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Cloning and expression of Hsp40 variants to study the functional association of human disaggregase

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CONTENTS

1. INTRODUCTION

1.1. MOLECULAR CHAPERONES AND PROTEIN AGGREGATION

Once a protein has been synthesised, it has to reach its native, stable, active conformation through a more or less complex process known as protein folding. Although some newly translated proteins are able to fold spontaneously, a significant fraction of proteins, particularly multidomain ones with complex structures, may expose hydrophobic residues to the polar solvent during folding, which might cause intermolecular non-native interactions that lead to aggregation. Furthermore, as a consequence of the flexibility requirement to function, proteins are only marginally stable in their physiological environment and can therefore be easily denatured when they are subjected to stress conditions, becoming susceptible to aggregation. Protein aggregation causes deleterious effects to all cells, as their functional units can be seized by a growing mass of partially (un)folded, aggregated polypeptide chains that eventually collapse the cell. To avoid this situation, cells have evolved a multicomponent protein quality control system responsible for the maintenance of protein homeostasis (or proteostasis), an essential process for cell fitness. Different components of this system are finely coordinated and include molecular chaperones with their accessory proteins, and the ubiquitin-proteasome and autophagy systems that remove misfolded and aggregated proteins (Hipp et al., 2014, Labbadia et al., 2015).

Molecular chaperones are central to the function of this network, and are classified in different families according to their molecular masses (Kim et al., 2013). In particular, they can counter the toxic properties of aggregates of different protein sequences that are formed when the effort to sustain cellular protein homeostasis fails. Protein conformational diseases such as Alzheimer, Parkinson and Huntington underscore the importance of protein quality control mechanisms for cell survival. In neurons, there are significant demands on cellular folding events such as the regulation of dynamic protein complexes involved in synaptic transmission, and of conformational changes in specific proteins that modulate their activity. Therefore, quality control mechanisms are required to avoid the detrimental effects of functionally impaired proteins and protein complexes. The regulation of molecular chaperones is thus essential to ensure cell survival and fitness.

A mechanistic understanding at the molecular level of chaperone action is required to unravel how they associate to form functional complexes, and to understand how they remodel and disentangle toxic protein oligomers and aggregates. (Jeng et al., 2015)

1.2. THE BACTERIAL AND HUMAN DISAGGREGASE SYSTEMS

The DnaK system in bacteria and the Hsp70 system in human cells are the chaperone networks able to prevent aggregation of partially (un)folded substrates, thus favouring their proper folding, and to remodel and reactivate protein aggregates formed during prolonged harsh conditions. The *E. coli* chaperone DnaK, along with the co-chaperone DnaJ and the Nucleotide Exchange Factor (NEF) GrpE build up the DnaK system. This system is engaged in *de novo* protein folding, prevents protein aggregation, and disaggregates and reactivates protein aggregates in cooperation with the disaggregase ClpB (Mayer, 2013).

The homologous system in human cells is the Hsp70 system, which works in a similar way than the bacterial one. The functional cycle of this system starts with the binding of Hsp40s to their Hsp70 partner, with the simultaneous transfer of unfolded substrates and activation of its ATPase activity. Moreover, this system is able to disentangle and refold aggregated polypeptides when powered by an Hsp110 co-chaperone that functions as a NEF and could also have foldase activity (Nillegoda NB. et al., 2015). The higher intrinsic disaggregation capacity of the human Hsp70 system compared to the yeast and bacterial systems might be an adaptation of metazoan Hsp70s to the lack of a dedicated disaggregase, i.e., a member of the Hsp100 family such as ClpB in *E. Coli* and Hsp104 in yeast (Aguado et al., 2015).

1.3. HSP40 AND DNAJ

It has been recently recognized that Hsp70 serves as a central hub and that Hsp40s or J proteins are the switches for diverse proteostasis events, thus fuelling further investigations to understand the specific role of the different J proteins. Hsp40 molecular chaperones constitute a large and diverse family of 40 kDa molecular chaperones classified into four different types (I, II, III and IV). They all share a J domain with a conserved His-Pro-Asp (HPD) motif that is located in the N-terminus and stimulates the ATPase activity of Hsp70. Type I and II also have the conserved

ability to bind and deliver non-native proteins to Hsp70, which is essential for life. Type I Hsp40s are descendants of bacterial DnaJ and contain the J domain followed by a flexible glycine/phenylalanine rich region (G/F), a zinc finger like region (ZFLR), and a conserved C-terminal domain. The main difference between type I and type II Hsp40 members is the presence of two conserved zinc-finger motifs within Type I but not Type II, that interact with non-native substrates along with the peptide binding fragment. The C-terminal peptide binding fragment is involved in protein dimerization and in substrate transfer to Hsp70 partner (Li et al., 2009). Type III Hsp40s contain the J domain, but none of the other conserved domains found in Types I and II. Instead, they often have specialized domains that localize them to certain areas of the cell and provide specificity in substrate binding. Type IV Hsp40 members, only contain the J domain without the HPD motif, and therefore do not interact with Hsp70.

In many instances Hsp40s dimers, either homo or hetero-dimers, interact with Hsp70, but a general requirement for dimerization in Hsp70 function has not been demonstrated. Among the different types of Hsp40s, Type I and II act independently of Hsp70 as chaperones, as evidenced by their ability to bind denatured proteins and prevent their aggregation. They show different substrate specificity and direct Hsp70 to perform different functions *in vivo*. Sequence analysis reveals two possible regions that may be responsible for this functional specialization: the G/F region, which when present can have different length, and the protein modules located at the middle of their sequences, which can be a ZFLR or a G/M rich region. Therefore, it is plausible that either the G/F or the central protein domains serve to specify their *in vivo* function (Fan et al., 2003).

In human cells, there are more than 50 members, with common functions but independent and different efficacies. Two members are involved in protein folding and aggregate reactivation, DnaJA2 and DnaJB1. It has been recently described that they differ in their preference for different aggregates, which could drive Hsp70 to remodel specific unfolded substrates or aggregates. They also differ in the length of their G/F region, which could regulate a different interaction with substrates and/or Hsp70 that might explain their functional specificity (Celaya et al., 2016 and Nillegoda et al., 2015). To prove this hypothesis is the general aim of this project.

2. OBJECTIVE OF THE WORK

The general aim of this project is to determine whether the G/F region of human DnaJA2 and DnaJB1, the Hsp40s involved in protein folding and aggregate reactivation, regulates the functional specificity of Hsc70 and Hsp70. To this aim, it is necessary to clone, express and purify full-length and ΔG/F variants of these human members of the Hsp40 family. To test if the role of this specialized protein region has been conserved during evolution, the same task will be performed with the bacterial DnaJ.

3. MATERIALS AND METHODS

3.1. *E. COLI* STRAINS

The *E. Coli* strains DH5α and XL1-Blue were used for the maintenance of the plasmids, while Rosetta pLys and BL21DE3 strains were employed for expression of the chaperones. The specific strains utilized for each protein are indicated in **Table 1**.

3.2. PLASMIDS

The plasmids containing the WT variants of each DnaJ protein were pE-SUMO (LifeSensors) and pET-22b (Novagen) (**Table 1**). pE-SUMO expresses the recombinant protein as a fusion protein with SUMO (Smt3), a strategy that improves the solubility and folding in the host bacterial cell. Moreover, the pE-SUMO vector facilitates the purification process as the fusion recombinant protein includes an N-terminal histidine tag.

3.3. PCR

PCR (*Polymerase Chain Reaction*) was used to amplify the sequence of proteins contained in a template DNA. The reaction consisted of a mixture of a polymerase, dNTPs (250μM) and primers (0.5 μM), to amplify the cDNA sequence of a protein that was later inserted in a vector. Annealing temperatures and extension times were adjusted according to the features of the samples amplified (see **Table 1**). We always used 30 cycles and a first denaturation step of 30 seconds at 93ºC for amplification. PCR products were purified using the “PCR clean-up protocol” kit (Macherey-Nagel).

3.3.1. Generation of the Tetracysteine full-length, deletion and single point DnaJ variants

Single point mutant: the single point mutant DnaJB1 G278C was obtained using the "*Quickchange II Site-Directed Mutagenesis kit*" (Agilent Technologies). Primers were designed with two regions of at least 10 nucleotides flanking the mutation site and a total length of 25 nucleotides. The primers used are indicated in **Table 1**. All the sample reactions were prepared according to the manufacturer instructions, with the following modifications: the maximum number of cycles -18- indicated in the protocol was used; the elongation time was increased 1 minute; 4 μL of the *DpnI*-treated DNA were used for transformation; cells were incubated 10 minutes in ice after the heat pulse; and LB (Luria-Bertani) broth was used instead of the NZY^+ broth.

Tetracysteine full-length variants: To take advantage of the possibility to add the tetracysteine (CCPGCC) tag into the N-terminus of any protein for fluorescence labelling, we modified the pE-SUMO vector to create one that carries the CCPGCC tag and thus can be used to easily clone any protein. First, we used PCR to insert the CCPGCC into pE-SUMO vector. Two primers were designed, one of them carrying the CCPGCC sequence just after the SUMO fusion protein, and the other one at the start of the SUMO and His-tag sequence:

SUMOLF: CATGCCATGGGTCATCACCATC
SUMOLR: GCTCTAGAGGTCTCAACAGCAACCCGGACAACATCCACCAATCTGTTCGCGG

The PCR product was cloned into the pE-SUMO vector, creating a new one that carries the CCPGCC sequence (pE-SUMO4Cys). Second, we cloned human DNAJA2 and DNAJB1 into the pE-SUMO4Cys vector following classical cloning procedures: PCR of the protein coding sequences (cDNAs of DnaJA2 and DnaJB1), digestion of the PCR product and the vector carrying the CCPGCC tag with two endonucleases, and ligation of the fragments.

G/F deletion variants: The G/F domain of human DnaJA2 and DnaJB1 and bacterial DnaJ was eliminated using the "*Phusion Site-Directed Mutagenesis Kit*" (Thermofisher Scientific). This approach consisted of an inverse PCR that amplifies the whole DNA sequence of the plasmid except the deleted region using 5'-phosphorylated primers, followed by a ligation that generated the new plasmid containing the sequence of our protein with the deletion. Primers used for these mutants are shown in **Table 1**.

Table 1. Summary of the plasmids, primers, PCR conditions and *E. Coli* strains used during the cloning process.

Protein obtained	Plasmid	Primers	*Annealing T[a] (ºC)*	*Extension Time (s)*	*E. Coli* strain	Type of process	Future use
CCPGCC-DnaJA2	pE-SUMO4Cys	**HJA2LF** ATCGTCTCACTGTGGTAAAGACTACTACCAG **HJ3R** GCGGATCCTTACTGATGGGCACACTG	48	60	Maintenance: DH5α Expression: Rosetta pLys	Classical cloning vector + insert	FRET studies
CCPGCC-DnaJB1	pE-SUMO4Cys	**HJB1LF** ATGGTCTCACTGTGCTAACGTGGCTGACAC **HJ1LR** GCGGATCCCTATATTGGAAGAACCTGC	55	150	Maintenance: Xl1-Blue Expression: Rosetta pLys	Classical cloning vector + insert	FRET studies
DnaJB1 G278C	pE-SUMO	**JB1G278CF:** 5' CCCACTCTGGACTGCAGGACGATAC 3' **JB1G278CR :** 5' GTATCGTCCTGCAGTCCAGAGTGGG 3'	55	150	Maintenance: DH5α Expression: Rosetta pLys	Mutagenesis protocol (Quickchange kit)	FRET studies
DnaJA2ΔG/F	pE-SUMO	**JA2DGFPHOF** 5'PHO GCCTTCCCGAAGACCTTGC 3' **JA2DGFPHOR** 5'PHO GGCAATCAGAGTAGAAGTCGAAATGG 3'	63.5	225	Maintenance: DH5α Expression: Rosetta pLys	Mutagenesis protocol (thermofiser Phusion Mutagenesis kit)	ATPases Refolding Interaction
DnaJB1ΔG/F	pE-SUMO	**JB1DGFPHF** 5'PHO CCCCTTTAGGCCTTCCTCCC 3' **JB1DGFPHR** 5'PHO GGCCGCTCCCGCTCTG 3'	68.6	210	Maintenance: DH5α Expression: Rosetta pLys	Mutagenesis protocol (thermofiser Phusion Mutagenesis kit)	ATPases Refolding Interaction
DnaJΔG/F	pET-22b	**JECODGFPHOF** 5'PHO ACCTTGCTCAAACGCAGCAT 3' **JECODGFPHOR** 5'PHO GGACGTGGTCGTCAACGTG 3'	68.5	195	Maintenance: DH5α Expression: BL21DE3	Mutagenesis protocol (thermofiser Phusion Mutagenesis kit)	ATPases Refolding Interaction

3.3.2. Analysis of the selected colonies

After transformation with the ligation product, the colonies obtained were analyzed by digestion of the plasmidic DNA.

A small overnight liquid culture in LB was prepared from single colonies. A fraction of saturated culture was used for plasmid extraction and purification using the "*Miniprep protocol kit*" (Nucleospin Plasmid, Macherey-Nagel). Plasmidic DNA was digested with restriction endonucleases and the resulting fragments analyzed by electrophoresis. All mutations were also confirmed by DNA sequencing performed at the UPV/EHU SGIKER service. The remaining fractions of the cultures corresponding to positive colonies were stored in glycerol (80% v/v) at -80ºC after the analysis.

3.4. DNA ELECTROPHORESIS

DNA electrophoresis in agarose gels was used to separate DNA fragments/molecules according to their size. DNA is a negatively charged molecule at neutral pH, so that under an electric field it migrates towards the positive electrode through a polymerized agarose matrix. This matrix functions as a molecular sieve that separates DNA molecules according to their size. DNA samples were prepared in loading buffer containing 0.25% (w/v) bromophenol blue and 30% (v/v) glycerol and loaded into the gel. The electrophoresis was run at 80 V in TAE buffer, DNA was visualized in an ultraviolet transiluminator labelled with a relation 1:10000 (v/v) of SYBR-Safe (Invitrogen). Size band was compared with a standard DNA 1Kb (Roche) ladder.

3.5. EXPRESSION SYSTEM

3.5.1. Transformation of selected strains

XL1-Blue cells were transformed by heat-shock, while DH5α, BL21DE3 and Rosetta pLys were transformed by electroporation in a Gene Pulser Xcell (BioRad) electroporator. Cells (DH5α or XL1Blue) with the recombinant DNA product of the ligation were selected on LB medium agarose plates supplemented with ampicillin (100 µg/ml). Once the sequencing service had confirmed the sequence, the purified plasmidic DNA was used to transform expression electrocompetent cells (Rosetta pLys/BL21DE3). For Rosetta pLys, selection was performed with ampicillin (100

µg/ml) and chloroamphenicol (35 µg/ml), while BL21DE3 cells were selected with ampicillin (100 µg/ml)

3.5.2. Expression and solubility studies

Cell cultures of Rosetta pLys/BL21DE3 carrying each protein construct were grown at 37 ºC over night under shaking in LB medium (25 mL) supported with ampicillin (100 µg/ml) and chloroamphenicol (35 µg/ml). The next day, 1 mL of saturated cell culture was employed to inoculate fresh LB medium (25mL) containing ampicillin (100 µg/ml) and chloroamphenicol (35 µg/ml) until the $O.D._{600}$ of 0.6. Induction was performed by the addition of 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and the culture was maintained at 30 ºC for 4 hours. A sample was analyzed every hour during the induction period. This procedure was carried out with all the proteins used in this work.

To examine protein solubility, cells induced 4 h with IPTG at 30ºC were harvested by centrifugation at 6000 x g at 4 ºC for 15 minutes, and cell pellets were resuspended in 2 mL of lysis buffer (50 mM Tris HCl pH 8, 550 mM NaCl, 1 mM β-mercaptoetanol, 0.2% Triton-X100, 1 mM imidazole, 1 mM PMSF, 20% sucrose). Cells were disrupted by 4 cycles of sonication for 20 seconds with 30 seconds intervals between pulses. The cell lysate was centrifuged at 35000 x g at 4ºC for 45 minutes. After centrifugation, the sample was divided in pellet and supernatant, being in the supernatant the soluble form of the protein of interest and in the pellet the insoluble fraction. The supernatant of fusion proteins (those expressed fused to SUMO) was treated with the protease Ulp1, which removed SUMO from the final Hsp40 protein, for 30 min at 4 ºC. This procedure is summarized in **Figure 1**.

3.6. SDS-PAGE ELECTROPHORESIS

Samples obtained after the overexpression of the corresponding protein and centrifugation of the cell lysates were analyzed by 12.5% polyacrylamide gel electrophoresis. Samples were prepared in loading buffer containing 50 mM Tris-HCl, pH 6.8, 5% glycerol (v/v), 4% SDS (w/v), 0.02% bromophenol blue (w/v) and 100 mM DTT. The electrophoresis was run in 25 mM Tris-HCl, pH 8.3, 200 mM glycine, and 1% SDS (w/v) buffer at 180 V for 45 min. Proteins were visualized using Coomasie staining.

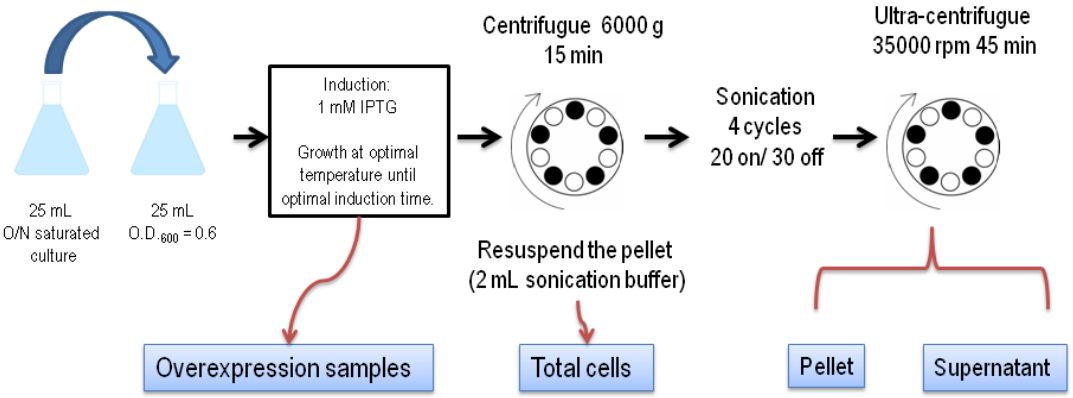

Figure 1. Overexpression and solubility protocol. 1 mL of a saturated cell culture was added to 25 mL LB, and incubated until the OD_{600} reached 0.6. Protein expression was triggered by the addition of 1 mM IPTG at 30ºC. An aliquot of the culture was taken every 1 h and analyzed by SDS-PAGE to test protein expression. Cells were harvested by centrifugation 6000g during 15 min and resuspended in 2 mL of sonication buffer. After cell disruption they were ultracentrifuged at 35000 rpm for 45 min. The resulting supernatants and pellets were analyzed by SDS-PAGE to prove protein solubility.

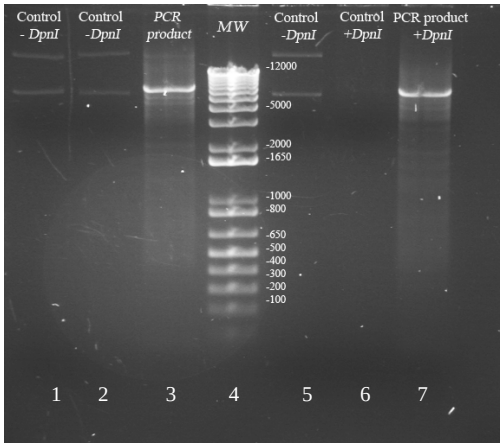

Figure 2: Mutagenesis of Hsp40 DnaJB1 to generate DnaJB1G278C. Lane1. Template control. Lane 2. Template control. Lane 3. Sample, PCR product. Lane 4. 1kb ladder. Lane 5. Control without *DnpI* treatment. Lane 6. Control treated with *DpnI*. Lane 7. Sample treated with *DonI*.

4. RESULTS AND DISCUSSION

4.1. PROTEIN CLONING

4.1.1. Single point mutant DnaJB1G278C

As it can be seen in **Figure 2** (Lane 3), the PCR resulted in an amplified band of the corresponding linearized plasmid size that contained the mutated product. *DpnI* digestion was effective, as evidenced by the disappearance of the super-coiled DNA template in the control and sample bands. (**Figure 2**, lanes 6 and 7).

4.1.2. DnaJA2 and DnaJB1 containing the -CCPGCC motif at the N-terminus

4.1.2.1. pE-SUMO modification

The aim of this task was to generate pE-SUMO plasmid containing the tetracysteine (CCPGCC) motif to overexpress any protein of interest with this motif at the N-terminus, for fluorescence labelling. PCR with the primers for the pE-SUMO modification gave a product of approximately 350 bps, as expected (**Figure 3A**). Digestion of the vector (*NcoI/BsaI*) and the PCR product (*NcoI/XbaI*) led to the

expected fragments (**Figure 3B**). The pE-SUMO vector digestion with *BsaI* yields one *XbaI* cutting site that matches the one in the digested PCR product. Ligation was performed overnight at 16 ºC with T4 Ligase (New England Biolabs). The relation vector:insert was adjusted to 1:10 from the intensities observed in an agarose gel (**Figure 3B**). Analysis of colonies by digestion with *XbaI,* which yielded two cuts in the pE-SUMO modified plasmid (pE-SUMO4Cys), revealed positive clone results (**Figure 3C**), which were confirmed by DNA sequencing.

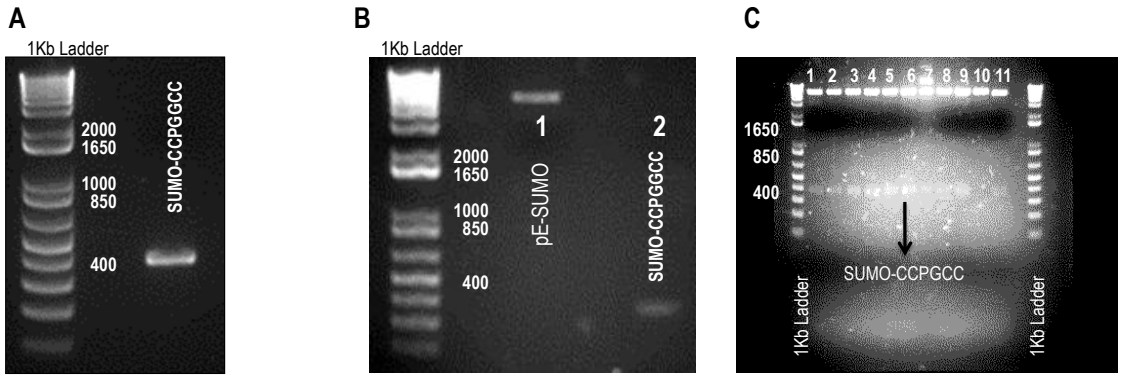

Figure 3. Cloning of the CCPGCC tag into the pE-SUMO vector. PCR product for the addition of the CCPGCC tag to the pE-SUMO vector (A). Digestion product of the pE-SUMO plasmid (*NcoI/BsaI,* Lane 1) and of the PCR product (*NcoI/XbaI,* Lane 2) (B). Analysis of different colonies -1 to 11- harbouring the pE-SUMO4Cys plasmid after digestion with *XbaI*. (C) 1.5% agarose gels in TAE buffer.

4.1.2.2. DnaJA2 and DnaJB1 insertion into pE-SUMO4Cys

pE-SUMO4Cys was used to clone the human DnaJA2 and DnaJB1 cDNAs. To this aim, we amplified the coding sequences by PCR with primers that allow a digestion with *BsaI* (DnaJA2) and *BsmBI* (DnaJB1) to insert the proteins into the pE-SUMO4Cys vector. The PCR products (**Figure 4A**) was digested with two enzymes (*BsaI* and *BamHI* for DnaJA2 and *BsmBI* and *BamHI* for DnaJB1), and pE-SUMO4Cys was digested with *BsaI* and *BamHI*. The digestion products were purified from an agarose gel and quantified (**Figure 4B and C**). Ligation was performed overnight at 16ºC at a vector:insert ratio of 1:10. The ligation product was transformed into *E. coli* DH5α competent cells that were grown overnight in selective agar plates containing ampicillin at 37ºC. Analysis of the colonies was performed by digesting the DNA (extracted with *Minipreps kit*) with *NdeI* and *XbaI* in the case of DnaJA2 and with *NcoI* and *BamHI* in the case of DnaJB1 (**Figure 4E and D,** respectively).

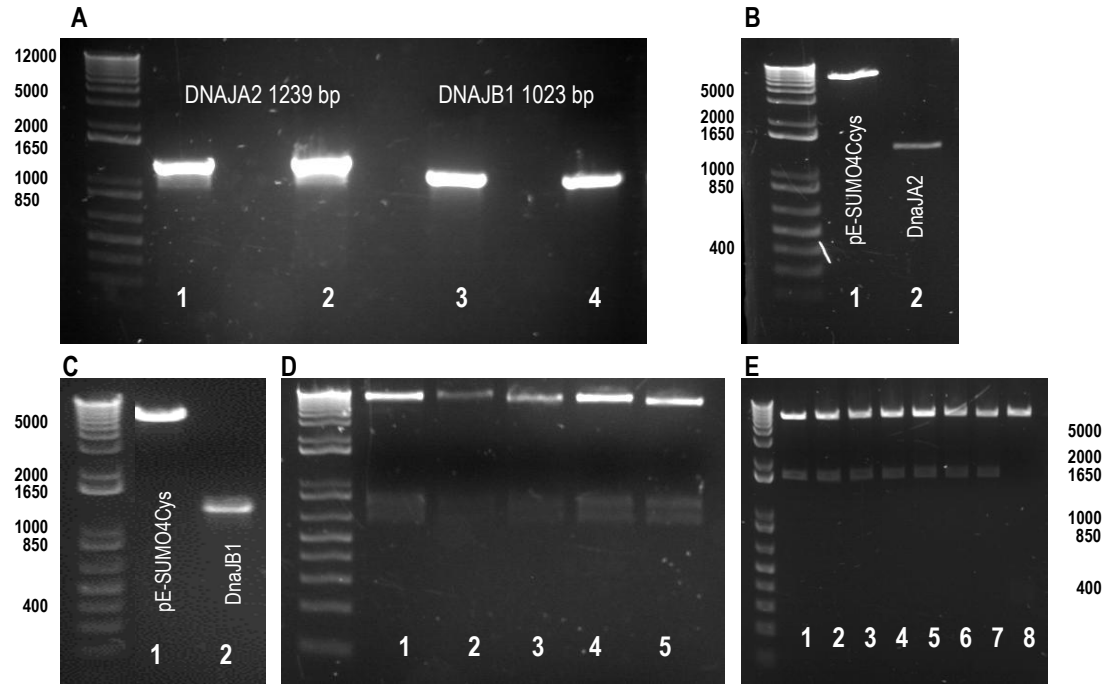

Figure 4. Cloning of DnaJA2 and DnaJB1 into the pE-SUMO4Cys. PCR product for DnaJA2 (lanes 1 and 2) and DnaJB1 proteins (lanes 3 and 4) (A). Digestion products used for the ligation reaction of DnaJA2 (B, Lane 2) and DnaJB1 (C, Lane 2) with pE-SUMO4Cys (Lane 1 in B and C). Analysis of different colonies obtained for CCPGCC-DnaJB1 (lanes 1-5 in D) and CCPGCC-DnaJA2 (Lanes 1-8 in E) by digestion with *NcoI/BamHI* (CCPGCC-DnaJB1, D*) and NdeI* and *XbaI* (CCPGCC-DnaJA2, E). 1.5% agarose gels in TAE buffer.

4.1.3. ΔG/F variants of human DnaJA2, DnaJB1 and bacterial DnaJ

With the aim of cloning the ΔG/F variants of human and bacterial Hsp40s, different strategies were attempted. First we tried to clone the downstream and upstream DNA fragments flanking the G/F domain separately. A suitable restriction endonuclease site was introduced by silent base substitutions in the 3' and 5' site of the downstream and upstream fragments, respectively, to join them later in a ligation reaction with the vector. This process was not successful as no positive colonies were obtained after several attempts.

One possible reason whereby this approach did not work is the fact that ligation of DNA fragments with a large difference in size (i.e. 5.6 Kb with 0.2 Kb and 0.8 Kb) is not as effective as ligation of fragments of similar sizes. Moreover, ligation reactions in which three fragments have to assemble are more difficult than classical ligations with a vector and a single insert, since it is not easy to calculate the optimum vector and inserts concentrations for the DNA ligation reaction.

Second, we used the product of a three-fragment ligation as a template for a PCR reaction, as described previously (An et al., 2010). If DNA molecules assemble properly in a ligation reaction, a PCR with the primers for the full-length protein could amplify the sequence of the deletion variant contained within the ligation product vector. Then, in our case we would observe a band corresponding with the size of the cDNA ΔG/F mutant. However, products of the size corresponding for the ΔG/F proteins were not detected in the agarose gels.

Finally, the strategy based on a commercial mutagenesis protocol (**Materials and Methods, 3.3.1**.) led to positive results. Analysis of the colonies obtained by digestion of plasmidic DNA revealed positive clones. In the case of DnaJB1ΔG/F, they can be identified by digestion with restriction endonucleases, as the G/F domain of this protein has one *NcoI* site. After treating the corresponding DNAs with *NcoI* and *XhoI*, WT DnaJB1 gave rise to two bands in the agarose gel, while the ΔG/F mutant generated just one (**Figure 5B**). For DnaJA2ΔG/F and DnaJΔG/F, the assessment of the efficiency of the mutagenesis from a restriction endonuclease digestion is not so clear, as the G/F domains of these proteins do not contain sites for any available endonuclease.

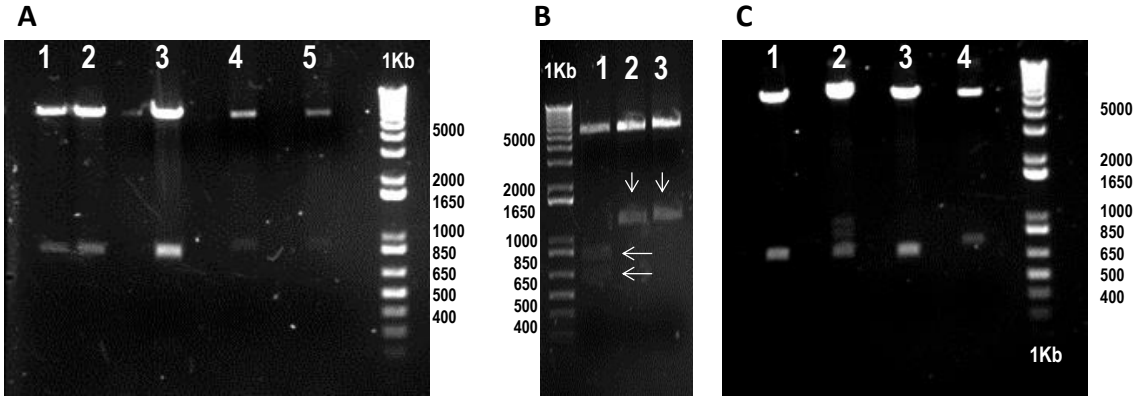

Figure 5. Analysis of the plasmids encoding the ΔG/F protein variants by digestion with restriction enzymes. pE-SUMO-DnaJA2ΔG/F. Lanes 1, 2 and 3: Digestion with *HindIII* of plasmidic DNA obtained from bacterial colonies. Lanes 4 and 5: digestion of the WT plasmidic DNA (A). **pE-SUMO-DnaJB1ΔG/F.** Lane 1: Digestion of the WT plasmidic DNA. Lanes 2 and 3: Digestion with *NcoI* and *XhoI* of plasmidic DNA (B). **pET-22b-DnaJΔG/F.** Lanes 1, 2 and 3: Digestion with *NdeI* and *HindIII* of plasmidic DNA. Lane 4: Digestion of the WT plasmidic DNA (C). 1.5% agarose gels in TAE buffer. 1kb ladder is used to compare sizes of the bands.

Nevertheless, the same digestion protocol was performed and the DNA bands obtained for the ΔG/F mutants seemed to be slightly smaller than those of the full-length forms, as expected. For human DnaJA2, a digestion with *HindIII* gave a band of 798 bp for the ΔG/F variant and 877 bp for the full-length, WT protein (**Figure 5A**). For bacterial DnaJ, digestion with *HindIII* and *NdeI* generated a band of 811 bp in the ΔG/F mutant

and of 880 bp for the WT protein (**Figure 5C**). In any case, the correctness of the three sequences was checked by sequencing the three DNAs.

4.2. PROTEIN OVEREXPRESSION AND SOLUBILITY

Cells transformed with the plasmids encoding the different Hsp40 variants were treated with IPTG to induce protein overexpression, as described above. The protein components of these cell cultures and control ones, which were not treated with IPTG, were analyzed by SDS-PAGE. The comparison demonstrated a good expression level of all protein variants after 4 h induction with 1 mM IPTG at 30ºC (***Figure 6***).

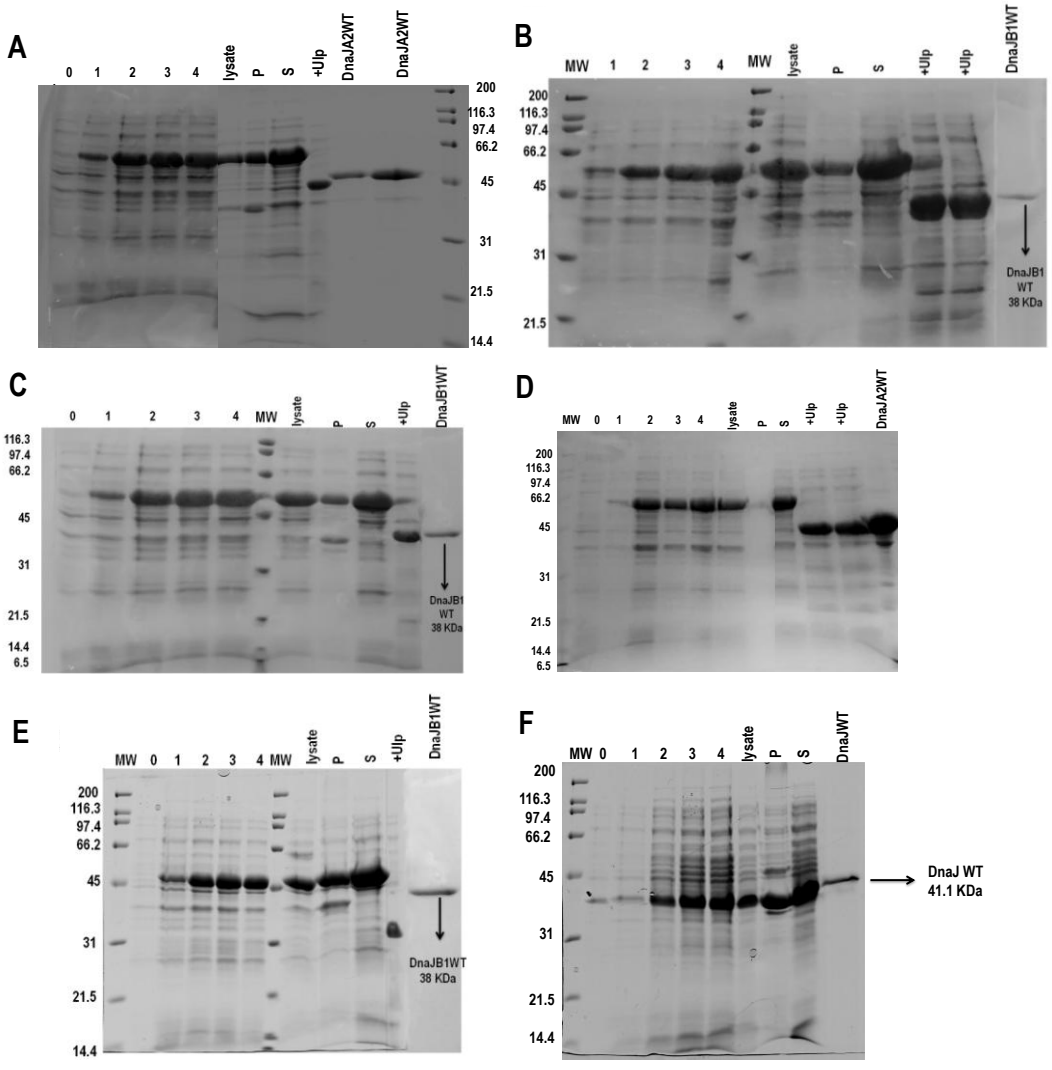

Figure 6. Overexpression and solubility of the variants of Hsp40/DnaJ analyzed by SDS-PAGE: CCPGCC-DnaJA2 (A),CCPGCC-DnaJB1 (B), DnaJB1G278C (C), DnaJA2ΔG/F (D), DnaJB1ΔG/F (E) and DnaJΔG/F (F). In all cases, lanes 0, 1, 2, 3 and 4 indicate protein overexpression 0, 1, 2, 3 and 4 hours after induction. Proteins contained in the total cell lysates (lysate), pellets (P) and supernatants (S) are also shown in the corresponding lanes. For proteins cloned following a fusion protein strategy (all except DnaJΔG/F) the digestion with the protease Ulp1 that removes SUMO is also shown. MW: molecular weight markers in KDa.

To determine the solubility of the overexpressed proteins, the cell lysates were centrifuged after sonication, and pellets and supernatants analysed by SDS-PAGE. The amount of protein found in the supernatant was significantly higher in all cases, thus demonstrating that the fusion proteins were predominantly soluble **(Figure 6**).

Fusion proteins cloned in the pE-SUMO and pE-SUMO4Cys vector also were correctly digested with the protease Ulp1 as shown in **Figure 6**. The protease releases the Hsp40 variants in all cases after a 30 min digestion of the supernatants.

4.3. FUTURE EXPERIMENTS

4.3.1. ATPases and refolding with the ΔG/F variants

The Hsp40 variants generated in this study will be used to analyze the effect of the G/F domain in the interaction of the corresponding J protein variant with protein substrates, Hsc70 and Hsp70. The interaction with aggregated proteins will be studied by fluorescence anisotropy, using the CCPGCC variants labelled with fluorescence probes. Binding to Hsc70 or Hsp70 will be assayed by monitoring the ability to stimulate their ATPase activity at increasing Hsp40 concentration. Finally, these abilities will be compared with the effect on the chaperone activity of the whole disaggregase system, composed of Hsp40, Hsp70 and Hsp110.

4.3.2. FRET (Förster Resonance Energy Transfer)

Formation of heterodimers of different human Hsp40s has been recently proposed as a mechanism to improve the efficiency of the human disaggregase system. With the use of the CCPGCC-DnaJA2 and CCPGCC-DnaJB1 variants labelled with a donor dye (FlAsH), and DnaJB1G278C with the acceptor dye Alexa 588, we will be able to detect formation of heterodimers by FRET (Fluorescence/Förster Resonance Energy Transfer). Experiments will be performed as previously reported (Granier et al., 2007).

5. CONCLUSION

We have successfully cloned and overexpressed several human and bacterial Hsp40 soluble variants that will allow us to dissect the role of the G/F domain and protein association on the chaperone activity of the disaggregase machinery.

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