dGTP (Fig. 3A). On the other hand, the amount of DNA synthesized with 8-oxo-dGTP was at the same level as that without 8-oxo-dGTP in the presence of staurosporine (Fig. 3A). This suggests that activation of a protein kinase may be necessary for the delay of DNA synthesis with 8-oxo-dGTP. One of the well-known protein kinases involved in checkpoint control is the ataxia telangiectasia (AT)-related protein kinase family, including ATM and ATR. On the other hand, staurosporine is a potent inhibitor of the protein kinase C family, although it is a less specific protein kinase inhibitor (18). We further tested the effects of two other protein kinase inhibitors, bisindolylmaleimide I, which is a potent and selective protein kinase C inhibitor (19), and caffeine, which inhibits the ATM family (20–22). The results are shown in Figure 4A. bis-Indolylmaleimide I but not caffeine showed a recovery effect on the delay of DNA synthesis with 8-oxo-dGTP. This suggests that the protein kinase C family rather than ATM family may be involved in the delay of DNA synthesis with 8-oxo-dGTP. Then, we examined whether protein kinase C really affected DNA synthesis in *Xenopus* egg lysates. DNA synthesis in the lysates was performed with a single-stranded DNA template in the presence of different amounts of protein kinase C. The results are shown in Figure 5. The amount of newly synthesized DNA was reduced according to the increasing amount of protein kinase C (Fig. 5A) and this effect was not seen with protein kinase C pre-heated for 10 min (Fig. 5B). Staurosporine also diminished the effect of protein kinase C on the delay of DNA synthesis (Fig. 5B). These results suggest that phosphorylation of some proteins in the lysates by exogenous protein kinase C may be required for the delay of DNA synthesis on the single-stranded DNA template in *Xenopus* egg lysates.

Figure 2. Pulse labeling analysis of products synthesized in *Xenopus* egg lysates without or with 8-oxo-dGTP. (A) DNA synthesis reactions without or with 50 μ M 8-oxo-dGTP were performed at 23°C. Newly synthesized DNA in each 5 min reaction period was labeled with 32 P by adding [α - 32 P]dCTP. Products were purified and subjected to 0.7% agarose gel electrophoresis and detected with a Fuji BAS-1500 phosphorimager. Incubation period and concentration of 8-oxo-dGTP are shown at the top and bottom of the panel, respectively. I, II and ss on the left indicate the position of form I and II M13 double-stranded DNA and M13 single-stranded DNA, respectively. (B) Total amounts of products synthesized in each 5 min period shown in (A) were determined and are represented as a bar graph. The amounts of products without 8-oxo-dGTP or with 50 μ M 8-oxo-dGTP are indicated as a shaded bar or a black bar, respectively.

Figure 3. Effect of staurosporine on the delay of DNA synthesis by 8-oxo-dGTP and by UV-irradiated single-stranded DNA. DNA synthesis in lysates with various concentrations of 8-oxo-dGTP and staurosporine (A) or UV-irradiated single-stranded DNA and staurosporine (B) was performed for 30 min at 23°C. The 32 P-labeled products were analyzed by agarose gel electrophoresis as described in Materials and Methods. Amounts of 8-oxo-dGTP and staurosporine and UV-irradiated single-stranded DNA and staurosporine are shown at the top of (A) and (B), respectively. The relative amounts of products are at the bottom. I, II and ss on the left indicate the positions of form I and II M13 double-stranded DNA and M13 single-stranded DNA, respectively.

Comparison of mechanism of delay by 8-oxo-dGTP and UV-irradiated single-stranded DNA

We also asked whether the mechanism of delay of DNA synthesis with the oxidized nucleotide is common to that with other DNA lesions. DNA lesions arising from irradiation with UV are known to cause cell cycle arrest (23). Recently, it was reported that UV-irradiated single-stranded DNA also causes a delay of DNA synthesis on an undamaged single-stranded DNA template in *Xenopus* egg lysates (11). We compared the mechanism of delay of DNA synthesis between 8-oxo-dGTP and with UV-irradiated single-stranded DNA. As reported, the amount of newly synthesized DNA on the undamaged template was reduced according to the increasing amount of UV-irradiated single-stranded DNA (Fig. 1B). Time course analysis revealed that the reduced amount of product in the presence of UV-irradiated single-stranded DNA might be due to a delay of DNA synthesis similar to the case of 8-oxo-dGTP (data not shown). However, contrary to the case of 8-oxo-dGTP, the protein kinase inhibitor staurosporine had no effect on the delay of DNA synthesis with UV-irradiated single-stranded

DNA (Fig. 3B). The protein kinase C selective inhibitor bis-indolylmaleimide I also showed no recovery effect (Fig. 4B). On the other hand, the delay of DNA synthesis by UV-irradiated single-stranded DNA was recovered by addition of caffeine (Fig. 4B). This result suggests that a caffeine-sensitive protein kinase, such as ATM or ATR, rather than a protein kinase C family member, may contribute to the delay of DNA synthesis by UV-irradiated single-stranded DNA. Therefore, although two different types of DNA lesions, 8-oxo-dGTP, which damages DNA by being incorporated into DNA, and UV light, which directly damages DNA by forming thymine-thymine dimers or 6-4 photoproducts, similarly cause a delay of DNA synthesis, the mechanisms appear to be different.

DISCUSSION

In the present paper we describe the effect of a mutagenic nucleotide, 8-oxo-dGTP, on DNA replication using a cell-free system derived from *Xenopus* egg lysates with a single-stranded DNA template. Although both double-stranded and single-stranded DNA are replicated in the egg lysates, the

Figure 4. Effects of several protein kinase inhibitors on the delay of DNA synthesis by 8-oxo-dGTP and by UV-irradiated single-stranded DNA. DNA synthesis in lysates with various concentrations of 8-oxo-dGTP and bisindolylmaleimide or caffeine (A) or UV-irradiated single-stranded DNA and bisindolylmaleimide or caffeine (B) was performed for 30 min at 23°C. The 32 P-labeled products were analyzed by agarose gel electrophoresis as described in Materials and Methods. Amounts of 8-oxo-dGTP and inhibitors and UV-irradiated single-stranded DNA and inhibitors are shown at the top of (A) and (B), respectively. The relative amounts of products are at the bottom. I, II and ss on the left indicate the positions of form I and II M13 double-stranded DNA and M13 single-stranded DNA, respectively.

system with a single-stranded DNA template does not require several replication factors involved in origin recognition or double-strand unwinding, such as Cdc6p and DNA helicase (24,25). Therefore, the results may reflect the effect of this nucleotide analog on the DNA chain elongation step, including the initiation step by the DNA polymerase α -primase complex. As shown in Figure 1A, the newly synthesized DNA in the extracts was reduced according to the increasing concentration of 8-oxo-dGTP. Pulse labeling analysis revealed that 8-oxo-dGTP could delay DNA synthesis by reducing the rate of chain elongation (Fig. 2). It has been shown that at least two kinds of DNA polymerases may contribute to DNA synthesis on the single-stranded DNA template in the lysates (26). We tested the inhibitory effects of 8-oxo-dGTP on two replicative DNA polymerases (α and δ) purified from *Xenopus* egg lysates, however, this nucleotide analog did not show any inhibitory effects on the activities of these two DNA polymerases, including DNA primase associated with polymerase α (data not shown). Therefore, the reduced rate of DNA synthesis with 8-oxo-dGTP is not the result of inhibiting DNA polymerases or primase directly. Interestingly, the inhibition of DNA synthesis by 8-oxo-dGTP was recovered by addition of a protein kinase inhibitor, staurosporine (Fig. 3A). Furthermore, bisindolylmaleimide I, a potent and selective inhibitor of protein kinase C, also showed the same effect (Fig. 4A). Indeed, DNA synthesis on the single-stranded DNA template in *Xenopus* egg lysates was inhibited by exogenous protein kinase C in a dose-dependent manner (Fig. 5). This result indicates that phosphorylation by a protein kinase with a protein kinase C-like spectrum may be necessary for the delay of DNA synthesis with 8-oxo-dGTP. However, at present little evidence that a protein kinase C family member is involved in

Figure 5. Effect of protein kinase C on DNA synthesis in *Xenopus* egg lysates. DNA synthesis in lysates was performed with the indicated amounts of protein kinase C (A) or under various conditions (B) for 0–20 min at 23°C. The 32 P-labeled products were analyzed by agarose gel electrophoresis as described in Materials and Methods. Amounts of protein kinase C and incubation times are shown at the top of (A) and reaction conditions and incubation times are shown at the top of (B). The relative amounts of products are given at the bottom.

checkpoint control has been reported. The possibility that a protein kinase other than protein kinase C is responsible for the 8-oxo-dGTP-dependent DNA lesion cannot be ruled out. As reported (11), UV-irradiated single-stranded DNA also inhibits DNA synthesis on an undamaged single-stranded DNA template in *Xenopus* egg lysates (Fig. 1B). However, neither staurosporine nor bisindolylmaleimide I recovered the inhibition by UV-irradiated single-stranded DNA (Figs 3B and 4B). It has been demonstrated that eukaryotic cells have several checkpoint pathways in order to deal with different types of DNA lesion (27). Different mechanisms of inhibition by 8-oxo-dGTP and UV-irradiated single-stranded DNA may reflect this observation. At present, the exact mechanisms of delay of DNA synthesis by 8-oxo-dGTP and UV-irradiated single-stranded DNA are still unknown. Further biochemical analyses are now going on.

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