Localization of a Portion of Extranuclear ATM to Peroxisomes*

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The gene mutated in the human genetic disorder ataxia-telangiectasia codes for a protein, ATM, the known functions of which include response to DNA damage, cell cycle control, and meiotic recombination. Consistent with these functions, ATM is predominantly present in the nucleus of proliferating cells; however, a significant proportion of the protein has also been detected outside the nucleus in cytoplasmic vesicles. To understand the possible role of extra-nuclear ATM, we initially investigated the nature of these vesicles. In this report we demonstrate that a portion of ATM co-localizes with catalase, that ATM is present in purified mouse peroxisomes, and that there are reduced levels of ATM in the post-mitochondrial membrane fraction of cells from a patient with a peroxisome biogenesis disorder. Furthermore the use of the yeast two-hybrid system demonstrated that ATM interacts directly with a protein involved in the import of proteins into the peroxisome matrix. Because peroxisomes are major sites of oxidative metabolism, we investigated catalase activity and lipid hydroperoxide levels in normal and A-T fibroblasts. Significantly decreased catalase activity and increased lipid peroxidation was observed in several A-T cell lines. The localization of ATM to peroxisomes may contribute to the pleiotropic nature of A-T.

Ataxia-telangiectasia (A-T)1 is a rare human autosomal recessive multisystem disease that is characterized by a wide range of features including, progressive cerebellar ataxia with onset during infancy, oculocutaneous telangiectasia, susceptibility to bronchopulmonary disease and neoplasia, onset of advanced cerebellar ataxia during childhood, and decreased catalase activity. The gene mutated in the human genetic disorder ataxia-telangiectasia codes for a protein, ATM, the known functions of which include response to DNA damage, cell cycle control, and meiotic recombination. Consistent with these functions, ATM is predominantly present in the nucleus of proliferating cells; however, a significant proportion of the protein has also been detected outside the nucleus in cytoplasmic vesicles. To understand the possible role of extra-nuclear ATM, we initially investigated the nature of these vesicles. In this report we demonstrate that a portion of ATM co-localizes with catalase, that ATM is present in purified mouse peroxisomes, and that there are reduced levels of ATM in the post-mitochondrial membrane fraction of cells from a patient with a peroxisome biogenesis disorder. Furthermore the use of the yeast two-hybrid system demonstrated that ATM interacts directly with a protein involved in the import of proteins into the peroxisome matrix. Because peroxisomes are major sites of oxidative metabolism, we investigated catalase activity and lipid hydroperoxide levels in normal and A-T fibroblasts. Significantly decreased catalase activity and increased lipid peroxidation was observed in several A-T cell lines. The localization of ATM to peroxisomes may contribute to the pleiotropic nature of A-T.

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2 The abbreviations used are: A-T, ataxia-telangiectasia; DNA-PK, DNA-dependent protein kinase; PTS, peroxisomal targeting signal; PBS, phosphate buffered saline; kb, kilobase(s); bp, base pair(s).

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confirmed by DNA sequencing.

A partial catalase clone was used in a polymerase chain reaction using primers

**Preparation of Antibodies**—The ATM4BA antibody has been described previously (24). A mouse monoclonal antibody (CT1) to the 491 bp 3′-end was subcloned by isolating a 280-bp fragment. pSL1180 containing full-length catalase was used in a polymerase chain reaction using NsiI/AseI fragment of full-length ATM cDNA into the yeast vector pAS2. This fragment contains the C-terminal of ATM corresponding to amino acid residues 1666–3056 plus DNA-binding domain. This fragment was cloned into the NcoI/EcoRI site of pAS2-1 (12). The sequence integrity of catalase was monitored by DNA sequencing.

**Subcellular Fractionation and Western Blotting**—Two 150-cm² flasks of each of neonatal foreskin and GM13267 fibroblasts were trypsinized to remove the cells from the culture dish, and the cells were suspended in 2 ml/flash of ice-cold homogenization buffer. The procedures for preparation of nuclear and post-mitochondrial pellet fractions and Western blotting for ATM were as described previously (24).

**Lipid Peroxidation** Measurements—Determination of lipid peroxides was performed on cell homogenates of normal and A-T fibroblasts using a thiobarbituric acid method. The membranes were then incubated with primary antibody in PBS containing 2% fetal calf serum at 4 °C overnight. This was followed by five washes in PBS at 4 °C and then blocked for 2 h at room temperature. The antibodies were used at the following dilutions: ATM-4BA 1:200; CT-1 culture supernatant (undiluted); CT-1 ascites fluid (1:200); and anti-catalase (1:200). Coverslips were mounted using Vectashield fluorescence mounting medium (Vector Labs) and viewed with a Bio-Rad MRC500 confocal microscope using K1/K2 filters.

**Yeast Two-hybrid Analysis**—A partial catalase cDNA clone (309301) was obtained from the IMAGE Consortium cDNA clone library (Genome Systems Inc.). A 1.4 kb nearly full-length EcoRI-NorI catalase fragment was subcloned into the EcoRI/NorI sites of pSL1180. The remaining 5′-end was subcloned by isolating a 280-bp EcoRI fragment from pCAT 2.2 (a gift from Dr. Nick Hayward of this institute) and inserting it into the EcoRI site of pSL-CAT14. Clones were screened for correct orientation of the 280-bp fragment. pSL1180 containing full-length catalase was designated pSL-catalase. Catalase was then subcloned into pGBT9 by polymerase chain reaction. The sense oligonucleotide 5′-GGAAGATC-CTTCATGGTCAGTACGCCCCG-3′, which encodes a BgII restriction site, and the antisense oligonucleotide 5′-ACTCGATCATGACGTTTGCGCTTCCCTCC-3′, which encodes an NsiI restriction site, were used in a polymerase chain reaction using Pfu DNA polymerase with pSL-catalase as the template. The polymerase chain reaction product was purified and digested with BgIII and NsiI and cloned into pGBT9 digested with BamHI and PstI. The sequence integrity of catalase was confirmed by DNA sequencing.

The pGAD10 plasmid containing a cDNA insert for Pex5p was kindly provided by Dr. S. Gould (27). The plasmid ATM 4.01 was obtained by cloning the NsiI/AseI fragment of full-length ATM cDNA into the yeast DNA-binding domain vector pAS2. This fragment contains the C-terminal of ATM corresponding to amino acid residues 1666–3056 plus some untranslated 3′-sequence (12). To remove 491 bp from the 3′-end of ATM, the plasmid was digested with NcoI (restriction site at 8964 bp) and NorI (site in vector). The linearized plasmid (3.51 kb) was ligated, purified after transformation. An 8.7-kb ATM fragment (corresponding to amino acid residues 1–2934) was cloned into the NorI site of pAS2-1 (12).

The Matchmaker two-hybrid system from CLONTECH (Palo Alto, CA) was used in which the combination of genetic selection for histidine prototrophy and the assay for β-galactosidase activity is applied to identify positive clones. Growth, maintenance, and transformation of S. cerevisiae were carried out according to the CLONTECH Matchmaker two-hybrid system protocol using 25 µM 3-amino(trimethyl) to eliminate many of the false positives that can occur. The S. cerevisiae strain Y190 (MATa, ura 3-52, his3-200, lys2-801, ade 2-101, trp1-901, leu2-3, 112, gal4Δ, gal1Δ, Cyn2 r2, URA3::GAL1α/α GAL1β/β, LacZ, LYS2::GAL1α/α HIS3/3::URA3::HIS3) was used, and it expresses the reporter genes lacZ (E. coli) and HIS3 (S. cerevisiae) under the control of the GAL1 promoter.

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bation on ice for 10 min, Triton X-100 was added to a final concentration of 0.1%. Following a further 5-min incubation on ice, the mixture was vortexed and used for assay of catalase activity as measured by the decrease in absorbance of hydrogen peroxide at 240 nm (28).

RESULTS

The C Terminus of ATM Is Similar to That of Catalase—The heterogeneous size of the vesicles labeled with ATM antibodies, the presence of a defined single membranous structure, and the punctate immunofluorescence, which we observed previously was suggestive of peroxisomes (24). Peroxisomes are single-membrane-bound organelles present in virtually all eukaryotic cells, and they contain a range of enzymes involved in a variety of metabolic processes including peroxide-based respiration, oxidation of very long chain fatty acids, and synthesis of plasmalogens and bile acids. Furthermore, comparison of the C-terminal sequences of the peroxisomal matrix enzyme catalase, which contains sequence important for its localization to this organelle, with that of ATM revealed some sequence conservation (Fig. 1A). This relatedness is evident in two regions as indicated by bold type in Fig. 1A. It is noteworthy that the C-terminal sequence (last 16 amino acids) shows 100% conservation between mouse and human ATM. A monoclonal antibody (CT-1) was raised against the extreme C-terminal 16 amino acids. The specificity of the CT-1 antibody was determined by immunoblotting with tissue extracts from normal and atm-/- mice (Fig. 1B).

Colocalization of ATM and Catalase—To confirm the localization of ATM to peroxisomes, we performed double-labeling experiments using an antibody to human catalase and two different antibodies to ATM, the polyclonal antibody ATM-4BA as described previously (24) and the monoclonal antibody (CT-1). When catalase was stained using a rhodamine-labeled secondary antibody (red) and ATM with a fluorescein isothiocyanate-labeled secondary antibody (green), it is evident that colocalization (yellow staining) occurs (Fig. 2A), demonstrating that ATM is localized to peroxisomes. However, it is also clear that ATM is present in other cytoplasmic vesicles not stained with anti-catalase. This is consistent with the report of Lim et al. (29), which shows that ATM binds to β-adaptin and β-neuronal adaptin-like protein, components of clathrin-coated and secretory vesicles. Confocal microscopy was used to confirm the colocalization of catalase and ATM using the CT-1 antibody (Fig. 2B).

ATM Is Present in Purified Peroxisomes—Because peroxisomes are readily purified from mouse liver using Nycodenz gradients, we used this approach to further demonstrate that ATM is present in peroxisomes. The results of such a gradient analysis appear in Fig. 3A, demonstrating that catalase activity peaks in fraction 3. Small amounts of mitochondria are also present elsewhere on the gradient as evidenced by the presence of succinate dehydrogenase peaking in fraction 6. Fractions were run on a SDS gel and checked for the presence of ATM by immunoblotting with CT-1 antibody. It can be seen that ATM is detected in the peak fraction (#3) from the gradient, coinciding with the peak of catalase (Fig. 3B). It is evident that the recovery of ATM in these fractionated peroxisomes is low, but this protein was reproducibly observed to coincide with the catalase peak. This is also not due to nuclear contamination because the much more highly abundant protein DNA-depend-
ent protein kinase (DNA-PK) was not detected in any of the gradient fractions (Fig. 3, lower panel).

**ATM Binds to the Peroxisomal PTS1 Receptor**—The C-terminal sequence similarity of ATM to catalase suggested that ATM might be imported into peroxisomes in a manner analogous to catalase. The import of proteins into peroxisomes requires a host of peroxisome assembly factors or peroxins that include specific PTS receptors, which recognize the targeting signals (30). In the case of catalase the PTS1 signal was thought to be the SHL sequence (positions 11–9 from the C terminus), which is in the corresponding position to a related sequence in ATM, SRL (Fig. 1A). More recently it has been shown that the extreme C-terminal sequence KANL is sufficient to target catalase to the peroxisome (31, 32). The C-terminal sequence of ATM is KAWV; however, it is not yet known whether this constitutes a PTS1 signal. Recognition of the PTS1 signal is mediated by the PTS1 receptor, now known as Pex5p (30). In our study the yeast two-hybrid assay was used to show that Pex5p binds to ATM. A 4.01-kb construct corresponding to the C-terminal half of ATM was cloned into the yeast vector pAS2, which contains the GAL4 DNA-binding domain (12, 24). Pex5p cloned into the pGAD10 vector was used to co-transform yeast cells, and interaction between the two proteins was assayed using a liquid culture β-galactosidase assay. As a control, the coding sequence of human catalase was also cloned into the pGBT9 vector. The results show that both proteins bind the PTS1 receptor (Table I). Although the observed binding was weak, it nevertheless represents a true interaction because a similar degree of binding was observed with catalase and may reflect the low affinity expected of a transient interaction in vivo. When the 8.7-kb and 3.51-kb ATM cDNA constructs in pAS2, both lacking the extreme C terminus, were used with Pex5p there was no interaction. The 8.7-kb construct expresses almost full-length ATM protein (corresponding to amino acids 1–2934), and we have previously used it to demonstrate an interaction between ATM and p53 (12). The 3.51-kb construct lacks 491 bp at the C terminus. In toto, these data suggest that ATM may be imported into peroxisomes via the PTS1 pathway.

**Reduced Extra-nuclear ATM in Peroxisome Biogenesis Disorder Cells**—It would be predicted that, where there is a PTS1 import defect, ATM should be absent or reduced in peroxisomes but present in the nucleus. Peroxisome biogenesis disorders result from failure to assemble normal peroxisomes. Peroxisome biogenesis disorder patients can be divided into at least 10 complementation groups, reflecting the complexity of the import process (33). We therefore performed subcellular fractionation, followed by immunoblotting for ATM, on Zellweger complementation group 4 cells (GM13267) compared with normal fibroblasts. Complementation group 4 cells contain a defect in an ATPase (Pex6p), which is required for stability of the receptor, Pex5p (30). It is clear that the relative amount of ATM in the post-mitochondrial pellet fraction versus nuclear ATM in GM13267 cells is reduced compared with that in normal cells (Fig. 4). Immunoblotting with antibody to DNA-PK was used to show that there was no contamination of nuclear material in the post-mitochondrial pellet fraction (data not shown). ATM was also found in the cytoplasm of this peroxisome biogenesis disorder line (data not shown), similar to that observed previously for the ATM protein in the A-T cell line (AT1ABR) expressing mutant ATM protein (24).

**Decreased Catalase Activity and Increased Lipid Hydroperoxide Levels in A-T**—There have been reports in the literature (although controversial) of defects in catalase activity in some A-T cell lines (34). The significance of these observations required reinvestigation in view of our finding of ATM in the peroxisome. We found significantly reduced catalase activity (50–70% of normal) in all the A-T fibroblasts tested (Table II). The reduction in activity does not appear to be due to reduced protein levels because no obvious change in the amount of catalase was detected by immunoblotting with anti-catalase antibodies (data not shown). In addition the distribution of catalase in A-T cells as assessed by fluorescence immunohistochemistry appears normal (data not shown). A consequence of reduced catalase activity would be increased hydrogen peroxide levels in the cell, which would in turn be expected to result in increased levels of lipid hydroperoxides. Lipid hydroperoxides were therefore measured in several normal and A-T fibroblasts, and the results are shown in Table III. It is evident that the levels of peroxides are significantly higher than controls in all A-T lines tested.

**DISCUSSION**

Our previous studies on the localization of ATM indicated that a proportion of the protein is localized outside the nucleus in vesicular structures (24). Using several different independent techniques and different anti-ATM antibodies, this study has shown that ATM is present in peroxisomes. We further demonstrated binding of the C-terminal half of ATM to Pex5p, the receptor for proteins containing a PTS1 signal. In addition we showed that ATM is markedly reduced in the post-mito-
chondrial pellet of GM13267 cells, which have a defect in the import of proteins into peroxisomes. The nuclear and peroxisomal forms of ATM appear to be identical in as far as the C terminus is concerned, because both are recognized by the monoclonal anti-C-terminal peptide antibody, CT-1. The mechanism of nuclear localization is likely to involve nuclear localization signals similar to those of peroxisomes. A recent study by Rotman and Shilo (36) has shown that ATM is also present on nonperoxisomal vesicles in a T-antigen/GAL4 activation domain hybrid in pGAD9p, respectively.

As pointed out by Rotman and Shilo (36), several lines of evidence suggest that the hypersensitivity of A-T cells to ionizing radiation may not result solely from defective cell cycle checkpoints and DNA damage processing. The constitutive activation of several IR-responsive signaling pathways in unirradiated A-T cells, which resembles but does not achieve the same extent as that in irradiated control cells, indicates that these cells are in a state of continuous oxidative stress. In addition, red blood cells from A-T heterozygotes have been reported to contain a lower content of sulphhydril groups, increased membrane fluidity and increased lipid peroxidation (37). We have now demonstrated decreased levels of catalase and increased levels of lipid hydroperoxides in several A-T cell lines. The major mechanism for disposal of hydrogen peroxide are catalase and glutathione peroxidase, both of which occur in peroxisomes as well as in the cytoplasm. The continuous state of oxidative stress in A-T could be explained by a deficiency in the detoxification of reactive oxygen species, and this may result from the decreased catalase activity observed here. Intriguingly, catalase activity in the livers of atm−/− mice (38–41) and requires further investigation. It should be pointed out that ATM is also present on nonperoxisomal vesicles co-localizing with β-adaptin (29), and ATM binds in vitro to the neuronal homologue of β-adaptin, β-neuronal adaptin-like protein. Because it has been suggested that β-NAP plays an essential role in synaptic vesicle transport in neuronal cells, the absence of ATM from these vesicles could also contribute to neuronal degeneration. Indeed there is evidence that defects in synaptic vesicle transport give rise to ataxia in humans (42). It is intriguing to suggest that the function of the noncatalase vesicles and peroxisomes may be interrelated, in that components of the peroxisome importation machinery (peroxins) have been localized to vesicles distinct from mature peroxisomes (43), and the endoplasmic reticulum plays an essential role in peroxisomal biogenesis (44).

Many of the features of A-T, for example defects in cell cycle checkpoints and aberrant meiosis, are consistent with the

### Table I

| Plasmid co-transformations | Units |
|---------------------------|-------|
| 4.01 kb ATM in pAS2       |       |
| Catalase in pGBT9         |       |
| pVA3*                     |       |
| pAS2                      |       |
| 4.01 kb ATM in pAS2       |       |
| 3.51 kb ATM (Neo1-Not1) in pAS2 |       |

* pVA3 and pTD1 are positive control plasmids encoding a murine T-antigen/GAL4 activation domain hybrid in pGAD9p, respectively.

\* Only a few colonies were detected.

\* The value is significantly different at the \* p < 0.005 level from that of normal fibroblasts.

### Table II

**Catalase activity in normal and A-T fibroblasts**

| Cell type                  | Specific activity |
|----------------------------|-------------------|
| Normal fibroblasts         |                   |
| NFF                        | 7.78 ± 2.77       |
| PGPF                       | 6.33 ± 2.55       |
| JRRF                       | 8.02 ± 2.79       |
| A-T fibroblasts            |                   |
| AT5B/VA                    | 2.53 ± 1.39*      |
| AT13A                      | 4.48 ± 1.72*      |
| GM3395                     | 3.72 ± 2.09*      |
| GM3487A                    | 4.48 (n = 1)      |

**The value is significantly different at the \* p < 0.005 level from that of normal fibroblasts.**

### Table III

**Lipid hydroperoxides in fibroblasts**

| Lipid peroxides | Units (nmol/mg) |
|-----------------|----------------|
| NFF             | 1.20 ± 0.006   |
| PGPF            | 1.17 ± 0.006   |
| JRRF            | 1.19 ± 0.014   |
| A-T             |                 |
| AT5B/VA         | 1.74 ± 0.036*  |
| AT13A-SV        | 1.77 ± 0.030*  |
| GM3395          | 1.71 ± 0.039*  |
| GM3487A         | 1.87 ± 0.027*  |

**The value is significantly different at the \* p < 0.001 level from normal fibroblasts.**

**FIG. 4. Reduced extranuclear ATM in fibroblasts from patients with peroxisome biogenesis disorders.** Western blot of ATM in nuclear (N) and post-mitochondrial organelle fractions (M) from complementation group 4 cells (GM13267) and normal fibroblasts (NFF). Equal amounts of protein (30 μg) were loaded in each lane and verified by Ponceau S staining before immunoblotting.

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**GM13267**

**NFF**

**ATM**

**ATM in Peroxisomes**

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clear localization of ATM. However, an explanation for the basis of the most debilitating and characteristic symptoms of the disease, the neurological dysfunction, has so far been elusive. Some peroxisome biogenesis disorders show similarity with A-T, for example, ataxia that occurs in infantile Refsum’s disease (45) and demyelination, which occurs in X-linked adrenoleukodystrophy (46). ATM is found predominantly outside the nucleus in Purkinje cells (47). Thus in noncycling cells the primary function of ATM would be in the maintenance of cellular homeostasis. In summary, we have demonstrated that ATM is associated with peroxisomes, which provides a new direction for investigating the role of ATM.

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