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The small heat shock protein, HSP30, is associated with aggresome-like inclusion bodies in proteasomal inhibitor-, arsenite-, and cadmium-treated *Xenopus* kidney cells

Saad Khan, Imran Khamis, John J. Heikkila *

Department of Biology, University of Waterloo, Waterloo, ON, Canada N2L 3G1

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ABSTRACT

In the present study, treatment of *Xenopus laevis* A6 kidney epithelial cells with the proteasomal inhibitor, MG132, or the environmental toxicants, sodium arsenite or cadmium chloride, induced the accumulation of the small heat shock protein, HSP30, in total and in both soluble and insoluble protein fractions. Immunocytochemical analysis revealed the presence of relatively large HSP30 structures primarily in the perinuclear region of the cytoplasm. All three of the stressors promoted the formation of aggresome-like inclusion bodies as determined by immunocytochemistry and laser scanning confocal microscopy using a ProteoStat aggresome dye and additional aggresomal markers, namely, anti-γ-tubulin and anti-vimentin antibodies. Further analysis revealed that HSP30 co-localized with these aggresome-like inclusion bodies. In most cells, HSP30 was found to envelope or occur within these structures. Finally, we show that treatment of cells with withaferin A, a steroidal lactone with anti-inflammatory, anti-tumor, and proteasomal inhibitor properties, also induced HSP30 accumulation that co-localized with aggresome-like inclusion bodies. It is possible that proteasomal inhibitor or metal/metalloid-induced formation of aggresome-like inclusion bodies may sequester toxic protein aggregates until they can be degraded. While the role of HSP30 in these aggresome-like structures is not known, it is possible that they may be involved in various aspects of aggresome-like inclusion body formation or transport.

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1. Introduction

Eukaryotic cells have developed strategies to ameliorate or remove potentially toxic stress-induced misfolded or aggregated proteins (Morimoto, 1998; 2008; Richter et al., 2010; Tyedmers et al., 2010). One of the first lines of defense is the stress-induced accumulation of heat shock proteins (HSPs), which are molecular chaperones that bind to unfolded protein, prevent their aggregation, and promote proper folding/refolding once the stress has been alleviated. Damaged proteins that cannot be refolded are degraded by the ubiquitin-proteasome system (UPS). Proteins destined for degradation are marked by the addition of ubiquitin molecules followed by their hydrolysis via the 26S proteasome in an ATP-dependent mechanism. Exceeding or inhibiting the ability of the proteasome to degrade protein can lead to an accumulation of unfolded and aggregated proteins. In fact, UPS impairment has been described in a range of human diseases including Alzheimer's, Parkinson's, Huntington's, and amyotrophic lateral sclerosis (Masliah et al., 2000; Taylor et al., 2002; Ross and Pickart, 2004). One mechanism by which the cell deals with this issue is by transporting protein aggregates along microtubules employing dynein motors, facilitated by histone deacetylase 6 (HDAC6), to the perinuclear region (Kopito, 2000; Garcia-Mata et al. 2002; Ito et al., 2002; Bauer and Richter-Landsberg, 2006; Rodriguez-Gonzalez et al., 2008; Bolhuis and Richter-Landsberg, 2010; Shen et al., 2011; Driscoll and Chowdhury, 2012; Hao et al., 2013; Nakajima and Suzuki, 2013; Richter-Landsberg and Leyk, 2013). Aggresome formation is a dynamic process in which small inclusion bodies coalesce to form larger aggresomes until they can undergo proteolysis by autophagy. These microtubule-dependent inclusion bodies contain γ-tubulin and vimentin, a type III intermediate filament, which forms a cage-like structure around the exterior of aggresomes. The process of aggresome formation reduces the cytotoxic effects of scattered cellular protein aggregates.

The impairment of the UPS in yeast, *Drosophila*, and mammalian cultured cells was reported to induce the accumulation of HSPs, including members of the small HSP superfamily (Lee and Goldberg, 1988; Ito et al., 2002; Lundgren et al., 2005; Noonan et al., 2008). Small HSPs, which are divergent in a range of organisms, are molecular chaperones with roles in actin capping/decapping, cellular differentiation, and prevention of apoptosis (MacRae, 2000; Van Montfort et al., 2002; Arrigo, 2005; Sun and MacRae, 2005; Acunzo et al., 2012; Garrido et al., 2012). During stress, small HSPs form multimeric complexes that bind to unfolded protein, inhibit their aggregation, and facilitate their refolding. Small HSP synthesis or their mutations were associated with

* Corresponding author. Tel.: +1 519 888 4567x33076; fax: +1 519 746 0614.
E-mail address: heikkila@uwaterloo.ca (J.J. Heikkila).

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diseases such as Alzheimer's, Charcot-Marie-Tooth, hereditary motor neuropathy type 2, Parkinson's, cancer, and desmin-related myopathy (Sun and MacRae, 2005; Chang et al., 2009; Acunzo et al., 2012; Carra et al., 2012; Garrido et al., 2012; Kampinga and Garrido, 2012). Interestingly, in Parkinson's disease animal models, small HSPs plus HSP70 prevented neurodegeneration by inhibiting protein misfolding and aggregation (Bruinsma et al., 2011; Chaari et al., 2013). Also, sHSP overexpression facilitated the clearance of misfolded proteins in motor neuron disease and reduced the number of Alzheimer's amyloid plaques in APP/PS1 mice (Carra et al., 2012; Toth et al., 2013).

In the amphibian model system, *Xenopus laevis*, the most intensively studied stress-inducible small HSP is HSP30 (Heikkila et al., 1987; Krone et al., 1992; Lang et al., 1999; Heikkila, 2004; Heikkila, 2010). In *Xenopus* A6 kidney epithelial cells, HSP30 accumulation was induced by a range of stresses including heat shock, and the environmental toxicants, sodium arsenite, and cadmium chloride (Gellalchew and Heikkila, 2005; Voyer and Heikkila, 2008; Woolfson and Heikkila, 2009; Young et al., 2009; Heikkila, 2010; Khamis and Heikkila, 2013). HSP30 molecules have a conserved C-terminal α-crystallin domain that is flanked by a poorly conserved C-terminal extension containing hydrophilic amino acids and an N-terminal region (Krone et al., 1992; Fernando and Heikkila, 2000). HSP30 functions as a molecular chaperone since it could bind to unfolded client protein, inhibit their aggregation, and maintain them in a folding competent state (Fernando and Heikkila, 2000; Abdulle et al., 2002; Fernando et al., 2002; Fernando et al., 2003). Interestingly, HSP30 has counterparts in other frogs as well as birds and fish but not in mammals (Norris et al., 1995; Helbing et al., 1996; Katoh et al., 2004; Elicker and Hutson, 2007; Mulligan-Tuttle and Heikkila, 2007; Heikkila, 2010). Previously, we determined that inhibition of the UPS in *Xenopus* A6 kidney epithelial cells by the proteasomal inhibitor, MG132, induced the accumulation of HSP30 (Young and Heikkila, 2010). Furthermore, we determined that treatment of A6 cells with sodium arsenite or cadmium chloride induced HSP30 accumulation and inhibited UPS function (Brunt et al., 2012). Immunocytochemical analysis revealed that MG132 and cadmium chloride induced the accumulation of HSP30 in the cytoplasm in a punctate pattern as well as larger HSP30 structures, some of which were detected in the perinuclear region (Woolfson and Heikkila, 2009; Young and Heikkila, 2010). A re-evaluation of the immunocytochemical images of cells treated with sodium arsenite also indicated the presence of large HSP30 structures primarily in the perinuclear region (Gauley et al., 2008).

In this study, we investigated whether these large HSP30 perinuclear structures induced by MG132, sodium arsenite, or cadmium chloride were associated with aggresome-like inclusion bodies. An examination of this possibility was indicated since recently we observed the association of HSP30 with tentatively identified aggresome-like structures (using a ProteoStat aggresome dye) in heat-shocked A6 cells that had recovered for 24 h (Khan and Heikkila, 2014). In the present study, initial studies examined the presence of HSP30 in the soluble and insoluble protein fractions isolated from A6 cells treated with either MG132, sodium arsenite, or cadmium chloride. This was followed by immunocytochemical analysis to determine whether these aforementioned stressors promoted the accumulation of aggresome-like inclusion bodies using ProteoStat aggresome dye, and anti-γ-tubulin and anti-vimentin antibodies. We then examined whether these stress-induced aggresome-like antibodies were associated with HSP30. Finally, we extended this analysis to determine whether withaferin A treatment of A6 cells induced aggresome-like inclusion bodies and whether they were associated with HSP30. Withaferin A is a steroidal lactone with anti-inflammatory and anti-tumor properties. For example, this compound demonstrated therapeutic properties in a cystic fibrosis in vitro model system as well as inhibiting tumor growth in mice (Yang et al., 2007; Mandal et al., 2008; Maitra et al., 2009). Recently, we found that withaferin A inhibited the UPS in *Xenopus* A6 cells and induced the accumulation of HSP30 (Khan et al., 2012).

2. Materials and methods

2.1. Maintenance and treatment of cultured cells

Xenopus laevis A6 kidney epithelial cells were obtained from the American Type Culture Collection (CCL-102; American Type Culture Collection (ATCC), Rockville, MD). The cells were grown in 55% Leibovitz L-15 Media containing 10% (v/v) fetal bovine serum (100 U/ml) and 1% penicillin/streptomycin (100 μg/ml; all purchased from Sigma–Aldrich, Oakville, ON) at 22°C in T75 cm^2 BD falcon culture flasks (BD Biosciences, Mississauga, ON). A6 cells were maintained at 22 °C (control) or treated with 30 μM MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; Sigma–Aldrich; stock solution of 21 mM was dissolved in DMSO and stored at −20 °C) or 20 μM sodium arsenite or 100 μM cadmium chloride (both from Sigma–Aldrich) for 16 h at 22 °C. After the different treatments, cells were rinsed using 65% HBSS and removed via scraping in 1 ml of 100% HBSS. Cells were centrifuged at 21,900 × *g* for 1 min and the resulting pellets were stored at −80 °C until protein isolation.

2.2. Protein isolation, quantification, and immunoblot analysis

Total protein was isolated from A6 cells as previously described (Young et al., 2009; Khan et al., 2012). Protein was quantified using a bicinchoninic acid protein assay kit following the manufacturer's instructions (Thermo Scientific, Rockford, IL). Protein samples (20 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a BioRad Mini Protean III gel system (BioRad). Electrophoresed proteins were transferred to a nitrocellulose membrane with a Trans-Blot Semi-Dry Transfer Cell (BioRad) at 25 volts for 20 min. Blots were then stained with Ponceau S (Sigma–Aldrich) to determine transfer efficiency. Immunodetection was carried out using either the polyclonal rabbit anti-*Xenopus* HSP30 antibody (Fernando and Heikkila, 2000; 1:1000 dilution) or the polyclonal rabbit anti-actin antibody (Sigma–Aldrich; 1:200 dilution). The polyclonal anti-*Xenopus* HSP30 antibody was produced by our laboratory and shown to cross-react with all *Xenopus* HSP30 family members (Fernando and Heikkila, 2000). The membranes were then incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (1:3000). For detection, the membranes were incubated at room temperature in alkaline phosphatase detection buffer (100 mM Tris base, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) with 0.3% 4-nitro blue tetrazolium (NBT; Roche) and 0.17% 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP; Roche).

2.3. Isolation of soluble and insoluble protein

In order to isolate soluble and insoluble protein fractions under non-denaturing conditions, cell pellets were re-suspended in 400 μL of solubilizing buffer (50 mM Tris (pH 7.5), 1% Nonidet P-40, 2 mM EDTA, 100 mM NaCl, 50 mM $MgCl_2$, 10 mM N-ethylmaleimide, and 1X Protease inhibitor cocktail (Promega). Re-suspended cell pellets were dispersed using a 20-G needle followed by incubation for 2 h at 4 °C in a nutator. Insoluble fractions were pelleted using a refrigerated Eppendorf microcentrifuge at 21,900 × *g* at 4 °C for 10 min. The soluble protein fractions (supernatants) were adjusted to a 1 X loading buffer (0.0625 M Tris (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.00125% (w/v) bromophenol blue) using a 5 X concentrate. The insoluble protein fractions (pellets) were resuspended in 400 μL of 1 X loading buffer. Immunoblot analysis of equivalent volumes of soluble and insoluble protein fractions was performed as described above.

2.4. Immunocytochemistry and laser scanning confocal microscopy

Immunofluorescence analysis was carried out as previously described (Manwell and Heikkila, 2007; Young et al., 2009; Khan and

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