Aprataxin, poly-ADP ribose polymerase 1 (PARP-1) and apurinic endonuclease 1 (APE1) function together to protect the genome against oxidative damage

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INTRODUCTION

Ataxia with Oculomotor Apraxia Type 1 (AOA1) is an autosomal recessive neurological disorder which is characterized by progressive cerebellar ataxia, peripheral neuropathy, oculomotor apraxia, and elevated serum albumin levels (1–6). Mutations in the APTX gene were identified as causal for AOA1 by two simultaneous linkage studies (1,2). Identified APTX mutations include single nucleotide insertions and deletions and single nucleotide changes which result in substitution or premature translation termination mutations (1–6) and result in destabilization and subsequent deficiency of the protein product, aprataxin (7). Cell lines derived from AOA1 patients are hypersensitive to a range of DNA damaging agents that generate single-strand breaks (SSBs) such as hydrogen peroxide, camptothecin and dl-buthionine-(S,R)-sulfoximine (BSO), indicating a role for aprataxin in the cellular response to DNA damage (8–11).

Aprataxin contains three functional domains, an N-terminal Forkhead Associated (FHA) domain, a centrally located Histidine Triad (HIT) nucleotide hydrolase domain and a C-terminal C2H2 zinc finger (1,2). The FHA domain of aprataxin interacts with XRCC4 and XRCC1, scaffold proteins involved in double-strand break repair (DSBR) and SSB repair (SSBR), respectively (8–10,12,13). XRCC1 interacting proteins include DNA ligase 3α (Lig3α), DNA polymerase β (polβ), poly-ADP ribose polymerase 1 (PARP-1), proliferating cell nuclear antigen (PCNA) and apurinic endonuclease...
lated DNA, a ligation intermediate which can accumulate at DNA breaks with 3'-deoxy termini and 'dirty' breaks induced by oxidative DNA damage (18–20). Cell extracts from AOA1 patients lacking aprataxin protein display no DNA-adenylate hydrolase activity, indicating a lack of redundancy in DNA adenylate hydrolase activity (18–20). DNA ligation is an essential process for the repair of DNA lesions to maintain genomic integrity. It has been shown that DNA ligation at H₂O₂-induced SSBs, which often lack appropriate 3'-hydroxyl termini, can cause adenyllylation of the 5' terminus and that aprataxin can remove this modification (19,21). Thus the 'proofreading' activity of aprataxin and its interaction with both XRCC1/Lig3α and XRCC4/LigIV complexes suggest a requirement for aprataxin in multiple repair pathways (8–10,13,18–20).

DNA repair generally proceeds in a sequential manner, and is initiated by the detection of the primary lesion followed by recruitment of DNA repair complexes, processing of the lesion and is finally completed by ligation of the nick which restores the integrity of the DNA molecule (22,23). An early step in the repair of DNA SSBs is the recruitment of PARP-1, a molecular sensor of DNA breaks, which plays a pivotal role in the spatial and temporal organization of DNA repair (24). The activation of PARP-1 at DNA breaks results in the localized synthesis of poly-ADP ribose (PAR), which recruits repair proteins such as XRCC1 (24,25). The importance of this recruitment is highlighted by the multiple repair defects displayed by PARP-1−/− cells (26–30). PARP-1-dependent recruitment of XRCC1 to breaks probably serves to concentrate XRCC1-associated repair factors such as PNKP, Lig3α, polβ, APE1 and aprataxin to sites of DNA damage (8–10,16,31). SSBs can also be generated indirectly by incision at abasic sites by abasic lyases, predominantly APE1, during BER (22,23). In addition to its well-characterized abasic lyase activity (32), APE1 can also hydrolyse 3'-phosphoglycolate and 3'-8-oxo-dG termini (33,34) and stimulate the activity of many BER proteins including polβ, FEN1, DNA ligase 1 and the bifunctional DNA glycosylases UDG1 and ΩGG1 (35–38). The protein interactions and stimulatory effects of APE1 on multiple BER processes have led to the proposal that APE1 has a central role in the coordination of BER (39,40).

To obtain further insight about how aprataxin operates in the repair of DNA SSBs, we investigated further the functional implications of the aprataxin-PARP-1 interaction, and mapped the site of interaction between these proteins. This interaction was supported by evidence of co-dependent expression of aprataxin, PARP-1 and APE1. PARP-1 deficiency resulted in deficiency of aprataxin and as a consequence a deficit in DNA-adenylate hydrolase activity. Furthermore, we demonstrated that PARP-1 is not only required for the expression of aprataxin, but also for its targeting to sites of DNA damage. Measurement of the DNA repair efficiency of AOA1 cells revealed attenuated 8-oxo-dG excision repair and reduced gap filling repair, in agreement with the critical roles of APE1 and PARP-1 in BER (26,28,38,41,42). This highlights the synergistic functions of aprataxin, PARP-1 and APE1 in the cellular response to DNA damage and the modulating function of aprataxin on base excision and long patch repair.

RESULTS

3' DNA damage inhibits ligation and causes 5' DNA adenylation

Previous studies uncovered a novel biochemical role for aprataxin in hydrolysing 5' adenylated DNA at a subset of DNA breaks (18–20). To determine if 5' adenylation could occur at DNA SSBs that arise in vivo under conditions of oxidative stress, we examined the effect of 3' modifications on ligation of SSBs (schematic Fig. 1A). The presence of 3' 8-oxo-dG at the 3' OH terminus reduced the efficiency of DNA ligation and triggered formation of 5' adenylated DNA (Fig. 1B). While the presence of a phosphate at the 3' end resulted in reduced ligation efficiency, 5' adenylated DNA was not detected. The presence of an abasic site, an intermediate of 8-oxo-dG repair which can also be generated by spontaneous N-glycosyl bond hydrolysis, also inhibited ligation and caused 5' adenylation (Fig. 1B). The formation of 5' adenylate at both 3' 8-oxo-dG and 3' abasic site ligation was confirmed by its hydrolysis following addition of purified recombinant aprataxin protein to the reaction (Fig. 1B). It is unlikely that the 5' adenylated DNA generated by abortive ligation at 3' 8-oxo-dG or abasic termini could be converted to ligation products. Recent work has demonstrated that DNA ligases can reverse 5' adenylation, but only if the ligase is in the non-adenylated form (18). The stalling of DNA ligation and the generation of 5' adenylated termini by biologically relevant 3' break modifications suggest that the hypersensitivity of AOA1 cells to hydrogen peroxide, a well-known inducer of oxidative DNA damage, could be due to the accumulation of adenylated breaks.

Aprataxin is a DNA-adenylate hydrolase

Having shown that modification of the 3' terminus of an SSB can generate 5' adenylated DNA, we subsequently examined the activity of aprataxin against a 5' adenylated SSB. Using an in vitro assay with a substrate mimicking a 5' adenylated break (Fig. 2A), we measured DNA-adenylate hydrolysis. We found that aprataxin is active in nuclear extracts from control cells (C2ABR and C3ABR), but extracts from AOA1 cells with point mutations in APTX (L938: P206L/P206L and L939: P206L/V263G) lacked DNA-adenylate hydrolyase activity (Fig. 2A–C). Time course experiments showed a rapid 5'-AMP hydrolysis in C3ABR extracts while no hydrolysis occurred in L938 extracts up to 10 min incubation (Fig. 2B and C). As shown in Supplementary Material, Figure S1, mutation of APTX results in deficiency of aprataxin protein, as demonstrated by the absence of aprataxin in AOA1 cell lines. Recombinant aprataxin also efficiently bound and hydrolysed adenylated DNA (Supplementary Material, Fig. S2A and B). Furthermore, the deficient 5' adenylate hydrolyase activity of AOA1 extracts was complemented by addition of
recombinant aprataxin (data not shown). Incubation of control cell extracts (C2ABR) with affinity purified anti-aprataxin antibody reduced the rate of DNA-adenylate hydrolysis compared with equivalent pre-immune serum (Fig. 2D), providing conclusive evidence that the hydrolase activity of wild-type extracts is attributable to aprataxin. The specificity of this affinity-purified antibody was confirmed by western blotting and immunostaining of corrected and uncorrected AOA1 cells (Supplementary Material, Fig. S3).

Given that S’ adenylated DNA can be generated by abortive ligation at breaks possessing physiologically relevant 3’ modifications such as 8-oxo-dG (Fig. 1), we next examined the effect of 3’ 8-oxo-dG on repair of 5’ adenylated termini by endogenous aprataxin. Deadenylation by control nuclear extracts was not affected by 3’ 8-oxo-dG modification (Fig. 2E), suggesting that adenylates which arise due to failed ligation attempts at such oxidized breaks would be efficiently excised by aprataxin.

Takahashi et al. (43) reported that aprataxin also possesses 3’ repair activities—hydrolysis of 3’ phosphate and α/β unsaturated aldehyde termini. To explore this further, the 3’ phosphatase activity of recombinant aprataxin was investigated. Incubation of dsDNA containing a 3’ phosphate modified nick with a 5-fold molar excess of recombinant aprataxin failed to detect 3’ phosphate hydrolysis after extended incubation, while parallel reactions containing 5’ adenylated DNA substrate were hydrolysed efficiently (Supplementary Material, Fig. S2C). Thus, aprataxin does not exhibit 3’ phosphatase activity.

Mapping PARP-1 and aprataxin interaction domains

Earlier studies showed that aprataxin is present in cells in a preformed complex with the scaffold protein XRCC1, Lig3α, polβ and the multifunctional DNA repair protein PARP-1 (8–10,12,13). The functions of PARP-1 at DNA breaks include detection of strand interruptions and subsequent synthesis of negatively charged ADP ribose polymers that serve as a recruitment signal for additional repair factors (25). To further characterize this interaction and understand the role of aprataxin in SSBR, we employed GST-pull downs to map the interacting domains of both proteins. GST fusion proteins covering the entire length of aprataxin (Fig. 3A) were used to confirm that PARP-1 interacts with the FHA domain of aprataxin (Fig. 3B), in agreement with a previous report (9). A series of GST fusion proteins encompassing the entire PARP-1 protein (Fig. 3C) were used to map the interaction of aprataxin to the BRCT motif of PARP-1 (Fig. 3D).

Reduced levels of PARP-1, APE1 and OGG1 in AOA1

There are many DNA repair complexes in which mutation or deficiency of one component destabilizes other members of the complex. For example, the MRN (Mre11, Rad50, Nbs1) complex is destabilized by mutations in mre11 and rad50 (44,45). Given that aprataxin and PARP-1 interact, we investigated whether the absence of one protein affects the stability of the other. Immunoblotting of total cell extracts from PARP-1−/− and PARP-1+/− mouse embryonic fibroblasts (MEFs) revealed the absence of aprataxin and APE1, a key player in base excision repair, in response to PARP-1 deficiency (Fig. 4A). A slight reduction in Lig3α expression was observed in PARP-1-deficient cells, while DNA polβ was unaffected (Fig. 4A). Similarly, knockdown of PARP-1 with small interfering RNA (siRNA) in HeLa cells resulted in a reduction in aprataxin and APE1 levels (Supplementary Material, Fig. S4A). Knockdown of APE1 caused a reduction in PARP-1 levels but aprataxin was unaffected (Supplementary Material, Fig. S4B). Additionally, uncorrected AOA1 fibroblasts (FD105 M20) had low levels of PARP-1 and APE1 compared with the full length aprataxin cDNA complemented cell line (FD105 M21) (Fig. 4B). Levels of Lig3α were not affected by the absence of aprataxin, while a slight reduction in the level of polβ was observed in FD105 M20 compared with corrected cells (Fig. 4B). This is in contrast to the situation with XRCC1 deficient (EM9) cells where DNA ligase 3 activity is reduced (46). These results suggest co-dependence between aprataxin, PARP-1 and APE1 protein levels. In order to determine whether the reduced levels of PARP-1 and APE1 observed in AOA1 cells were due to protein degradation or reduced transcription, transcript levels were analysed. Reduced expression of both PARP-1 and APE1 mRNA was observed in AOA1 cells compared with the aprataxin-complemented counterparts (Fig. 4C, upper panel). Consistent with these findings, treatment of AOA1 cells with proteasome inhibitor MG132 did not affect PARP-1 or APE1 protein levels (data not shown). Given the established role of APE1 in stimulation of bifunctional DNA glycosylases (38,41) and our observation that APE1 expression was aprataxin-dependent, we also measured the expression of OGG1. Expression of this gene was undetectable in AOA1 cells but increased to detectable levels on complementation with full-length aprataxin cDNA (Fig. 4C, lower panel). While Apto, Ape1 and Ogg1 expression was detected in PARP-1+/− cells, expression of these genes was not detected in the PARP-1−/− cells (Supplementary Material, Fig. S5),
Figure 2. Aprataxin is a DNA-adenylate hydrolase. (A) Schematic of 5' DNA adenylated substrate, Duplex A. Hydrolysis of 5' DNA adenylate was assessed in increasing concentrations of control (C2ABR and C3ABR) and AOA1 (L938 and L939) lymphoblastoid cell extracts (0.1, 0.2, 0.5, 1.0 and 1.5 μg/μl). Reactions were initiated by addition of Duplex A and allowed to proceed for 2 min before termination and analysis by 20% denaturing PAGE. (B) Time course of 5' DNA
Figure 3. Interaction between aprataxin and PARP-1. (A) Schematic of aprataxin GST fusion proteins. (B) Aprataxin GST pull downs identified the FHA domain as the PARP-1 interacting motif. HeLa cell extracts were incubated with aprataxin fragment-GST fusion proteins. GST fusions and their interacting proteins were precipitated using glutathione agarose beads and analysed by 12% SDS–PAGE and immunoblotting with anti-PARP-1 antibody. Coomassie staining shows equivalent input of GST fusion proteins. WCE indicates 5% input of whole cell extract used for pulldowns. (C) Schematic of PARP-1 GST fusion proteins. (D) Mammalian GST vectors expressing PARP-1 fragments were transfected into HeLa cells. After cell lysis PARP-1 GSTs and interacting proteins were precipitated with glutathione agarose beads. Proteins were eluted in SDS-PAGE loading buffer and resolved by denaturing protein gel electrophoresis. Precipitated proteins were detected by immunoblotting for aprataxin. WCE indicates 5% input of untransfected whole cell extract.

adenylate hydrolase activity in control (C3ABR) and AOA1 (L938) extracts. Two microgram of extract was incubated with 2 pmol of Duplex A for the indicated times. N.E is a no extract negative control. (C) Plot representing the kinetic of 5′ DNA adenylate hydrolysis in control (C3ABR) and AOA1 (L938) extracts derived from the time course (B). (D) Inhibition of DNA adenylate hydrolysis by addition of anti-aprataxin antibody. Four microgram of control (C2ABR and C3ABR) and AOA1 (L938) extract was incubated with 400 ng of anti-aprataxin antibody (Aptx Ab) or pre-immune serum (pre-I) in a 18 μl volume for 20 min. Reactions were initiated by addition of 2 pmol of Duplex A (1 pmol/μl) prior to termination after the indicated times. N.E is a no extract negative control. (E) Modification of the 3′ extremity does not affect aprataxin 5′ DNA adenylate hydrolase activity. Five micrograms of control (C2ABR and C3ABR) LCL nuclear extract was incubated with 2 pmol of 5′ adenylated DNA, with or without an adjacent 3′ 8-oxo-dG as indicated. N.E is a no extract negative control. Reactions were terminated after the indicated times and resolved by 20% denaturing PAGE.
consistent with their low levels of aprataxin and APE1 proteins (Fig. 4A).

Aprataxin activity is dependent on PARP-1

Based on the absence of aprataxin in PARP-1−/− cells, we predicted that they would have deficient 5′ DNA adenylate hydrolase activity. As shown in Figure 5A, nuclear extracts from PARP-1−/− cells display a marked impairment in the rate of DNA adenylate hydrolysis compared with wild-type cells. Having shown that aprataxin protein level, and therefore activity, is dependent on PARP-1, we investigated whether PARP activity impacts on DNA-adenylate hydrolysis. Nuclear extracts from control lymphoblastoid cells were treated with the PARP inhibitors PJ-34 (20 μM), 3AB (10 μM) or DMSO vehicle only control for 30 min at room temperature. Subsequently, DNA-adenylate hydrolyase reactions were initiated by addition of substrate (Duplex A, 2 pmol). Final reaction volumes were 20 μl (in Ligase Buffer) and extract concentrations are indicated. Hydrolase reactions were terminated after 5 min and analysed by denaturing PAGE. N.E is a no extract negative control.

These findings reveal that PARP-1 protein is required for the maintenance of normal aprataxin levels. In contrast, PARP activity appears dispensable as inhibition of PARP activity did not affect 5′ adenylated DNA hydrolysis.
XRCC1 focus formation is reduced by mutation of its PARP-1 interacting BRCT domain (24). Moreover, XRCC1 foci are not detected in PARP-1−/− MEFs after treatment with H2O2 (24). Biological imaging of charged particle tracks represents an alternative approach for producing discrete regions of sub-nuclear damage to investigate the association of repair proteins with chromatin (49,50). Using a remote-controlled system coupled to a beamline microscope that allows the acquisition of fluorescence images of living cells, in real-time during heavy ion irradiation (Fig. 6A), we previously observed that GFP-aprataxin is rapidly translocated to particle-induced DNA damage (9,49). Since aprataxin interacts with both XRCC1 and PARP-1, and both PARP-1 protein and PARP activity are required to recruit XRCC1 to DNA breaks (24), we investigated whether PARP-1 and PARP activity are required to recruit aprataxin to breaks. Given the lack of aprataxin protein in PARP-1−/− cells (Fig. 4A), transiently transfected GFP-aprataxin was used to study the role of PARP-1 in recruitment of aprataxin to breaks. While GFP-aprataxin re-localized to sites of DNA damage in PARP-1+/+ cells, this recruitment did not occur in PARP-1−/− cells (Fig. 6B). Under these conditions we also confirmed PARP-1 dependent recruitment of XRCC1 to sites of particle-induced DNA damage (Supplementary Material, Fig. S6). This is in agreement with previous reports where H2O2 treatment and laser irradiation were used to induce breaks (24,49,50). Immunoblotting of nuclear extracts confirmed the absence of PARP-1 protein and the presence of XRCC1 in PARP-1 KO MEFs (Fig. 6C). In contrast to reduced levels of aprataxin (Fig. 4A), XRCC1 levels were normal in PARP-1 deficient cells (Fig. 6C).

Subsequently, we examined the effect of PARP inhibition on recruitment of GFP-aprataxin to DNA breaks. HeLa cells transfected with GFP-aprataxin were either untreated or pre-treated with 3AB or PJ-34 prior to heavy ion irradiation. The use of GFP-aprataxin allows real-time and dynamic monitoring of aprataxin movements in live cells. Inhibition of PARP activity by 3AB in HeLa cells did not prevent aprataxin re-localization; however accumulation of aprataxin at sites of DNA damage was delayed relative to untreated cells (Fig. 6D and Supplementary Material, Fig. S7A). Maximum signal intensity was observed ~60 s after irradiation in untreated cells, compared with ~120 s in 3AB-treated cells (Fig. 6D). PJ-34 had a similar effect on aprataxin recruitment to sites of DNA damage in HeLa cells (Fig. 6E and Supplementary Material, Fig. S7B). Maximum signal intensity was again observed approximately at 50 s in untreated cells compared with 120 s in PJ-34-treated cells. Effective inhibition of PARP activity in these conditions was demonstrated by lack of poly-ADP ribose (PAR) foci formation after H2O2 treatment (data not shown). Taken together these results indicate that PAR synthesis stimulates not only the recruitment of XRCC1 but also that of aprataxin, and that PARP-1 protein is essential for targeting aprataxin to breaks.

**Figure 6.** PARP-1-dependent recruitment of aprataxin to sites of DNA damage. (A) Methodology employed to study the recruitment of aprataxin to sites of DNA damage in vivo. (B) PARP-1+/+ and PARP-1−/− MEFs were transfected with a GFP-aprataxin fusion construct using Amaca Nucleofection. DNA damage was induced by irradiation with xenon ions (4.5 MeV/u; LET 8815 keV/μm). Representative images of the recruitment of GFP-aprataxin to DNA breaks (γH2AX foci) in PARP-1+/+ and PARP-1−/− cells 10 min after irradiation are shown. ToPro3 shows nuclei. (C) Immunoblotting of PARP-1−/− cells confirming the absence of PARP-1 in these cells. XRCC1 is unaffected by PARP-1 deficiency and β-tubulin was used as loading control. (D) HeLa cells stably expressing GFP-aprataxin were either untreated or pre-treated with 5 mM 3AB before irradiation with samarium ions (4.2 MeV/u; LET 10290 keV/μm). Relative GFP fluorescence intensity at the DNA breaks was monitored over time. (E) HeLa cells stably expressing GFP-aprataxin were either untreated or pre-treated with 10 μM PJ-34 before irradiation with xenon ions (4.7 MeV/u; LET 8655 keV/μm). Relative GFP fluorescence intensity at the DNA breaks was monitored over time.

**Heightened levels of oxidative DNA damage in AOA1 cells**

Hirano et al. (11) have shown that post-mortem brain sections from AOA1 patients display elevated levels of oxidative DNA damage (8-oxo-dG staining) compared with matched controls.

Here we provide additional evidence for this, by showing significantly elevated levels of 8-oxo-dG in primary fibroblasts derived from an AOA1 patient (FD105) compared with normal fibroblasts (NFF) (Fig. 7A and B). Furthermore, complementation of AOA1 fibroblasts with APTX full length cDNA (FD105 M21) resulted in a significant decrease in...
basal levels of 8-oxo-dG (Fig. 7C and D). Together, these data suggest that AOA1 cells have a defect in the removal of oxidative DNA damage (8-oxo-dG). This is consistent with the failure to detect OGG1 mRNA in aprataxin deficient cells (Fig. 4C, lower panel). To investigate the relationship between aprataxin and OGG1 in more detail, we investigated the effect of aprataxin on OGG1 activity using recombinant proteins. Using an oligonucleotide duplex containing a single 8-oxo-dG, we first confirmed that recombinant OGG1 excised the 8-oxo-dG and hydrolysed the resulting abasic site (Supplementary Material, Fig. S8A), consistent with previous reports (42). As predicted, both full length and a truncated form of aprataxin lacking the zinc finger domain had no activity on that substrate (Supplementary Material, Fig. S8A). Addition of aprataxin to OGG1 reactions stimulated substrate hydrolysis by OGG1 (Supplementary Material, Fig. S8B and C). Deletion of the aprataxin zinc finger reduced this stimulation indicating an aprataxin-DNA complex is involved (Supplementary Material, Fig. S8B and C).

**Aprataxin deficiency causes multiple BER defects**

Recent work has highlighted a role for aprataxin in short patch repair of 5’ adenylated DNA breaks (18). A defect in the excision phase of BER, supported by increased endogenous levels of 8-oxo-dG in AOA1 cells, prompted us to investigate the efficiency of subsequent repair steps: namely DNA end processing; gap filling and ligation. DNA ligation is a common step among all DNA repair mechanisms, and requires adjacent 3’ hydroxyl and 5’ phosphate termini in order to be successful. Given that DNA SSBs produced in cells via either direct or indirect mechanisms often lack such termini, end-processing is an important feature of many repair mechanisms. Takahashi et al. (43) reported that recombinant aprataxin possesses 3’ phosphatase activity in addition to its well-characterized 5’ DNA adenylate hydrolase activity (18–20), although we failed to detect such activity here (Supplementary Material, Fig. S2C). To examine the 3’ end-processing activity in aprataxin-deficient cells, control and AOA1 nuclear extracts were incubated with a DNA substrate containing a 5’ hydroxyl, 3’ phosphate modified nick (Fig. 8A). Control and AOA1 nuclear extracts display equal rates of 3’ phosphate hydrolysis (Fig. 8B), indicating that 3’ phosphate processing is unaffected by aprataxin deficiency. This is consistent with the lack of detectable 3’ phosphatase activity in recombinant aprataxin protein (Supplementary Material, Fig. S2C).

To further investigate the role of aprataxin in BER, we focused on long patch extension, given the role of PARP-1
in this mechanism and the interaction between aprataxin and PARP-1 (9,26,28). We first examined gap filling in PARP-1+/− and PARP-1−/− cells using an oligonucleotide-based substrate containing a single-strand gap of six nucleotides (Fig. 9A). PARP-1−/− nuclear extracts were able to extend a single nucleotide from a 3′ hydroxyl terminus but did not efficiently polymerize additional nucleotides compared with PARP-1+/− extracts, where all intermediates in the reaction were observed (Fig. 9B). This indicates that PARP-1 deficiency results in a defect in gap filling, consistent with the long patch repair defect previously reported (26,28).

Examination of gap filling in control and AOA1 lymphoblastoid nuclear extracts revealed an impaired ability to extend multiple nucleotides in AOA1 cells (Fig. 9C and D, Supplementary Material, Fig. S9). Immunoblotting of the nuclear fractions used in the gap filling assays confirmed a lack of PARP-1 and APE1 proteins in AOA1 extracts, while DNA polβ was unaffected by aprataxin deficiency (Fig. 9E). This statistically significant defect in long patch repair efficiency of AOA1 nuclear extracts (48% reduction, n = 4, P < 0.02) was not corrected by the addition of recombinant aprataxin (Fig. 9F), supporting the idea that this defect is indirect and most likely due to insufficiency of PARP-1 (Figs 4B and 9E).

Finally, we examined the effect of aprataxin deficiency and nucleotide oxidation on the final stage of DNA SSB repair, ligation (Fig. 9G). Consistent with the findings of Reynolds et al. (18), ligation of conventional (5′ P, 3′ OH) SSBs was not impaired in AOA1 nuclear extracts. Given the elevated level of 8-oxo-dG in AOA1 cells (Fig. 7) and the capacity of this modification to lead to 5′ DNA adenylation (Fig. 1B), we also examined the impact of 3′ 8-oxo-dG on ligation by AOA1 nuclear extract (Fig. 9G). 3′ 8-oxo-dG modification inhibits ligation in both control and AOA1 nuclear extract to an equivalent extent, indicating that ligation of oxidized SSBs is not aprataxin-dependent. Ligation at 3′ 8-oxo-dG SSBs by AOA1 extracts did not induce detectable 5′ adenylation (Fig. 9G). Ligation at 3′ oxidized breaks by AOA1 extracts may be undetected due to a combination of low substrate turnover, a low frequency of adenylation at 3′ oxidized breaks and high levels of 3′–5′ exonuclease activity in cell extracts (evident by the indicated ‘degraded ligation products’ in Fig. 9G). In summary, we have analysed several aspects of DNA SSB repair in AOA1 cells and provide evidence for defects in the excision and long patch DNA polymerization phases of BER in AOA1 cells due to reduced expression of PARP-1, APE1 and OGG1.

**DISCUSSION**

AOA1, one of the most common autosomal recessive cerebellar ataxias in some countries, is caused by mutation of the APTX gene which results in destabilization of the protein product aprataxin (1,2,7,51). Previous studies demonstrated that aprataxin is a DNA processing protein that hydrolyses 5′ adenylated DNA (18–20). Here we confirmed these findings and furthermore demonstrate that 5′ adenylation can occur via abortive ligations at DNA SSBs with physiologically relevant oxidized or abasic 3′ termini. This provides a link between the in vitro enzymatic activity of aprataxin and the observed sensitivity of AOA1 cells to agents that induce oxidative DNA damage and abasic site formation (8–11). This is particularly relevant to the pathology of AOA1, where the brain is the major affected tissue (1–6). Indeed, high energy production and oxygen consumption in the brain are likely to lead to elevated levels of free radicals, oxidative stress and as a consequence oxidative DNA damage (52). As adenylated DNA is unlikely to be produced by ligation at ‘clean’ DNA breaks possessing 5′ phosphate and 3′ hydroxyl termini, it was important to determine whether the ability of aprataxin to hydrolyse 5′ adenylates at DNA breaks is impaired by the presence of damage on the adjacent 3′ terminus. Having shown that adenylation can occur at breaks with 3′ 8-oxo-dG termini in vitro we then demonstrated that endogenous aprataxin was able to remove 5′ adenylates adjacent to 3′ 8-oxo-dG or unmodified termini with equal proficiency. This indicates that hydrolysis of 5′ adenylated DNA by aprataxin is not hindered by the presence of base damage at the adjacent 3′ terminus.

A recent study by Reynolds et al. (18) revealed that processing of 3′ phosphate termini by PNKP was unaffected by adenylation of the 5′ terminus. We report here that 3′ phosphate hydrolysis (which is predominantly catalysed by PNKP) is unimpaired in AOA1 cells, indicating that cells can effectively process 3′ phosphate ends independent of aprataxin or 5′ adenylation. The ability of AOA1 cells to process 3′ phosphate termini suggested that they may be able to indirectly excise 5′ adenylates using BER. Indeed, Reynolds et al. (18) found that fill-in of a single nucleotide gap by polβ is unaffected by 5′ adenylation, but that short patch BER ultimately fails due to an inability of endogenous DNA ligases to reverse 5′ adenylation in the absence of aprataxin.

Protein–protein interactions have the potential to regulate DNA repair reactions. To explore the impact of protein–protein interactions on recruitment and activity of aprataxin, we examined the physical and functional relationship between aprataxin and PARP-1, a critical player in the repair of DNA SSBs (22,23). Remarkably, aprataxin levels were dramatically reduced in the absence of PARP-1 protein, causing a deficit in the DNA-adenylate hydrolase
Figure 9. Impaired long patch extension in PARP-1 and AOA1 cells. (A) Schematic of long patch repair substrate and assay schematic. (B) PARP-1+/+ or PARP-1−/− MEF nuclear extracts (30 μg) were incubated with the patch repair substrate (1 pmol) for the indicated times prior to sample denaturation and electrophoresis. Polymerization from the 3′ terminus is visible as discrete single nucleotide increases in molecular weight of the radiolabelled band. N.E is a no extract negative control showing the input of substrate. (C) Control (C2ABR and C3ABR) and AOA1 (L938 and L939) nuclear extracts (30 μg) were incubated with the patch repair substrate (1 pmol) for the indicated times prior to sample denaturation and electrophoresis. N.E is a no extract control showing the input of substrate. (D)
activity of PARP-1<sup>-/-</sup> cells. Furthermore, APE1 and OGG1 were also deficient in PARP-1<sup>-/-</sup> cells. Thus the well-documented hypersensitivity of PARP-1 knockout cells to a range of DNA damaging agents may in fact result from a combined deficiency of aprataxin, APE1 and OGG1. PARP-1<sup>-/-</sup> cells lack PARP-1 protein and thus could not be employed to determine whether the observed destabilization of aprataxin is due to a lack of PARP-1 protein or PAR synthetic activity. However, while lack of PARP-1 led to loss of aprataxin protein, treatment of control cells with the PARP inhibitors 3AB and PJ-34 did not affect aprataxin activity. This indicates that PARP-1 protein is critical for maintaining aprataxin protein levels but that PARP activity is dispensable for DNA-adenylate repair by aprataxin.

The well-described role of PARP-1 as a sensor of SSBs and DNA repair recruitment factor (22–24) led us to investigate the role of PARP-1 in recruitment of aprataxin to breaks. We have previously demonstrated the co-localization of aprataxin with XRCC1 along heavy ion particle tracks in chromatin (9,49). Using a similar approach, we showed that GFP-aprataxin did not re-localize to sites of DNA breaks in PARP-1<sup>-/-</sup> cells, whereas it did in wild-type cells. While PARP activity is not required to maintain aprataxin levels, synthesis of poly-ADP ribose is crucial in the recruitment of DNA repair factors to the sites of DNA breaks (24,49,50). To explore the role of PARP activity in the recruitment of aprataxin to breaks, we measured the impact of PARP inhibition on the recruitment of GFP-aprataxin to sites of DNA damage. Inhibition of PARP activity delayed recruitment of aprataxin to DNA breaks, in keeping with the failure of PARP-1<sup>-/-</sup> cells and the reduced efficiency of long patch repair in PARP-1<sup>-/-</sup> cells (26,28,30) suggested that AOA1 cells could possess an additional long patch BER defect. The reduced levels of PARP-1 in AOA1 cells and the defective long patch repair in PARP-1<sup>-/-</sup> cells (26,28,30) are consistent with the present findings of an impaired gap filling capacity in AOA1 cells. Although estimates of DNA SSB repair efficiencies of AOA1 cells have provided conflicting results (9,11,18,53,54), which may depend on the specific AOA1 cells and methodology employed, the present study identified that AOA1 cells have compounding partial defects in DNA SSB repair.

In summary, we propose that the AOA1 disorder resulting from aprataxin deficiency exhibits a combination of intermediate DNA repair defects (Fig. 10). These include (i) the inability to hydrolyse 5<sup>`</sup> DNA adenylates due to aprataxin deficiency, (ii) a defect in the excision of 8-oxo-dG due to APE1 and OGG1 deficiency, (iii) a reduced gap filling efficiency caused by low levels of PARP-1 and (iv) a defective ligation step in short patch BER due to non-adenylated DNA ligase insufficiency (18). These findings highlight the intricacies and synergy between the various factors of the DNA SSBR machinery and uncovered a novel role for aprataxin in the transcriptional regulation of PARP-1, APE1 and OGG1. These combined defects, in addition to the direct

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(D) The 20 minute time points of four independent patch repair experiments were quantified. For each experiment the abundance of fully extended product was determined for the control (C2ABR+C3ABR) and AOA1 (L938+L939) cell lines. The mean of the control cell lines was denoted ‘100 percent’ and the AOA1 activity expressed as a proportion of this. A significant impairment in synthesis of fully extended product by AOA1 extract was demonstrated by Student’s t test (48% reduction, n = 4, P = 0.001). (E) Immunoblotting of the protein extracts used in the long patch repair assay described in (C) showing the absence of PARP-1 and APE1 in AOA1 fractionated nuclear extracts. DNA polβ is unaffected by aprataxin deficiency in these cells. (F) Control (C2ABR) and AOA1 (L938) cell extracts were incubated with the patch repair substrate (1 pmol) for the indicated times, with or without recombinant aprataxin (1 pmol). (G) Nicked duplex DNA radiolabelled on the 5` break terminus and possessing either hydroxyl or 8-oxo-dG 3` break termini (2 pmol) were incubated with 30 μg of control (C2ABR) or AOA1 (L938) nuclear extracts for the indicated times prior to reaction termination and analysis. Full length ligation product, degraded ligation products and unligated oligo (oligo A) are indicated.
A cell line, derived from FD105. FD105 M20 and M21 M20 is an uncorrected vector only control immortalized cDNA under a constitutive expression promoter. FD105 primary cell line, corrected with full-length primary AOA1 patient fibroblast cell line. FD105 M21 is an P206L/P206L and L939: P206L/V263G). FD105 is a have substitution mutations within the HIT domain (L938: blood of healthy Caucasian males. Both of the AOA1 patients lines C2ABR and C3ABR were derived from peripheral blood of male Japanese AOA1 patients and the control cell The cell lines L938 and L939 were derived from peripheral blood of healthy Caucasian males. Both of the AOA1 patients have substitution mutations within the HIT domain (L938: L939: P206L/V263G) and L939: P206L/V263G). FD105 is a have substitution mutations within the HIT domain (L938: blood of healthy Caucasian males. Both of the AOA1 patients lines C2ABR and C3ABR were derived from peripheral blood of male Japanese AOA1 patients and the control cell

M20 is an uncorrected vector only control immortalized AOA1 patient fibroblast cell line. FD105 M20 is an uncorrected vector only control immortalized AOA1 cell line, derived from FD105. FD105 M20 and M21 were kind gifts from Keith Caldecott. PARP-1 \(^{+/+}\) and PARP-1 \(^{−/−}\) MEF cell lines were derived from wild-type and PARP-1 knockout mice by Valérie Schreibers’ laboratory. Nuclear extracts were generated as published by Audebert et al. (29), described briefly below. Lymphoblastoid cells (C2ABR, C3ABR, L938 and L939) were grown in RPMI 1640 (GIBCO BRL) containing 6% FCS (Lonza), 2 mM l-glutamine (Life Technologies) and 100 units of penicillin/streptomycin (GIBCO BRL). Adherent cells were grown in DMEM (GIBCO BRL) with 12% FCS (Lonza) and 100 units of penicillin/streptomycin (GIBCO BRL). All cells were cultured at 37°C in a humidified atmosphere supplemented with 5% CO\(_2\). The PARP inhibitors PJ-34 and 3-aminobenzamide were purchased from Sigma.

Recombinant proteins

Full length and truncated recombinant aprataxin proteins were expressed using pGEX constructs as described previously (55). Recombinant human OGG1, APE1, UDG1 and T4 DNA ligase and polynucleotide kinase were obtained from NEB. Aprataxin fragment-GST fusion proteins have been described previously (9,56). PARP-1 GSTs were produced as previously described (15).

Preparation of nuclear extracts

All protein extraction steps were performed at 4°C. A total of 2–3 × 10\(^{08}\) cells were pelleted by centrifugation for 5 min at 1500g. The cell pellet was washed twice with PBS and resuspended in 2–5 ml of Hypotonic Buffer (10 mM Tris–HCl pH 7.5, 10 mM KCl, 10 mM MgCl\(_2\), 1 mM EDTA, 13% glycerol, 0.1 M potassium glutamate and 1 mM PMSF and 1× complete inhibitor) and incubated on ice for 15 min. Cell membranes were broken by dounce homogenization (15 strokes), and the nuclei pelleted at 2000g for 3 min. Nuclei were resuspended in 0.5–2 ml of Extraction Buffer (10 mM Tris–HCl, pH 7.5, 10 mM KCl, 450 mM MgCl\(_2\), 1 mM EDTA, 13% glycerol, 0.1 M potassium glutamate and 1× complete inhibitor) and incubated on a rotating wheel for 45 min. Lysate was cleared by centrifugation at 16 100g for 10 min and the supernatant was precipitated by addition of 0.313 g of ammonium sulphate per milliliter, neutralized with 3.13 μl 1 M NaOH per milliliter and incubated on a wheel for 30 min. Precipitated proteins were recovered by centrifugation at 16 100g for 20 min, resuspended in Dialysis Buffer (50 mM Tris–HCl pH 7.5, 1 mM EDTA, 13% glycerol, 0.1 M potassium glutamate and 1 mM DTT) and incubated on a rotating wheel for 30 min prior to dialysis against Dialysis Buffer. Extract was then centrifuged at 16 100g for 10 min and the supernatant stored in aliquots at –80°C.

In vitro SSB repair

SSB repair substrates were generated by annealing three oligonucleotides (Oligo A: CCCTCAATTCCGATAGTGAC-TACA, Oligo B: 5′ CATATCCGTGTCG and Common Strand: 5′ TGTAGTCTATCCGGAATGAGGCACGCA GATATG). The 3′ terminal nucleotide of Oligo B has been terminal nucleotide of Oligo B has been terminal nucleotide of Oligo B has been substituted with an 8-oxo-dG or abasic site or phosphorylated as indicated. The effect of 3′ DNA damage on ligation was

MATERIALS AND METHODS

Cell lines, inhibitors

The cell lines L938 and L939 were derived from peripheral blood of male Japanese AOA1 patients and the control cell lines C2ABR and C3ABR were derived from peripheral blood of healthy Caucasian males. Both of the AOA1 patients have substitution mutations within the HIT domain (L938: P206L/P206L and L939: P206L/V263G). FD105 is a primary AOA1 patient fibroblast cell line. FD105 M21 is an immortalized AOA1 patient fibroblast line, derived from the FD105 primary cell line, corrected with full-length APTX cDNA under a constitutive expression promoter. FD105 M20 is an uncorrected vector only control immortalized AOA1 cell line, derived from FD105. FD105 M20 and M21 were kind gifts from Keith Caldecott. PARP-1 \(^{+/+}\) and PARP-1 \(^{−/−}\) MEF cell lines were derived from wild-type and PARP-1 knockout mice by Valérie Schreibers’ laboratory. Nuclear extracts were generated as published by Audebert et al. (29), described briefly below. Lymphoblastoid cells (C2ABR, C3ABR, L938 and L939) were grown in RPMI 1640 (GIBCO BRL) containing 6% FCS (Lonza), 2 mM l-glutamine (Life Technologies) and 100 units of penicillin/streptomycin (GIBCO BRL). Adherent cells were grown in DMEM (GIBCO BRL) with 12% FCS (Lonza) and 100 units of penicillin/streptomycin (GIBCO BRL). All cells were cultured at 37°C in a humidified atmosphere supplemented with 5% CO\(_2\). The PARP inhibitors PJ-34 and 3-aminobenzamide were purchased from Sigma.

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examined using 5’ P-32 labelled Oligo A annealed to 3’ unmodified, phosphate, abasic or 8-oxo-dG-modified Oligo B and the Common Strand. This generated a nick with 5’ P-32 phosphate and 3’ unmodified, phosphate, abasic or 8-oxo-dG termini. These substrates were incubated with recombinant T4 DNA ligase [2 pmol of substrate with 1 unit of enzyme in Ligase Buffer (50 mM Tris pH 7.5, 25 mM MgCl₂, 0.5 mM DTT, 1 mM ATP)] at 22°C for the indicated times before denaturation in sequencing gel loading buffer (50 mM EDTA pH 8.5 with bromophenol blue in formamide) and analysis by denaturing PAGE (20% 19 : 1 acrylamide, 42% urea, 1 x TBE). Ligation of an SS by nuclear extract was assessed using the same set of oligonucleotides, with the radiolabel on the 5’ terminus of Oligo B. Ten micrograms of control or AOA1 nuclear extract was incubated with 2 pmol of nicked DNA with either an unmodified or 8-oxo-dG 3’ terminus in Ligase Buffer (total volume 10 µl) at room temperature for the indicated times. Reactions were denatured and analysed by denaturing polyacrylamide gel.

Generation of adenylated DNA

Adenylated DNA was generated similarly to as previously reported (19). The oligonucleotide ‘5’ 18-mer’ (ATTCCTAT AGTGACTACA) was 5’ radiolabelled using T4 kinase. This was then annealed with equal amounts of ‘3’ 18-mer’ (CAT ATCCGTGTCGCCCT-dideoxyC) and the Common Strand. Adenylation was achieved using T4 DNA ligase (50 units of ligase to 200 pmol of substrate in Ligase Buffer at 22°C for 24 h). The abortive ligation was terminated by boiling and the three strands were re-annealed to generate a 5’ adenylated 3’ dideoxy SSB. This structure is referred to as Duplex A. For experiments examining the effect of a 3’ modification on de-adenylation, Oligo A was 5’ radiolabelled and annealed to the Common Strand. This structure was adenylated as described earlier, heat denatured and subsequently annealed with Oligo B (3’ unmodified or 3’8-oxo-dG) to generate a 5’ adenylated structures with different 3’ termini.

DNA-adenylate hydrolysis assays

Unless stated otherwise, 10 µl reactions were performed in Ligase Buffer at room temperature using 2 pmol of substrate. Experiments using MEFs nuclear extract also contained 5 mM EDTA to reduce non-specific nuclease activity. Where relevant, inhibition of the adenylate hydrolase activity of nuclear extract was achieved by pre-incubation of extracts with the affinity purified rabbit polyclonal anti-prataxin antibody. Four micrograms of nuclear extract were incubated with 200 ng of purified anti-prataxin antibody or pre-immune serum at room temperature for 30 min in a total volume of 18 µl in Ligase Buffer. After antibody binding, reactions were initiated by addition of 2 pmol of Duplex A (to yield a final volume of 20 µl). Reactions were terminated as previously described after the indicated times.

Gene expression of PARP-1, APE1 and OGG1

Total RNA was extracted with TriReagent (Sigma) and DNase treated with RQ1 RNase-free DNase (Promega) according to the manufacturer’s instructions. First strand cDNA was synthesized from 2 to 3 µg of DNase treated RNA using 250 ng of random primers (ABgene) and 1 mM dNTP mix in a final reaction volume of 13 µl made up with DEPC treated water. The reaction mixture was then heated for 65°C for 5 min, chilled on ice for 2 min, then 4 µl of 5 x First-Strand Buffer (Invitrogen), 1 µl of 0.1 M DTT (Invitrogen), 1 µl of RNaseOUT™ recombinant RNase inhibitor (GibcoBRL) and 400 U of Superscript III RNase H⁻ Reverse Transcriptase (Invitrogen) were added. The contents were mixed and incubated at 25°C for 5 min. The samples were then incubated at 55°C for 60 min and then heat inactivated at 70°C for 15 min. Prior to PCR amplification, RNA complementary to the cDNA was degraded by adding 1 µl of the cDNA (2 units) of Escherichia coli RNase H (New England Biolabs) and incubated at 37°C for 20 min. The cDNA samples were finally ethanol precipitated and stored at −20°C until further use. The PCR reactions for PARP-1, APE1, OGG1 and β-2M were made up to final volume of 25 µl and contained: 200 ng of cDNA template; 1 unit of AmpliTaq Gold DNA Polymerase (Applied Biosystems); 20 mM Tris–HCl, pH 8.8; 2 mM MgSO₄; 10 mM KCl; 10 mM (NH₄)₂SO₄; 0.1% triton X-100; 0.1 mg/ml BSA; 200 µM dNTPs (Fisher Biotech) and 20 µM of each forward and reverse primer. The PCR mixtures were then thermocycled on a GeneAmp™ 2700 PCR machine (Perkin-Elmer) using a touch-down protocol from 64 to 58°C: initial denaturation at 95°C for 2 min, 95°C for 30 s, 64°C for 30 s, 72°C for 1 min (2 cycles); 95°C for 30 s, 62°C for 30 s, 72°C for 1 min (2 cycles); 95°C for 30 s, 60°C for 30 s, 72°C for 1 min (2 cycles); and 95°C for 30 s, 58°C for 30 s, 72°C for 1 min for 35 cycles. For all PCR, an appropriate negative control of no template was used. The resulting PCR products were subsequently run on a 2% TAE agarose gel and visualized under a UV lamp after staining with ethidium bromide at a final concentration of 100 ng/ml. The list of primers used for PCR amplification is provided in Supplementary Material, Table S1.

Apratxin-GST and PARP-1 GST pull-down assays, antibodies and immunoblotting

Apratxin-GST fusion proteins have been described previously (9,56). Pull-down assays were performed as previously described (56). Proteins were separated on 10% SDS–PAGE, transferred onto nitrocellulose membranes (Hybond C, Amer-sham) and detected using the relevant antibody. Anti-apratxin polyclonal sera have been described previously (9,56). The affinity purified apratxin antibody used here was generated using serum from a rabbit immunized with the full-length apratxin protein. A cross-linked apratxin (amino acid 1–319)-GST fusion protein glutathione resin was generated according to the method used by Bar-Peled et al. (57). Serum was pre-cleared of GST reactive antibody using GST-cross-linked glutathione resin. Anti-GST cleared serum was bound to the affinity column, washed and eluted as described (57). Confirmation of the specificity of the eluted antibody is shown in Supplementary Material, Figure S3. For immunoblotting, antibodies were used at dilutions of: apratxin (1/2000), XRCC1 (1/4000, cat# AHP428, Serotec), PARP-1 (1/1000, cat# MCA1522T,
Serotec), anti-GST (1/2000), β-tubulin (1/2000, cat# 2-28-33, Sigma), PAR (1/1000, cat# 10H, Becton Dickinson), APE1 (1/2000), UBF (1/1000, cat# sc-13125, Santa Cruz), AIF (1/1000, cat# 4642, Cell Signalling Technologies) followed by the relevant species-specific horseradish peroxidase conjugated secondary antibody from Chemicon or Sigma (1/10 000).

Recruitment of GFP-aprataxin to DNA damage after heavy ion irradiation and immunofluorescence

Cells were transfected with Amaza Nucleofection technology (Amaza). Transfection of MEFs was performed with Amaza Nucleofector Kit VDP-1004 and protocol A-023. All transfections were done according to the manufacturers’ instructions (Amaza). For heavy ion irradiation experiments, cells were seeded on sterilized glass coverslips and irradiated at the UNILAC beamline at GSI (Darmstadt, Germany). Directly before the irradiation, the coverslips were mounted into sample holders and placed in a medium filled container. For the ion irradiation, the sample holders were automatically taken from the medium tank, exposed to the ion beam at an angle of 15° between the axis of the ion beam and the plane of the cell monolayer and placed back again. For the applied fluence of $3 \times 10^8$ P/cm², the whole procedure takes <30 s. Briefly, wild-type (PARP-1+/−) and PARP-1−/− MEFs expressing GFP-XRCC1 or GFP-aprataxin were grown to confluence in a 75 cm² flask and treated with 0.3 µg/ml RNase in 150 mM NaCl with 15 mM sodium citrate for 1 h at 37°C. After RNA digestion, coverslips were sequentially washed in PBS, 35% ethanol, 50% ethanol and 75% ethanol for 2 min each. DNA was denatured by incubating the coverslips with 0.15 N NaOH in 70% ethanol for 4 min. A series of washes were performed starting with 70% ethanol containing 4% v/v formamide, then 50% ethanol, 35% ethanol and finally PBS for 2 min each. Proteins were digested with 5 µg/ml proteinase K in TE pH 7.5 for 10 min at 37°C. After several PBS washes, coverslips were incubated with an anti-8-o xo-dG antibody ( Trevigen, cat # 4355-MC-100) in PBT20 (1 x PBS/1% BSA/0.1% Tween 20) for 1 h at room temperature. Following several washes with 0.1 x PBS, 8-oxoG was detected using an AlexaFluor488 secondary antibody (Invitrogen, 1/500 in PBT20). Nuclei were counterstained with DAPI and slides were mounted for immunofluorescence. Images were captured using a digital camera (Carl Zeiss, Axiocam MRm) attached to a fluorescence microscope (Carl Zeiss Axioskop2 mot plus (Carl Zeiss) using Plan Apochromat 1.4 oil DIC (63 x magnification). Zeiss software (Axiovision LE 4.3) was used to capture the individual images that were assembled using Adobe Photoshop 7.0. Fluorescence intensity was quantitated on the RAW images using the public domain software Image J version 1.34s (NIH, USA).

OGG1 activity assays

An 8-oxo-dG containing 39mer (ATATACCGCGGCAGGA [8oxo]GCAAGCTTATTGGGTACCGA) was 5′ P-32 labelled and annealed to its complimentary strand to generate an 8-oxo-dG containing duplex. Recombinant OGG1 was obtained from NEB. One unit of OGG1 hydrolyses the N-glycosyl bond of 1 pmol of 8-oxo-dG containing duplex per hour at 37°C. To examine the activity of OGG1, the 2 ng 8-oxo-dG duplex was incubated with OGG1 for the indicated periods and quantities, in a 20 µl final volume of 1 x NEBuffer 4 supplemented with 0.1 µg/µl BSA. Where applicable, full length or C-terminally truncated (amino acids 1–319) recombinant aprataxin (4 pmol) were also added prior to reaction initiation by substrate addition. Reactions were terminated by addition of formamide-EDTA denaturing loading buffer and analysed by denaturing PAGE and autoradiography.

Phosphatase assays

Radiolabelled nicked 3′ phosphate SSBs were generated by 5′P-32 labelling Oligo B with a phosphate 3′ end and annealing it to Oligo A and the Common Strand. To examine the 3′ phosphatase activity of recombinant aprataxin, the indicated quantities of full-length aprataxin were incubated with 1 pmol of 3′ phosphorylated duplex in Ligase Buffer (total volume 10 µl) at room temperature for 75 min. As a positive control for aprataxin activity, aprataxin was incubated in parallel with 1 pmol of Duplex A. The phosphatase activity of nuclear extracts was examined in a similar manner. Extracts were incubated in the indicated quantities with 1 pmol of 3′ phosphorylated substrate in 10 µl reactions for 10 min at 22°C before reaction termination.

8-Oxo-dG immunostaining

For 8-oxo-dG detection, Normal (NFF), AOA1 (FD105, FD105 M20) and corrected AOA1 (FD105 M21) fibroblasts were grown on coverslips for 48 h and processed for immunofluorescence as described in (58). Briefly, cells on coverslips were fixed with 100% pre-chilled methanol for 5 min and immersed in 100% pre-chilled acetone for 5 min. Coverslips were subsequently air dried, treated with 0.05 N HCl for 5 min on ice and washed three times with PBS. RNA was digested by incubating the coverslips in 100 µg/ml RNase in 150 mM NaCl with 15 mM sodium citrate for 1 h at 37°C. After RNA digestion, coverslips were sequentially washed in PBS, 35% ethanol, 50% ethanol and 75% ethanol for 2 min each. DNA was denatured by incubating the coverslips with 0.15 N NaOH in 70% ethanol for 4 min. A series of washes were performed starting with 70% ethanol containing 4% v/v formaldehyde, then 50% ethanol, 35% ethanol and finally PBS for 2 min each. Proteins were digested with 5 µg/ml proteinase K in TE pH 7.5 for 10 min at 37°C. Following several washes with 0.1 x PBS, 8-oxoG was detected using an AlexaFluor488 secondary antibody (Invitrogen, 1/500 in PBT20). Nuclei were counterstained with DAPI and slides were mounted for immunofluorescence. Images were captured using a digital camera (Carl Zeiss, Axiocam MRm) attached to a fluorescence microscope (Carl Zeiss Axioskop2 mot plus (Carl Zeiss) using Plan Apochromat 1.4 oil DIC (63 x magnification). Zeiss software (Axiovision LE 4.3) was used to capture the individual images that were assembled using Adobe Photoshop 7.0. Fluorescence intensity was quantitated on the RAW images using the public domain software Image J version 1.34s (NIH, USA).
Long patch extension repair

5’ radiolabelled Oligo B (unmodified) was annealed with Common Strand and ‘Oligo C’ (5’ TTCCGATAGTGAACA) to generate a duplex containing a central single-stranded region of six nucleotides. Nuclear extracts were incubated with the patch repair substrate (30 μg of protein, 1 pmol of substrate) in Ligase Buffer without ATP but supplemented with dTNPs (10 μM each) at 22°C for the indicated times. Recombinant aprataxin was added as indicated. Reactions were terminated and resolved as previously. Gap filling is observed as several single nucleotide increases in molecular weight of the substrate. Reactions are quantified by integration of the intensity of each band in the 20 min lanes using Image-Quant 5.1. The sum of these values provided the total intensity of each lane. The relative abundance of each band was determined by division of its intensity by the total lane intensity. To quantify the repair defect in AOA1 cells, the relative abundance of the full extended product (Oligo B–6nt) was determined for each of the four cell lines used in four independent replicates. These relative abundances for the control (C2ABR and C3ABR) and AOA1 (L938 and L939) reactions were pooled. In each instance the control data was designated ‘100%’ and the AOA1 pooled relative abundance was expressed as a proportion of this. This data was subjected to Student's t-test (n = 4, assuming unequal variances).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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REFERENCES


