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Y-family DNA polymerase-independent gap-filling translesion synthesis across aristolochic acid-derived adenine adducts in mouse cells

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ABSTRACT

Translesion DNA synthesis (TLS) operates when replicative polymerases are blocked by DNA lesions. To investigate the mechanism of mammalian TLS, we employed a plasmid bearing a single 7-(deoxyadenosine-N^6-yl)-aristolactam I (dA-AL-I) adduct, which is generated by the human carcinogen, aristolochic acid I, and genetically engineered mouse embryonic fibroblasts. This lesion induces A to T transversions at a high frequency. The simultaneous knockouts of the *Polh*, *Poli* and *Polk* genes did not influence the TLS efficiency or the coding property of dA-AL-I, indicating that an unknown DNA polymerase(s) can efficiently catalyze the insertion of a nucleotide opposite the adduct and subsequent extension. Similarly, knockout of the *Rev1* gene did not significantly affect TLS. However, knockout of the *Rev3l* gene, coding for the catalytic subunit of polζ, drastically suppressed TLS and abolished dA-AL-I to T transversions. The results support the idea that Rev1 is not essential for the cellular TLS functions of polζ in mammalian cells. Furthermore, the frequency of dA-AL-I to T transversion was affected by a sequence context, suggesting that TLS, at least in part, contributes to the formation of mutational hot and cold spots observed in aristolochic acid-induced cancers.

1. Introduction

DNA damage, generated by endogenous and environmental agents, often blocks DNA synthesis catalyzed by replicative DNA polymerases [1]. Under this situation, translesion DNA synthesis (TLS) catalyzed by specialized DNA polymerases operates across a lesion, often resulting in a mutation. Among mammalian DNA polymerases, the Y-family polymerases, polη, polι, polκ and Rev1, play important roles in TLS [1]. A defect in human polη, the *XPV* gene product, is responsible for the xeroderma pigmentosum variant syndrome, an inherited disorder in individuals highly predisposed to sunlight-induced skin cancer [2,3]. Polη catalyzes accurate TLS across UV-induced cyclobutane pyrimidine dimers [2,3] to avoid mutation induction. Polι, together with polη, suppresses the development of skin cancer in mice [4,5]. It also plays a role in protecting human cells against oxidative damages [6]. Polκ protects mouse cells against genotoxicity of benzo[*a*]pyrene dihydrodiol epoxide-derived lesion [7]. It also plays a role in the bypass of cholesterol-induced guanine lesions in mice [8]. Rev1 has deoxycytidyl transferase activity [9,10] and catalyzes TLS across a certain class of lesions [11,12]. Rev1 also plays a non-catalytic role in TLS [13] by physically interacting with other Y-family polymerases [14,15] and the Rev7 subunit of polζ [16,17]. Polζ, consisting of Rev3, Rev7, Pold2 and Pold3 subunits (Pold2 and Pold3 subunits are shared with DNA polymerase δ) [18–21], belongs to the B family and also plays an important role in TLS [1]. This pol is especially competent for extending a primer from a 3′-terminal nucleotide pairing to a template DNA lesion [1]. Although Rev1 plays a critical non-catalytic role in the polζ activity in yeast [22], this role is questioned in mammalian cells: Rev1 is critical for the activity of Y-family polymerases, but not polζ [23].

Although many recently discovered specialized polymerases can catalyze TLS *in vitro*, Y-family polymerases likely play a major

Abbreviations: AA, aristolochic acid; dA-AL-I, 7-(deoxyadenosine-N^6-yl)-aristolactam I; MEF, mouse embryonic fibroblast; pol, DNA polymerase; TKO, triple-gene knockout; TLS, translesion DNA synthesis; PCNA, proliferating cell nuclear antigen.

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role. If a recruited polymerase cannot extend a primer following nucleotide insertion, a second polymerase such as polζ and polκ extends from the newly formed primer terminus [1]. In this case, TLS is accomplished by two specialized polymerases, often called two-step TLS. However, our previous study questioned the essential role for the Y-family polymerases in TLS: neither the TLS efficiency nor the coding properties was greatly affected in the polη/polι/polκ triple-gene knockout (TKO) mouse embryonic fibroblasts (MEFs) when TLS across a single benzo[*a*]pyrene-derived dG was studied [24]. To further explore the mechanism of mammalian TLS, we employed another environmental human carcinogen (aristolochic acid)-derived bulky adenine adduct in this study.

Aristolochic acid (AA), a nephrotoxin and human carcinogen, is found in *Aristolochia* plants and associated with both chronic kidney disease and urothelial carcinomas of the upper urinary tract [25,26]. Following metabolic activation, a metabolite(s) reacts with DNA to form covalent aristolactam-DNA adducts [27,28]. The aristolactam-dA adducts persist in the renal cortex for many years and are also found in urothelial tissues, where they initiate cancers bearing characteristic mutations in oncogenes and tumor suppressor genes [25,26,29,30]. The mutational spectrum in the urothelial carcinomas associated with AA exposure is dominated by A to T transversions (73% of single-base substitutions) of a non-transcribed strand [29,30]. The A to T transversions are rare in other cancers (4.4%) [31]. A sequence preference has also been observed for 5′pyrimidine**A**G, which coincides with the splicing acceptor sequence of a non-transcribed strand [29,30]. In this study, we have again observed that the Y family polymerases, including Rev1, are not essential for the efficient TLS across this adduct, but Polζ is.

2. Materials and methods

2.1. Cell lines

Rev1$^{-/-}$ MEFs [32], *Rev3l*$^{-/-}$ *Trp53*$^{-/-}$ MEFs [33] and *Polh*$^{-/-}$ *Poli*$^{-/-}$ *Polk*$^{-/-}$ TKO MEFs [24,34] have been described. The genomic reconfirmation of these knockouts is presented in Supplementary Fig. S1. Fig. S2 of reference 24 shows the UV sensitivity of TKO MEFs.

2.2. Construction of gapped, site-specifically modified plasmid containing 7-(deoxyadenosin-N^6-yl)-aristolactam I (dA-AL-I, **A**)

The 27-mer oligonucleotides containing dA-AL-I (Fig. 1A) were synthesized as described previously [35,36]. The oligonucleotide, 5′CCATCATCTCCAGAC**A**GATCCTCACAC (Fig. 1C) or 5′CCATCATCTCCAGAA**A**TATCCTCACAC (Fig. 1D), was annealed to a complementary, uracil-containing 27-mer, 5′TTCCGUGUGAGGAUAGAUCUGGAGAUG. The annealing resulted in the formation of four-nucleotide overhangs on both ends with two or three base mismatches opposite and adjacent to the adduct site (Fig. 1C and D). The annealed oligonucleotides were incorporated into pMTEX4 by ligating to the BsaI and BsmBI sites of the vector (Fig. 1B). Closed circular DNA was separated by ultracentrifugation in a cesium chloride-ethidium bromide continuous density gradient. To generate a gap opposite dA-AL-I, 200 ng of a modified construct was incubated, prior to transfection, with 2.5 units of uracil-DNA glycosylase (NEB) for 30 min at 37 °C, followed by treatment with 25 units of apurinic/apyrimidinic endonuclease I (NEB) for 30 min at 37 °C (Fig. 1C and D). These treatments rendered dA-AL-I resistant to nucleotide excision repair.

2.3. Transfection of MEFs with a modified construct and recovery of plasmid

Cells were cultured under 5% (v/v) CO_2 at 37 °C in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%, v/v), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells (1×10^6) were plated in a 25-cm^2 flask, cultured overnight, and then transfected overnight with 200 ng of a freshly prepared, gapped construct together with 400 ng of internal control plasmid, pMTKm, using the X-tremeGene 9 DNA transfection reagent (Roche). pMTKm was constructed by replacing the blasticidin S and ampicillin resistance genes in pMTEX4 with the kanamycin resistance gene [24]. The following day, cells were detached by treating with trypsin/EDTA, transferred to a 150-cm^2 flask and cultured for 3 days. Progeny plasmids were recovered from cells by the method of Hirt [37].

2.4. Determination of TLS efficiencies

Progeny plasmids were analyzed for TLS events. Recovered plasmids were treated with DpnI (10 units) and BglII for 1 h to remove unreplicated input DNA and progeny derived from the residual complementary strand, respectively. NEB 10-beta electrocompetent *E. coli* (*Δ(ara-leu)7697 araD139 fhuA ΔlacX74 galK16 galE15 e14-ϕ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL* (Str^R) *rph spoT1 Δ(mrr-hsdRMS-mcrBC)*) (NEB) was transformed with progeny plasmid and plated on YT (1x) agar plates containing both ampicillin (100 μg/ml) and blasticidin S (50 μg/ml) for the detection of progeny derived from the modified construct or kanamycin (50 μg/ml) alone for progeny of the internal control, pMTKm. Because the adduct incorporation site is located very close to the blasticidin resistance gene (Fig. 1B), *E. coli* transformants carrying a progeny plasmid with deletions around the adduct site do not grow on a blasticidin S-containing plate and are therefore excluded from the analysis. The ratio of the number of ampicillin/blasticidin S-resistant colonies (TLS products) to the number of kanamycin-resistant colonies (internal control) was determined for each MEF line, and the relative TLS efficiency was determined by setting the ratio obtained in experiments with wild-type MEFs to 100% [24].

2.5. Analysis of TLS events

E. coli colonies on plates containing ampicillin and blasticidin S were picked up individually and analyzed for a sequence of the adducted region by oligonucleotide hybridization using probes shown in Fig. 1C and D. Probes L and R were used to confirm the presence of the oligonucleotide insert and to detect untargeted mutations and small deletions around the adduct site. These mutants were also excluded from the analysis. Probes A1, A2, T1, T2, and C1 detect targeted base substitutions. Probes D1 and D2 detect targeted one-base deletions. An example of oligonucleotide hybridization was presented in Fig. 1E. DNA sequencing was performed when none of these probes hybridized or when the confirmation of hybridization results was necessary.

3. Results

3.1. Efficient TLS in the absence of the three Y-family polymerases, polη, polι, and polκ

When considering the structure and size of dA-AL-I, Y-family polymerases were anticipated to be involved in TLS across this lesion. Therefore, we analyzed for TLS in TKO MEFs, using the 5′C**A**G sequence context. Unexpectedly, neither TLS efficiency (Fig. 2A) nor coding specificity (Table 1) was markedly affected in TKO MEFs. The TLS efficiency remained greater than 50% when compared with