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Chaperones in autophagy

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Abstract

Cells continuously turn over proteins through cycles of synthesis and degradation in order to maintain a functional proteome and to exert a tight control in the levels of regulatory proteins. Selective degradation of proteins was initially thought to be an exclusive function of the ubiquitin-proteasome system however, over the years, the contribution of lysosomes to this selective degradation, through the process of autophagy, has become consolidated. In this context, molecular chaperones, classically associated with protein folding, unfolding and assembling, have been revealed as important modulators of selectivity during the autophagic process. Here, we review this relatively new role of chaperones in mediating selective autophagy and comment on how alterations of this function can lead to human pathologies associated to proteotoxicity.

Keywords

Aging; chaperones; lysosomes; membrane proteins; protein degradation

1. Introduction: Selectivity in autophagy

Since its discovery in the mid-1950s, autophagy was considered a non-selective process by which whole regions of the cytosol were degraded “in bulk” inside lysosomes [1]. The idea of selectivity in degradation of proteins was not considered until later when the ubiquitin-proteasome system was identified and demonstrated to be capable of eliminating only a specific subset of proteins, those previously tagged with ubiquitin [2].

Although for a while selective degradation was solely linked to the ubiquitin-proteasome system, studies from Dice and colleagues in the late 1970s started to point towards some level of selectivity in lysosome-dependent degradation. These early studies showed that different cytosolic proteins were degraded by lysosomes at very different rates and that some proteins were preferentially degraded by this organelle under particular conditions, such as starvation and diabetes [3]. This initial concept of selective protein degradation by the lysosomes has matured over the years into a pathway, now known as chaperone-mediated autophagy (CMA) – the first form of autophagy for which selectivity for a specific pool of cytosolic proteins was demonstrated (Figure 1) [4–6]. Selectivity in this type of autophagy is

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achieved through the recognition and targeting to lysosomes of only the subset of proteins bearing a specific motif in their amino acid sequence. This one-by-one delivery to lysosomes permits for the degradation of the targeted proteins without affecting surrounding ones.

In recent years, selectivity has also been identified in other forms of autophagy, namely macroautophagy and microautophagy (Figure 1). The way in which cargo is delivered to lysosomes through these pathways made it difficult at first to conceive how some cytosolic proteins could be chosen over others for degradation. In both pathways the cargo delivered to lysosomes is sequestered inside vesicles that entrap cytosol as they close. In the case of macroautophagy these vesicles form in the cytosol and the transfer of cargo to lysosomes occurs upon fusion of both compartments. In microautophagy, the vesicles form from the lysosomal membrane that as it invaginates toward the lumen sequesters areas of the surrounding cytosol. It was thus difficult to envision how a forming autophagosome in the process of macroautophagy, or protrusion of the lysosomal membrane in microautophagy, could engulf or entrap cytosolic proteins selectively. However, growing number of studies in recent years support the selective degradation of cytosolic components - protein aggregates and organelles - through macroautophagy. In fact, different subtypes of macroautophagy depending on the cargo degraded, such as, mitophagy (for mitochondria), pexophagy (for peroxisomes), reticulophagy (for endoplasmic reticulum), lipophagy (for lipid droplets), or xenophagy (for intracellular pathogens), have now been identified [7–11]. The same is true for microautophagy, where selective degradation of peroxisomes, glycogen or even of nuclear material directly through entrapment by the lysosome has now been described [12,13]. Likewise, cytosolic proteins have also been recently shown to undergo selective degradation by macroautophagy (mainly in the form of aggregates, giving rise to aggrephagy) and by microautophagy [14,15].

The determinants of selectivity in the different autophagic pathways are as varied as the substrates themselves and range from peptide motifs, covalent modifications (such as ubiquitin), post-translational modifications (such as acetylation, phosphorylation), and recognition via specific receptor proteins (such as p62 or NBR1) [16–19]. A recent addition to this growing list of targeting determinants for selective autophagy is molecular chaperones, which have been revealed capable of delivering cytosolic proteins for degradation through each of the three autophagic pathways.

Molecular chaperones form an integral part of the cellular protein quality control mechanisms along with the proteolytic systems. Proteins that fail to fold or lose their native functional conformation are detected by chaperones that through direct interaction with the misfolded proteins prevent their aggregation [20]. Assisted by co-chaperones, the chaperones can then either assist in refolding of the protein back to its correct conformation, or target it to degradation when refolding is unsuccessful [21]. Therefore, chaperones have been revealed to be of great importance in the prevention of the proteotoxicity that associates with protein conformational disorders, such as neurodegenerative diseases. In these conditions, prone-to-aggregate proteins require continuous assistance from chaperones, as otherwise the proteins tend to organize into irreversible toxic oligomeric species and aggregates. For instance, the heat shock protein of 70kDa (hsp70) has been tightly linked with the defense against proteotoxicity in very different neurodegenerative disorders [22–24]. In most of these cases, hsp70 along with its co-chaperones has been shown to target unfolded proteins for degradation via the ubiquitin-proteasome system, thus decreasing their aggregation [25–27]. However, only recently, the contribution of hsp70 to the removal of these pathogenic proteins through autophagy has been recognized.

Some of the molecular chaperones seem to not only determine the fate of a protein regarding its degradation by the ubiquitin-proteasome system or the lysosome, but also help to decide among the different autophagic pathways. Thus, for example, the constitutive member of the hsp70 family of chaperones, the heat shock cognate protein of 70kDa (hsc70), was initially discovered to provide selectivity to CMA [28], but it has recently been also linked to a form of microautophagy in late endosomes known as endosomal microautophagy (eMI) [15], and to a form of macroautophagy mediating the degradation of protein aggregates, chaperone-assisted selective autophagy (CASA) [29]. This mini-review focuses on the role of chaperones in mediating selectivity through the different types of mammalian autophagy and in the implications that this sharing of chaperones among autophagic pathways may have for their coordinated functioning.

2. Chaperone-mediated autophagy: cytosolic and lysosomal chaperones

2.1. Concept

CMA degrades only those soluble cytosolic proteins that contain a targeting motif related to the pentapeptide KFERQ [4,30]. This motif is recognized by a cytosolic chaperone, hsc70, which mediates the delivery of the substrate protein to the surface of the lysosome [30,31]. CMA is upregulated in response to stressors, such as oxidative stress, hypoxia, starvation and exposure to toxic compounds [32–35]. During prolonged starvation, by degrading no-longer needed proteins, CMA contributes to replenish the pool of free amino acids available for protein synthesis. Activation of CMA in conditions that have associated protein damage, allows for the selective removal of the affected proteins by lysosomes. Lastly, constitutive CMA activity, detected also in most cells, contributes to the continuous degradation of altered or prone-to-aggregate proteins, which makes CMA an essential component of the cellular quality control mechanism. Interested readers are referred to other published reviews for a comprehensive description of the physiological and pathological roles of CMA [5,6,36].

2.2. Main steps of CMA

CMA is a multi-step pathway that starts with the recognition of the substrate proteins in the cytosol and ends with their degradation in the lysosomal lumen (Figure 1). The steps in CMA are, (1) binding of hsc70 to the KFERQ-like targeting motif in substrate protein; (2) delivery of the substrate-chaperone complex to the CMA receptor at the lysosomal membrane, lysosome-associated membrane protein type 2A (LAMP-2A); (3) unfolding of the substrate protein; (4) organization of LAMP-2A into a multimeric translocation complex; (5) translocation of the substrate protein across the lysosomal membrane assisted by the lysosomal resident form of hsc70; and (5) degradation of the substrate protein by the lysosomal proteases [6,36].

The amount of monomers of LAMP-2A at the lysosomal membrane is limiting for substrate binding, whereas the rate of substrate transport into lysosomes is a determinant of the ability of this single span membrane protein to assemble into the active translocation complex [37]. Once the substrates have crossed the lysosomal membrane, the translocation complex is quickly disassembled to generate monomers of LAMP-2A that can then participate in another round of substrate recognition [37]. Several proteins, including two chaperones, lysosomal hsc70 and hsp90, act as regulators of this assembly and disassembly steps [38]. In fact, as the name of this pathway indicates, almost all steps in CMA are dependent on chaperones, localized either in the cytosol, at the cytosolic side of the lysosomal membrane, or in the lysosomal lumen.

2.3. Cytosolic hsc70 and CMA

Cytosolic hsc70 recognizes and binds substrate proteins in the cytosol through the KFERQ-like motifs in their sequence. This binding and targeting of the substrate proteins to the lysosomal membrane are the essence of the selectivity of this autophagic pathway (Figure 2) [28]. Binding of hsc70 to the KFERQ-targeting motif is sufficient and necessary for lysosomal targeting of the substrate protein. In fact, experimental mutation of this targeting motif in bona fide CMA substrates has been shown to abolish their degradation through this pathway. Conversely, fusion of this motif to a protein not usually degraded in lysosomes redirects it to this compartment via CMA. The motif is necessary for the binding to the chaperone and consequently it is essential for lysosomal targeting, however it is not utilized for binding of the substrate to LAMP-2A. Four positively charged amino acids in the cytosolic C-terminus of LAMP-2A are the ones mediating the binding of the substrate to this receptor protein [39]. Which amino acids in the substrate proteins are necessary for binding LAMP-2A remain unidentified till date.

Although further experimental evidence needs to be gathered, hsc70 has also been proposed to participate in the unfolding of the substrate proteins at the lysosomal membrane, a prerequisite before their translocation into the lumen. Interestingly, unfolding is not required for binding as chimeric forms of CMA substrates with a protein in which unfolding can be chemically blocked, fails to undergo translocation but is able to bind to the lysosomal membrane. This implies that unfolding occurs after the substrate proteins have bound the lysosomal membrane [40].

As described for other chaperone functions, the binding of hsc70 to the substrate proteins is modulated by cycles of ATP to ADP hydrolysis. The presence of ATP at the nucleotide-binding site of hsc70 accelerates substrate binding and release, whereas conversion of ATP to ADP slows down this process and stabilizes the substrate-bound conformation [41]. As detailed in later sections, several co-chaperones have been found associated to the hsc70-substrate protein complex in the cytosol and are likely to modulate this step of CMA [41].

2.4. Multiplicity of functions for lysosomal hsc70

CMA substrate translocation is dependent on the presence of a form of hsc70 in the lysosomal lumen, usually referred to as lysosomal hsc70 or lys-hsc70. The participation of lys-hsc70 in CMA was demonstrated by the fact that anti-hsc70 antibodies internalized and delivered to the lysosomal lumen by endocytosis abrogated CMA activity in cells in culture [42]. In fact, only a subset of cellular lysosomes (ranging from 20–80% depending on the cell type and cellular conditions) is active for CMA, and the distinctive feature of this lysosomal subpopulation is the presence of hsc70 in their lumen [43]. Although the exact mechanism by which lysosomal hsc70 facilitates translocation of CMA substrates across the lysosomal membrane is not known, it has been proposed that lys-hsc70 binds the substrates as they enter through the translocation complex and actively ‘pulls’ them into the lumen. However, it is also possible that binding of lys-hsc70 to the entering substrate acts passively, ‘locking’ the protein in order to prevent any retrotranslocation into the cytosol.

The amount of lys-hsc70 becomes limiting for translocation and in fact, levels of luminal lys-hsc70 increase or decrease with changes in CMA activity to accommodate to the volume of entering substrate. When CMA is upregulated as for example during starvation [34] or oxidative stress [32], levels of lys-hsc70 per lysosome also increase. Although in most conditions the increase in levels of LAMP-2A and lys-hsc70 in lysosomes occurs simultaneously to assure effective binding and translocation of substrate proteins, in circumstances when levels of LAMP-2A are abnormally reduced, such as during aging, an increase in the lysosomal content of hsc70 is often noticeable [44]. This higher abundance of

lys-hsc70 is believed to be compensatory for the reduced levels of the receptor and has been shown to be the result of an increase in the amount of chaperone per lysosome and of the presence of a higher percentage of lysosomes positive for this chaperone [44]. The fact that the receptor protein LAMP-2A is present in both CMA -competent and -incompetent lysosomes, but that only the former contain hsc70 in their lumen makes this chaperone a direct determinant of CMA activity. Thus, cells can rapidly augment their CMA capability by increasing the number of CMA-active lysosomes out of the inactive pool, which become competent for CMA as soon as hsc70 reaches their lumen. These findings support the existence of highly interrelated lysosomal subtypes that can be diverted towards one function or another inside cells depending on the cellular requirements. Thus, for example, although CMA-active lysosomes are for the most part engaged in CMA and have lower propensity to fuse with autophagosomes as part of macroautophagy, under conditions of high macroautophagy activation they become the terminal compartment for autophagosomes [45].

How hsc70 is delivered to the lysosomes is not well understood. Although experimentally, cytosolic hsc70 can be internalized by lysosomes via CMA because this chaperone contains two CMA-targeting motifs in its amino acid sequence [43], blockage of CMA in cultured cells does not reduce the amount of hsc70-positive lysosomes [46], suggesting that this pathway does not contribute, for the most part, to hsc70 enrichment in the lysosomal lumen. Blockage of macroautophagy does not interfere with the delivery of hsc70 to lysosomes either, and in fact, cells with compromised macroautophagy often display higher percent of lysosomes active for CMA [47]. This leaves microautophagy as the most probable way in which cytosolic hsc70 reaches the lysosomal lumen. In this respect, two-dimensional electrophoresis analysis of cytosolic and lysosomal hsc70 has revealed that out of the 7 different species of hsc70 detectable in cytosol (isoelectric points ranging from 5 to 8), only the most acidic isoform (pI 5.3) can be observed in the lysosomal matrix. Whether only this particular isoform of hsc70 is capable to access the lysosomal lumen, or if all isoforms of hsc70 reach the lumen and only the most acidic one remains stable in this compartment, requires further investigation.

In addition to this luminal form of hsc70, a small fraction (approximately 20%) of hsc70 is detectable at the cytosolic side of the lysosomal membrane [41,42]. Membrane-associated hsc70 has an isoelectric point of 5.5, suggesting that its binding to the membrane may occur upon post-translational modification of the cytosolic variant [42]. Although it is possible that this variant modulates or actively participates in substrate unfolding, so far it has been shown to play a key role in the disassembly of LAMP-2A from the multimeric translocation complex that occurs as substrate translocation is completed. *In vitro* studies with isolated lysosomes have demonstrated that addition of this chaperone is sufficient to promote active LAMP-2A disassembly [37]. This implies that in the absence of substrates, LAMP-2A disassembly is favored and suggests the transitory rather than stable nature of the translocation complex at the lysosomal membrane, possibly to prevent the leakage of acidic hydrolases into the cytosol.

2.5. Hsp90 and lysosomes

Another chaperone associated with lysosomes that affects CMA activity is hsp90. Similar to hsc70, a fraction of cellular hsp90 can be detected at the lysosomal surface [41]. This chaperone was considered to reach this compartment as part of the substrate/chaperone complex and contribute to stabilize the substrate as it undergoes unfolding previous to translocation. However, a more detailed biochemical characterization of the lysosomal hsp90 has recently revealed that a fraction of this protein is resistant to manipulations that remove all the proteins present on the cytosolic side of the lysosomal membrane, suggesting that part of lysosomal hsp90 is inside lysosomes [37]. Contrary to hsc70 that can be

localized in the lysosomal lumen, hsp90 inside lysosomes is only detectable as a form bound to the luminal side of the lysosomal membrane. It is still unclear whether this lysosomal form of hsp90 is a result of the internalization of the cytosolic variant of this chaperone or if, as other cellular compartments, lysosomes have a dedicated hsp90, and that the homology with the cytosolic hsp90 makes it recognizable by the same antibodies. Thus, lysosomal hsp90 could be a new member of the hsp90 family of chaperones that include Grp94 in the endoplasmic reticulum or mhsp90 in mitochondria.

Future studies are required to determine the possible functions of lysosomal hsp90, but in the context of CMA this chaperone contributes to stabilize LAMP-2A molecules while they transition from monomeric to multimeric state. Addition of 17-DMAG, a semi-synthetic derivative of the irreversible hsp90 inhibitor geldanamycin [48], to isolated lysosomes leads to a marked decrease in the levels of lysosomal LAMP-2A and, consequently, to reduced CMA activity [37]. The reduction in LAMP-2A levels when hsp90 is inhibited originates from the rapid degradation of this receptor by the luminal proteases. It has been proposed that the interaction of the membrane-associated luminal variant of hsp90 with LAMP-2A stabilizes this protein at the different transient conformations that it may require to adopt during the process of multimerization [37].

Contrary to this inhibitory effect of the hsp90 inhibitors on CMA, when added directly to isolated lysosomes [37], in the cellular context, inhibition of hsp90 has been shown to either increase or decrease CMA activity depending on the cell type [37,49]. This apparent opposite effect seems to be related with the capability of the cells to respond to CMA blockage. Thus, although it is now accepted that inhibition of hsp90 reduces CMA at the lysosomal level in all contexts, some cell types, in particular cancer cells that are known to be highly dependent on CMA activity [50], respond to this initial CMA blockage with a compensatory upregulation of this pathway through both a transcriptional increase in LAMP-2A and by conversion of CMA-incompetent lysosomes into active ones [37]. In cells capable to elicit this response to the hsp90 inhibitors, total CMA, at least transiently, increases; whereas in cells unable to activate this compensatory response, CMA activity decreases [37]. It is also possible that inhibition of other functions of cellular hsp90 with these treatments could have different impact on CMA depending on the cell type.

2.6. Possible regulatory roles of co-chaperones in CMA

The activity of molecular chaperones is often modulated by their association to specific co-chaperones capable to enhance or reduce their ability to bind to nucleotides and substrate proteins [51]. Cytosolic hsc70, in the context of CMA, has been shown to associate to particular co-chaperones that accompany the substrate/chaperone complex to the lysosomal membrane [41]. For example, hsp40, hip (hsc70-interacting protein), Bag1 (BCI₂-associated anthogene 1), hsc70-hsp90 organizing protein (hop) and hsp90 can all be co-immunoprecipitated with the hsc70/substrate complex and also be detected at the lysosomal membrane, and indeed, incubation of lysosomes with blocking antibodies for some of these co-chaperones has been shown to have a negative impact on CMA activity [41].

Further studies are needed to specifically dissect the role of each of these co-chaperones on CMA, but by analogy to the functions that these exert on other functions of chaperones, it has been proposed that hsp40 and hip may contribute to stabilize the CMA substrate-hsc70 complex by hydrolyzing ATP to ADP, and by blocking binding of new ATP molecules, respectively. On the other hand, Bag1 may favor substrate release from the chaperone by promoting ATP binding. The co-chaperone hsp90 (in this case the lysosomal variant associated to the cytosolic side of the lysosomal membrane) could prevent unwanted aggregation of the substrate protein as it unfolds prior to translocation, by directly binding to the unfolded segments of the substrate in order to stabilize them [41]. By analogy to other

cellular functions, it is anticipated that hsp90 and hsc70 at the lysosomal membrane act coordinately, providing a rationale for the lysosomal presence of hop that promotes the interaction between hsc70 and hsp90. The fact that some of these co-chaperones exert an inhibitory effect on chaperone function and others an activating effect, but that at a given time both activators and inhibitors can be detected at the lysosomal membrane suggest that at a particular instance, there are probably multiple substrate-chaperone complexes at different steps in the CMA process associated with lysosomes.

Since CMA activity is dependent on the targeting of substrate proteins via hsc70, it might seem plausible to deplete the levels of this chaperone in order to decrease CMA. However, the multiple functions exerted by hsc70 inside cells make this approach very non-specific and infeasible for CMA inhibition. Therefore, modulating the interactions of hsc70 with its co-chaperones, specific for CMA seems a more attractive option to increase or decrease CMA activity for therapeutic benefits. In this respect, the cytosolic co-chaperone CHIP (carboxyl terminus of hsc70-interacting protein) has attracted some attention as it has been shown key for the decision as to whether a cytosolic protein undergoes full refolding or it is directed for degradation. Although most studies have established a link between CHIP and degradation through the ubiquitin-proteasome system [52], there are some reports that demonstrate a connection of CHIP with lysosomal degradation in the context of pathogenic protein removal [53]. Future studies should aim at identifying which of the autophagic pathways is behind this CHIP-mediated lysosomal degradation and whether this function is dependent or independent of hsc70.

Unlike the cytosolic counterpart, there are no known co-chaperones identified for the luminal isoform of hsc70 yet. Two possible explanations are that either hsc70 enters the lysosomes alone or that it enters complexed with the co-chaperone(s), but that they become unstable at the acidic pH and undergo rapid degradation.

3. Microautophagy: hsc70-dependent selectivity

Traditionally, microautophagy has been described as a process that involves the entrapment of cytosol by invaginations of the lysosomal membrane and subsequent degradation of the cargo-containing vesicle after it pinches off from the lysosomal membrane [54]. In mammalian cells, of the three forms of autophagy, microautophagy has been the least studied, mainly due to the lack of information on the molecular components that participate in this process. In fact, in most early studies the identification of this process relied solely on morphological evidences [55]. In yeast, however, microautophagy has been better characterized and a number of proteins have been described to participate in the formation of the projections that originate from the vacuole (yeast equivalent of lysosome) to entrap the cytosolic cargo [56]. Microautophagy-related genes, contrary to macroautophagy genes, are not conserved from yeast to mammals.

Recently, a microautophagy-like process has been described to take place in late endosomes/multivesicular bodies (MVB) (Figure 1). This form of autophagy, now known as endosomal microautophagy or eMI relies on the formation of invaginations in the surface of endosomes taking advantage of a well-characterized mechanism that was previously considered to participate only in the degradation of plasma membrane proteins in endosomes [15].

3.1. Concept and main steps

Cytosolic proteins have been recently shown to reach the endosomal lumen and undergo degradation in this compartment [15]. Blockage of CMA, by knockdown of LAMP-2A, or of macroautophagy, by knock-down of different genes essential for this pathway, did not modify the internalization and degradation of these cytosolic proteins in late endosomes, and

this process could be reconstituted with isolated endosomes, suggesting that endosomal uptake was taking place directly at the membrane of this organelle [15]. In fact, internalization was occurring inside the small vesicles that form in the surface of endosomes (MVB vesicle) and it was dependent on the complex machinery that usually participates in the biogenesis of these vesicles [15] (Figure 1).

The different steps that participate in the process of eMI include: 1) sequestration of the cytosolic material as MVB vesicles form; and 2) closing of the vesicles and pinching off into the late endosomal lumen. Once inside the endosomes, the fate of the MVB is multiple and include, a) degradation in this compartment probably by the digestion of the limiting membrane (eMI), b) transfer to lysosomes through endosome/lysosomes fusion and degradation by lysosomal enzymes, or c) release of the contents outside the cell in the form of exosomes, when late endosomes fuse with the plasma membrane [15]. How much of the internalized cytosolic proteins undergo degradation and what percentage is released in the form of exosomes probably varies depending on the cell type and cellular conditions and it is currently a subject of intensive characterization.

3.2. In-bulk versus selective eMI

In yeast, in addition to in-bulk forms of microautophagy in which ‘random’ areas of the cytosol are degraded, selective removal of organelles, such as peroxisomes (termed as micropexophagy) or specific regions of the nucleus (piecemeal microautophagy of the nucleus) have also been described [12,13]. Similarly, in-bulk and selective internalization of cargo has also been recently described for eMI [15] (Figure 2). Although artificial proteins, such as green fluorescent protein, when introduced in the cytosol can be retrieved in the multivesicular bodies inside endosomes at similar concentrations as those in the cytosol (suggesting an “in bulk” sequestration), proteomic analyses of these vesicles revealed enrichment in the subset of cytosolic proteins bearing KFERQ-related motifs in their sequences [15]. This motif, until then associated only to CMA, seems to be shared by both pathways. However, while the KFERQ-motif is necessary and sufficient for CMA, in the case of eMI it is only necessary – when eliminated, proteins are no longer concentrated in MVB – but it is not sufficient, because contrary to CMA, addition of this motif to a cytosolic protein does not target it for eMI (Figure 2). These findings suggest the participation of additional yet-to-be identified factors for substrate targeting to eMI [15]. In addition, the mechanisms by which these KFERQ-containing cytosolic proteins are recognized, targeted, and internalized by late endosomes via eMI are distinct from the ones described for their CMA degradation.

3.3. Contribution of hsc70 to selective eMI

In selective eMI, the KFERQ-containing proteins are recognized by the cytosolic chaperone hsc70 (as in the case of CMA), but the chaperone-substrate complex does not require a protein receptor at the MVB membrane. Instead, lipids in the outer leaflet of the MVB membrane (phosphatidylserine) mediate the binding and recruitment of the eMI substrates to late endosomes [15]. In fact, it is hsc70 that directly binds to phosphatidylserine in the membrane through a positively charged pocket that when disrupted eliminates selective eMI [15]. It is yet unclear if hsc70 binds the substrates in the cytosol and then associates to the membrane of the MVB or if hsc70 bound at the MVB membrane acts as a receptor where cytosolic proteins bearing the targeting motif bind. In the former case, the co-chaperones associated to the cytosolic hsc70 would plausibly be the decision-makers to target the proteins to CMA or eMI [57]. If binding occurs directly to membrane-bound hsc70, the discrimination between CMA and eMI targeting may depend on the differences in the conformation of the molecules of hsc70 bound to the membrane and those in solution in the cytosol.

Another key difference between eMI and CMA is in the way the substrates are internalized into late endosomes or lysosomes, respectively. While unfolding is a prerequisite for proteins that undergo CMA, eMI substrates do not require unfolding (Figure 2). Experimental chemical blockage of unfolding does not have an impact on the internalization of eMI substrates [15] and recent studies have identified the presence of microaggregates of irreversibly cross-linked oxidized proteins in MVB, in further support of the independence of eMI to unfolding [58]. This ability of eMI to internalize and degrade proteins even if they are organized into irreversible oligomeric complexes leads to the proposal that eMI may be the pathway of choice for proteins that have formed aggregates and are hence not amenable for CMA.

Multiple questions remain regarding this relatively new form of autophagy: is hsc70 the only chaperone capable to mediate eMI targeting? Are there co-chaperones involved in this process? What determines the fate of the substrates once inside the MVB? Does eMI occur simultaneously with the ESCRT-mediated internalization of plasma membrane proteins or can it be initiated with the sole purpose of removal of cytosolic cargo?

4. Macroautophagy: CASA

Added to the role of hsc70 in targeting of proteins for CMA and eMI described in previous sections, recent studies have revealed the contribution of this chaperone in the removal of aggregated proteins via macroautophagy in a process known as chaperone-assisted selective autophagy (CASA) [29].

4.1. Concept and main steps

The first description of CASA focused on the involvement of Bag3 (BCI₂-associated anthogene 3) and hspB8, a small heat shock protein, in mediating the degradation of the aggregate-prone polyglutamine-expanded huntingtin protein via macroautophagy in cultured cells. It was shown that Bag3 forms a stable complex with hspB8, and stimulates macroautophagy [59]. Hsp70 was suggested to cooperate with the chaperone activity of hspB8 in this macroautophagy-mediated selective degradation of mutant huntingtin but the specific role of the chaperone in this process was not fully elucidated [59]. A couple of years later, the identification that Bag3 coordinated the activity of hsc70 along with hspB8 during the selective macroautophagic degradation of another protein, namely the muscle component filamin led to the reconstitution of the different steps of CASA (Figure 1) [29].

The selectivity of CASA is attributed to the chaperone, hsc70, which binds to the cargo protein along with the small heat shock protein hspB8. Bag3 acts as a co-chaperone to couple these two chaperones, and facilitates the interaction with CHIP, the dual-function co-chaperone/ubiquitin ligase that ubiquitinates the cargo protein. Upon ubiquitination, the cargo protein is recognized by p62, a macroautophagic ubiquitin adapter protein, which also interacts with components of the isolation membrane of the autophagosome, namely LC3 (light chain 3), allowing for the formation of the autophagosome around the aggregated protein. Once the membrane seals and the formation of the autophagosome is completed, the sequestered aggregate is delivered to lysosomes through vesicular fusion [29] (Figure 1).

The signaling mechanisms regulating CASA are just beginning to be discovered. In this regard, NF- κ B signaling was recently shown to be a major regulator of CASA [60]. During recovery after heat stress, the induced NF- κ B signaling increased the expression levels of Bag3 and hspB8 and the formation of Bag3-hspB8 complexes, which in turn increased the autophagic clearance of irreversibly damaged proteins [60]. By selectively degrading damaged or aggregated proteins, CASA seems to be more important in non-dividing cells

such as neurons and myocytes. Consequently, several myopathies have been linked to a defect in this pathway (detailed in Section 5).

4.2. CASA substrates

The CASA substrates identified so far include the polyglutamine-huntingtin [59], and large bulky proteins, such as superoxide dismutase 1 (SOD1) [61] and the muscle Z-disk protein filamin [29]. Mutant SOD1, known to misfold and form insoluble aggregates, has been linked to familial forms of amyotrophic lateral sclerosis (ALS). Interestingly, hspB8 was upregulated in a transgenic mouse model of ALS (G93A-SOD1 mice) where it was shown to selectively remove the neurotoxic mutant SOD1 from the motor neurons via CASA [61]. Another interesting substrate is filamin, a 280kDa protein that acts as an extensible anchor between actin and integrins through its 24 immunoglobulin-like domains that can undergo reversible unfolding [62]. Continuous rounds of unfolding and refolding during muscle contractions make filamin prone to irreversible damage, making, therefore, CASA necessary for its degradation.

4.3. Unique features of CASA and relation with aggrephagy

CASA is one of the most recently described forms of selective macroautophagy and therefore, many questions still remain unanswered about this pathway. There are, however, features that already differentiate this process from the chaperone-independent removal of aggregated proteins via aggrephagy (Figure 2). Although the presence of chaperones is not unusual in the aggregates removed via aggrephagy, they have not been shown to be required for the active sequestration of the aggregate by macroautophagy [17,63]. It is possible that CASA and aggrephagy in mammalian cells are linked to the two quality control compartments described for aggregating proteins in yeast [64]. Unfolded and misfolded proteins in yeast can organize into dynamic aggregates amenable for degradation by the proteasome, or into a more static type of aggregate no longer susceptible to proteasomal degradation and proposed to be targeted to macroautophagy. These aggregate structures differ in their level of ubiquitination and associated components. In this context, CASA could be related with the more dynamic aggregate from which proteins can still be selectively removed and targeted to the proteasome. Thus, Bag1 and Bag3 could act as molecular switches that determine proteasomal or lysosomal degradation via CASA of the aggregated proteins, respectively [65].

The role of CASA has been tightly linked so far to the maintenance of muscle homeostasis but it is likely that this process also participates in the removal of aggregated proteins in other organs and tissues. The question remains then whether CASA and aggrephagy are co-existing independent pathways or if there is a functional co-dependence between both the processes. It is possible that CASA evolved to guarantee the elimination of specific highly abundant proteins, such as filamin in muscle, without compromising the removal of other proteins that aggregate inside cells under different conditions. The occurrence of specific chaperones solely dedicated to the maintenance of the homeostasis of a single protein is not a foreign concept in the chaperone field. Thus, although chaperones such as hsc70 are relatively promiscuous and assist in the folding of a large variety of intracellular proteins, other chaperones become partners with a single specific protein - usually abundant or difficult to fold - and are exclusively dedicated to its folding and the maintenance of a homeostatic balance [51].

Also of interest is the interplay of CASA with eMI and CMA through hsc70, the chaperone common to the three pathways. In contrast to these two pathways where little is known about the co-chaperones that modulate hsc70 and its interaction with the substrates, in the case of CASA, Bag3 has been demonstrated to coordinate the assembly of the chaperone

complex required for targeting and removal of the aggregated protein by macroautophagy [29]. Whereas hsc70 and its direct interaction either with LAMP-2A or specific lipids at the endosomal membrane are the determinants of selectivity and degradation in the case of CMA and eMI, respectively, Bag3 is the one that mediates the interaction of the chaperone complex with the autophagy adaptor p62 and promotes aggregate degradation by macroautophagy in CASA. In this respect a feature shared between CASA and eMI is the fact that chaperones and co-chaperones are “consumed” through these autophagic processes as they undergo degradation with the sequestered cargo, whereas in CMA the chaperone dissociates from the substrate once at the lysosomal membrane and is likely recycled back to the cytosol for the recognition of additional substrates. The possible implications of this more conservative handling of chaperones in CMA remain unknown.

5. Pathological implications of the contribution of chaperones to autophagy

As alluded to earlier, the selective degradation of proteins via autophagy, in the form of CMA, eMI or CASA, is critical to maintain proteostasis in normal physiological conditions [66]. In fact, when the activity of any of these pathways is compromised due to pathology and/or aging, cellular homeostasis is severely affected and the occurrence of proteotoxicity leads often to cellular malfunction and in many instances to cell death. In the initial stages of failure of one pathway, there is usually a compensatory activation of the other pathways, however, due to the inherent nature of the pathways - requirement for unfolding of substrates (in the case of CMA), ability to degrade aggregated proteins (in the case of eMI and CASA) - this might give only a temporary benefit to the cell.

Rapidly dividing cells can handle the presence of aggregating proteins even when intracellular degradation is impaired through a dilution mechanism (aggregates or toxic oligomers distribute between the two resulting cells) [66]. In contrast, non-dividing cells, such as neurons and myocytes, are in general more susceptible to proteotoxicity following the failure to remove misfolded or aggregated proteins that remain inside these cells for long periods of time. Consequently, conditions associated to proteotoxicity in these two important types of non-dividing cells, neurons and myocytes, that give rise to common neurodegenerative disorders and myopathies, have been closely linked to malfunctioning of the cellular quality control mechanisms. There is therefore, a growing interest in the possible chemical manipulation of the autophagic pathways with therapeutic purposes in these predominant debilitating diseases.

5.1. Selective autophagy mediated by chaperones and neurodegeneration

Several of the proteins implicated in neurodegenerative disorders, such as alpha-synuclein (in Parkinson's disease) or tau (in tauopathies) have been established as bona fide CMA substrates [67–69]. In these two examples, CMA degrades the wild-type version of these proteins efficiently, whereas the pathogenic counterpart - resulting from either mutations or post-translational modifications - fail to undergo CMA and often interfere with the normal functioning of this pathway [67,68]. In these two examples, targeting of the pathogenic proteins to the lysosomal membrane by hsc70 seems to be unaffected, but they fail to translocate across the lysosomal membrane. Inefficient translocation is associated with abnormally enhanced binding of the pathogenic proteins to LAMP-2A that often becomes a seed for the organization of these proteins into oligomeric toxic complexes at the lysosomal surface. In the case of alpha-synuclein, oligomerization does not affect the stability of the lysosomal membrane but severely compromises uptake of other CMA substrates through this pathway [67,68]. In contrast, the pathogenic variants of tau that fail to translocate into lysosomes via CMA exert a severe destabilizing effect on lysosomes when they organize into irreversible oligomers, leading to lysosomal leakage and failure not only of CMA but of also other lysosome-dependent cellular processes [69].

The primary defect responsible for the poor uptake of these pathogenic proteins into lysosomes via CMA has not been identified yet. It is possible that alterations in the chaperone-dependent steps at the lysosomal membrane that precede translocation could be behind the failure. Thus, poor unfolding or disassembly of the pathogenic proteins by the lysosomal chaperones/co-chaperones, inability of the chaperones to release the substrates once at the lysosomal membrane, or failure of the chaperones to cover the hydrophobic regions of these proteins as unfolding/disassembling occurs, could all contribute to the defective translocation. The fact that in addition to their poor removal by CMA, large part of the toxic effect of these pathogenic proteins could be due to their negative impact on this pathway, has led to the proposal that preventing the interaction of these proteins with hsc70, and consequently their targeting to the lysosomal membrane could indeed be beneficial by at least eliminating the lysosomal toxicity [70]. However, considering the recently discovered role of hsc70 on eMI, this type of intervention may also compromise their targeting through this pathway.

Although the contribution of eMI to the degradation of some of these pathogenic proteins has not been directly analyzed, their elimination through this pathway could be a good solution when CMA is compromised as eMI has the capability to sequester and eliminate proteins even when they organize into high order molecular weight complexes or small aggregates [58]. Interestingly, while mild oxidation of alpha-synuclein enhances its degradation through CMA, as also demonstrated for other CMA substrates [32,68], severely oxidized forms of this protein fail to undergo degradation through CMA [68]. The recent finding that intracellular oxidized proteins once organized into oligomeric/small aggregate complexes can be actively removed by eMI [58] makes even more appealing the possibility that the fact that both CMA and eMI share the same targeting motif and main targeting chaperone, hsc70, may not be a coincidence but instead a way for cells to guarantee that if a protein is not amenable for degradation via CMA because of its organization into irreversible oligomeric complexes, it can still be efficiently removed from the cells by eMI.

Although CASA has been shown capable to eliminate pathogenic proteins related to neurodegeneration such as huntingtin [59], the interplay of this pathway with CMA and eMI in this context has not been explored yet. It is possible that physical characteristics of the aggregating protein - such as size, composition of the aggregate or organization of the chaperones inside the aggregating complex -determine their recognition by either eMI or CASA. Similar factors may also play a role in the decision of the removal of these toxic species by CASA instead of by conventional autophagy. In this respect, recent studies have revealed that intrinsic properties of the forming aggregates, namely the mobility of the proteins in and out of the aggregate, determine their susceptibility to undergo degradation by macroautophagy. Thus, assembly of the autophagic machinery around the aggregate occurs more efficiently in those aggregates with lower mobility of proteins on their surface [71]. The role of chaperones in this process has not been analyzed, but the fact that chaperones have been previously described to actively contribute to the disassembly of proteins from protein aggregates [72] would suggest that, in an indirect way, their association to the aggregates may modulate whether proteasome or autophagy get involved in the removal.

5.2. Selective autophagy mediated by chaperones and myopathies

Protein quality control is essential for the maintenance of the skeletal and cardiac muscle, and alterations in this process have been linked to muscle wasting and atrophy, sarcopenia (age-related degeneration of the muscle fibers) and myopathies [73–76]. To date no direct connections have been established between muscle pathology and CMA or eMI. In fact, although mutations in the *lamp2* gene underlie the basis of a severe myopathy known as Danon disease [77], and it is possible that CMA is also compromised in these patients as a result of the loss of LAMP-2A, the characteristic vacuolar phenotype in the muscle of these

patients is primarily the consequence of defective macroautophagy. This defect originates from the loss of the other LAMP-2 proteins generated through alternative splicing from this gene, namely LAMP-2B and LAMP-2C, shown to participate in macroautophagy and lysosomal biogenesis, respectively.

The third autophagic pathway dependent on chaperones, CASA, has been recently linked to muscle dysfunction. Deficiency of an important CASA component, Bag3, in mice leads to lethality shortly after birth due to progressive muscle weakness [78] and mutation in *Bag3* gene (P209L) causes rapidly progressing myopathy in affected children [79]. In fact, the contribution of CASA to muscle maintenance may be an evolutionary conserved function because starvin, a functional ortholog of Bag3 in *Drosophila* also participates in muscle homeostasis in flies [29]. Homozygous flies deficient for starvin die during larval development, while heterozygous flies reach adult stage but develop an age-dependent muscle weakness [29]. Other CASA components are not essential for the function of this pathway in muscle homeostasis, since CHIP-deficient cells have unaltered CASA activity [80] and CHIP knockout mice do not suffer from myopathy [81]. These findings suggest that ubiquitin ligases other than CHIP are likely involved in this selective autophagic pathway. Among the possible candidates, Parkin is one of the most appealing ones, as this ligase has been shown to associate to protein aggregates [71], has already been linked to protein degradation via proteasome [82] and to autophagy as a determinant of the selective degradation of mitochondria or mitophagy [83,84].

6. Concluding remarks

The important role that chaperones play in modulating different forms of selective autophagy is just beginning to be recognized. Despite the need for additional dissection of the different steps and regulators that participate in the different types of autophagy dependent on chaperones, a series of common themes have started to be delineated. Thus, the hsp70 family of chaperones and in particular, the constitutive variant of this family hsc70, is shared by the three types of autophagy (Figure 3). The other commonality between the three pathways is that hsc70 interacts directly with the substrate protein, and in CMA and eMI the region recognized by this chaperone in the substrates is also common. The challenge ahead is to decipher the code that determines targeting of substrates to each of these pathways and even to the ubiquitin-proteasome system by the same chaperone. In this respect, co-chaperone modulators of hsc70 function may heavily weigh on the decision of the degradative pathway for the substrate proteins. Intrinsic properties, post-translational modifications, as well as the conformational state of the substrate proteins may all contribute to the decision for delivery towards one autophagic pathway or another. For example, while soluble single monomeric proteins could be degraded by any of the proteolytic pathways, once they organize into irreversible oligomeric complexes or aggregates, pathways such as CMA or the ubiquitin-proteasome system are no longer a viable option for their degradation, leaving CASA, aggrephagy and eMI as suitable available options (Figure 3). Other factors that could influence the decision process as to where a particular substrate is targeted by the chaperones are the cell type, cell conditions and the volume of the protein that needs to be removed from the cell.

Disease-dependent factors and aging may also modulate the targeting function of the chaperones. In this regard, it has been shown that Bag3 levels increase whilst Bag1 levels decrease with age in neuronal cells, favoring thus the switch toward targeting of damaged and altered proteins characteristic of aging, from the ubiquitin-proteasome system to macroautophagic degradation [65]. This switch may be of compensatory nature because experimental Bag1 deficiency in cultured cells is sufficient to trigger upregulation of Bag3 expression and *vice versa* [65]. Because both macroautophagy and CMA activity decrease

with age [85] it would be interesting to know if eMI becomes the degradative mechanism of choice under these conditions and how hsc70 senses the failure of the other pathways to favor eMI delivery.

Chaperones could become ideal therapeutic targets to promote removal of specific toxic protein products through a particular autophagic pathway. Considering the recent advances in the development and implementation of chemical chaperones to assist in protein folding in different pathologies, it could become of interest to determine whether aberrant proteins could also be re-routed to a particular autophagic pathway through the use of these chemical modulators.

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Abbreviations

CASA	chaperone-assisted selective autophagy
CMA	chaperone-mediated autophagy
hsc	heat shock cognate protein
hsp	heat shock protein
LAMP-2A	lysosome-associated membrane protein 2A

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Figure 1. Chaperones in selective types of autophagy

Molecular chaperones participate in the recognition of cargo by three different autophagic pathways. **Chaperone-mediated autophagy:** where chaperones bind a KFERQ-like peptide motif in the substrate protein and deliver it to the surface of lysosomes; upon binding to the LAMP-2A receptor the substrate translocates across the membrane and is degraded.

Endosomal microautophagy: where chaperones bind the same targeting motif in the soluble protein but deliver it to the surface of late endosomes; once there, internalization occurs through the formation of vesicles through invagination of the membrane and subsequent pinch-off.

Chaperone-assisted selective autophagy: where chaperones bind substrate proteins organized in the form of aggregates and attract components of the autophagic machinery for the formation of an autophagosome around the aggregate. Degradation occurs upon fusion of the autophagosome with the lysosome.

		CMA	eMI	CASA

COMPARTMENT		Lysosomes	Endosomes	Autophagosomes/ Lysosomes
SELECTIVITY		Selective	Selective/ In Bulk	Selective/ In Bulk
DELIVERY		Transporter	Vesicles (MVB)	Vesicles (APG)
TARGETING	Motif	KFERQ	None/KFERQ	None
	Chaperone	hsc70	hsc70	hsc70/ Bag3
CARGO	Organelles	NO	YES	YES
	Proteins	YES	YES	YES
	Unfolding	YES	NO	NO
Hsc70 BINDING		LAMP-2A	Lipids	Autophagy effectors

Figure 2. Comparison of the different types of autophagy that require participation of molecular chaperones

The three autophagic pathways in which participation of chaperones have been described until date differ in the compartment in which cargo sequestration/degradation takes place, type of substrates, markers for substrate targeting and the need for substrate unfolding. They all share the same chaperone (hsc70) that binds directly to the substrates but to different components of the autophagic machinery.

Figure 3. Chaperones determine the fate of intracellular proteins

This scheme depicts some of the functions of chaperones in the regulation of proteostasis. Molecular chaperones participate in folding, aggregation, refolding and degradation of proteins through autophagic and non-autophagic pathways.