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Differential phosphorylation-based regulation of αB-crystallin chaperone activity for multipass transmembrane proteins

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ABSTRACT

We have previously shown that αB-crystallin (CRYAB), a small heat shock protein (sHsp) that prevents irreversible aggregation of unfolded protein by an ATP-independent chaperone activity, plays a pivotal role in the biogenesis of multipass transmembrane proteins (TMPs) assisting their folding from the cytosolic side of the endoplasmic reticulum (ER) (D'Agostino et al., 2013). Here we present evidence, based on phosphomimetic substitutions, that the three phosphorytable serine residues at position 19, 45 and 59 of CRYAB play a different regulatory role in this novel chaperone activity: S19 and S45 have a strong inhibitory effect, either alone or in combination, while S59 has not and counteracts the inhibition caused by single phosphomimetic substitutions at S19 and S45. Interestingly, all phosphomimetic substitutions determine the formation of smaller oligomeric complexes containing CRYAB, indicating that the inhibitory effect seen for S19 and S45 cannot be ascribed to the reduction of oligomerization frequently associated to a decreased chaperone activity. These results indicate that phosphorylation finely regulates the chaperone activity of CRYAB with multipass TMPs and suggest a pivotal role for S59 in this process.

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1. Introduction

CRYAB is member of the sHsp family endowed of ATP-independent chaperone activity [1–5]. It is a 175-residues polypeptide that assembles into polydisperse and dynamic protein complexes ranging between 200 and 1000 kDa [2,4,6]. The protein consists of an N-terminal domain, a conserved central α-crystallin domain and a short C-terminal domain [4,7]. It forms soluble complexes with partially unfolded proteins preventing them from irreversible aggregation ("holdase" activity) and keeping them ready for the function of other chaperones that assist in the folding [8]. Besides the crucial role made in the lens in association with αA-crystallin [3], CRYAB is an extensively expressed sHsp that play a role in a variety of cellular functions such as cell cycle, differentiation, apoptosis, gene expression and has been associated with several pathological conditions [9–14]. This variety of functions most likely relies upon structural and functional changes, largely depending on post translational modifications [15]. A major role in CRYAB function is played by phosphorylation occurring at three serine residues at positions 19, 45 and 59 of the N-terminal domain [16,17]. S45 (and possibly S19) is phosphorylated by ERK1/2 and S59 by p38-mitogen-activated protein kinase (MAPK) [16,18,19]. The effect of phosphorylation of these serines on the chaperone activity of CRYAB is disputed, and several examples of increased as well as decreased activity have been reported [15,17,19–24]. It is agreed that phosphorylation (or pseudo-phosphorylation) leads to the formation of smaller oligomeric complexes that exhibit higher dynamic of subunit exchange and several reports ascribe the decreased chaperone activity to the smaller size of the oligomers [23–25]. In contrast, it has been proposed that phosphorylation-dependent induction of small oligomeric structure enhanced CRYAB chaperone activity by increasing binding affinity for target proteins [17,26]. In particular, it was shown that phosphorylation increases the rate of CRYAB subunit exchange influencing its flexibility and determining structural changes that lead to expose more

Abbreviations: CRYAB, αB-crystallin; ER, endoplasmic reticulum; TMP, transmembrane protein; FEVR, Familial exudative vitreoretinopathy; MAPK, mitogen-activated protein kinase; sHsp, small heat shock protein.

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substrate binding sites, eventually enhancing the chaperone activity. Therefore, phosphorylation impacts CRYAB structure and function in a complex fashion.

We have recently reported that CRYAB assists the folding of multipass transmembrane proteins from the cytosolic face of the ER. It binds and prevents the oligomerization-dependent retention in the ER of ATP7B-H1069Q, a mutant form of the copper transporter associated to the Wilson disease, and of Frizzled4-L501fsX533, a frame-shift mutant associated to a rare form of Familial exudative vitreoretinopathy (FZ4-FEVR) [27]. In both instances, the mutated proteins accumulate in the ER and are not transported to their final destination (trans-Golgi network and plasma membrane, respectively), but in the presence of CRYAB they rescue proper folding and localization, and the rescued ATP7B-H1069Q moves to post-Golgi locations in response to copper overload similarly to the wild-type counterpart [27]. Notably, even endogenous level of CRYAB were sufficient to rescue overexpressed ATP7B-H1069Q to the trans-Golgi [27]. Thus, given the interesting therapeutic perspectives opened by these findings, in particular for the Wilson disease, we asked whether phosphorylation of the three serine residues of CRYAB would affect this chaperone activity towards misfolded multipass TMPs. Our results, obtained with phosphomimetic substitutions, show that phosphorylation of S19 and S45 has a clear inhibitory effect while phosphorylation of S59 has not. Intriguingly, the latter protects the chaperone activity from the partial phosphorylation of the other reesidues, while in all instances the size of the oligomeric complexes containing CRYAB is decreased.

2. Material and methods

2.1. Reagents

All reagents for cells culture were from Gibco Life Technologies, Grand Island, U.S.A.; Corning, Manassas, U.S.A,; Lonza, Verviers, Belgium. Solid chemicals and liquid reagents were from Sigma-Aldrich, Milan, Italy; Bio-Rad, Segrate, Italy; Roche, Indianalpolis, U.S.A.; Promega, Madison, U.S.A.

2.2. Antibodies

The following antibodies were used: mouse monoclonal anti-HA, rabbit polyclonal anti-HA, mouse monoclonal anti-FLAG and rabbit polyclonal anti-Golgin (GOLGA2) from Sigma Aldrich, Milan, Italy); mouse monoclonal anti-CRYAB (Enzo Life Sciences, Rome, Italy); Alexa Fluor-conjugated goat anti-mouse and anti-rabbit IgG (Termo Fisher Scientific, U.S.A); HRP-conjugated goat anti-mouse and anti-rabbit IgG for immunoblotting (Santa Cruz Biotechnology, Dallas, U.S.A.

2.3. cDNA cloning and plasmid construction

See supplementary material.

2.4. Cell culture, transfection, immunofluorescence and sedimentation analysis in glycerol gradient

All performed as previously detailed [27].

2.5. Preparation of cell extracts, SDS-PAGE and western immunoblotting

Preparation of cell extracts, SDS-PAGE, and western immunoblotting were performed as previously detailed [28].

3. Results

3.1. Mutagenesis

In order to investigate the role of phosphorylation in the chaperone function of CRYAB toward misfolded multipass TMPs, we substituted the relevant serine residues with aspartic acid. This is a widely used tool to mimic serine-phosphorylation of protein for performing functional studies with CRYAB as well as many other proteins [23]. As shown in Fig. S1, the serine residues in position 19, 45 and 59 were all substituted with aspartic acid (CRYAB-S3D) or with alanine residues (CRYAB-S3A) to prevent phosphorylation. In addition, CRYAB-S3A construct was further mutagenized to generate either the single pseudo-phosphorylated CRYAB-S19D, -S45D and -S59D mutants or the double pseudo-phosphorylated CRYAB-S19/45D, -S19/59D and -S45/59D to ascertain the functional contribution of each phosphomimetic residue or of couple of residues, having prevented phosphorylation at the other relevant serines. Finally, as a negative control for CRYAB activity, we generated and used throughout this study the mutant CRYAB-R120G that binds to the client proteins but is largely defective in the chaperone activity [29].

3.2. Effect of serine pseudo-phosphorylation on CRYAB chaperone function

We have previously shown that CRYAB, but not CRYAB-R120G, is able to rescue the localization to the Golgi complex of the mutant copper transporter ATP7B-H1069Q expressed in transfected cells [27]. This mutant accumulates in the ER, presumably as the result of misfolding, and has a shorter half-life [30]. Confocal immunofluorescence microscopy of transfected COS-7 cells allowed to evidentiate the mislocalization of ATP7B-H1069Q in comparison of ATP7B, whose great majority resides in the Golgi complex (besides a minority in putative post-Golgi locations, Fig. 1A,B and Fig. S2). When CRYAB or CRYAB-S3A, -S3D or -R120G mutants were co-transfected with ATP7B-H1069Q, a clear-cut result was obtained: CRYAB-S3A was as good as CRYAB in rescuing the localization of the mutant transporter (Fig. 1C,E and Fig. S2), while CRYAB-S3D showed only residual rescuing activity, close to the inactive mutant -R120G (Fig. 1D,F and Fig. S2). Next, we asked whether the three serines could equally and/or additively contribute to the negative regulation of chaperone activity seen for the -S3D mutant. Intriguingly, the two pseudo-phosphorylated CRYAB-S19D and -S45D single mutants showed only residual activity (Fig. 1G,H and Fig. S2), while the -S59D mutant resulted fully active (Fig. 1I and Fig. S2). Co-immunoprecipitation experiments indicated that phosphomimetic S19, S45, S59 and S3D, as well as R120G, bind to ATP7B-H1069Q (data not shown) [27], thus the lack of activity could not be ascribed to loss of binding. Therefore, these results strongly suggested that phosphorylation at the three serine residues differently influences CRYAB chaperone activity toward ATP7B-H1069Q in living cells.

Next, in order to generalize the results obtained, we performed the same experimental design using the mutant FZ4-FEVR receptor instead of ATP7B-H1069Q. In this mutant, a frameshift mutation (L501fsX533) generates a different and shorter C-terminal cytosolic tail of the receptor that accumulates in the ER of transfected cells [31], not reaching the plasma membrane as FZ4 wild-type. This mutation is associated to a very rare dominant form of Familiar exudative vitreoretinopathy (FEVR) through a molecular mechanism not clarified yet [32,33]. In previous work we demonstrated that FZ4-FEVR aggregated in the ER forming not-native inter-chain disulfide bridges [34], but the overexpression of CRYAB prevented aggregation and allowed plasma membrane localization of this

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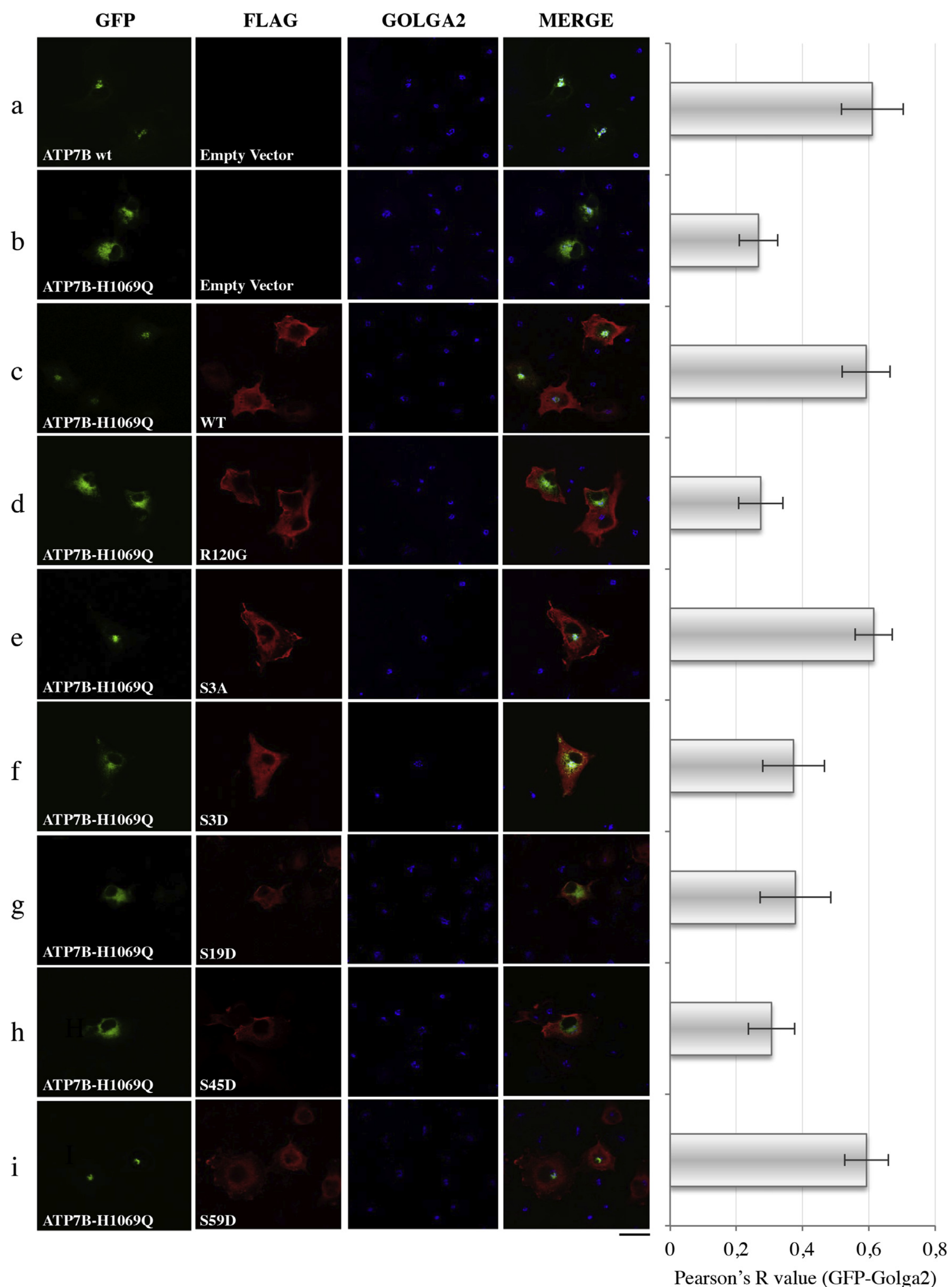

Fig. 1. CRYAB-S59D restores ATP7B-H1069Q localization in the Golgi complex while CRYAB-S19D, CRYAB-S45D and the triple pseudo-phosphorylated CRYAB-S3D do not. Parallel cultures of COS-7 cells grown on coverslips were co-transfected to express the indicated GFP-ATP7B forms (first column on the left) and the indicated 3xFLAG-tagged CRYAB forms or the control empty vector (second column from the left). 48 h post-transfection the cells were processed for confocal immunofluorescence microscopy. The anti-Golgin polyclonal Golga2 antibody was used to visualize the Golgi complex (third column from the left). Colocalization of GFP and Golga2 signals was determined with the ImageJ colocalization plugin and the obtained Pearson's R value is shown on the right (mean ± s.d., n. = 10 cells of randomly selected fields from two independent experiments). Scale bar: 10 μm.

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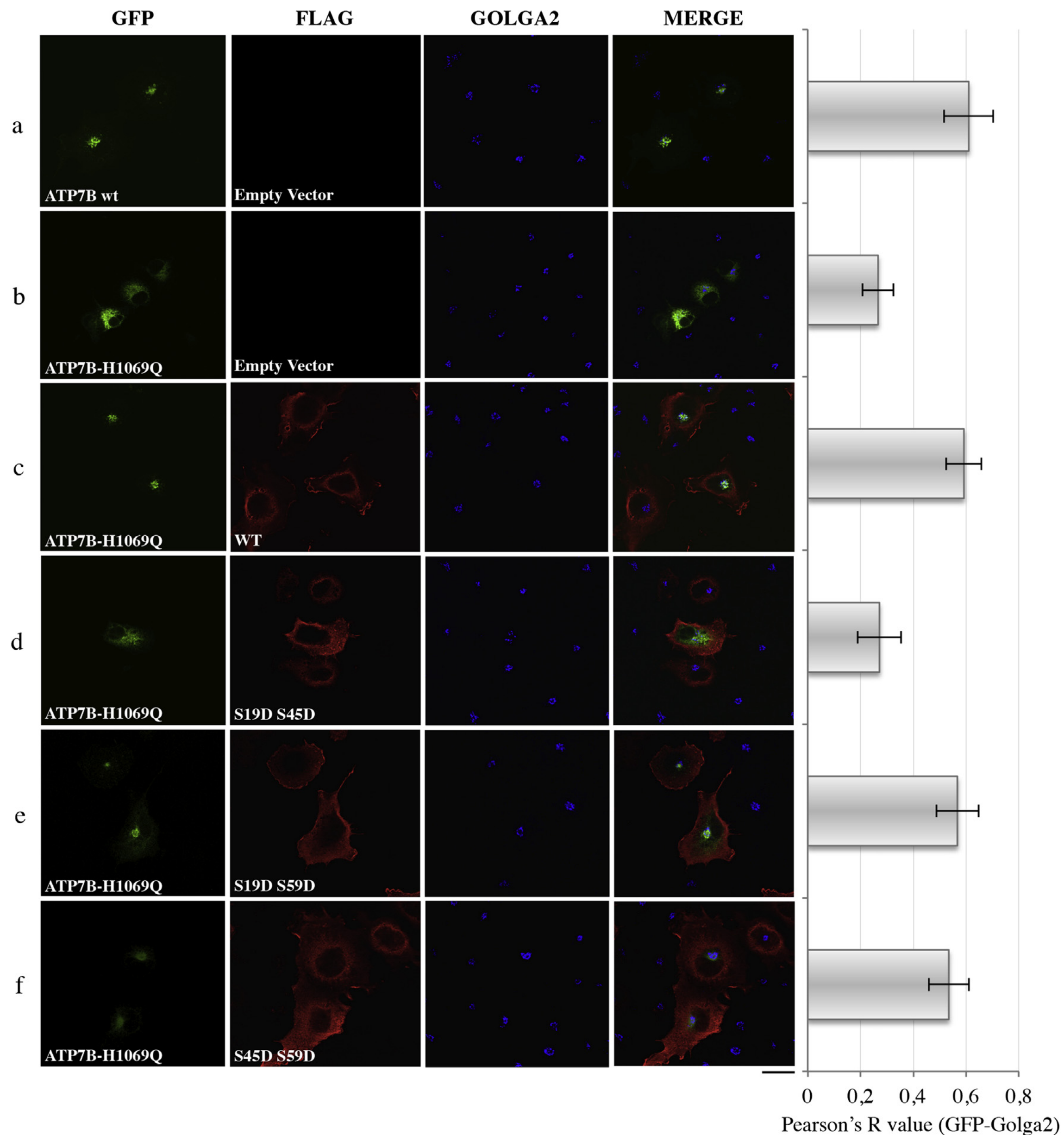

Fig. 2. The pseudo-phosphorylated S59D protects CRYAB chaperone activity in double pseudo-phosphorylated mutants. Cells were manipulated and analyzed as in Fig. 1.

mutant [27]. As shown in Fig. S3A and B, the great majority of FZ4 and only background level of FZ4-FEVR was localized on the surface of Huh-7 transfected cells. As expected, in the presence of CRYAB, FZ4-FEVR was almost fully rescued at the plasma membrane (Fig. S3C). In contrast, only a minor rescue was detected when CRYAB-S3D, -S19D or -S45D substituted CRYAB (Fig. S3E-G), while both CRYAB-S3A and -S59D had a strong rescue effect, only slightly below the wild-type (Fig. S3D,H). Thus, these results fully confirmed those obtained with ATP7B-H1069Q, clearly suggesting that phosphorylation results in a differential control of CRYAB chaperone activity toward multipass TMPs.

3.3. Chaperone activity of CRYAB double pseudo-phosphorylated mutants

Since CRYAB serine residues are phosphorylated by kinases downstream to different signaling pathways [19,24] that could differently control the final phosphorylation pattern of CRYAB, we asked whether different combinations of pseudo-phosphorylation could differently influence CRYAB chaperone activity. To this end, we generated double pseudo-phosphorylated mutants of CRYAB (Fig. S1) that were assayed for chaperone activity by co-transfection with the ATP7B-H1069Q mutant. As shown in Figs. 2D and Fig. S2, the S19/45D resulted almost not active, while both S19/59D and S45/59D (Fig. 2E,F and Fig. S2) were close to the full active CRYAB control (COS-7 cells. and Fig. S2). These results support the exclusive inhibitory role of pseudo-phosphorylated serines 19 and 45, but also indicate that pseudo-phosphorylation at S59 has an interesting protective effect toward single pseudo-phosphorylation of either S19 or S45, but not of both residues at the same time.

3.4. Pseudo-phosphorylation decreases the size of the oligomeric complex containing CRYAB in transfected cells

It has been previously reported that CRYAB chaperone activity, similarly to Hsp27, is directly proportional to the size of its

oligomeric complexes [22,24,25]. Phosphorylation negatively impacts the oligomerization state of these chaperones, thus suggesting an indirect link between phosphorylation and chaperone activity [15]. For this reason, we asked whether the different contribution of phosphorylated serines for the chaperone activity could be due to a different impact on the oligomerization state of the chaperone. To this end, cells were transiently transfected with different phosphomimetic mutants and cell lysates were analyzed by sedimentation assay in glycerol gradient, SDS-PAGE and immunoblotting. As shown in Fig. S4, almost 2/3 of total CRYAB sedimented in the heavier region of the gradient, as well as CRYAB-R120G. Interestingly, CRYAB-S3D was slightly enriched in the lighter region while CRYAB-S3A was equally present in the two regions. Most importantly, all three single pseudo-phosphorylated mutants -S19D, -S45D and -S59D were clearly enriched in the lighter fractions. Thus, both the results observed with CRYAB-S3D and -S3A, and primarily the finding that CRYAB-S59D forms small oligomeric complexes although is endowed of almost full chaperone activity, do not support the simple correlation between loss of activity and reduced oligomerization. In conclusion, these results indicate that phosphorylation has a direct role in the regulation of CRYAB chaperone activity besides its role in the regulation of the oligomeric state of the chaperone.

4. Discussion

In this work we provide compelling evidences that in living cells phosphorylation plays an important role in the regulation of the chaperone activity of CRYAB to correct mislocalization of two disease-associated multipass TMPs. Mimicking phosphorylation of serine 19 and 45, either singularly or in combination, causes a pronounced reduction of CRYAB chaperone activity, while the single phosphomimetic S59 has no such effect. On the other hand, pseudo-phosphorylation on serine 59 prevents the 19/45 phosphorylation-dependent inhibitory effect, but this protecting effect is lost when all three serine residues are simultaneously pseudo-phosphorylated. Interestingly, the inhibition of chaperone activity does not correlate with the reduction of the oligomerization state of CRYAB, suggesting a direct role of phosphorylation in controlling CRYAB chaperone activity. In conclusion, our work demonstrates that phosphorylation of the serine residues 19, 45 and 59 finely regulates the chaperone activity of CRYAB with multipass TMPs and suggests a pivotal role for S59 in this process.

How does phosphorylation control CRYAB chaperone activity?

As previously reported, CRYAB can use its structural plasticity to expose different binding interfaces [35,36]. Indeed, in a proteomic study using HeLa cells subjected to heat stress, more than 300 proteins belonging to the heat-sensitive fraction of the proteome have been found to be protected from aggregation by CRYAB, indicating a rather promiscuous binding [26]. However, some proteins, like caspase-3 or Bax, that were shown to interact with CRYAB in physiological condition [37], have not been found in this interactome suggesting that stress conditions and/or extracellular stimuli may influence the specificity of CRYAB binding/chaperone activity [26]. On line with this observation, we speculate that phosphorylation controls CRYAB conformational state and plasticity so that changes of the phosphorylation pattern may differently change CRYAB activity. Accordingly, the phosphorylation pattern that inhibits chaperone activity of CRYAB for FZ4-FEVR and ATP7B-H1069Q (S19D, S45D, S19/45D, or 3D) has been previously reported to reduce its activity to prevent amyloid fiber formation of ccβ-Trp peptide but not of κ-casein where the same pattern was shown to confer higher protection [23]. Thus, the phosphorylation of CRYAB has a different effect according to the aggregation prone protein engaged. Thereby, aside its role in the regulation of CRYAB oligomeric size, phosphorylation plays an important role in controlling the activity and perhaps the targeting of this chaperone.

Does CRYAB phosphorylation play a role in the unfolded protein response (UPR) triggered by accumulation in the ER of multipass TMPs?

Recent findings have shown that MAPK signaling pathways have a fundamental role in the response to ER stress and UPR [38]. All three canonical MAPKs (ERK1/2, JNK and p38) are known to be activated with different kinetics and with different roles [38]. Indeed, the MAPK signaling network is known to regulate either cell cycle progression and survival or cell death [39–41]. Among them, ERK1/2 and p38 signaling pathways seem to have a relevant role in the regulation of several cytosolic HSPs including CRYAB [42–44]. Particularly, serine 45 (and possibly serine 19) is phosphorylated by ERK1/2 while serine 59 by p38 dependent MAPK [19]. Interestingly, p38 MAPK down-regulates ERK1/2 [45] suggesting that phosphorylation on serine 59 is followed by a negative regulation of the phosphorylation of serine 19 and 45. Thus, the protective role shown in the present paper of phosphomimetic S59 when CRYAB is also pseudo-phosphorylated at position 19 or 45 would reinforce the negative regulation of ERK1/2. Interestingly, it has been reported that overexpression of ATP7B-H1069Q in HEP-G2 cells activates p38, and the total level of ATP7B-H1069Q and the amount able to fold and reach the trans-Golgi network increase when the cells are incubated with inhibitors of p38 [46]. However, CRYAB is minimally expressed in hepatocytes and hepatoma cell lines [47–49], as confirmed by the comprehensive proteomic analysis performed to determine the interactome of ATP7B-H1069Q transfected in HEP-G2 cells that failed to reveal CRYAB [46]: thus, most likely, p38 does not hit CRYAB in HEP-G2 cells. Most interestingly, p38 kinase activity is required for the phosphorylation dependent nuclear translocation of the spliced form of Xbp1s [50]. This protein is produced during the UPR, which normally occurs when unfolded proteins accumulate in the ER. Because multipass TMPs expose domains on either the luminal as well as the cytosolic side of the ER, mutations affecting their folding and export from the ER most likely require the coordinated activation of chaperones acting from both sides of the ER membrane. Indeed, the MAPK signaling pathway may provide this coordination, participating to the UPR to generate the response in the lumen and protecting, at the same time, the cytosolic chaperone activity of CRYAB to assist misfolded multipass TMPs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.09.071.

Transparency document

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