Monitoring base excision repair proteins on damaged DNA using human cell extracts

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Abstract
BER (base excision repair) is a major pathway for the removal of simple lesions in DNA including base damage and base loss (abasic site). We have developed an assay, using formaldehyde cross-linking during repair in human cell extracts, to observe BER proteins involved in the repair of damaged DNA. This approach allows visualization of repair proteins on damaged DNA during BER in human cell extracts and provides a detailed view of the molecular events leading to repair.

Introduction
Spontaneously derived lesions, such as uracil and apurinic/apyrimidinic sites (AP sites, abasic sites) and products of base deamination, oxidation and alkylation, are removed by the BER (base excision repair) pathway [1]. Several models for the mechanism of BER, involving different protein complexes and/or different temporal order of BER protein engagement, have been proposed [2–4]. These models are based on studies of multiple interactions between BER proteins (reviewed in [5]) and on studies of BER in cell extracts using radio-labelled oligonucleotide duplexes or single-lesion closed circular DNA substrates (reviewed in [6]). However, these techniques do not allow monitoring of the proteins involved in this process and, therefore, are unable to verify the BER mechanisms proposed. In the present study, we developed an assay whereby BER proteins physically interacting with damaged DNA bound to streptavidin beads were cross-linked by formaldehyde. This approach provides us with information as to which proteins were involved and when they are engaged during BER.

Results and discussion
A new method was designed to reveal the real-time engagement of BER proteins during the repair of damaged DNA. This method employs oligonucleotides containing a 3′-biotinylated end that are used to form a uracil or a cytosine control containing duplex oligonucleotide, complete with a hairpin loop to prevent nuclease digestion (Figure 1). The oligonucleotides are subsequently bound to streptavidin magnetic beads and incubated with human WCEs (whole cell extracts). After incubating for the indicated time periods, the reactions were stopped by the addition of formaldehyde. The beads were subsequently washed, the cross-links reversed and the released proteins were separated by gel electrophoresis and identified by immunoblotting with the corresponding antibodies.

Using HeLa WCE and a uracil hairpin substrate, we were able to cross-link several DNA repair proteins analysed, including Pol β (DNA polymerase β), DNA ligase IIIα–XRCC1 (X-ray cross-complementing protein 1) complex and poly(ADP-ribose) polymerase-1 (PARP-1; Figure 2A). Cross-linking of Pol β and DNA ligase IIIα–XRCC1 complex, unlike PARP-1 cross-linking, is damage-specific, since we were unable to significantly cross-link any of these proteins using a control substrate not containing uracil (Figure 2B). In comparison with the amount of damage-specific PARP-1 cross-linking, the levels are significantly lower throughout the time course using the control oligonucleotide, as PARP-1 eventually dissociates from the non-specific binding sites (Figure 2B). This indicates that, during uracil repair, the DNA is being continually incised during the initial stages of repair, creating high-affinity binding sites for PARP-1. Therefore these results suggest that PARP-1 binding to repair intermediates is involved in BER and that poly(ADP-ribosyl)ation-dependent dissociation of PARP-1 is required for other BER proteins to bind. Formaldehyde cross-linking is essential for observing protein interactions with DNA-bound streptavidin magnetic beads, since no interactions were observed without formaldehyde treatment.

Key words: base excision repair, DNA polymerase β, DNA repair, X-ray cross-complementing protein 1 (XRCC1).

Abbreviations used: BER, base excision repair; PARP-1, poly(ADP-ribose) polymerase-1; Pol β, DNA polymerase β; WCE, whole cell extract; XRCC1, X-ray cross-complementing protein 1.

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Figure 2 | Cross-linking of BER proteins during the repair of a uracil-containing substrate by HeLa WCE

A uracil-containing biotinylated hairpin substrate (A) or the corresponding control biotinylated hairpin substrate (B) was bound to magnetic streptavidin beads and incubated with 100 µg of HeLa WCE for the indicated time periods before cross-linking with 0.5% formaldehyde. DNA–beads were subsequently purified, washed and cross-links reversed before separation of the proteins by SDS/PAGE (10% gel). Proteins were transferred to PVDF membranes and analysed by immunoblotting with the indicated antibodies. A uracil-containing biotinylated hairpin substrate bound to streptavidin magnetic beads was incubated for 1 min with HeLa WCE and the proteins interacting with DNA were cross-linked with 0.5% formaldehyde (C). The beads were subsequently washed before incubation in the absence (lanes 1 and 2) or presence of DNase I (lanes 3 and 4). Beads were further washed and the samples were heated either to 65°C for 2 h (lanes 1 and 3) for reversal of cross-links or to 95°C for 3 min (lanes 2 and 4). Proteins were separated by SDS/PAGE (10% gel), transferred to PVDF membranes and analysed by immunoblotting with the indicated antibodies.

(results not shown). We therefore conclude that cross-linking does reflect damage repair activity in human cell extracts. Protein dynamics on damaged DNA revealed by the cross-linking assay are in good agreement with the repair kinetics of labelled oligonucleotide substrates studied under the same experimental conditions (results not shown).

To understand the nature of cross-linking in our assay, we compared the amount of XRCC1 and Pol β released from DNA with and without reversing cross-linking, and we found no XRCC1 or Pol β after electrophoresis of the complexes without reversing the reaction (Figure 2C, lanes 2 and 4). This result suggests that all the proteins observed in our assay are either individually cross-linked to DNA molecules or cross-linked to DNA as an intercross-linked complex. To discriminate between these possibilities, we pretreated samples before electrophoresis with an excess of DNase I. This treatment removes most of the DNA and should release individual proteins cross-linked. However, independent of DNase I treatment, we found release of XRCC1 and Pol β only after the reversal reaction (Figure 2C, lanes 1 and 3), suggesting that the BER proteins shown in Figure 2(A) were cross-linked to the same DNA molecule as a cross-linked complex. Therefore stable complexes of Pol β–XRCC1–DNA ligase IIIα are formed during the processing of a uracil-containing substrate.

In summary, our results demonstrate that formaldehyde cross-linking may be used to study the dynamics of BER proteins on damaged DNA.

References


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