Aprataxin, a novel protein that protects against genotoxic stress

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Ataxia-oculomotor apraxia (AOA1) is a neurological disorder with symptoms that overlap those of ataxia-telangiectasia, a syndrome characterized by abnormal responses to double-strand DNA breaks and genome instability. The gene mutated in AOA1, APTX, is predicted to code for a protein called aprataxin that contains domains of homology with proteins involved in DNA damage signalling and repair. We demonstrate that aprataxin is a nuclear protein, present in both the nucleoplasm and the nucleolus. Mutations in the APTX gene destabilize the aprataxin protein, and fusion constructs of enhanced green fluorescent protein and aprataxin, representing deletions of putative functional domains, generate highly unstable products. Cells from AOA1 patients are characterized by enhanced sensitivity to agents that cause single-strand breaks in DNA but there is no evidence for a gross defect in single-strand break repair. Sensitivity to hydrogen peroxide and the resulting genome instability are corrected by transfection with full-length aprataxin cDNA. We also demonstrate that aprataxin interacts with the repair proteins XRCC1, PARP-1 and p53 and that it co-localizes with XRCC1 along charged particle tracks on chromatin. These results demonstrate that aprataxin influences the cellular response to genotoxic stress very likely by its capacity to interact with a number of proteins involved in DNA repair.

INTRODUCTION

Ataxia-oculomotor apraxia (AOA) was first recognized in 1988 as a distinct human syndrome, characterized by early-onset cerebellar ataxia, oculomotor apraxia, early areflexia and late peripheral neuropathy (1). Ataxia, oculomotor apraxia and cerebral atrophy are major clinical features of AOA patients that are also observed in patients with ataxia-telangiectasia (A-T). A-T is caused by mutations in the ataxia-telangiectasia-mutated (ATM) gene (2,3). The most debilitating feature of this syndrome is the progressive neurodegeneration associated with loss of Purkinje cells and ectopic location of these cells in the molecular layer (4). The loss of Purkinje cells has also been associated with AOA (5). Although AOA shows strong similarities to A-T, extra-neurological features that are usually associated with A-T, such as telangiectasia, immunodeficiency and susceptibility to malignancies, have not been reported for AOA. One of the hallmarks of cells from A-T patients is their extreme sensitivity to ionizing radiation (IR) (6–8), the exact molecular basis of which remains unexplained but arises as a consequence of the failure of mutated ATM protein to recognize and signal breaks in DNA (9). ATM has multiple signalling functions to target DNA repair complexes to the sites of damage as well as to activate cell cycle checkpoints in response to genotoxic stress (9). Double-strand breaks in DNA activate ATM by autophosphorylation, which enables it to phosphorylate multiple substrates (10,11). ATM is intimately linked to the Mre11/Rad50/Nbs1 complex that is responsible for recognition and processing of double-strand breaks in DNA (12,13). Mutations in two members of this complex, Mre11 and

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Nbs1, also give rise to AT-like syndromes, A-TLD and Nijmegen breakage syndrome, respectively (14,15).

Two recent reports have linked one form of AOA (AOA1) to mutations in the APTX gene which is located on 9p13 (16,17). AOA1 is an autosomal recessive ataxia associated with hypoalbuminemia and hypercholesterolemia (18). This disease has been described primarily in Portuguese and Japanese families but is also found in other countries throughout the world (19). The APTX gene is proposed to code for a 342 amino acid protein named aprataxin. Sequence similarities link aprataxin to the family of histidine triad (HIT) domain proteins. Truncating and missense mutations have been described in APTX and are largely confined to the HIT domain (16,17). Members of the family of HIT proteins can be divided into two main groups: the Hint (histidine triad nucleotide binding)–related proteins, found in all forms of life, and the Fhit (fragile histidine triad)–related proteins, found in animals and fungi. Hint proteins bind nucleotides and display adenosine S'-monophosphoramidase activity (20), whereas the Fhit proteins possess di-nucleoside-polyphosphate-hydrolase activity (21).

In addition, the Fhit protein can act as a tumour suppressor (22). However, the exact mechanism involved remains unclear. Despite the similarity of aprataxin to the subgroup of Hint proteins, nothing is known about its cellular function or its role in preventing neurodegeneration. In addition to its central HIT domain, based on sequence similarities, the aprataxin protein also contains a putative forhead associated (FAH) domain at its N-terminus (23) and a predicted C-terminal zinc-finger (ZF) domain (16,17). Given the phenotypic similarity between A-T and AOA1, it was of interest to determine whether AOA1 cells, with specific aprataxin mutations, would show increased sensitivity to genotoxic agents similar to that observed in A-T cells. In contrast to A-T cells, AOA1 cells were not hypersensitive to IR but, instead, displayed increased sensitivity to genotoxic agents that induce single-strand breaks in DNA. This sensitivity was corrected by reintroducing a wild-type aprataxin expression construct into cells from AOA1 patients. Consistent with this sensitivity profile we provide evidence that aprataxin is a nuclear protein that is also a component of the DNA single-strand break repair complex with PARP-1, XRCC1 and p53.

RESULTS

Sensitivity of AOA1 cells to genotoxic agents

Because of the overlap of the AOA1 and A-T phenotypes we determined whether AOA1 cells also show hypersensitivity to DNA-damaging agents. The results in Figure 1A demonstrate that three AOA1 cell lines showed no increased sensitivity to IR compared to control cells, whereas an A-T cell line was hypersensitive. In contrast, five AOA1 cell lines showed significantly greater sensitivity to hydrogen peroxide (H$_2$O$_2$) than either a control or an A-T cell line (Fig. 1A). AOA1 cells were also more sensitive to another agent that causes single-strand breaks in DNA, methyl methane sulfonate (MMS). However, there was no increased sensitivity for these cells after exposure to mitomycin C (MMC). As an additional criterion for agent sensitivity, we assayed for induced chromosome aberrations (ICA). Exposure of control cells to H$_2$O$_2$ resulted in an average of two ICAs per metaphase, while the values for three AOA1 cell lines were 4.32, 4.16 and 5.6 (Fig. 1B). These results suggest that aprataxin is involved in the response to or repair of DNA single-strand breaks.

Expression of aprataxin in AOA1 cells

Previous reports have revealed that there are two major mRNA species that are potentially capable of encoding short and long forms of aprataxin (16,17). To study the expression at the protein level and determine the status of aprataxin in AOA1 cells we generated antibodies against different regions of the predicted protein (Fig. 1C). Polyclonal antibodies were prepared in sheep using glutathione S-transferase (GST)–fusion proteins corresponding to the FHA, HIT and ZF homology domains. As is evident from Figure 1D (lane 1), a single band of ~45 kDa was detected in normal control cells (C3ABR) after immunoblotting with the anti-ZF antibody F4. This size is in general agreement with that derived from the cDNA nucleotide sequence which predicts a molecular weight of ~40 kDa. In addition, it migrates at approximately the same position as a human recombinant form of the protein (Fig. 1D, lane 1). Under these conditions no aprataxin was detected in three AOA1 cell lines (lanes 3–5): L136 (689insT/840delT), L938 (P206L/P206L) and L939 (P206L/V263G). Aprataxin was also not detected in two other AOA1 cell lines: L990 and L991 (data not shown). However, it was possible to detect low amounts of aprataxin in AOA1 cells carrying missense mutations in the gene when the protein was first immunoprecipitated with anti-aprataxin antibody (data not shown). Normal levels of aprataxin were detected in an A-T cell line (AT25ABR). It is clear from these data that missense mutations in the APTX gene are sufficient to destabilize the aprataxin protein (Fig. 1D). Since loss of aprataxin gave rise to increased sensitivity to agents that cause single-strand breaks in DNA, we determined whether this would alter the expression of other proteins involved in single-strand break repair. The results in Figure 1D show that two such proteins, XRCC1 and PARP-1, are present at normal levels in AOA1 cells.

Subcellular localization of aprataxin

In order to determine the cellular localization of aprataxin, we employed immunostaining, enhanced green fluorescent protein (EGFP)-tagging and subcellular fractionation. Immunofluorescent antibody staining (F4 antibody) of normal human foreskin fibroblasts (NFF) revealed that aprataxin is a nuclear protein, distributed both uniformly throughout the nucleus and localized to subnuclear structures of varying size (Fig. 2A, left panel). On the contrary, in fibroblasts derived from an AOA1 patient (FG4003) with two truncating mutations in APTX (689insT/840delT), only very low levels of background staining were detected (Fig. 2A, right panel). To confirm this distribution we transfected NFF cells with an EGFP–aprataxin construct and monitored the transient expression of the fusion protein (Fig. 2B, left panel). The data confirm that aprataxin is distributed throughout the nucleus and to subnuclear structures as observed with antibody staining in NFF cells. When the EGFP–aprataxin construct was transfected into HeLa cells a similar pattern of distribution was...
observed (Fig. 2B, right panel). Since the size and number of the larger subnuclear structures were reminiscent of nucleoli, we looked for co-localization of aprataxin with the nucleolus-specific marker protein, nucleolin, in stably transfected HeLa cells using anti-nucleolin antibody staining (Fig. 2C). When this fluorescence pattern was merged with the EGFP–aprataxin signal, co-localization of these proteins was observed. In addition to the uniform nuclear and nucleolar distribution, small intense regions of EGFP–aprataxin were also present in the nucleus (Fig. 2C, arrows). These were not localized to nucleoli since they did not co-localize with nucleolin. To confirm the observed nuclear and nucleolar localization, cells were subfractionated and immunoblotted with anti-aprataxin antibody. As expected, aprataxin was found both in the nucleoplasm and in nucleoli (Fig. 2D). Detection of nucleolin confirmed the integrity of the fractions.

Localization and expression of mutant forms of aprataxin

Since we demonstrated that missense mutations destabilize aprataxin, it was of interest to examine whether deletions of any of the putative domains in the molecule would also destabilize the protein and/or subcellular distribution. A series of EGFP–aprataxin constructs were generated (Fig. 3). As predicted from data with AOA1 cell lines (Fig. 1D), the V263G mutation in the HIT domain destabilized the protein (Fig. 3B). For this construct, it was only possible to detect residual perinuclear/cytoplasmic EGFP-fluorescence using a 10-fold longer exposure time and digital enhancement, compared to wild-type fluorescence, which was localized to the nucleus (Fig. 3A). A series of other constructs deleting the FHA, NL, HIT and ZF domains led to a similar reduction in the amount of protein as well as abnormal distribution (Fig. 3C–G).

Localization of aprataxin in response to genotoxic agents

A typical feature of proteins involved in DNA repair is their re-localization to sites of damage (24). AOA1 cells are hypersensitive to H$_2$O$_2$ and since this agent gives rise to distinct XRCC1 foci (25) we looked for possible co-localization between a portion of the aprataxin and XRCC1. The results in Figure 4A,

Figure 1. Sensitivity to genotoxic agents and detection of aprataxin in AOA1 cells. (A) Cell survival of control (C3ABR), A-T (AT1ABR) and AOA1 (L136, L938, L939, L990, L991) cell lines to genotoxic agents (ionizing radiation; IR; methyl methane sulfonate; MMS; mitomycin C; MMC; hydrogen peroxide; H$_2$O$_2$). (B) Chromosome aberrations in response to H$_2$O$_2$. After treatment with 2 mM H$_2$O$_2$, 50 metaphases (10 for L136) were scored for each cell line for chromosome aberrations (sb, chromatid breaks; cb, chromosome breaks; Int, interchanges; ICA, induced chromosome aberrations per metaphase). (C) Schematic representation of the predicted functional domains of aprataxin (forkhead associated homology domain, FHA; nuclear localization signal, NL; histidine triad domain, HIT; zinc-finger motif, ZF) and the four aprataxin–GST constructs generated. Three of those (GST–FHA, GST–HIT, GST–ZF) were used to generate polyclonal antibodies (F1, F3 and F4). (D) Aprataxin, XRCC1 and PARP-1 expression in control (C3ABR), A-T (AT25ABR) and AOA1 (L136, L938, L939) cell lines. Recombinant aprataxin (F4) expressed in S. cerevisiae was used as a positive control. Expression of PCNA was used as a loading control.
upper panel demonstrate the presence of multiple H2O2-induced XRCC1 foci in HeLa cells. There was no evidence of such foci for EGFP–aprataxin, but it is clear that both proteins are also uniformly distributed throughout the nucleus under these conditions. Exposure of cells to H2O2 failed to induce any PARP-1 foci (Fig. 4A, lower panel). We also showed that other genotoxic agents, IR, MMS and MMC, failed to alter the
uniform nuclear distribution of aprataxin or the extent of nucleolar fluorescence (Fig. 4B).

**Involvement of aprataxin in XRCC1 foci formation**

Since AOA1 cells show increased sensitivity to H₂O₂ and since treatment with this agent normally leads to formation of XRCC1 foci, we determined whether the loss of aprataxin in AOA1 cells would compromise the induction of XRCC1 foci. No foci were observed in untreated control fibroblasts, but at 10 min after exposure to H₂O₂, multiple, intensely labelled nuclear XRCC1 foci were observed in ~80% of those cells (Fig. 5). These foci decreased in number with time and by 60 min had formed larger aggregates. In contrast, multiple XRCC1 foci of more irregular shape and lower intensity were constitutively present in untreated AOA1 fibroblasts (Fig. 5), and this pattern of labelling was largely unchanged post-treatment. None of the small intensely stained foci was evident.

**Figure 4.** Localization of aprataxin in response to genotoxic agents. (A) HeLa cells expressing EGFP–aprataxin (green) were treated with H₂O₂ for 30 min before fixation and immunostaining for XRCC1 (red, upper panels) and PARP-1 (red, lower panels). (B) HeLa cells expressing EGFP–aprataxin were untreated (−) or treated (+) with 10 Gy of IR, 1 μg/ml MMS and MMC and 10 mM H₂O₂. Cells were analyzed 30 min after treatment.
Complementation of H$_2$O$_2$ sensitivity in AOA1 cell lines

To establish the importance of aprataxin in the cellular resistance to genotoxic agents, we transfected AOA1 cells (L938, L939) with full-length aprataxin. Transient transfections were carried out with the EGFP–aprataxin expression vector (A) and the empty vector as a control (V). Exposure of L938 AOA1 cells, transfected with EGFP–aprataxin, to H$_2$O$_2$ revealed a pattern of survival similar to that observed in control cells (Fig. 6A). Correction of H$_2$O$_2$ sensitivity was also observed in a second AOA1 cell line (L939) transfected with EGFP–aprataxin. AOA1 cells transfected with empty vector remained hypersensitive to H$_2$O$_2$. Complementation of H$_2$O$_2$ sensitivity in AOA1 cells by full-length aprataxin was also determined by correction of induced chromosome aberrations. The results in Figure 6B show that AOA1 cells transfected with full-length EGFP–aprataxin (L939/A) display similar numbers of chromosome aberrations when compared with H$_2$O$_2$-treated control cells (C1ABR). However, transfection of L939 AOA1 cells with an expression construct that encodes EGFP–aprataxin containing a point mutation within the HIT domain (V263G), previously described in an AOA1 patient (16), failed to normalize the number of H$_2$O$_2$-induced chromosome aberrations. Introduction of the EGFP–aprataxin construct into control cells did not alter the number of induced aberrations (data not shown).

Interaction of aprataxin with DNA repair proteins and mapping of interaction domains

While it was not possible to localize aprataxin to subnuclear structures with XRCC1 and PARP-1 it was still possible that these proteins interacted, given their uniform nuclear distribution and the sensitivity of AOA1 cell lines to agents that cause single-strand breaks in DNA. In addition, Caldecott (23) has reported an interaction between aprataxin and XRCC1 using the yeast two-hybrid system. Constitutive association was evident between aprataxin and XRCC1 when control extracts were immunoprecipitated with an anti-aprataxin antibody and subsequently immunoblotted with anti-XRCC1 (Fig. 7A, left panel). Exposure of cells to 10 mM H$_2$O$_2$ did not alter this association (Fig. 7B). This interaction was also seen in A-T (AT3ABR) cells. Co-immunoprecipitation also revealed that aprataxin was bound to both p53 and PARP-1 (Fig. 7A). Reverse co-immunoprecipitation using anti-XRCC1 and PARP-1 antibodies confirmed the association with aprataxin (Fig. 7A), and we also detected p53 in these co-immunoprecipitates. The H$_2$O$_2$-induced increase in p53 bound to aprataxin (Fig. 7B) is due to DNA-damage-induced p53 stabilization. As observed previously XRCC1 immunoprecipitates also contained PARP-1.
apartaxin. The results in Figure 7C show that nucleolin bound most strongly to the FHA domain.

**Repair of breaks in DNA**

We demonstrated that AOA1 cells are sensitive to agents that cause single-strand breaks in DNA and since we have also shown that apartaxin interacts with the repair protein XRCC1, it was possible that the increased sensitivity in these cells was due to a defect in single-strand break repair. We tested this possibility using exposure to H2O2, which gives rise primarily to single-strand breaks in DNA at concentrations >10 mM (28). Single-strand break repair was measured using analysis of intracellular NAD(P)H levels with a water soluble tetrazolium salt method, which represents a reliable method to monitor imbalance of break repair in base excision repair deficient cells (29). When cells were exposed to H2O2 a time-dependent depletion of NAD(P)H was observed and this occurred to approximately the same extent in control and AOA1 (L939) cells, indicating that there was no gross imbalance of single-strand break repair in the AOA1 cells (Fig. 8).

**Dynamics of apartaxin binding to chromatin**

Biological imaging of charged particle tracks represents a new approach to investigating the association of repair proteins with chromatin (30,31). In this method a monolayer of cells is exposed to heavy ions where individual particle traversals can be visualized in single cells when a small angle (<5°) is utilized between the beam direction and the monolayer (30). Under these conditions the track structure leads to clustering of damage which includes single- and double-strand breaks and base damage (32). This approach has the potential to visualize events associated with single-strand break repair. Based on data described above, our prediction was that apartaxin would co-localize with XRCC1 at the break sites. Exposure of a monolayer of HeLa cells expressing EGFP–apartaxin to uranium ions gave rise to EGFP–apartaxin aggregates distributed along the particle trajectory as discrete tracks (Fig. 9). It seemed likely that these aggregates localized to sites of DNA damage. In order to confirm this, we investigated whether XRCC1 was distributed in a similar fashion using immunostaining. Again, in this case, discrete tracks of XRCC1 aggregates were distributed across the nucleus. Merging the fluorescent images demonstrated that apartaxin and XRCC1 showed complete co-localization across the same sites of DNA damage. We also demonstrated that apartaxin was tightly associated with chromatim in response to oxidative damage since these tracks were resistant to detergent extraction (data not shown).

**DISCUSSION**

The gene defective in the human genetic disorder AOA1 was predicted to code for a 342 amino acid protein, apartaxin (16,17). We have demonstrated here that apartaxin is predominantly expressed as a 45 kDa nuclear protein. Under these conditions it was not possible to detect a short form of the protein which is also predicted from the open reading frame
Immunoblotting failed to reveal the presence of the long form of aprataxin in extracts from five AOA1 cell lines but protein was detected in the cell lines carrying aprataxin missense mutations after immunoprecipitation. These data demonstrate that in the case of L938 (homozygous for P206L) and L939 (compound heterozygote for P206L/V263G) the presence of missense mutations destabilize the protein. This was further substantiated by the generation of an EGFP-tagged V263G aprataxin mutant that was expressed at almost undetectable levels in HeLa cells. The deletion of the various putative domains for the molecule also resulted in its destabilization. These results highlight the difficulty of expressing mutant forms of aprataxin for functional studies.

Use of immunofluorescence, EGFP-tagging, cellular fractionation and immunoblotting demonstrated that aprataxin was present both in the nucleoplasm and in the nucleolus. In the nucleoplasm, aprataxin was uniformly distributed with the exception of intense labelling in small discrete foci. These are not fragments of nucleoli since nucleolin did not localize in these foci (Fig. 2C). Nucleoli are multifunctional subnuclear organelles in which ribosome biogenesis and RNA processing take place (33). Other functions associated with these organelles include tumour suppression, cell cycle control, viral replication and DNA repair (33,34). Immunoblotting suggests that as much as 50% of the cellular aprataxin is localized to nucleoli, indicating a greater concentration per unit volume in this organelle than throughout the nucleoplasm as a whole. This is supported by antibody staining and EGFP fluorescence. Under conditions of asynchronous cell growth the relative amounts of nucleolar and nucleoplasmic aprataxin appear to be stable, and this distribution is not influenced by exposing cells to DNA damaging agents.

Early-onset ataxia with ocular motor apraxia is common to the autosomal recessive neurodegenerative disorders AOA1 and A-T (4,35). The gene product defective in A-T cells, ATM, recognizes double-strand breaks in DNA and signals this damage to cell cycle checkpoints (2,9). In the absence of ATM, a portion of induced double-strand breaks remain unrepair which may contribute to the hypersensitivity to radiation that is characteristic of A-T cells (2,8). While AOA1 cells are not sensitive to radiation they show increased sensitivity to MMS and H2O2, agents that cause single-strand breaks in DNA (36,37). The different effects of H2O2 and radiation on AOA1 cell survival can be explained by the observation that H2O2 only gives rise to single-strand DNA breaks at concentrations up to 10 mM while radiation gives a mixture of lesions at any dose (28). Furthermore, the exact nature of the lesion may vary between the two treatments. In addition it is evident that H2O2 causes not only DNA damage but also membrane and mitochondrial damage which add further complexity to the outcome (38). It is also clear that the radiosensitive A-T cells show a normal response to H2O2 as has been reported previously (39).

The sensitivity to agents that cause single-strand breaks in DNA is a direct result of the loss of functional aprataxin since transfection of AOA1 cells with full-length aprataxin cDNA reversed this hypersensitivity to H2O2 and also decreased the genome instability observed in these cells after treatment with the same agent. Further evidence for a role for aprataxin in some aspect of DNA strand break repair is provided by its interaction with two proteins, XRCC1 and PARP-1, both of which have been shown to play central roles in repair of breaks in DNA (40–42). Interaction with XRCC1 was demonstrated by co-immunoprecipitation and GST pull-down with an N-terminal region containing the putative FHA domain of aprataxin. This domain is a phosphopeptide-binding module found in a range of proteins involved in signal transduction, RNA binding and DNA repair (43). We also showed that PARP-1 binds to a region of aprataxin extending from the FHA to the HIT domains. Since PARP-1, XRCC1, DNA polymerase β and DNA ligase Iα all form part of a complex involved in base excision repair (26,41) it suggests that aprataxin may also have a role in this repair process. However, the data described here provide no evidence for a gross defect in the rate of rejoining of DNA single-strand breaks in AOA1 cells. Nevertheless, it is clear that aprataxin accumulates along the path of heavy ion trajectories in the nuclei of irradiated cells, and the pattern of clustering coincides exactly with that of XRCC1. It is not possible to define the exact lesion recognized but track structure causes damage clusters that include single- and double-strand breaks (32). While XRCC1 is involved primarily in single-strand break repair there is evidence for a defect in rejoining of radiation-induced DNA double-strand breaks in the hamster cell lines EM9 and EM-C11, mutated in XRCC1 (44). It appears that mutation in XRCC1 leads to DNA ligase III deficiency. In the present case, loss of aprataxin does not destabilize XRCC1 or PARP-1. In addition another partner protein of XRCC1, PARP-1, binds to both single- and double-strand breaks and when disrupted in a defective Ku80 background leads to lethality (23). Thus it is possible that the aprataxin/XRCC1 association on chromatin is at single- and/or double-strand breaks in DNA.

The agent sensitivity phenotype of AOA1 and XRCC1-deficient cells is similar but not identical. XRCC1-deficient cells show strongest sensitivity to EMS and MMS, moderate sensitivity to H2O2 and MMC and weak sensitivity to IR and UV (23,45). Here we demonstrated that AOA1 cells were...
sensitive to MMS and H$_2$O$_2$ but not to radiation or MMC. These observations might well explain the difference in single-strand break rejoining in the two mutant types. Failure to observe increased sensitivity to IR and MMC suggests that aprataxin is not involved in recognizing double-strand breaks in DNA or breaks arising due to failure to resolve blocked replication forks. The weak sensitivity of XRCC1 mutants to MMC could be due to some partial involvement of this protein in double-strand break repair for which there is some evidence (44). A more specialized role of aprataxin in break recognition or processing or in relation to emphasis at specific stages of the cell cycle might not be manifested as a gross defect in rate of repair.

A characteristic of proteins recognizing damage in DNA is that they form discrete foci at either short and/or long times after DNA damage (46,47). It seems likely that these foci represent aggregates of proteins deposited to facilitate DNA repair or assist in the signalling of the DNA damage to cell cycle checkpoints (24). Exposure of CHO cells to H$_2$O$_2$ causes the appearance of XRCC1 foci which peak at $\sim$30 min post-treatment (25). We have observed multiple small intensely labelled XRCC1 foci in 80% of control cells almost immediately after exposure to H$_2$O$_2$ and these appear to aggregate and form larger foci with time. In the case of AOA1 cells, less well-defined foci were constitutively present and showed little alteration after H$_2$O$_2$ treatment. Thus, in the absence of aprataxin there is an abnormal distribution of XRCC1 in the nucleus and the normal response to H$_2$O$_2$ is attenuated. Co-localization of aprataxin with XRCC1 at sites of DNA damage (tracks) after radiation exposure would appear to be in conflict with a failure to observe co-localization of aprataxin and XRCC1 in H$_2$O$_2$-induced foci. This might be explained by complex forms of DNA damage inflicted by high-LET (linear energy transfer) radiation. By necessity, high-LET radiation was used here to visualize track structure. In this context aprataxin and XRCC1 may recognize a specific form of damage only possible to detect with high-energy radiation. On the other hand, the oxidative damage caused by H$_2$O$_2$ would be expected to be recognized by XRCC1 and if only a subset of this damage requires the involvement of aprataxin, this protein would not be detected as co-localizing with XRCC1. This is supported by single-strand break repair data which appears to be grossly normal for AOA1 cells.

Based on the presence of several putative functional domains [FHA, polynucleotide kinase 3'-phosphatase (PNKP), HIT and ZF] in aprataxin, it may function in different aspects of single-strand break repair. As demonstrated, it binds to XRCC1 through its FHA domain which may tether it to the repair complex. This region of the molecule also shares homology with PNKP which again implicates aprataxin in single-strand break repair. The ZF domain might enable aprataxin to bind to a specific DNA structure (e.g. ends of the break) as part of the
recognition of lesions in DNA. Aprataxin is also included in the Hint family of proteins (48). It possesses a minimum Hint domain which may confer adenosine 5’-monophosphoramidase (AMP) activity. Hint proteins hydrolyze the AMP–NH₂ bond and AMP linked to a lysine side chain (49). It is conceivable that aprataxin, while not essential for the release of AMP from the ligase III–AMP complex during the process of sealing of breaks in DNA plays a role in enhancing the process. The appearance of p53 in co-immunoprecipitates with aprataxin, PARP-1 and XRCC1 is in keeping with a role for this molecule in DNA repair (50). A requirement for p53 in nuclease repair exiton repair has previously been demonstrated (51,52). Furthermore, there is also evidence for the direct involvement of p53 in base excision repair. Ofer et al. (53) and Zhou et al. (54) have shown that p53 stimulated base excision repair in a reconstituted assay, possibly by binding to the APE-1 endonuclease. Finally, co-localization of nucleolin and aprataxin to the nucleoli, together with data revealing binding between nucleolin and the FHA domain of aprataxin, provide additional evidence for a role for aprataxin in the stress response, since Yang et al. (27) have demonstrated that activation of the RNA-binding properties of nucleolin is part of the cellular response to genotoxic stress. It is also intriguing to note that activation of nucleolin is achieved through phosphorylation by SAPK p38 which is consistent with binding of nucleolin to the FHA domain of aprataxin.

The similarity in clinical phenotype between AOA1 and A-T suggests that the two gene products have at least some degree of mechanistic overlap. It is still not clear what is responsible for the neurodegenerative phenotype in A-T but failure to completely repair double-strand breaks in DNA, aberrant apoptosis and oxidative stress resulting from loss of ATM may all contribute (2,55,56). It is clear that ATM is not primarily a repair protein since the vast majority of DNA breaks are repaired in A-T cells after radiation exposure (57,58). ATM signals these breaks to the cell cycle machinery to ensure stability of the genome (2). Our data suggest that aprataxin is also not directly involved in the rate of single-strand break rejoining but its association with proteins involved in DNA repair suggests that it may have a recognition or more subtle processing role in the cellular response to single-strand breaks. The defect in response in AOA1 cells to DNA single-strand breaks partially addresses that issue since there is evidence for association between repair genes and neurodegeneration (59–61). However, since the clinical defect in AOA1 is confined largely to the nervous system, it requires the aprataxin response to DNA strand breaks is more critical to this tissue. Any accompanying oxidative stress, confined largely to the brain as observed with A-T, would help to explain the specificity of the effect in AOA1. Failure to observe increased cancer incidence in AOA1 patients might be explained by differences in cell cycle regulation compared with that in other syndromes with cerebellar ataxia (e.g. A-T) or might be explained by the nature of the repair defect, i.e. single-strand breaks in AOA1 versus double-strand breaks in A-T. The recent description of senataxin, mutated in patients with AOA2, adds further support for an involvement of a DNA repair defect in the neurodegenerative phenotype (61). Senataxin is homologous to the yeast sen1p proteins that possess RNA helicase activity, which could also implicate this protein in DNA repair.

**MATERIALS AND METHODS**

**Cell culture and treatment**

Lymphoblastoid cells lines (LCL) from control (C3ABR, C1ABR), A-T (AT1ABR, AT25ABR) and AOA1 (L136, L938, L939, L990, L991) patients were cultured in RPMI 1640 medium (GIBCO BRL) containing 10% fetal calf serum (FCS) (JRH Biosciences), 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin (GIBCO BRL) and 100 U/ml streptomycin (GIBCO BRL) and maintained in a humidified incubator at 37°C/5% CO₂. NFF, fibroblasts from an AOA1 patient (FG4003) and cervical adenocarcinoma cells (HeLa) were cultured under identical conditions. For all experiments LCL were used at a density of 1 x 10⁶ cells/ml or at 75% confluency for adherent cell lines. Genotoxic agents MMS (Sigma) and MMC (Boehringer) were added to the cell culture medium using concentrations and time periods as indicated. Cells were treated with H₂O₂ (Sigma) for 30 min for survival and 10 min for cell staining before culture medium was replaced with fresh medium. All irradiations were performed at room temperature using a ¹³⁷Cs source (Gammacell 40 Exactor, MDS Nordion, dose rate 1.1 Gy/min).

**Cell survival and chromosome aberrations in response to genotoxic agents**

Cell survival and chromosome aberrations were measured as described previously (62). For complementation of H₂O₂ sensitivity, a PCR product corresponding to full-length human aprataxin was cloned into the EGFP expression vector EGFP-C2 (Invitrogen). Control and AOA1 cell lines were transiently transfected with this construct and left for 24 h. Subsequently, cells were treated with H₂O₂ concentrations as indicated and the surviving fraction was measured after 48 h as described previously (63). For complementation of H₂O₂-induced chromosome aberrations, AOA1 cells were transiently transfected with full-length EGFP–apratxin. A previously described AOA1 mutant, V263G, (16) was introduced into full-length EGFP–apratxin expression vector as described previously (64). This construct was used as a negative control. EGFP–apratxin expression was monitored by fluorescence microscopy.

**Purification of recombinant human aprataxin**

Full-length human APTX was cloned into a modified pCYB2 (New England Biolabs) plasmid, pMH1, containing a self cleaving 3'chitin-binding domain and a galactose-inducible promoter for overexpression in yeast (65). Specifically, the SalI–SmaI fragment of the pMH1 construct was replaced with SalI–human APTX coding region (long form). This construct, pMHA, was transformed into Saccharomyces cerevisiae, BJ2168, and overexpression induced using 2% galactose. Human aprataxin was affinity purified using a chitin resin (New England Biolabs) according to Hall and Kunkel (65).
Anti-aprataxin antibodies

Polyclonal antibodies against different domains of human aprataxin were generated in sheep using methods described previously (11,66). Briefly, GST–aprataxin fusion proteins corresponding to amino acids 1–110 (F1), 175–272 (F3) and 266–342 (F4) were expressed in Escherichia coli and used for immunization. The corresponding sheep antisera were pre-cleared on GST columns, before antibodies were affinity purified using the appropriate GST–aprataxin columns. Antibodies were tested using recombinant full-length human aprataxin protein.

Aprataxin–GST fusion proteins and binding studies

Four overlapping aprataxin–GST fusion proteins (GST–FHA, aa 1–110; GST–NL, aa 98–176; GST–HIT, aa 175–272; GST–ZnF, aa 266–342) and GST alone were generated by cloning the corresponding PCR products into pGEX5X-1 (Amersham). Recombinant proteins were induced and purified as recommended by the manufacturer (Amersham). For GST pull-down studies the fusion proteins were cross-linked to glutathione–sepharose beads as previously described (66). Total cell extracts were prepared by lysing 3 × 10^9 control cells (C3ABR) in 100 ml of lysis buffer L [50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM ethylene diamine tetra-acetic acid (EDTA), 1% NP-40, 5 mM dithiothreitol supplemented with protease and phosphatase inhibitors]. Cleared lysate was adjusted to 1 mg/ml and loaded onto the various columns simultaneously (GST, GST–FHA, GST–NL, GST–HIT and GST–ZF). Binding was performed overnight at 4°C. Columns were subsequently washed with 10 column volumes of phosphate-buffered saline (PBS) and subsequently 10 volumes of buffer PBS/0.15 m NaCl. Proteins were eluted with five volumes of buffer E (0.1 M glycine pH 2.5, 2 m NaCl, 1 mM phenylethylthiourea fluorode) Eluted fractions were pooled, concentrated by centrifugal filtration and then separated by 10% SDS–PAGE. Proteins were transferred onto nitrocellulose membranes and detected using rabbit polyclonal anti-XRCC1 (HP428, Serotec, 1/5000), mouse monoclonal anti–PARP-1 (MCA1522T, Serotec, 1/1000), mouse monoclonal anti-p53 (DO-1, BD PharMingen, 1/1000) and mouse monoclonal anti-nucleolin antibodies (M0193, MBL, 1/1000) and the species-specific horseradish peroxidase conjugated secondary antibody from Chemicon (1/10 000).

Cellular fractionation, immunoprecipitation and western blotting

Cells were separated into cytoplasmic, nucleoplasmic and nucleolar fractions as previously described (67). For immunoprecipitations, cells were washed in PBS before resuspension in lysis buffer [50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, supplemented with protease and phosphatase inhibitors and Benzonase (Novagen)] for 1 h at 4°C. Insoluble components were removed by centrifugation (16 000 g for 10 min). For immunoprecipitation, 1 mg of total cell extract was incubated for 4 h at 4°C with 1 µg of antibody and 40 µl of protein G-Sepharose beads (Amersham). After washing the beads three times with lysis buffer and once with high-stringency buffer (50 mM Tris pH 7.5, 0.5 mM NaCl) they were resuspended in 20 µl of sample loading buffer before separation of the proteins by SDS–PAGE. Proteins were transferred to nitrocellulose and immunoblots were performed using the appropriate antibody described above.

EGFP–aprataxin and immunostaining

APTX cDNA corresponding to full length (aa 2–342) or parts of aprataxin (aa 25–342, aa 99–342, aa 173–342, aa 252–342, aa 2–300; Fig. 3) were cloned into pEGFP-C2 (Clontech) via the XhoI and BamHI sites. HeLa cells were transfected with these constructs by electroporation (200 V, 10 ms) and stable cell lines selected using G418 and FACS-enrichment. For immunostaining, cells were grown on coverslips for 48 h before treatment with genotoxic agents for time intervals as indicated. Subsequently, coverslips were washed with PBS/5 mM MgCl2, fixed in 4% PFA/PBS/5 mM MgCl2 at room temperature for 10 min and permeabilized in 0.1% Triton X-100/PBS/5 mM MgCl2 for 10 min. Non-specific binding was blocked by incubating the coverslips in 5% FCS/PBS/5 mM MgCl2 for 1 h at 37°C. Coverslips were covered with various antibody solutions (all 1:200 in 5% FCS/PBS/5 mM MgCl2) overnight at 4°C before detection with species-specific AlexaFluor488-conjugate (Molecular Probes). Images were captured using a digital camera (Zeiss Axiocam MRc) attached to a fluorescence microscope (Zeiss Axiovert).

Single-strand break repair assay

Single-strand break repair was measured using a recently described method relying on analysis of intracellular NAD(P)H levels after treatment with H2O2 (29). The reduction of NAD(P)H to a brown coloured formazan dye was monitored using a water-soluble tetrazolium salt (CellTiter 96AQueous, Promega). Briefly, 96-well plates were seeded with control C3ABR and AOA1 (L939) cells (1 × 10^5 cells/well) and cultured in 100 µl of medium containing 10% FCS and antibiotics as described above. Cells were untreated or treated with 10 mM H2O2 as described above. Cells were then washed and resuspended in medium containing 20 µl of CellTiter 96 AQueous solution [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) and 1-methoxy-5-methylphenazinium methylsulfate (PMS)]. The amount of formazan dye produced by the living cells in the medium was determined every 30 min by measuring absorbance at 490 nm in a 96-well plate spectrophotometer with 650 nm as a reference. A blank was prepared with only medium and CellTiter 96AQueous (MTS/PMS) solution. The depletion in NAD(P)H was assessed by comparing the absorbance of H2O2-treated cells with PBS-treated cells over time. Error bars represent the standard deviation of 12 parallel measurements of one typical experiment.

Generation of tracks of ion-induced DNA damage

HeLa cells were irradiated with low-energy ions entering the cell monolayer at a small angle (<5°) as described previously (30). The uranium ions used (3.5 MeV/u, 1 × 10^7 P cm⁻² corresponding to an average of four traversals per nucleus) were
generated at the UNILAC facility at the Gesellschaft für Schwerionenforschung (Darmstadt, Germany). Cells were grown for 72 h in 35 mm Petri dishes. Prior to irradiation the outer rim of each dish was removed and cells were irradiated for 2 min. After a subsequent incubation for 3 min in conditioned medium at 37°C the cells were fixed and immunostained as described above. Imaging was performed using confocal laser microscopy (LEICA TCS) as described previously (30).

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REFERENCES


