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The Copper Transport Protein Atox1 Promotes Neuronal Survival*

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Atox1, a copper transport protein, was recently identified as a copper-dependent suppressor of oxidative damage in yeast lacking superoxide dismutase. We have previously reported that Atox1 in the rat brain is primarily expressed in neurons, with the highest levels in distinct neuronal subtypes that are characterized by their high levels of metal, like copper, iron, and zinc. In this report, we have transfected the Atox1 gene into several neuronal cell lines to increase the endogenous level of Atox1 expression and have demonstrated that, under conditions of serum starvation and oxidative injury, the transfected neurons are significantly protected against this stress. This level of protection is comparable with the level of protection seen with copper/zinc superoxide dismutase and the anti-apoptotic gene bcl-2 that had been similarly transfected. Furthermore, neuronal cell lines transfected with a mutant Atox1 gene, where the copper binding domain has been modified to prevent metal binding, do not afford protection against serum starvation resulting in apoptosis. Therefore, Atox1 is a component of the cellular pathways used for protection against oxidative stress.

Characterizing the proteins that are involved in neuronal survival and protection against injury is of paramount importance to understand and ultimately control neurodegenerative processes that lead to disease. Proteins that function in cellular oxidative processes such as Cu/Zn superoxide dismutase (SOD) continue to be intensely investigated. This is significant because many of the underlying mechanisms associated with neurodegeneration involve oxidative damage (1, 2). This is true in the case of amyotrophic lateral sclerosis (3–5) and Alzheimer's disease (2).

Some of the mechanistic components in neurodegeneration can be attributed to the regulation of metal ions. In both Wilson's and Menkes diseases, the aberrant regulation of copper ions initiates the neurodegenerative process. The Menkes protein is a P-type ATPase characterized by a copper binding site, a phosphorylation site, a phosphatase domain, an ATP binding site, and a transmembrane cation channel (6). Mutations in the Menkes protein result in copper deficiency. The neurodegeneration seen in this disease may be because of a decreased activity of several copper-dependent enzymes in the brain. In contrast, in Wilson's disease copper overload leads to toxic levels of this metal and contributes to neurodegeneration (7).

We have investigated the biological role of a recently reported copper transport protein Atox1. Originally identified in yeast, Atox1 was shown to protect superoxide dismutase (SOD1)-deficient yeast from oxidative damage induced by superoxide anions (O_2^-) and hydrogen peroxide (H_20_2) (8). Others have further demonstrated that, in yeast, Atox1 transfers copper to Ccc2 (9), a protein that is homologous to the Menkes protein in mammals. The copper-bound Ccc2 then transfers the copper ion to Fet3, a component of the high affinity iron-uptake system. The mammalian counterpart of Fet3 is the ceruloplasmin protein (10).

In this report, we demonstrate that overexpression of Atox1 can increase neuronal viability under stress conditions such as serum deprivation and oxidation. The protection afforded by overexpression of Atox1 is comparable with that seen with the overexpression of the anti-apoptotic protein Bcl-2. Furthermore, overexpression of an Atox1 metal binding mutant is detrimental to cell viability.

EXPERIMENTAL PROCEDURES

Cell Culture—NT-2, a teratocarcinoma cell line (11), SK-N-MC, a neuroblastoma cell line which has cholinergic characteristics (12), and GT-1 (13), a hypothalamic neuronal cell line, were utilized in this study. The Atox1 and mutant Atox1 transfected cell lines were maintained in 100-mm tissue culture plates containing high glucose-Dulbecco's modified Eagle's medium (HG-DMEM,[1] Life Technologies, Inc., UK), 1 mM sodium pyruvate (Sigma, UK), 10% fetal bovine serum (Life Technologies, Inc., UK), and 100 IU/ml penicillin and 100 mg/ml streptomycin (Life Technologies, Inc., UK). Cells were maintained in a humidified 5% carbon dioxide, 95% air 37 °C incubator.

cDNA Libraries—cDNA libraries were generated from rat astrocyte mRNA with the SuperScript plasmid system for cDNA synthesis and plasmid cloning from Life Technologies, Inc. (Gaithersburg, MD) essentially as described by the manufacturer. One modification to the procedure was the substitution of *Bst*XI adapters (Invitrogen, San Diego, CA) for the *Sal*I adapters provided with the kit. The resultant cDNA was used to generate libraries in the plasmid pJFE14 SR alpha (14). The cDNA was cloned in the *Bst*XI-*Not*I polylinker and was used to transform the DH10B strain of *Escherichia coli*. Plasmid was isolated and purified with the Qiagen system (Chatsworth, CA) and was used to generate a subtracted cDNA library.

Transfection Constructs—The retroviral expression construct pBabe-Puro, containing the puromycin gene for selection, was used for rat Atox1 and Bcl-2 expression. Cells were infected with either the vector or

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF127137 and XAAD27844.

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[1] The abbreviations used are: HG-DMEM, high glucose-Dulbecco's modified Eagle's medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MPP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridium ion; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium salts.

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with a positive control construct in which the bcl-2 gene was inserted or the construct containing the rat Atox1 gene or Atox1–12S15S. The expression construct pCB6 containing the neomycin gene for selection was used for expression of SOD, SOD-G37R, and SOD-G85R.

DNA Transfections—pBabePuro control vector, pBABE-Atox1, or pBABE-mutant Atox1 cDNAs were used for the transfection. At least 10 μg of each construct was transfected into the two cell lines using the DOTAP liposomal transfection kit (Roche Molecular Biochemicals). In the DOTAP liposomal method, at least 10 μg of DNA in Tris-HCl-EDTA buffer (pH 7.4) was mixed with HEPES buffer (pH 7.4) (final volume of 50 μl) and added into the mixture containing 30 μl of DOTAP and 70 μl of HEPES buffer. This mixture was added to cells of approximately 80% confluence in 100-mm tissue culture plate and incubated in 37 °C incubator over night. The selection of transfectants was followed with 200 μg/ml puromycin (Sigma, UK). The antibiotic-resistant cells were chosen and cultured for further experiments.

Assay of the Effect of Rat Atox1 on Neuronal Cell Survival—The neuronal cell lines GT-1 (13), SKNMC, and NT-2 were cultured in DMEM with 10% fetal calf serum in 5% CO_2. Cells were grown to confluence, harvested, and used for transfection. For experiments, cells were harvested and plated on 100-mm diameter culture plates at 15–20% confluence and then allowed to grow to 50–70% confluence. To induce cell death, media without serum was added to the cultures. The cells were then assayed on each day for viability using Trypan blue exclusion. Percentage viability was determined by comparing the average total cell number of a given treatment group with the average total cell number from the group treated for the identical time with control medium. For all conditions, viability assays were performed on triplicate dishes. The tetrazolium salt MTT assay detects living cells, and the signal generated is dependent on the degree of mitochondrial activity in cells (15). This substance can be detected by absorbance at 550 nm. Cells (2×10^5) were trypsinized (2 ml of 0.1% trypsin) and washed twice with phosphate-buffered saline buffer (pH 7.4) and grown in 96-well plates overnight. They were then incubated with MTT (Sigma, UK), and the absorbance at 550 nm was measured in the spectrophotometer.

Gel Electrophoresis of Apoptotic DNA Laddering—Cells (10^7-10^8) were trypsinized (2 ml of 0.1% trypsin) and washed two times with phosphate-buffered saline buffer (pH 7.4), and then a lysis buffer containing 10 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), and 3% Triton X-100 was added. The lysates were incubated at a 37 °C incubator for at least 1 h and then were centrifuged (12000 × *g*, 20 min). They were then treated with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) two times each and precipitated with 0.6 volume of isopropyl alcohol and 0.1 volume of 3 M sodium acetate (pH 5.2) and then placed at −20 °C for at least 30 min. The precipitates were centrifuged (12000 × *g*, 20 min) and washed with 70% ethanol and dissolved with TE buffer (pH 8.0) containing 2 mg/ml RNase (Sigma, UK). DNA was electrophoresed in 10 μg/ml ethidium bromide-containing 2% agarose gel to identify the DNA laddering.

Data Analysis—Statistical differences were analyzed by two-way ANOVA followed by the using the *t* test with Bonferroni correction of multiple comparisons. His test allows the comparison of several *t* tests. The aim of Bonferroni *t* test was to determine the magnitude of the two differences between control and each treatment. For example, when two different significance scores were obtained between the control and pBABE-puro and between the control and pBABE-ATOX1 5 days after serum deprivation conditions, the Bonferroni *t* test was employed to determine the magnitude of the two significance scores. The test was employed for all values having significance to find out the effect of the treatment condition on the viability of ATOX1 and mutant ATOX1 transfectants.

RESULTS

We have cloned a gene from rat glial cells which shares significant identity with the human Atox1 gene HAH1 (16). The nucleotide sequence of rat Atox1 is 423 base pairs. The deduced protein is 68 amino acids. Sequence alignment of the rat, human (16), and yeast (8) Atox1 protein (Fig. 1) indicates that the rat and yeast proteins share 35% identity, the human and yeast proteins share 40% identity, and the rat and human proteins share 89% identity. Furthermore, the metal binding motif in the yeast Atox1 protein, which is common to bacterial metal transporter proteins (17, 18), is conserved in both the rat and human Atox1 protein. This motif is characterized by the amino acid sequence MTC*XX*C, where *X* can be any amino acid. The conservation of this motif from yeast to human suggests that it plays a critical role in the function of the protein.

FIG. 1. **Atox1 amino acid sequence alignment.** Human (GenBank™ accession number AAC51227), rat (accession number AAD27844.1), and yeast (accession number AAC37428) Atox1 amino acid sequences were aligned. *Boxed* residues indicate amino acids that are conserved among the species. The *black boxed* residues represent the amino acids in the metal binding motif MTCXXC that are conserved, and the *gray* residues represent those amino acids in the metal binding motif that do not require conservation.

We have mapped the chromosomal location of the human Atox1 gene. Fluorescence *in situ* hybridization (FISH) indicated that human Atox1 is located on chromosome 5q32. To date, this region has not been implicated in any diseases. However, this chromosomal information may help illuminate some currently unresolved genetic linkage. In the brain, Atox1 expression was detected by polymerase chain reaction in primary cultures of neurons, microglia, and astrocytes. In addition, expression of Atox1 was found in all rat peripheral organs analyzed by polymerase chain reaction. Based on this analysis, it is likely that Atox1 is ubiquitously expressed.

The yeast Atox1 gene product was shown to protect yeast against oxidative damage (8). To determine whether overexpression of Atox1 could promote neuronal survival under conditions of injury, several neuronal cell lines were retrovirally transfected with either the rat Atox1 cDNA or a metal binding mutant Atox1 cDNA. Because the metal binding motif of Atox1 is conserved from yeast to human, we believe that this sequence is mediating an important function of the protein, and therefore we have generated a rat Atox1 gene that has serine substitutions at cysteines 12 and 15 (Atox1–12S15S). These substitutions should eliminate the affinity of copper for this binding site because the cysteines are involved in coordinating metal ions (19).

Atox1 was transfected into three different types of neuronal cells: SKNMC, a neuroblastoma cell line (Fig. 2*A*) which has cholinergic characteristics; NT-2, a teratocarcinoma cell line (Fig. 2*B*); and GT-1, hypothalamic neuronal cells (Fig. 2*C*). In all three cell lines, the overexpression of Atox1 resulted in enhanced viability as compared with the vector-only transfected cells under conditions of serum deprivation (Fig. 2). For example, in the absence of serum the Atox1 transfected SKNMC cells demonstrated about 50% viability as compared with the untransfected control cells, which were in serum-containing medium, after 5 days in culture (Fig. 2*A*). In contrast, there were essentially no viable cells in the vector-transfected and -untransfected SKNMC cells in the absence of serum after 5 days. Interestingly, both Atox1 and the anti-apoptotic gene Bcl-2 retrovirally transfected GT-1 neurons showed between 50–60% viability after 3 days under conditions of serum deprivation (Fig. 2*C*). The magnitude of protection observed with Bcl-2 in these experiments is consistent with the magnitude of protection reported for CSM14.1 neuronal cells transfected with Bcl-2 and subjected to serum starvation (20). The Atox1–12S15S metal binding mutant-transfected cells demonstrated decreased viability as compared with the vector-only transfected cells under conditions of serum starvation. In fact, after 2–3 days in culture without serum, the viability of the metal binding mutant-transfected cells were between 15–45% as compared with the control cells (Fig. 2, *A* and *B*). Even under growth conditions containing serum, the Atox1 metal binding

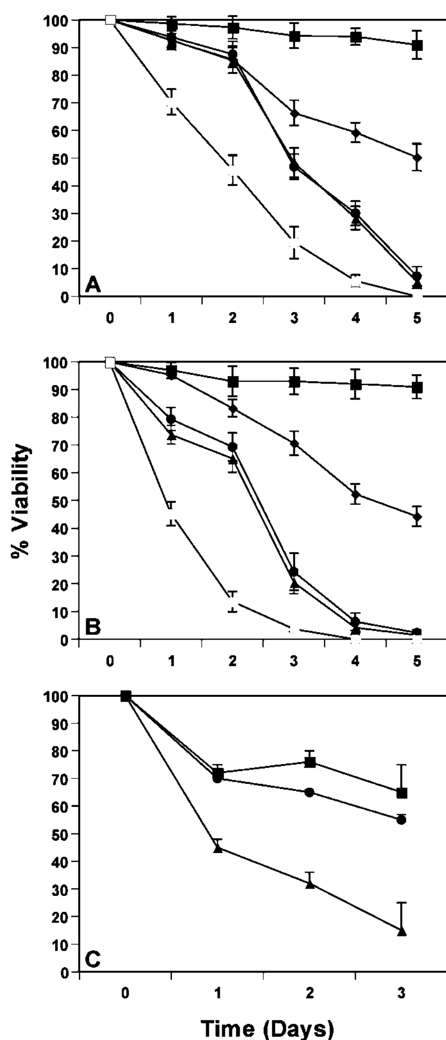

FIG. 2. **Atox1 cell viability under conditions of serum starvation.** SKNMC (*A*), NT-2 (*B*), and GT-1 (*C*) transfected cells were cultured in DMEM supplemented with 10% serum. At day 0, cells were washed and cultured with or without serum in the media. Viable cells were counted every 24 h with Trypan blue. All data are represented as percent cells viable as compared with day 0. For *panels A* and *B*: ■, untransfected (10% serum); ●, untransfected (0% serum); ▲, pBabe (0% serum); ♦, pBabe-Atox1 (0% serum); □, pBabe-Atox1–12S15S (0% serum). For *panel C*: ■, pBabe-Bcl-2; ●, pBabe-Atox1; ▲, pBabe.

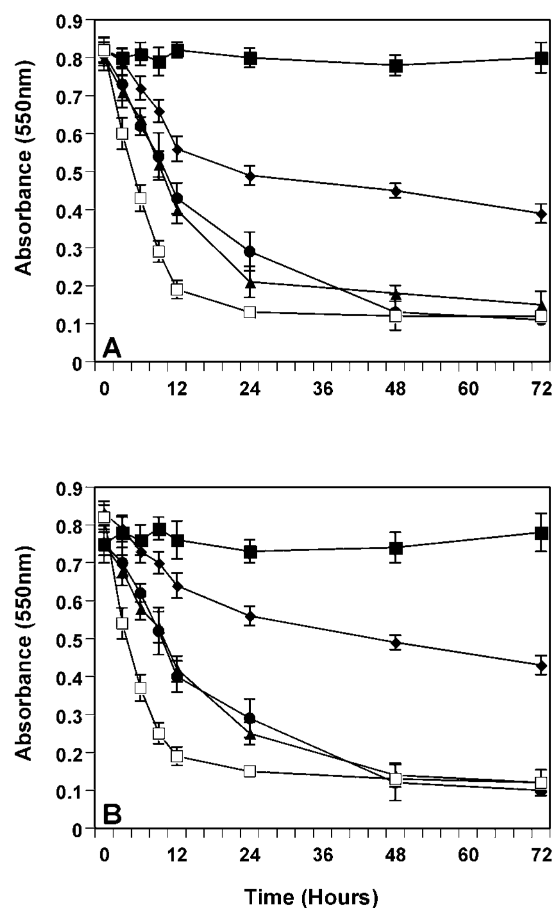

FIG. 3. **Atox1 cell metabolic rate under conditions of serum starvation.** NT-2 (*A*) and SKNMC (*B*) transfected cells were cultured in DMEM supplemented with 10% serum. At day 0, cells were washed and cultured with or without serum in the media. Metabolic rate was determined by MTT assay over 72 h. All data are represented as the absorbance at 550 nm. For *panels A* and *B*: ■, untransfected (10% serum); ●, untransfected (0% serum); ▲, pBabe (0% serum); ♦, pBabe-Atox1 (0% serum); □, pBabe-Atox1–12S15S (0% serum).

mutant is significantly less metabolically active than both the Atox1 and the vector-only transfected cells (data not shown). This dominant negative phenotype with the Atox1 metal binding mutant suggests that the mutant protein may be competing with the endogenous protein for functionality in a yet unidentified pathway.

In addition to determining viability, metabolic rate was assayed with MTT. The Atox1 transfected cells were metabolically more active than either the vector only or the metal binding mutant transfected cells under conditions of serum starvation (Fig. 3). To determine whether Atox1 transfected cells were more resistant than control cells to the effects of other insults such as oxidation, the SKNMC and NT-2 Atox1 transfected cells were treated with hydrogen peroxide or with MPP+, an electron transport chain complex I inhibitor. Both Atox1 transfected SKNMC and NT-2 cells were metabolically more active than vector-only transfected cells in the presence of the oxidative agent hydrogen peroxide (Fig. 4). Similar results were observed when the agent was MPP+. Furthermore, the Atox1 transfected cells demonstrated increased viability as measured by the trypan blue assay compared with the vector-only transfected cells when treated with hydrogen peroxide or MPP (data not shown).

To address whether the mechanism of cell death in serum-starved cells was apoptosis or necrosis, DNA laddering of the transfected SKNMC and NT-2 cells under conditions of serum starvation was investigated. The characteristic DNA laddering observed in cells undergoing apoptosis was observed in both the vector only and Atox1–12S15S metal binding mutant transfected cells but not the Atox1 transfected cells (Fig. 5). Similarly, DNA laddering was observed in the vector only and Atox1 metal binding mutant-transfected cells but not the Atox1 transfected cells under conditions of oxidative injury induced by hydrogen peroxide or MPP (data not shown). Therefore, Atox1 protected the transfected cells from apoptosis.

To confirm that the transfected Atox1 protein was expressed in the retrovirally transfected neurons, we isolated protein from the transfected SKNMC and NT-2 cells. All Atox1 and Atox1 metal binding mutant transfected clones were designed to incorporate a carboxyl-terminal FLAG sequence that allows detection of the transfected protein with FLAG-specific antibodies without detecting the endogenous protein. As presented in Fig. 6, Western blot analysis indicates a band at between 8–9 kDa corresponding to the size of the Atox1 protein in both SKNMC and NT-2 cells that is not seen in the vector control-transfected cells. In summary, the expression of the transfected Atox1 protein increases the viability of neurons against serum

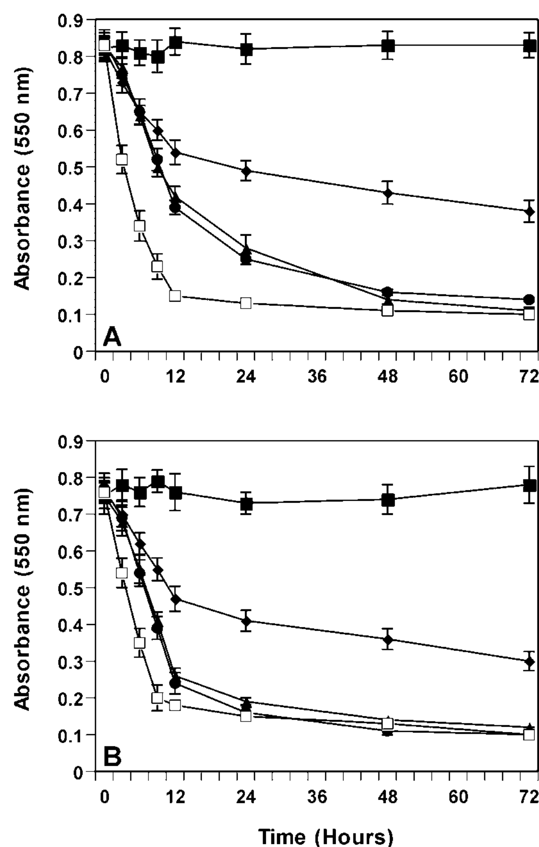

FIG. 4. **Atox1 cell metabolic rate in the presence of oxidative agents.** NT-2 (*A*) and SKNMC (*B*) transfected cells were cultured in DMEM supplemented with 10% serum. At day 0, cells were washed and cultured with or without 100 μM hydrogen peroxide. Metabolic rate was determined by MTT assay over 72 h. All data are represented as the absorbance at 550 nm. For *panels A* and *B*: ■, untransfected; ●, untransfected (hydrogen peroxide); ▲, pBabe (hydrogen peroxide); ♦, pBabe-Atox1 (hydrogen peroxide); □, pBabe-Atox1–12S15S (hydrogen peroxide).

deprivation and oxidative injury. In addition, the metal binding motif is critical for this protective mechanism.

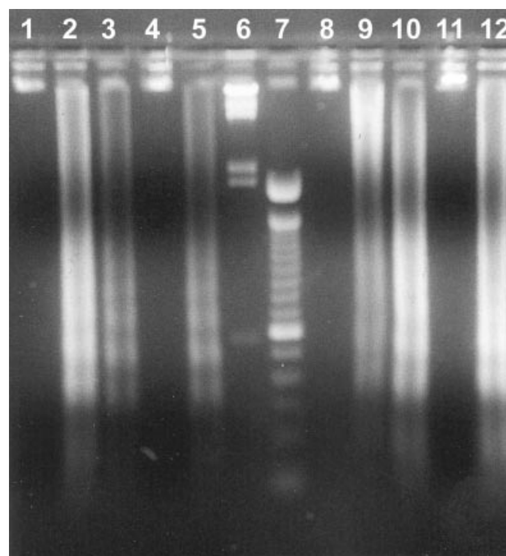

FIG. 5. **Apoptotic laddering of Atox1 transfected cells under conditions of serum starvation.** DNA from cell lysates was electrophoresed on an agarose gel. *Lanes 1*, NT-2 (10% serum); *2*, NT-2 (0% serum); *3*, NT-2 pBabe (0% serum); *4*, NT-2 pBabe-Atox1 (0% serum); *5*, NT-2 pBabe-Atox1–12S15S (0% serum); *6*, λ *Hin*dIII digest ladder; *7*, 100-bp ladder; *8*, SKNMC (10% serum); *9*, SKNMC (0% serum); *10*, SKNMC pBabe (0% serum); *11*, SKNMC pBabe-Atox1 (0% serum); *12*, SKNMC pBabe-Atox1–12S15S (0% serum).

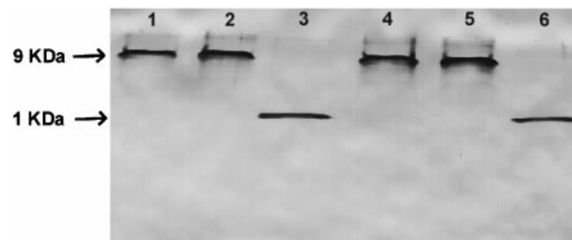

FIG. 6. **Atox1 protein expression in transfected cells.** Cell extracts were used to detect FLAG epitope on Atox1 expressed protein in transfected cells. M2 antibody which recognizes the FLAG epitope was used. *Lanes 1*, SKNMC pBabe-Atox1–12S15S; *2*, SKNMC pBabe-Atox1; *3*, SKNMC pBale-FLAG; *4*, NT-2 pBabe Atox1–12S15S; *5*, NT-2 pBabe-Atox1; *6*, NT-2 pBabe-FLAG.

DISCUSSION

We have cloned the rat Atox1 gene from rat brain. This gene and a metal binding mutant of this gene have been transfected into several neuronal cell lines to increase Atox1 expression level over endogenous levels. The overexpression of Atox1 in NT-2 cells, SKNMC cells, and GT-1 cells protects the cells from serum starvation. Interestingly, the magnitude of protection against serum starvation in the Atox1 transfected GT-1 cells is comparable with that seen in GT-1 cells transfected with the anti-apoptotic gene Bcl-2. Furthermore, the NT-2 and SKNMC Atox1 transfected cells are protected against oxidative stress-induced death from agents such as hydrogen peroxide and MPP. The ability of Atox1 to protect these cells from several different oxidative injuries suggests a fundamental role for Atox1 in the cellular response to oxidative stress.

In contrast, transfection of the metal binding mutant is significantly more detrimental to cell viability than the vector only transfected cells under these injurious conditions. In fact, the expression of the metal binding mutant is detrimental to cell viability under growth conditions where no injurious event was induced. This dominant negative phenotype of the metal binding mutant suggests that the protein is interfering or competing with the function of the endogenous Atox1 protein. This inhibition may be because of a competition for interactions with other protein partners that are involved in the pathway. This may be occurring at the level of copper uptake or at the level of copper transfer to ceruloplasmin, the high affinity iron uptake protein. At the level of copper uptake, Atox1 may signal the transfer of a copper ion into the cell for transport by Atox1. In this hypothesis, the metal binding mutant would be unable to bind the copper through the M*X*C*XX*C motif. This perturbation in function of the endogenous Atox1 protein by the metal binding mutant protein may result in free cytosolic copper ions that can cause auto-oxidation of proteins, lipids, and nucleic acids. In support of this theory, it has been shown that Atox1 acts as a metal ion chaperone and delivers the potentially reactive copper ions to their target proteins such as the Menkes and Wilson's diseases protein (21). These proteins are members of the P-type adenosine triphosphatase (ATPase) cation transporter family (22). Alternatively, but not mutually exclusive, the detrimental effect of the mutant Atox1 protein on cell viability may be because of competition with the endogenous copper ion-bound Atox1 for its binding to ceruloplasmin. This may result in inefficient copper transfer and downstream consequences. Recently, evidence has been reported that the copper chaperone function of Atox1 is distinct from the protein's ability to suppress oxidative damage (23). This would support the idea that the metal binding mutant might be competing with the endogenous Atox1 for interactions with proteins in a

pathway involved in suppression of oxidative damage. Because all of the components in the pathway for Atox1 function have not been completely elucidated, it is not clear at what other stages of copper ion transport the metal binding mutant may be interacting.

Based on the previously reported neuronal distribution of Atox1 in rat brain, it appears that Atox1 may play a role in protecting cells sensitive to oxidative damage from death. We have previously reported the distribution of Atox1 expression in the rat brain as determined by *in situ* hybridization (24). The highest level of Atox1 expression was associated with neuronal cell populations that sequester copper (24). It is not clear what the significance of this distribution is. There are several neurological diseases where the role of copper is fundamental to the function of the proteins involved in the disease mechanism. For example, in Wilson's disease the liver does not efficiently clear copper ions. This accumulation of copper extends to the brain and results in neurodegeneration. Conversely, in Menkes disease the Menkes Cu-ATPase does not function correctly resulting in the inability of Cu ions to cross the intestinal mucosa. The downstream affect of this is that Cu ions are deficient in peripheral organs. Such a deficiency in copper leads to neurodegeneration. Furthermore, the possible role of Cu/Zn superoxide dismutase (SOD1) in amyotrophic lateral sclerosis suggests that the neurodegeneration that accompanies the progression of this disease is mediated at least in part by inappropriate regulation of the copper protein.

Atox1 has been shown to act as a specific copper chaperone where it transports copper ions in the cytosol to the copper-dependent iron oxidase. Others have reported Atox1 functions in at least two independent pathways (9, 23). Atox1 is involved in the transport of copper to the secretory pathway and is involved in mechanisms of anti-oxidation. In this report we have shown that overexpression of Atox1 protects neurons from oxidative injuries. Furthermore, mutations in the copper binding motif of Atox1 result in a dominant negative phenotype where the cell viability is diminished. Together these results suggest that Atox1 plays a fundamental role in maintaining cell viability and the maintenance of this viability may be dependent on the role of Atox1 in protecting against oxidative damage.

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