**The RAD30 cancer susceptibility gene**

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Abstract

The human skin cancer-prone disease xeroderma pigmentosum variant (XPV) results from a mutation in RAD30, which encodes the novel lesion bypass DNA polymerase η. XPV cells are characterized by delayed completion of DNA replication and increased mutagenesis following UV irradiation. In cell-free extracts of XPV lymphoblasts, functional DNA polymerase η is required for the complete replication of a double-stranded plasmid containing either a single (6–4) photoproduct or a cyclobutane pyrimidine dimer (CPD), the major mutagenic UV-induced lesion. In cultured XPV cells, replication arrest activates downstream signalling pathways, leading to hyperphosphorylation of the 34-kDa subunit of the trimeric single-stranded DNA-binding protein, RPA (replication protein A). Many of the RAD30 mutations identified in XPV cells result in truncation and inactivation of DNA polymerase η. To examine whether polymorphisms in the RAD30 gene that result in altered polymerase η function, rather than enzyme inactivation, might contribute to individual susceptibility to skin cancer, methods to screen for sequence changes in the RAD30 gene in human genomic DNA have been developed.

Introduction

The genome is constantly exposed to DNA-damaging agents, both from endogenous sources such as oxidation and hydrolysis, and from environmental sources such as UV irradiation and ionizing radiation, as well as from chemicals in, for example, cigarette smoke [1,2]. While cells have dedicated pathways that repair damaged DNA, not all DNA damage is removed before DNA synthesis occurs in dividing cells [1,2]. Replication of DNA containing unrepaired damage is an important source of the mutations in tumour suppressor genes, such as p53, and in oncogenes, such as ras, that contribute to the initiation of human cancer [1–3]. Understanding the biochemical events involved in mutation fixation is an important goal of cancer research, and one that has made considerable strides in recent years. The best known examples are those where there is a clear link between exposure to the DNA-damaging agent and cancer incidence; in particular, the relationship between exposure to UV light and the development of skin cancer. Recent progress in this research area has come from two directions. First, the study of rare human genetic diseases in which the processing of DNA damage is altered, leading to increased cancer incidence, e.g. xeroderma pigmentosum (XP), where altered processing of UV-induced DNA damage leads to a vastly increased risk of skin cancer, has provided considerable insight into the relationship between DNA damage and cancer [4–7]. Secondly, the recent identification, using molecular genetic and biochemical approaches, of novel DNA polymerases that are specifically involved in processing damaged or aberrant DNA structures, has provided a molecular basis for mutation fixation, and its integration with other cellular processes, such as replication arrest following DNA damage [8–11]. A more complete understanding of this process may ultimately provide novel therapeutic targets for treating cancer.

XP

In the rare genetic disease XP, mutations in genes required for repair or replication of UV-damaged DNA lead to a 1000-fold increase in the incidence of skin cancer, and especially basal and squamous cell carcinomas [12]. In XP complementation groups A–G, inactivating mutations in one of the genes encoding proteins in the nucleotide excision repair pathway, the major pathway for the repair of UV-induced DNA damage, lead to an increased frequency of mutations in the genome, and ultimately to cancer. In addition to complementation groups A–G, a form of XP, termed XP variant (XPV) is characterized by increased skin cancer susceptibility in the presence of normal levels of nucleotide excision repair. Instead, in XPV, a mutation in the RAD30 gene, which is required for accurate replication at sites of UV-induced DNA damage, leads to accumulation of errors in the genome, providing the initiating step in cancer [3–6].

XPV patients, who account for approx. 25% of XP cases, show the clinical symptoms of sun sensitivity and an accelerated onset of sun-induced skin cancer [3]. The phenotype of cultured XPV cells includes hypersensitivity to UV irradiation (especially in the presence of caffeine [13,14]), hypermutability [15,16] and delayed recovery of DNA
replication following UV irradiation, with a reduction in the size of nascent DNA fragments [17,18]. The biochemical basis for these characteristics was elucidated with the identification of mutations in the RAD30 gene in XPV patients [9,10].

**RAD30 and DNA polymerase η**

Replicative DNA polymerases, such as polymerase δ, are blocked by lesions in the template, which leads to replication arrest. In recent years, a number of novel DNA polymerases that play specialized roles in allowing bypass of lesions induced by environmental or endogenous agents have been identified in mammalian cells [19] (Table 1). These DNA polymerases are generally characterized by low processivity, and synthesize DNA on undamaged templates with relatively low fidelity. It is therefore likely that the novel DNA polymerases normally only carry out short stretches of DNA synthesis at lesion sites, inserting one or a few nucleotides before being displaced by a higher processivity, higher fidelity polymerase such as polymerase δ, following lesion bypass [8]. From the point of view of cancer susceptibility, the RAD30 gene is particularly interesting, because of the relationship between mutations in RAD30 and UV-induced skin cancer in XPV. Human RAD30, located on chromosome 6p21.1–6p12, consists of 11 exons, of which exon 1 is untranslated [20,21]. RAD30 shows sequence homology with the *Escherichia coli* umuC gene, and encodes DNA polymerase η [9,10], a 713-amino-acid, 78-kDa protein belonging to the Y family of DNA polymerases which are involved in replication at damaged DNA sites in *E. coli*, yeast and mammalian cells [8].

DNA polymerase η is normally required for error-free bypass of the major UV-induced lesion, the cyclobutane pyrimidine dimer (CPD) [9,10]. Polymerase η reduces the frequency of mutations during bypass of CPD sites (translesion synthesis), by correctly inserting two adenine residues opposite a thymine–thymine pyrimidine dimer. In the absence of active polymerase η in XPV patients, UV-induced lesions in the DNA are replicated by additional DNA polymerases, such as polymerase ε (REV3), which frequently misinsert a base at the lesion site, leading to a greatly increased incidence of mutations in XPV cells [8]. This has been termed error-prone DNA synthesis. Mechanistically, crystal structure evidence reveals that the active site of DNA polymerase η can accommodate Watson–Crick base-pairing at helix-distorting adducts that would not be possible with other major replicative DNA polymerases [22]. Purified DNA polymerase η also carries out efficient replication on DNA containing a number of different adducts, including an 8-oxo-deoxyguanine adduct (a major product of oxidative damage to DNA [23]), a G–G adduct induced by the anti-cancer drug cisplatin [24], an N-(deoxyguanosin-8-yl)-acetylamino-sulfonuore adduct [25] and adducts induced by 1,3-butadiene metabolites [26]. Purified human DNA polymerase η is relatively inaccurate when copying benzo[a]pyrene 7,8,9,10-epoxide-induced adducts at guanine residues, and it frequently misincorporates purine bases opposite the lesion [27]. The role of polymerase η in replication of DNA containing these lesions *in vitro* remains to be clarified.

### Specificity of UV-induced lesion bypass by DNA polymerase η

The observation that DNA replication by polymerase η can be error-free or error-prone, depending on the type of lesion, raises the question of the specificity of novel DNA polymerases in replication of different types of damaged DNA. In the case of DNA polymerase η and replication of DNA containing UV-induced damage, this is important because the two major UV-induced lesions, the *cis–syn* CPD and the (6–4) pyrimidine–pyrimidone photoprodut [(6–4)PP] are structurally distinct, and would be expected to impose different constraints on a polymerase involved in bypass of the lesion. The *cis–syn* CPD results from a covalent linkage between C5 and C6 of adjacent purines, while the (6–4)PP is formed by a bond between C6 of a 5′ pyrimidine and C4 of the adjacent pyrimidine [28]. While UV irradiation induces 3–5 times more CPDs than (6–4)PPs in DNA, the latter are repaired more rapidly than are CPDs in normal human cells [29]; however, unrepaired (6–4)PPs are more mutagenic than CPDs in bacterial, yeast, and mammalian cells [30,31]. Cell extracts prepared from XPV cells have a defect in replication of a plasmid containing a single CPD on the leading strand *in vitro*, demonstrating that DNA polymerase η is required for this process [32–34]. Purified human DNA polymerase η can carry out efficient bypass of a single CPD, but not of a single (6–4)PP, in a primed oligonucleotide template *in vitro* [35,36].

### Table 1 | Eukaryotic polymerases involved in bypass of lesions

<table>
<thead>
<tr>
<th>DNA polymerase</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>α</td>
<td>DNA replication/priming</td>
</tr>
<tr>
<td>β</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>γ</td>
<td>Mitochondrial DNA replication</td>
</tr>
<tr>
<td>δ</td>
<td>Chromosomal replication/excision repair</td>
</tr>
<tr>
<td>ε</td>
<td>Chromosomal replication/repair</td>
</tr>
<tr>
<td>ζ</td>
<td>REV3: error-prone bypass synthesis</td>
</tr>
<tr>
<td>η</td>
<td>RAD30: error-free bypass of UV-induced CPDs</td>
</tr>
<tr>
<td>η*</td>
<td>DNA repair</td>
</tr>
<tr>
<td>ζ*</td>
<td>RAD30B: bypass synthesis</td>
</tr>
<tr>
<td>κ</td>
<td>DinB: bypass synthesis</td>
</tr>
<tr>
<td>λ</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>μ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>σ</td>
<td>Sister chromatid cohesion</td>
</tr>
<tr>
<td>REV1*</td>
<td>Deoxyctydyl transferase</td>
</tr>
</tbody>
</table>

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Requirement for DNA polymerase η in replication of double-stranded DNA containing a single (6–4)PP

To investigate whether DNA polymerase η was required for complete replication of DNA containing a single (6–4)PP or a single CPD, we used the approach of in vitro replication in low-salt, cell-free extracts of normal and XPV lymphoblast cells that lack functional polymerase η [37]. A series of plasmid DNA constructs, containing either a single -T–T- (6–4)PP or a -T–T- CPD, on either the leading or lagging strand for replication, were generated by molecular cloning techniques, and used as templates for in vitro replication in low-salt, cell-free extracts of normal and XPV lymphoblasts that contain the proteins required for SV40 origin- and T-antigen-dependent plasmid DNA replication [37–39]. The efficiency of replication of the site-specifically modified plasmids in cell extracts was determined based on incorporation of [α32P]dCTP into closed circular (form I) plasmid DNA. In the absence of DNA polymerase η in XPV extracts, plasmid replication is inhibited by a single -T–T- (6–4)PP on the leading strand, as well as by a single -T–T- CPD [37]. Thus, complete in vitro replication of double-stranded plasmid DNA containing either of the major UV-induced lesions requires functional polymerase η. While purified human DNA polymerase η can bypass a -T–T- CPD in a primed oligonucleotide template with the insertion of two adenine residues, this enzyme does not bypass a -T–T- (6–4)PP, but inserts a nucleotide opposite the 3′ thymine of the photoproduct [35,36,40]. Consistent with the mechanism already outlined, to complete bypass, an additional DNA polymerase, possibly the REV3 gene product, polymerase ζ [11], may insert a base opposite the 5′ thymine of the photoproduct, and extend the resulting primer terminus [40]. Polymerase ζ will be displaced subsequently by a processive replicative polymerase, such as polymerase δ, to complete synthesis of the nascent strand [8] (Figure 1). This two-step mechanism has been demonstrated for bypass of a (6–4)PP in vitro involving the combined action of DNA polymerases ι and ζ [41].

Inhibition of replication by a single (6–4)PP in vitro is associated with arrest of nascent strand synthesis at the lesion site [37]. By using isolated, labelled, nascent strands as probes to hybridize to individual leading and lagging strand sequences, it was found that when the replication fork encounters a lesion on the leading strand, uncoupling of leading and lagging strand replication occurs. Thus, the consequences for DNA replication may differ depending on whether the lesion is encountered by the replication fork on the leading or lagging strand [32,33].

DNA polymerase η and other replication proteins

DNA polymerase η interacts with proliferating cell nuclear antigen (PCNA), which normally acts as a sliding clamp that tethers replicative DNA polymerases to the DNA template [42,43]. Interaction occurs through the conserved PCNA-interaction domain, at amino acids 702–708 (QTLESFF) in the C-terminus of polymerase η [43]. The C-terminal domain (120 amino acids) is also essential for the nuclear localization of polymerase η, and for its accumulation into replication foci following UV-irradiation of cultured cells [42]. PCNA interaction stimulates the incorporation of a nucleotide opposite an abasic lesion by polymerase η, but does not increase the processivity of the enzyme [43]. The trimeric, mammalian, single-stranded DNA-binding protein RPA (replication protein A) is also required for PCNA-dependent stimulation of polymerase η activity in bypass of an abasic site.
Figure 2 | Phosphorylation of RPA p34 in XPV cells

XP30RO cells were treated with 10 or 20 J/m² of UV-C. At the indicated times, whole cell lysates were prepared. Equivalent amounts of proteins were separated on a 12% SDS/polyacrylamide gel, and RPA p34 was detected by Western immunoblotting, using anti-RPA p34 (Ab-3, Oncogene Science) as primary antibody, and chemiluminescent detection. The arrow indicates the hyperphosphorylated form of RPA p34. A lysate prepared from untreated normal cells was used as a control (N).

in vitro [43]. After replication arrest as a result of UV and ionizing radiation, the 34-kDa subunit of replication protein A (RPA p34) is hyperphosphorylated in an ataxia telangiectasia mutated- and DNA-dependent protein kinase (DNA-PK)-dependent manner [44,45]. Given the role of RPA in replication, repair and recombination and the fact that RPA can also stimulate DNA synthesis by DNA polymerase η in vitro, we investigated whether RPA p34 was hyperphosphorylated in response to UV irradiation in XP30RO cells that lack DNA polymerase η. Cells were treated with 10 or 20 J/m² of UV-C light, and the phosphorylation state of RPA p34 was examined in cell extracts, using Western blotting. RPA p34 was found to be hyperphosphorylated in a UV-dependent manner in XPV cells up to 18 h post-treatment (Figure 2). The relationship between RPA p34 phosphorylation, and the arrest of DNA replication at damage sites in the absence of DNA polymerase η is under investigation. Phosphorylation of RPA p34 may play a role in the processing of replication intermediates at arrested forks by recombination [46], a process which is activated in XPV cells [47].

Analysis of sequence changes associated with the RAD30 cancer susceptibility gene

The majority of RAD30 mutations identified to date in XPV patients result in truncation of DNA polymerase η in the conserved N-terminal catalytic domain; however, missense mutations in the catalytic domain have recently been identified, as have mutations in the C-terminal domain, which may preserve catalytic activity, but prevent nuclear localization of the protein in vivo [9,10,42,48]. Polymorphisms in the RAD30 gene in the general population, which result in amino acid substitutions and reduced polymerase η function, rather than complete inactivation of the enzyme, could contribute to individual susceptibility to sporadic cancers induced by DNA-damaging agents. To determine whether sequence changes in RAD30 gene that result in altered polymerase η function in the general population contribute to individual susceptibility to skin cancer, we have applied the technique of glycosylase-mediated polymorphism detection [49], to screen human genomic DNA for polymorphisms and mutations in RAD30. By using PCR primers fluorescently labelled with TET and HEX, the analysis can be carried out using automated sequencing. The glycosylase-mediated polymorphism detection system for detection of RAD30 sequence changes has been validated by demonstrating that a 13-bp deletion in exon 2 (in the XPV cell line XP30RO), a 2-bp deletion in exon 5 (in the cell line XP7TA) and a single-base transition (C→T) in exon 4 (in the cell line XP2CH), can be readily detected by analysis of the peaks in the electrophoretogram. The individual exons of RAD30 have been amplified successfully from genomic DNA derived from white blood cell samples from skin cancer patients, with the aim of determining if there is an association between specific RAD30 alleles and skin cancer incidence, and to investigate whether somatic mutations in RAD30 occur in skin tumours.

Conclusion

The RAD30 gene product, DNA polymerase η, plays a critical role in preventing mutations at sites of UV-induced DNA damage. Loss of DNA polymerase η function in XPV patients leads to a large increase in skin cancer incidence. Using biochemical approaches, we have found that polymerase η is required for complete replication of DNA containing a UV-induced lesion on the leading strand. Further investigation of the role of RAD30 in the cellular response of DNA damage, and the role of rare alleles of RAD30 in contributing to skin cancer incidence, may provide further insight into the role of this important cancer susceptibility gene.

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References


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