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Recombinant human Tat-Hsp70-2: A tool for neuroprotection

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ABSTRACT

Human Hsp70-2 is a chaperone expressed mainly in the nervous system. Up to now, no study has reported on the recombinant expression of this important human chaperone. Herein, we describe the successful purification and characterization of recombinant human Hsp70-2 in *Escherichia coli* in both the full-length and the chimeric protein containing the protein transduction domain corresponding to the trans-activator of transcription (Tat) from HIV. Under optimized conditions, the Tat-Hsp70-2 was expressed in a soluble form and purified by two chromatographic steps (in a 3.6 mg/L fermentation broth yield): recombinant Tat-Hsp70-2 was folded and showed ATPase activity. In contrast, the full-length recombinant protein was only expressed in the form of inclusion bodies and thus was purified following a refolding procedure. The refolded Hsp70-2 protein was inactive and the protein conformation slightly altered as compared to the corresponding Tat-fused variant. The Tat-Hsp70-2 protein (100 nM), when added to human neuroblastoma SH-SY5Y cells subjected to hydrogen peroxide or 6-hydroxydopamine stress, partially protected from the deleterious effect of these treatments. This work describes an approach for the functional expression of human Tat-Hsp70-2 that provides sufficient material for detailed structure-function studies and for testing its ability to protect neuroblastoma cells from oxidative stress.

1. Introduction

Heat shock proteins (Hsp) are classified according to their molecular mass (in kiloDaltons): Hsp110, Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small Hsps (typically 20–25 kDa). Hsp proteins play different functional roles, which include assisting nascent protein folding, protein translocation, modulating assembly/disassembly of protein complexes, and degradation of unstable and/or misfolded proteins [1,2]. Hsp proteins play key roles in maintaining cellular homeostasis and driving cell fate in response to physical or chemical stress. Interacting protein-binding affinity is regulated through conformational changes of Hsp resulting from ATP binding/hydrolysis.

The Hsp70 family is one of the most evolutionarily conserved protein families [1], present in a wide array of different species [3]. It consists of at least 14 isoforms encoded by 17 different genes: their sequence conservation extends to functional overlap within the family, with cellular localization serving, in part, as a means of regulating their functional activity and cellular role [4,5]. The Hsp70 family members are known to prevent the protein misfolding and aggregation associated with neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, as well as to protect cells against heat shock, oxidative stress, and apoptotic stimuli in numerous cell death pathways [6,7]. Hsp70s are composed of a highly homologous N-terminal ATPase domain, a substrate-binding domain, and a C-terminal variable domain [3]: their chaperone activity is dependent on close interaction between the substrate and the ATPase domains.

The functional characterization and application of human Hsp

Abbreviations: Tat, trans-activator of transcription from HIV; Hsp, heat shock protein; IPTG, isopropyl thio-β-D-galactoside; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; 6-OHDA, 6-hydroxydopamine.

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proteins have been hampered by difficulties associated with large-scale heterologous overproduction of purified protein. Actually, a large part of the data published on human Hsp70 family deal with Hsp70-1 (isoforms a and b), i.e., the main stress-induced members. We focused our interest on Hsp70-2 (84% sequence identity with Hsp70-1), which is constitutively expressed at low levels in most tissues but in high quantities in testis and brain (mainly in the nervous system, indicating a special role for this chaperone) [4]; it is located in the nucleus, lysosomes, and cytoplasm.

The sequences known as protein transduction domains or cell-penetrating peptides have been used to mediate the translocation of proteins and other compounds across the cellular membrane and into the brain [8,9]. The basic domain of the trans-activator of transcription (Tat) from HIV has been reported to effectively transduce different proteins, including Hsp70 proteins [10–12].

The goal of our study was to generate an efficient expression system for recombinant human Hsp70 corresponding to the neuronal form and to test whether Tat-mediated Hsp70-2 is an effective neuro-protectant in models of neurodegeneration. While the wild-type, full-length Hsp70-2 was produced in inclusion bodies and did not recover ATPase activity after protein renaturation, the Tat-fused version was efficiently expressed as soluble, active protein. We delivered Tat-Hsp70-2 into the SH-SY5Y neuroblastoma cells challenged with oxidant stimuli at increasing doses and examined whether cultured cells were protected.

2. Materials and methods

2.1. Design and cloning of Hsp70-2 genes

Two synthetic genes for human Hsp70-2, the first encoding the full-length protein and the second containing the additional sequence corresponding to the Tat transduction domain (GenBank accession number KU894588 and KU894589, respectively), were designed by *in silico* back translation of the amino acid sequence reported in the data bank (GenBank accession number NM_021979). The codon usage was optimized for expression in *Escherichia coli*; for details, see Suppl. Table 1. Genes were produced by GeneArt (Thermo Fisher Sci., Waltham, MA, USA).

In order to facilitate subcloning into the pRSET A plasmid (carrying the resistance to ampicillin, Invitrogen, Thermo Fisher Sci.), *Bam*HI (GGATCC) and *Hin*dIII (AAGCTT) sites were added at the 5′- and 3′-ends of the synthetic genes, respectively. The resulting expression cassette includes a sequence encoding six histidine residues at the N-terminal end of both Hsp70-2 protein forms (see Suppl. Fig. 1). The Hsp70-2 genes were inserted in the pRSET A vector using *Bam*HI and *Hin*dIII sites.

2.2. Optimization of Hsp70-2 expression conditions

For protein expression, recombinant pRSET A plasmids encoding Hsp70-2 and Tat-Hsp70-2 were first transferred to the BL21(DE3) pLysS *E. coli* host. In order to identify the best conditions for producing both Hsp70-2 variants in soluble form, the following parameters were investigated using LB medium [13]: IPTG concentration (0.1 or 1 mM), growth temperature after inducing protein expression (20 or 37 °C), and time of cell harvest after adding IPTG (up to 24 h). For Hsp70-2 only, three additional parameters were investigated: the *E. coli* host strain: BL21(DE3)pLysS, Origami(DE3)pLysS (which mutations in thioredoxin reductase and glutathione reductase genes enhance disulfide bond formation in the cytoplasm), or BL21 STAR(DE3) (which mutation in RNase E and outer membrane protease OmpT genes allow to increase mRNA stability and to reduce protease activity, respectively), the fermentation broth (LB + 5 g/L NaCl or Terrific broth, TB), induction of protein expression by IPTG (0.1 or 1 mM), and 25 g/L NaCl. See Suppl. Table 2.

Finally, to further improve the solubility of Hsp70-2 and Tat-Hsp70-2, IPTG was removed from the medium: cells were collected by centrifugation at 2600 *g* for 10 min at 25 °C and the cell pellet was re-suspended in 1/10 of fresh medium supplemented with 30 μg/mL kanamycin, 100 μg/mL ampicillin, and 34 μg/mL chloramphenicol. After another two hours of incubation of the culture at 20 °C, the cells were harvested by centrifugation at 8000 *g* for 10 min at 4 °C [14].

2.3. Purification of recombinant Hsp70-2 variants

Recombinant Hsp70-2 variants were expressed in the BL21(DE3)pLysS *E. coli* strain. Starter cultures were prepared by growing a single colony of *E. coli* cells carrying the recombinant plasmids overnight at 37 °C in flasks containing LB to which ampicillin and chloramphenicol (respectively 100 μg/mL and 34 μg/mL, final concentration) were added; this culture was diluted in the same medium to a starting OD_{600nm} = 0.1. For preparation purposes, 2 L baffled Erlenmeyer flasks containing 350 mL of liquid LB broth were used. Cells were grown at 37 °C with shaking (200 r.p.m.) until reaching an OD_{600nm}—0.6—0.8, at which time 1 mM IPTG was added and growth temperature was decreased to 20 °C. Cells expressing Hsp70-2 were collected after 5 h, whereas those expressing Tat-Hsp70-2 were collected after ~20 h. Then, in order to increase the solubility of Tat-Hsp70-2, cells were treated by using the procedure aimed at eliminating IPTG (see section on "Optimization of Hsp70-2 expression conditions") [14].

Cell pellets expressing Tat-Hsp70-2 were resuspended in freshly prepared lysis buffer (3.5 mL/g wet cells) containing 20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 150 mM NaCl, 1% (v/v) glycerol, 0.7 μg/mL pepstatin, 0.19 mg/mL phenylmethylsulfonil fluoride, and DNase and disrupted by sonication - eight cycles of 30 s each, on ice, using a Branson Sonifier 250 (Branson Ultrasonics, Danbury, CT, USA). The insoluble fraction was removed by centrifugation at 39,000 *g* for 1 h at 4 °C. Recombinant Tat-Hsp70-2 containing an N-terminal His-tag was purified from soluble extracts using Ni^{2+}-affinity chromatography (HiTrap Chelating column, GE Healthcare, Little Chalfont, UK) equilibrated with 50 mM sodium pyrophosphate, pH 7.5, and 1 M NaCl. The bound protein was eluted with 500 mM imidazole in 50 mM sodium pyrophosphate, pH 7.5, and 1% (v/v) glycerol. Imidazole was then removed by overnight dialysis at 4 °C against 10 mM Tris-HCl, pH 8.0, and 250 mM KCl. This Tat-Hsp70-2 preparation was subjected to a second step of purification by size-exclusion chromatography on a Superdex 200 Increase column (GE Healthcare) in 10 mM Tris-HCl, pH 8.0, and 250 mM KCl.

Cell pellets from *E. coli* BL21(DE3)pLysS cells expressing Hsp70-2 were resuspended in freshly prepared lysis buffer (3.5 mL/g wet cells) containing 20 mM Tris-HCl, pH 8.0, 0.7 μg/mL pepstatin, 0.19 mg/mL phenylmethylsulfonil fluoride and DNase, were disrupted by sonication (four cycles of 30 s each, on ice), and centrifuged at 39,000 *g* for 20 min at 4 °C. The pellet containing the inclusion bodies was resuspended in 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2% (v/v) Triton X-100 containing 2 M urea, sonicated (five cycles of 10 s each, on ice), and centrifuged at 39,000 *g* for 10 min at 4 °C. To isolate inclusion bodies, the pellet was subjected to a second round of urea wash and then washed further with the same buffer lacking urea. The inclusion bodies were solubilized in binding buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, and 1 mM 2-mercaptoethanol), stirred for 1 h at room temperature, and centrifuged at 39,000 *g* for 15 min at 4 °C. The remaining particles were removed by filtration through a 0.22 μm filter. Recombinant