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The CXXC motifs in the metal binding domains are required for ATP7B to mediate resistance to cisplatin ☆

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ABSTRACT

The copper (Cu) exporter ATP7B mediates resistance to cisplatin (cDDP) but details of the mechanism are unknown. We explored the role of the CXXC motifs in the metal binding domains (MBDs) of ATP7B by investigating binding of cDDP to the sixth metal binding domain (MBD6) or a variant in which the CXXC motif was converted to SXXS. Platinum measurement showed that cDDP bound to wild type MBD6 but not to the SXXS variant. Wild type ATP7B rendered ovarian 2008 cells resistant to cDDP. In 2008 and in HEK293T cells, wild type ATP7B trafficked from TGN to peripheral locations in response to Cu or cDDP. A variant in which the CXXC motifs in all 6 MBDs were converted to SXXS localized correctly to the TGN but failed to traffic when exposed to either Cu or cDDP. Deletion of either the first 5 MBDs or all 6 MBDs resulted in failure to localize to the TGN. Neither the SXXS variant nor the deletion variant was able to mediate resistance to cDDP. We conclude that cDDP binds to the CXXC motifs of ATP7B and that this interaction is essential to the trafficking of ATP7B and to its ability to mediate resistance to cDDP.

Nomenclature

ATP7B	ATPase, Cu++ transporting, beta polypeptide
GenBank ID	ENSG00000123191
PDB ID	P35670
EC	3.6

1. Introduction

Copper (Cu) transporters CTR1, ATP7A, ATP7B and the metallochaperone ATOX1 modulate the cellular pharmacology of the Pt containing drugs by controlling their uptake, efflux and subcellular distribution. CTR1 mediates the influx of cisplatin (cDDP) [1–4] and the exporters ATP7A (Menkes disease protein) and ATP7B (Wilson's disease protein) sequester this and other Pt drugs into secretory vesicles and mediate their eventual efflux [5–7]. ATP7A and ATP7B are P_{1B}-type ATPases that reside in the *trans*-Golgi network; they receive Cu^{1+} from ATOX1 that interacts with their N-terminal cytoplasmic domains and transport it across the vesicle membrane by utilizing ATP [8]. Each of the six N-terminal metal binding domains (MBD) of ATP7A and ATP7B contains a core CXXC motif to which Cu^{1+} becomes chelated. The structure of MBD is conserved in evolution with a ferredoxin βαββαβ fold [9,10] which is also found in ATOX1 and is the site to which cDDP binds [11,12]. NMR studies of ATOX1 [13], ATP7A [14] and ATP7B [15] show that MBD binds Cu^{1+} to the two surface-exposed cysteines in the metal binding loop and undergo structural changes that are similar in all of these proteins [10,16–20]. The CXXC motifs in the single MBD of ATOX1 and the six MBD units in the N-terminal cytosolic regions of ATP7A and ATP7B are required for the efflux of Cu. Among the 6 MBD units of ATP7A and ATP7B, mutational studies have identified a particularly important role for the sixth MBD which is the closest to the trans-membrane pore. The sixth MBD is likely to receive Cu from the fourth MBD and propel it toward the central pore [20–23]. In the case of ATP7B, the importance of the sixth MBD has been documented by the fact that missense mutations in this region, of which the G591D in turn 2 is the best known, cause Wilson's disease [24]. This mutation is likely to alter the stability of MBD6 and renders it insoluble [20,24].

The finding that the cysteines that bind Cu in ATOX1 also chelate cDDP [11,12] raises the possibility that the MBDs of ATP7A and ATP7B could potentially chelate this Pt-containing drug. cDDP has been reported to form complexes with several other proteins through cysteine and methionine residues [25–27] and thus can be expected to interact with ATP7A and ATP7B. Our previous study showed that cDDP can be transported by ATP7B into subcellular vesicles in Sf9

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cells thus indicating a direct interaction between cDDP and ATP7B but left unanswered the question of whether the interaction was mediated by the CXXC motifs.

To understand how ATP7A and ATP7B modulate the cellular pharmacology of cDDP, this study examined the role of cysteine residues in the CXXC motifs of ATP7B. We show here that the cDDP binds to the CXXC motif in the sixth MBD of ATP7B and causes its multimerization. Truncation of most of the MBDs of ATP7B, or conversion of the CXXC motifs of all 6 MBDs to SXXS, eliminated the ability of ATP7B to confer resistance to cDDP, to regulate the accumulation of this drug and to undergo Cu- and cDDP-induced trafficking.

2. Experimental section

2.1. Reagents

The lentiviral vector, pLVX-mCherry-C1, In-Fusion cloning kit, packaging kit and anti-mCherry antibodies were from Clonetech, Palo Alto, CA. pMAL/c2x vector, restriction enzymes, maltose binding resin, and antibodies to maltose binding protein were from New England Biolabs, Ipswich, MA. Secondary antibodies, fluorescent reagents, Superscript III, PCR kits, Genetailor mutagenesis kit, PCR2.1-TOPO vector, media and sera were from Invitrogen, Carlsbad, CA. P230 monoclonal antibody was from BD Biosciences, San Diego, CA. Cisplatin was from Teva Parental Inc., Irvine, CA. Antibodies to tubulin were from Sigma, St. Louis, MO. Complete protease inhibitor was from Roche, Nutley NJ. Electrophoresis gels and Bradford reagent were from Bio Rad, Richmond, CA. Other chemicals were from Sigma, St. Louis, MO.

2.2. DNA constructs

All constructs of ATP7B were generated from a 2008 ovarian carcinoma cell cDNA library generated by Superscript III and amplified by PCR using oligonucleotides listed in Table 1. The MBD6 sequence spanning from amino acids 561–633 was cloned into pCR2.1-TOPO from which it was excised by EcoR1 and cloned into pMAL/c2x. After transformation into *Escherichia coli* BL21 (DE3), and sequence verification, a bacterial clone was selected for the expression of MBD6 fused to the maltose binding protein which will be referred to herein as MBD6. Conversion of both cysteines in MBD6 to serines was accomplished with the Genetailor kit using the wild type pMAL-MBD6 as template. The wild type ATP7B, ATP7B Δ1–5 (in which MBDs 1–5 spanning from amino acids 1–539 were truncated), and ATP7B Δ1–6 (in which MBDs 1–6 spanning from amino acids 1–599 were truncated) were PCR amplified from a 2008 cDNA library. The ATP7B variant in which all the CXXC motifs were converted to SXXS was PCR amplified from the plasmid vector 0CMB398 that was generously provided by Dr. S. La Fontaine and Dr. J.F. Mercer (University of Melbourne, Melbourne Australia) [22]. All ATP7B variants were cloned into pLVX-mCherry-C1 vector using the In-Fusion cloning kit.

Table 1
Oligonucleotides used for cloning ATP7B variants.

MBD6	F[a] TCCGATGGCAACATTGAGCT
	R[a] TTACTGGGCCAGGGAAGCATGAA
SXXS	F GACAATCACAGGGATGACCTCTGCGTCCTCTGTCCACAAC
	R GTCATCCCTGTGATTGTCAGC
ATP7B	F ATGCCTGAGCAGGAGAGACAG
	R TCAGATGTACTGCTCCTCAT
ATP7B Δ1–6	F GCCACCAGCAAAGCCCTTGTTAAG
	R TCAGATGTACTGCTCCTCATC
ATP7B Δ1–5	F CTCGAGATAGCTCAGTTCATC
	R TCAGATGTACTGCTCCTCATC
ATP7B SXXS	F ATGCCTGAGCAGGAGAGACAG
	R TCAGATGTACTGCTCCTCAT

[a] F, forward; R, reverse.

2.3. Cell culture and expression of lentiviral constructs of ATP7B

Human ovarian carcinoma 2008 cells were maintained in RPMI medium containing 10% fetal calf serum; HEK293T cells were cultured in high glucose DMEM with 1 nM sodium pyruvate, and 1 nM essential amino acids. Cells were incubated at 37 °C, 5% CO_2. Lentiviral stocks of ATP7B variants were produced in HEK293T cells and used to transduce 2008 ovarian carcinoma cells or HEK293T cells [28]. Selection was made with 10 μg/mL puromycin. A pool of cells expressing high levels of the fluorescent mCherry tag was obtained by three rounds of FACS sorting.

2.4. Production and purification of recombinant MBD6

Plasmids expressing either maltose binding protein alone or maltose binding protein (MBP) fused to MBD6 were transformed into competent *E. coli* BL21 (DE3) and grown in LB containing 100 μg/mL ampicillin. For protein purification, cultures were grown in minimal medium M9 containing 3% LB medium and incubated at 37 °C at 260 rpm until OD600 reached ~0.6 after which the temperature was reduced to 30 °C and 0.3 μM IPTG (isopropyl-β-D-thiogalactoside) was added. The bacteria were harvested at 4 °C by centrifugation at 12,000×*g* for 45 min. The pellets were resuspended in 20 mL lysis buffer (10 mM HEPES, pH 7.6, 150 mM NaCl, 1% DMSO, 1 μg/mL DNAse 1, 0.25 mg/mL lysozyme, Complete protease inhibitor), incubated at room temperature for 30 min, sonicated on ice for 6 min and following centrifugation at 4 °C and 16,000×*g* for 30 min, incubated for 1 h with 200 mM of Cu chelators tetrathiomolybdate or bathocuproine sulfate and 0.5 mM of the reducing agent Tris-(hydroxypropyl)phosphine at 4 °C. The lysate was loaded onto amylose columns that were pre-equilibrated with 10 column volumes of binding buffer (100 mM NaCl, 10 mM HEPES, pH 7.5, 1 mM NaN_3, 20 mM β-mercaptoethanol (BME)). After 4 washes with 5–10 column volumes of the same buffer, MBP or MBP-MBD6 was eluted with binding buffer containing 10 mM maltose. For some experiments, MBD6 was excised from the maltose binding protein using Factor Xa in a buffer containing 100 mM NaCl, 50 mM HEPES, pH 7.5, and 20 mM BME. The cut protein was injected into an FPLC system (BIO-RAD, Richmond CA) and purified on a Superdex75 column (GE, Piscataway, NJ) and concentrated with an Amicon Ultra Cell filtration unit (Millipore, Billerica, MA). All samples were kept in the presence of 20 mM BME until use. BME was removed by washing the samples under anaerobic conditions with binding buffer in a Millipore filtration unit.

2.5. Analysis of the interaction of cDDP with MBD6 with UV spectrometry

The absorbance at 280 nm reflecting the formation of Pt–sulfur bonds and disulfides was measured as a function of time using a single beam spectrophotometer (Beckman model DU530). Triplicate samples of 1.0 mL each containing 50 μM protein in the binding buffer were used. The spectrophotometer was immediately zeroed after cDDP addition and the reaction progress was measured in a 1-cm path length CVD-UV disposable Cuvettes (Ocean Optics, Dunedin, FL) for 2 h at room temperature. The control sample contained the same reaction mixture without cDDP.

2.6. Analysis of the interaction of cDDP with MBD6 and MBD6-SXXS using gel filtration, ICP-MS or ICP-OES

250 μL aliquots of a reaction mixture containing 0.2 mg/mL protein in binding buffer were incubated with either native cDDP (4:1 molar ratio cDDP to protein) or with 1% volatile reducing agent triethylphosphene for 24 h at 37 °C. The reaction mixtures were placed in a Millipore filtration unit and washed with 100 volumes of binding buffer (without BME) under anaerobic conditions. The Pt levels were measured by ICP-MS. Control levels of Pt were obtained