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Glutaredoxin 1 is a major player in copper metabolism in neuroblastoma cells

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ABSTRACT

Background: Glutaredoxin 1 (Grx1), a small protein belonging to the thioredoxin family, is involved in redox-regulation since it catalyzes the reduction of protein disulfides and that of mixed disulfides. It was reported to modulate active copper extrusion from cells, by affecting the function of the pumps ATP7A and B. These are components of the network of protein chaperones involved in the control of the homeostasis of copper, an essential, though harmful, metal. However, the effect of Grx1 on copper levels, copper chaperones and copper-elicited cell toxicity was never investigated.
Methods: In order to investigate the effect of Grx1 on copper metabolism, we constitutively overexpressed Grx1 in human neuroblastoma SH-SY5Y cells (SH-Grx1 cells) and assessed a number of copper-related parameters.
Results: SH-Grx1 cells show a basal intracellular copper level higher than control cells, accumulate more copper upon $CuSO_4$ treatment, but are more resistant to copper-induced toxicity. Grx1 shows copper-binding properties and copper overload produces a decrease of Grx1 enzyme activity in SH-Grx1 cells. Finally, Grx1 overexpression decreases copper accumulation in mitochondria upon copper overload and modulates the expression of copper transporter 1 (Ctr1).
Conclusion: Altogether, these data demonstrate that Grx1 is a major player in copper metabolism in neuronal cells.

1. Introduction

Copper is an essential micronutrient for all aerobic organisms because it serves as the catalytic cofactor for many enzymes, including Cu,Zn-superoxide dismutase (SOD1), cytochrome c oxidase (COX), ceruloplasmin and dopamine β-monooxygenase [1]. All cells have evolved mechanisms strictly regulating intracellular copper level to support physiological requirements, while preventing copper accumulation that would lead to oxidative damage. In fact, when in excess or not properly bound to proteins, copper may catalyze the formation of reactive oxygen species that can adversely modify proteins, lipid and nucleic acids thus affecting the overall cell physiology including the function of organelles like mitochondria [2,3].

Copper homeostasis is controlled by protein chaperones, showing one or more copper-binding domains [4]. For the most part, copper enters cells through the high affinity transporter Ctr1, which upon multimerization forms a pore thus allowing the entrance of copper in an ATP-independent fashion, because of its methionine-rich copper-binding domain [5,6]. Ctr1 is regulated by copper concentrations, with the zinc-finger transcription factor Sp1 (Specific protein 1) mediating this response [7]. In the cytosol, copper can be distributed among three different routes, each specific for a cell district; the cytosolic compartment is mainly governed by CCS (copper chaperone for SOD1), which is responsible for the correct insertion of copper in the most abundant cytosolic copper-enzyme [4,6]. The mechanisms by which copper is delivered to mitochondria are not clear; a hypothesis is that it can pass through the mitochondrial membrane bound to a small non-proteinaceous complex. Then, it is transferred by several mitochondrial copper chaperones (COX17, COX11, SCO1 and SCO2) to the active site of COX [8–10]. Cytosolic chaperone Atox1 (also called HAH1) delivers copper in the *trans* Golgi network to the P-type ATPases ATP7A or ATP7B, possessing six CXXC copper-binding domains at the N-terminus. These ATPases are selectively expressed in different tissues and cell types, with ATP7B mainly expressed in liver. By cell trafficking and ATP-dependent catalytic activity, they achieve both the metallation of copper-dependent enzymes of the secretory pathway and efflux of excess copper from the cell [11,12].

Glutaredoxin 1 (Grx1) is a small (12 kDa) protein belonging to the thioredoxin family of proteins, that is abundant in the cytosol, but also

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present in the intermembrane space of mitochondria (IMS). It catalyzes both the reduction of protein disulfides and that of mixed disulfides formed between its substrate glutathione (GSH) and protein thiols (deglutathionylation). One or both redox-active cysteines located in the active site of Grx1 are involved in the catalytic mechanisms [13,14]. In both cases, oxidized GSH (GSSG) is produced, which is regenerated at the expense of NADPH. The reversible formation of mixed disulfides between protein thiols and GSH is a key mechanism in redox-regulation and signaling, comparable to reversible protein phosphorylation.

It was demonstrated that Grx1 interacts with the N-terminus of both ATP7A and ATP7B, and that this interaction requires copper and involves the CXXC motifs of the pumps [15,16]. Furthermore, Grx1 and GSH seem to be involved in the regulation of Atox1 [17]. However, no additional studies were performed to investigate the role played by Grx1 in copper homeostasis.

Here we report the effect of Grx1 on copper metabolism in an experimental model represented by human neuroblastoma SH-SY5Y cells; overexpression of Grx1 induces a number of modifications that suggest a major role for this protein in copper homeostasis.

2. Materials and methods

2.1. Plasmids

Mouse Glutaredoxin 1 (*glrx1*, accession no. NM053108) was cloned in plasmid pcDNA3 by RT-PCR from mouse brain cDNA as previously described [18].

2.2. Cell cultures and treatments

Human neuroblastoma SH-SY5Y cells were purchased from the European Collection of Cell Cultures (Salisbury, UK) and grown in Dulbecco's modified Eagle's F12 medium (Lonza, Milano, Italy) supplemented with 15% fetal calf serum at 37% in an atmosphere of 5% CO_2 in air. Cells were routinely seeded at a density of 2×10^5 cells/ml.

SH-SY5Y cells constitutively expressing Grx1 were obtained by transfection with Lipofectamine Plus (Life Technologies Italia, Monza, Italy) followed by selection with 400 μg/ml G418 (Gibco, Paisley, UK) as described in Ref. [18].

Treatment with copper was performed 24 h after plating by adding $CuSO_4$ to a final concentration of 50 μM or 150 μM for 24 h. Cells were detached in trypsin/EDTA, collected by centrifugation and washed with phosphate-buffered saline (PBS: 10 mM phosphate buffer, 2.7 mM KCl, and 137 mM NaCl, pH 7.4).

2.3. Mitochondrial isolation

Cells were homogenized in mitochondrial buffer (0.2 mM EDTA, 0.25 M sucrose, and 10 mM Tris/HCl, pH 7.4) and Protease Inhibitor Cocktail (Roche, Basel, CH) (1:1000, v:v) in a Potter glass homogenizer with a Teflon pestle. The samples were centrifuged at 600 ×*g* for 10 min at 4 °C and supernatants were then centrifuged at 7,000 ×*g* for 10 min at 4 °C to obtain the crude mitochondrial fraction.

2.4. Cell viability assessment

Cell viability was evaluated in a hemocytometer chamber under a phase contrast microscope by their capacity to exclude Trypan blue (0.2%, w:v). Viability was also estimated by measuring the capability of mitochondrial dehydrogenases to reduce MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethylphenyl]-2-[4-sulphophenyl]-2H-tetrazolium inner salt) (Promega, Madison, WI, USA) to formazan. For this assay, cells were grown and treated with $CuSO_4$ in 96-well plates and the formation of formazan was followed at 490 nm by a microplate reader (Benchmark, Bio-Rad, Hercules, CA, USA).

2.5. Cell lysis and Western blot analysis

Total cell lysates were obtained by resuspending pelleted cells in lysis buffer (10 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100) containing Protease Inhibitor Cocktail (Roche, Basel, CH), for 30 min on ice, followed by centrifugation at 14,000 ×*g* for 30 min. Protein content was assayed according to Ref. [19].

After standard reducing, denaturing SDS–PAGE, Western blot was performed on nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Filters were incubated with the appropriate antibody at the following dilutions: Grx1, goat polyclonal antibody (R&D Systems, Minneapolis, MN, USA), 1:1000; GAPDH, mouse monoclonal antibody (Santa Cruz Biotechnology, CA, USA), 1:1000; CCS, rabbit polyclonal antibody (Santa Cruz Biotechnology, CA, USA), 1:500; ATP7A, mouse monoclonal antibody (BD Biosciences, Franklin Lakes, NJ, USA), 1:2500; Nrf2, rabbit polyclonal antibody (Santa Cruz Biotechnology, CA, USA), 1:500; Ctr1, goat polyclonal antibody (Santa Cruz Biotechnology, CA, USA) 1:500.

Filters were then incubated with the proper peroxidase conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA) and developed using the ECL Advance Western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK).

2.6. Copper, iron and zinc content measurement

Cells in hypotonic PBS (1:2, v:v) were sonicated for 20 s. For copper, iron and zinc content assays, cell lysates were diluted 1:2 (v:v) with 65% nitric acid. After at least 1 week at room temperature, copper, iron and zinc contents were assayed by atomic absorption spectrometry using an AAnalyst 300 instrument equipped with a graphite furnace with platform (HGA800) and an AS-72 autosampler (Perkin-Elmer, Waltham, MA, USA).

2.7. SOD1 activity

SOD1 activity was determined by in-gel inhibition of the staining with nitro blue tetrazolium, as described [20].

2.8. Grx1 activity

Grx1 enzyme activity was determined using β-hydroxyethylene disulfide (HED) as a substrate according to Ref. [21]. The decrease in absorbance at 340 nm was followed using a Perkin Elmer lambda-25 spectrophotometer. Activity was expressed as μmol of NADPH oxidized/min/mg protein (Units/mg protein) using an extinction coefficient of 6,200 M^{-1} cm^{-1}.

2.9. Immobilized metal-affinity chromatography (IMAC)

Copper binding proteins in SH-Grx1 cells were separated using a copper-loaded IMAC column according to [22]. This method is routinely applied for isolation of copper-binding proteins [23]. Both the loading and elution buffer contain 1 M NaCl, avoiding the possibility of unspecific binding of Grx1 to Cu-IMAC. Control runs were performed in the presence of 2 mM EDTA. Nrf2 protein, which is not known to bind copper, and CCS protein, a copper-binding protein, were used as a negative and positive control, respectively.

2.10. RNA preparation and qRT-PCR

Total RNA from SH-SY5Y and SH-Grx1 cells, either untreated or treated with 150 μM $CuSO_4$, was extracted using TRIzol Reagent (Life Technologies Italia, Monza, Italy), according to the manufacturer's instructions. RNA was then reverse transcribed using ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA).