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Hsp70s and lysosomal proteolysis

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Abstract. Confluent cultured cells activate a lysosomal pathway of polypeptide breakdown in response to withdrawal of serum growth factors. The substrates for this proteolytic pathway are a restricted class of cytosolic polypeptides containing peptide sequences biochemically related to lysine-phenylalanine-glutamate-arginine-glutamine, or, in single amino acid abbreviations, KFERQ. The heat shock cognate protein of 73 kD (hsc73) binds to a variety of polypeptides via this molecular determinant and facilitates their lysosomal import and degradation. In addition, a portion of intracellular hsc73 resides within the lysosome and appears to be an essential component of the proteolytic machinery. Several potential mechanisms by which hsc73 mediates selective lysosomal import and degradation of polypeptides are discussed.

Key words. Lysosomes; proteolysis; heat shock proteins; protein targeting; translocation.

Introduction

Intracellular polypeptides are degraded by a variety of pathways in eukaryotic cells[10,19,33]. Proteolytic pathways exist in the cytosol[10,36], as well as in such organelles as the endoplasmic reticulum, mitochondria, and lysosomes[10,33]. These pathways are often highly regulated, responding to changing physiological conditions or environmental stress. In addition, specific molecular determinants within polypeptides often target these molecules to a particular proteolytic pathway. The subject of this brief review is an example of a proteolytic pathway that is activated in response to a defined environmental stress, that is specific for polypeptides containing a particular peptide motif and, interestingly, involves proteins of the heat shock 70-kD family (hsp70s).

Polypeptides of endogenous and exogenous origin are degraded in lysosomes[10]. Lysosomes internalize these molecules in a variety of ways. For example, exogenous polypeptides and certain plasma membrane polypeptides[18] are delivered to endosomes[12] and lysosomes[17] by the process of endocytosis. Lysosomes also internalize cytosolic and other endogenous polypeptides by the mechanisms of crinophagy[22,25,34,38], microautophagy[1,13,29,30,35], macroautophagy[14,15,29,35,44] and hsc73-mediated transport[3-6,9,11,27,31,42,43]. Crinophagy appears to involve a fusion of secretory granules with lysosomes and degradation of the granule contents[22,25,34,38]. Presumably, this pathway maintains intracellular hormone levels within certain limits, particularly when secretion is low. In some cases, the lysosome membrane appears to invaginate, and engulf areas of cytoplasm. This process, called microautophagy, displays little selectivity with respect to the polypeptides internalized[1,13,29,30,35]. Macroautophagy, also a nonselective process[29,35], involves a vesiculation of ribosome-free regions of the endoplasmic reticulum[14,15,44]. These nascent vesicles, containing isolated cytosolic material, then acquire lysosomal membrane proteins, acidify, and acquire lysosomal hydrolases. Phagophores may also give rise to autophagic vacuoles[39].

Lysosomes also internalize cytosolic polypeptides in a highly selective manner[3,4,6,11,27,31]. This pathway is mediated by hsc73, which recognizes a molecular determinant, specifically, peptide regions biochemically related to KFERQ, in cytosolic polypeptides[5,43]. Hsc73 then facilitates the lysosomal import and degradation of these substrates[5,42,43]. This proteolytic pathway appears to be active in certain tissues of starved animals as well as in cultured cells deprived of serum growth factors[6,47].

KFERQ as a lysosomal targeting signal

Radiolabeled polypeptides have been introduced into the cytosol of cultured cells by erythrocyte-mediated microinjection[28,37]. The stability of these polypeptides could then be determined under a variety of conditions. For example, the half-life of microinjected ribonuclease A (RNase A) was approximately 100 hours in serum growth factor supplemented cultures of human diploid fibroblasts[4]. However, RNase A's half-life was decreased to approximately 50 hours in response to serum withdrawal. This phenomenon was not observed with all polypeptides, that is, some were degraded at enhanced rates in response to serum withdrawal, whereas others were not[4,31]. The basis of this selectivity is discussed further below.

Based on a variety of criteria, the site of this enhanced proteolysis was determined to be the lysosome. For example, a portion of the microinjected RNase A was found associated with lysosomes after subcellular fractionation procedures[27]. No such lysosomal association was found for polypeptides that were equally stable in

the presence or absence of serum. Also, the enhanced degradation was sensitive to lysosomotropic agents[27]. Furthermore, cells that have degraded endocytosed RNase A release the same peptide fragments as those cells that have had RNase A microinjected[20].

In an attempt to identify the essential region within RNase A necessary for lysosomal targeting, a series of RNase A derivatives were prepared and individually microinjected. The amino-terminal 20 amino acids of RNase A, called RNase S-peptide, were found to be necessary and sufficient to target RNase A, as well as other polypeptides to which they were covalently conjugated, to the lysosome under serum deprivation conditions[3,4]. Further characterization identified residues 7 to 11, specifically, KFERQ, as the essential region[11]. KFERQ's degradation was serum regulated[11], and, when microinjected in increasing concentration along with RNase A, KFERQ could abolish RNase A's enhanced degradation[6]. This latter result suggested a specific and saturable proteolytic pathway. The importance of individual residues within the KFERQ region is currently being investigated.

Polyclonal antibodies were raised to KFERQ and, remarkably, were found to recognize 30% of the cytosolic polypeptides from human fibroblasts[6]. Of these immunoreactive polypeptides, nearly 80% were degraded within 24 hours of serum withdrawal. In contrast, non-immunoreactive polypeptides were not degraded at enhanced rates in response to serum withdrawal. Rat tissues were also examined for immunoreactive cytosolic polypeptides, and several were positive[6]. For example, liver, kidney, and heart contained a large number of KFERQ-containing polypeptides, and these molecules were largely depleted after 3 days of starvation. Brain and testes, tissues that are not thought to accelerate proteolysis rates, contained few immunoreactive cytosolic polypeptides. Thus, although the time course is slightly different, it appears clear that the KFERQ-selective lysosomal pathway of polypeptide degradation, first identified in human fibroblasts, also operates in several other cell types.

Role of hsp70s in lysosomal proteolysis

A specific peptide motif in cytosolic polypeptides destined for lysosomal catabolism under serum deprivation conditions implied the existence of a cellular protein that could recognize the motif. Such a binding protein was isolated from the cytosol of serum deprived fibroblasts by RNase S-peptide affinity chromatography[5]. This 73 kD protein, initially designated the peptide recognition protein (prp73), was identified as hsc73 by immunological and protein sequence data[5]. At the time, hsp70s were known to function in the import of polypeptides into the endoplasmic reticulum and mitochondria[7,8]; this was the first suggestion they might also be involved in the lysosomal import and degradation of intracellular polypeptides. Hsc73 bound to RNase S-peptide with a K_d of 8 μM[43]. The protein also bound RNase A, aspartate aminotransferase, and pyruvate kinase, all polypeptides with KFERQ-like regions[43]. No binding was observed to lysozyme, ovalbumin, or ubiquitin, polypeptides which lack a KFERQ-like region. Interestingly, binding to the KFERQ-motif was unique to hsc73. That is, of all the hsp70 family members tested, including hsp70, grp78, and dnaK, among others, only hsc73 bound to RNase S-peptide[43]. The precise binding specificities of hsc73, as well as these other hsp70 family members, is currently an area of much investigation.

In order to obtain a mechanistic understanding of how polypeptides are targeted to and translocated across lysosomal membranes, this pathway of proteolysis was reconstituted using isolated fibroblast lysosomes[5,42,43]. The import and degradation of RNase S-peptide was stimulated by hsc73 and ATP, but not by other hsp70 family members, including dnaK and grp78 (ref. 43). In addition, the in vitro proteolytic pathway was selective for KFERQ-containing polypeptides[42]. Rat liver lysosomes have also been used to examine the uptake and degradation of polypeptides[2]. As in the fibroblast system, hsc73 and ATP were stimulatory, and the process was selective (A. M. Cuervo, S. R. Terlecky, J. F. Dice and E. Knecht, unpubl. data). Thus, it was concluded that hsc73 binds polypeptides at KFERQ-like regions, and facilitates their lysosomal import and degradation. Further characterization has recently revealed several new aspects of the proteolytic pathway by isolated fibroblast lysosomes. For example, lysosomal import was saturated at micromolar concentrations of RNase S-peptide[42]. Furthermore, at 0 °C, RNase S-peptide bound to a lysosomal membrane protein, also in saturable fashion. The receptor-like protein on the surface of lysosomes, although not yet isolated, was sensitive to mild trypsin treatment[42].

Importantly, lysosomes incubated at 0 °C (ref. 5), or in the presence of ammonium-chloride[5] or leupeptin[42], were incapable of degrading RNase S-peptide. Also, the observed degradation required intact lysosomes since, in the incubation buffer maintained at pH 7.2, disrupted organelles were also devoid of RNase S-peptide hydrolyzing activity[42].

Since serum withdrawal from cultured cells stimulated lysosomal proteolytic degradation in vivo, lysosomes were isolated from serum-deprived cells and their ability to import and degrade RNase S-peptide and RNase A compared to lysosomes isolated from serum-supplemented cells. Not only were the lysosomes from serum-deprived cells twice as active in the in vitro assay, but they also contained increased amounts of hsc73 (ref. 42). Protease-protection assays showed the majority of this lysosomal hsc73 to be inside the organelle lumen (S. R. Terlecky and J. F. Dice, unpubl. data). Indirect

immunofluorescence using laser scanning confocal microscopy confirmed the subcellular distribution of hsc73 in serum-deprived cells (F. Agarraberes and J. F. Dice, unpubl. data). Hsc73 colocalized with the lysosomal marker proteins, LGP120 and β-hexosaminidase. Interestingly, hsc73 was not found associated with all lysosomes, perhaps suggesting that serum withdrawal activates only a subset of the population. These studies also showed that the hsc73-containing lysosomes fused to form a tubular network in response to serum withdrawal, the significance of which remains to be determined. Other investigators have also described hsp70 molecules within lysosomes[16,26].

To determine a functional role for intralysosomal hsc73, the effects of 13D3, an anti-hsc73 specific monoclonal antibody[23], introduced into cells by endocytosis were examined. Whereas this treatment had no effect on the intralysosomal degradation of endocytosed RNase S-peptide or other polypeptides, endocytosed 13D3 completely blocked the enhanced degradation of metabolically labeled polypeptides in response to serum withdrawal (S. R. Terlecky and J. F. Dice, unpubl. data). Lysosomal proteolysis in the presence of serum was unaffected by endocytosis of the antibody. Additional experiments showed that 13D3's inhibitory activity was abolished by preincubation with hsc73, and that

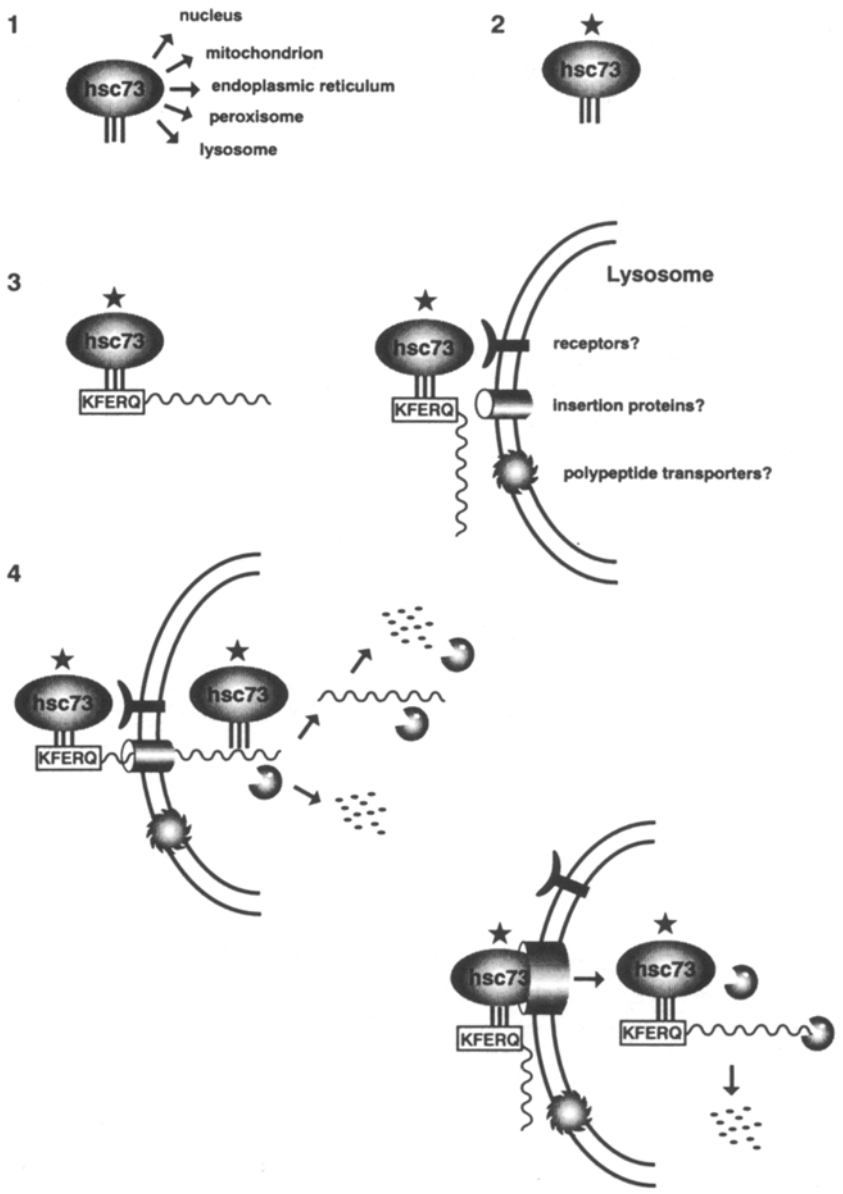

Figure. Hsp70s and lysosomal proteolysis. Hypothetical model for the acitvation of hsc73, the recognition of KFERQ-like peptide regions in polypeptides, and the subsequent transport into lysosomes for degradation. See text for details. The star indicates an activated hsc73 molecule, and the pie-shaped figures inside lysosomes denote proteases.

endocytosis of control antibodies or hsc73 itself were without similar effect. These results suggest that intralysosomal hsc73 may be required to pull substrate polypeptides across the membrane bilayer, a role previously identified for hsp70s within the endoplasmic reticulum[32,45] and mitochondria[21].

Conclusions and future directions

In conclusion, hsc73 appears to have multiple functions within cells. This fascinating molecule clearly plays a role in the import of polypeptides in the mitochondrion, endoplasmic reticulum, nucleus, and peroxisome[7,8,41,46]. The results summarized in this review suggest that hsc73 also plays a role in lysosomal import and degradation of intracellular polypeptides and therefore, may be correctly included in part 1 of the summary figure. A model for polypeptide import into lysosomes, consistent with the observations summarized herein, is also shown in the figure.

In response to serum withdrawal, hsc73 may be activated (fig., part 2) and become capable of binding polypeptides (fig., part 3). This activation could result from altered interaction with cellular components, from covalent or noncovalent modifications, or from a change in subcellular localization. Alternatively, substrate polypeptides might be induced to expose their KFERQ-like peptide regions by a regulatory step.

The interaction of hsc73 and substrate molecules could result in an altered polypeptide conformation such that the substrate may now interact with lysosomal membrane proteins. With analogy to polypeptide import into mitochondria and endoplasmic reticulum, these molecules might include membrane receptors, insertion proteins, or polypeptide transporters. Hsc73 may participate in substrate binding to the lysosome membrane (fig., part 4) and subsequently release for another cycle of transport. Alternatively, hsc73 may cross the lysosome membrane along with the substrate (fig., part 4, bottom). Indeed, hsc73 is known to shuttle into and out of the nucleus in such a manner[24]. Also, hsc73 does not contain two KFERQ-like regions and therefore, may be a substrate of the pathway. Finally, it is possible that hsc73 itself could act as a transporter protein in the lysosome membrane.

Although the mechanistic similarities between the various organellar import systems with respect to the role of hsc73 is striking, there are also differences. For example, the import of polypeptides into the endoplasmic reticulum, mitochondrion, peroxisome, and nucleus results in correctly folded and assembled molecules, whereas this may not be the case for molecules imported into the lysosome. Perhaps, in the lysosome lumen, polypeptides are maintained in a loosely folded state so as to permit access by proteases. Perhaps it is hsc73, or a closely related hsp70 family member which performs this function inside the lysosome (fig., part 4). The idea that a molecular chaperone is acting like a molecular undertaker is an idea already put forth[40]. Alternatively, lysosomal hsc73 may simply be involved in the import process, as discussed above.

The translocation apparatus is currently being explored in several ways (J. F. Dice, pers. commun.). One is a ligand blotting approach, whereby lysosomal proteins are separated by SDS-PAGE, transferred to nitrocellulose, and permitted to renature in the presence of a cocktail of lipids. The blot is then probed with a specially tagged RNase S-peptide molecule which can be followed. A cross-linking approach which has been successful in isolating components of the translocation apparatus in other systems including the mitochondria and endoplasmic reticulum will also be employed to identify transport molecules. It would be interesting to try to trap a translocating molecule and identify, by cross-linking, those molecules on either side of the membrane that interact with it.

The study of these and other issues related to the import and degradation of polypeptides in lysosomes should provide exciting avenues of research for years to come.

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