Autophagy and other vacuolar protein degradation mechanisms

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Abstract. Autophagic degradation of cytoplasm (including protein, RNA etc.) is a non-selective bulk process, as indicated by ultrastructural evidence and by the similarity in autophagic sequestration rates of various cytosolic enzymes with different half-lives. The initial autophagic sequestration step, performed by a poorly-characterized organelle called a phagophore, is subject to feedback inhibition by purines and amino acids, the effect of the latter being potentiated by insulin and antagonized by glucagon. Epinephrine and other adrenergic agonists inhibit autophagic sequestration through a prazosin-sensitive α_1-adrenergic mechanism. The sequestration is also inhibited by cAMP and by protein phosphorylation as indicated by the effects of cyclic nucleotide analogues, phosphodiesterase inhibitors and okadaic acid.

Asparagine specifically inhibits autophagic-lysosomal fusion without having any significant effects on autophagic sequestration, on intralysosomal degradation or on the endocytic pathway. Autophaged material that accumulates in prelysosomal vacuoles in the presence of asparagine is accessible to endocytosed enzymes, revealing the existence of an amphifunctional organelle, the amphisome. Evidence from several cell types suggests that endocytosis may be coupled to autophagy to a variable extent, and that the amphisome may play a central role as a collecting station for material destined for lysosomal degradation.

Protein degradation can also take place in a 'salvage compartment' closely associated with the endoplasmic reticulum (ER). In this compartment unassembled protein chains are degraded by uncharacterized proteinases, while resident proteins return to the ER and assembled secretory and membrane proteins proceed through the Golgi apparatus. In the *trans*-Golgi network some proteins are proteolytically processed by Ca^{2+}-dependent proteinases; furthermore, this compartment sorts proteins to lysosomes, various membrane domains, endosomes or secretory vesicles/granules. Processing of both endogenous and exogenous proteins can occurr in endosomes, which may play a particularly important role in antigen processing and presentation. Proteins in endosomes or secretory compartments can either be exocytosed, or channeled to lysosomes for degradation. The switch mechanisms which decide between these options are subject to bioregulation by external agents (hormones and growth factors), and may play an important role in the control of protein uptake and secretion.

Key words. Protein degradation; autophagy; lysosomes; endosome; salvage compartment; secretion; endocytosis; amphisome.

Introduction

The lysosome is the major site of intracellular protein degradation. Containing 15–20 proteolytic enzymes as well as numerous other hydrolases, lysosomes are well equipped to degrade the variety of proteins and other macromolecules which are continually transported to these organelles from other regions of the cell. The bulk of the material is delivered by the process known as *autophagy*, which involves a series of steps and several prelysosomal vacuoles[58]. Lysosomes also receive proteins for degradation from the endoplasmic reticulum[91] and from the secretory pathway[110]; there is also some evidence for direct lysosomal protein uptake by membrane invagination (microautophagy)[120] or by penetration through the lysosomal membrane, chaperoned by a heat shock protein[42]. In addition to their engagement in the turnover of endogenous cell components, lysosomes are responsible for the degradation of macromolecular material taken up from the outside of the cell by the processes of *endocytosis* and *phagocytosis*. Endocytic/phagocytic uptake, as well as some of the other delivery processes, implies a steady flux of membrane proteins through the lysosomes, associated with a certain rate of membrane protein degradation.

The present review will discuss the degradation of endogenous protein in the various vacuolar compartments of the cell. Intralysosomal degradation is considered in detail in the previous article, hence only the pathways of delivery to the lysosome will be considered here. The vast topic of endocytosis is not addressed except with regard to protein degradation in endosomes and interaction with other proteolytic pathways. The proteolytic processing of lysosomal enzymes, secretory proteins and viruses is dealt with by other articles in this issue.

Autophagic-lysosomal and non-lysosomal protein degradation

Autophagy is the major mechanism by which intracellular protein is broken down. The term is usually applied in a restricted sense (a practice which will be followed in the present context) to denote the nonselective bulk process – also called macroautophagy – whereby whole re-

gions of cytoplasm become enveloped to form closed vacuoles, from which the sequestered material is subsequently delivered to lysosomes for degradation. In addition, electron microscopic studies have suggested that lysosomes may have the ability to ingest cytosol by direct invagination of the lysosomal membrane (micro-autophagy)[2].

The concept of autophagy has been developed mainly on the basis of ultrastructural studies. Until recently, the biochemistry of autophagy could be approached only indirectly, by measuring the inhibitor-sensitive fraction of overall intracellular protein degradation. The tendency of a number of bioregulators and metabolic inhibitors to inhibit protein degradation to the same incomplete extent, with a larger effect on the more long-lived proteins, led to the idea of two major pathways of protein degradation: one inhibitor-sensitive pathway with preference for long-lived, normal proteins, and one inhibitor-resistant pathway with preference for short-lived, aberrant proteins[5,92,141]. Most importantly, the fact that inhibitors with widely different mechanisms of action had non-additive effects on protein degradation at their maximally effective doses[164,172] would seem to rule out the possibility of a single, incompletely inhibited degradation pathway. The specific effect of ammonia and other lysosomotropic weak-base amines on the inhibitor-sensitive pathway made it clear that the latter reflected lysosomal proteolysis[159,175], hence the major distinction between lysosomal and non-lysosomal protein degradation could be drawn[92,160,172].

The relative quantitative roles of lysosomal and non-lysosomal protein degradation vary greatly between different cell types, and – in particular – depending on the nutritional and hormonal conditions. While non-lysosomal degradation proceeds at a fairly invariant rate of 1.0–1.5%/h in most cell types, lysosomal degradation may vary from zero to 4%/h. Thus, scientists' views of the relative importance of these two processes tend to depend on the experimental conditions they employ. A lot of cell culture work is done on established cell lines with a low intrinsic capacity for lysosomal degradation of endogenous protein; the cells are, moreover, usually incubated in amino acid-rich, serum-containing media that suppress the lysosomal pathway effectively, thus giving the impression that the latter is unimportant[93,147]. At the other extreme, among those who work with normal tissues like the liver, where lysosomal degradation may sometimes account for 70–80% of the total degradation, it is sometimes asserted that all degradation of long-lived protein may be lysosomal[79,118].

In isolated rat hepatocytes incubated under conditions where the lysosomal pathway is maximally activated, amino acids and certain purines like 3-methyladenine (3MA) are able to inhibit the lysosomal degradation of endogenous, long-lived protein almost completely, while having no effect on the lysosomal degradation of certain exogenous proteins. Since these agents also suppress morphologically observed autophagy under the same conditions, the data suggest that autophagy is the main source of protein delivered to the lysosomal pathway in hepatocytes[98,99,162]. Non-autophagic delivery appears to be preferentially (but not exclusively) concerned with short-lived, i.e. newly synthesized protein[155,164]. The ability of the autophagic-lysosomal pathway to account for approximately two-thirds of the degradation of long-lived hepatocytic protein[160,162], makes degradation measurements a useful approximation to the study of autophagy. However, the remaining non-autophagic degradation could cause problems: for example, amino acids, widely investigated as inhibitors of autophagic protein degradation[157,169], may actually stimulate non-lysosomal proteolysis[155]. To obtain reliable information about the biochemistry and regulation of autophagy, more specific assay methods would therefore be desirable.

Biochemical measurement of autophagy

The basic principle of all autophagy assays is to measure the transfer of a soluble, membrane-impermeant probe from cytosol to sedimentable vacuoles of the autophagic-lysosomal pathway. The probe may be simply an endogenous cytosolic enzyme, e.g. lactate dehydrogenase (LDH), which will accumulate in such vacuoles provided a proteinase inhibitor like leupeptin is present to prevent lysosomal degradation of the enzyme. This approach has been used to measure enzyme accumulation in isolated lysosomes, separated from other organelles by virtue of their high density acquired upon leupeptin treatment[94], or, more simply, in cells made leaky for cytosolic enzymes by electrodisruption of the plasma membrane ('cell corpses')[95]. Apart from simplicity, the latter method has the advantage of recovering all autophagic-lysosomal vacuoles, not just the density-shifted fraction of the lysosomes.

The introduction of exogenous autophagy probes into cells may offer the convenience of radiolabelling as well as the possibility of studying autophagy and degradation of individual proteins. The problem that needs to be solved, however, is how to get membrane-impermeant compounds across the plasma membrane without compromising cellular viability. Fusion of cells with protein-loaded erythrocyte ghosts has been used in several studies; while these have given interesting information about the importance of structural determinants for protein stability[11,148,181], no investigations of protein autophagy appear to have been made. The tendency of microinjected cytosol proteins to aggregate[93,147] and to be extruded from the cells in intact form[87] may perhaps make them somewhat unsuitable as probes of normal protein degradation mechanisms.

Radiolabelled di- and trisaccharides are membrane-impermeant small molecules which can be used as autophagy probes without the artifacts associated with mi-

croinjected proteins[77]. Their small size, furthermore, enables their introduction into cells by the gentle method of electroloading[167]. By subjecting cells in suspension, such as isolated hepatocytes, to a short series of high-voltage electric shocks[60,62,163], tiny plasma membrane bleds are generated at the cell surface; these act as high-permeability regions for small (below 1000 dalton), neutral molecules like sugars[64]. The surface lesions are rapidly repaired at 37 °C, and as a result the electroloaded sugar molecules are trapped in the cytosol and become available for autophagic sequestration. Inert sugars like [^{14}C]sucrose[60,65] and, in particular, [^{3}H]raffinose (which unlike sucrose does not enter mitochondria)[171] are suitable probes for measurement of the initial autophagic sequestration step, while [^{14}C]lactose, which is hydrolyzed in lysosomes, can be used to study the later steps in the autophagic pathway[85]. By using electrodisruption (electric shock in a non-ionic medium) to break up the plasma membrane, and density cushion centrifugation to separate cytosolic from autophaged sugar[60], autophagy can be reliably assayed in a large series of samples.

Autophagy is a non-selective bulk process

Although ultrastructural studies suggest that autophagy is basically a bulk process, some gross selectivity due to steric factors (e.g. difficulties in enclosing large organelles in small autophagosomes) might be expected. There have been reports of autophagic selectivity in relation to differentiation and tissue involution[24,75,105,114], but the data presented are generally inadequate for distinguishing between differential autophagy and differential intralysosomal degradation. Other examples of selective protein degradation cannot be unequivocally ascribed to autophagy[43,101].

The use of native enzymes as autophagic sequestration probes has made it possible to address one of the long-standing issues in the field of protein degradation: can the wide range of enzyme half-lives[23] be accounted for by selective autophagy, or must non-autophagic mechanisms be invoked? To answer the question we measured the autophagic sequestration rates of a spectrum of cytosolic enzymes, differing in half-life from 1 h (ornithine decarboxylase) to 19 h (LDH). These enzymes were all found to be sequestered at the same rate[95], suggesting that autophagy is a non-selective bulk process, as previously indicated by morphological criteria. The enzyme sequestration rate (nearly 4%/h) was close to the sequestration rates for inert sugar probes measured under similar conditions[171], and to the previously calculated rate of autophagic-lysosomal protein degradation[172]. Similar hepatocytic degradation rates were observed for ribosomal RNA[14]. All data are thus is accordance with the concept of autophagy as a nonselective bulk process, proceeding at a maximum rate of about 4%/h in nutritionally deprived hepatocytes. This would be the expected minimum rate of degradation for all cytoplasmic macromolecules under those conditions. The reason why liver enzyme half-lives longer than 17 h are observed in vivo is that hepatocytic autophagy is not always running at the maximum rate: in well-fed animals, autophagy may be almost completely suppressed[90,118].

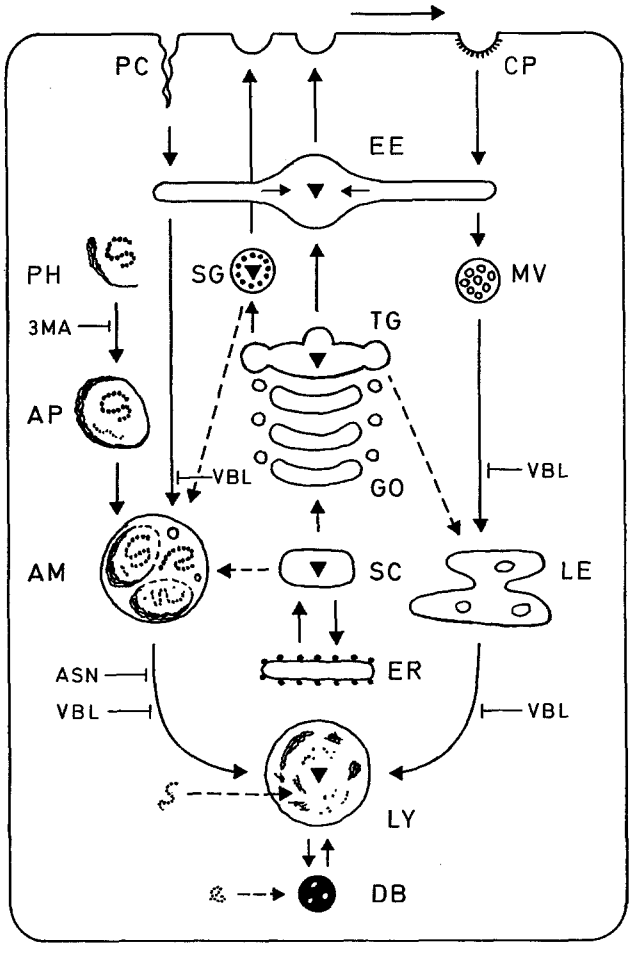
Since autophagy is non-selective, differential enzyme turnover must be due to non-autophagic proteolytic mechanisms. It is noteworthy that degradation of the cytosolic enzymes studied by Kopitz et al.[95] was inhibited by the proteinase inhibitor leupeptin only to an extent corresponding to the rate of autophagy, i.e. neither lysosomes nor other leupeptin-sensitive proteolytic systems (such as the calpains) would appear to contribute materially to the differential turnover of these enzymes. In the case of the most rapidly degraded enzyme (ODC), other studies have indicated that the enzyme half-life is determined by sequence information in the C-terminal region[54], or by post-translational arginylation at the N-terminal end[96], and that degradation occurs by a phenanthroline-sensitive, non-lysosomal mechanism which does not involve ubiquitin conjugation[149].

As a general rule, the available information suggests that all cytoplasmic constituents are degraded by the autophagic-lysosomal pathway to a certain extent, and at the same rate. A mere qualitative demonstration of the presence of a substance in lysosomes or prelysosomal autophagic vacuoles therefore tells nothing more than the obvious. For information about specific turnover of proteins or other macromolecules, one must turn to the non-autophagic pathways.

Initiation of autophagy: the sequestration step

The autophagic pathway for degradation of intracellular macromolecules includes several steps and organelles, as indicated by recent ultrastructural and biochemical studies (fig.). The various steps are difficult to distinguish morphologically, hence the term *autophagic vacuole* is used to denote all vacuoles that contain material of autophagic (cytoplasmic) origin, including lysosomes engaged in autophagic degradation.

In the first autophagic step, sequestration, a membranous organelle of unknown origin and composition excises and envelops a portion of the cytoplasm, eventually enclosing it in a vacuole called an *autophagosome*. Various known membranes have been proposed to perform the sequestration or to give rise to the sequestering organelle, in particular the endoplasmic reticulum (ER) membranes and the Golgi cisternae[136]. The frequent presence of ER marker proteins in the membranes surrounding early autophagic vacuoles was interpreted as evidence for an ER origin[49]. However, a large fraction of the autophagic vacuoles was always negative for the marker proteins studied, even in pictures that also included positively immunostaining vacuoles[49]. It is therefore unlikely that sequestration is performed by a modified,



Intracellular pathways of endocytosis, exocytosis and protein degradation. Known proteolytic compartments are indicated with an arrowhead. 3MA, 3-methyladenine; AM, amphisome; AP, autophagosome; ASN, asparagine; CP, coated pit; DB, dense body (passive lysosome); EE, early endosome; ER endoplasmic reticulum; GO, Golgi; LE, late endosome; LY, lysosome; MV, multivesicular endosome; PC, pinocytic (endocytic) channel; PH, phagophore; SC, salvage compartment; SG, secretory granule; TG, *trans*-Golgi network; VBL, vinblastine. Broken lines indicate pathways with a tentative destination. Transport vesicles and membrane flow not shown.

degranulated ER membrane[49]. The sequestering structure has in fact sufficiently distinctive morphological characteristics to warrant its being considered as a specific organelle[133], to which the name *phagophore* has been assigned[161]. Phagophores can be observed as condensed, non-sequestering structures in the cytoplasm, or at various stages of spreading, eventually forming the walls of the autophagosomes. They have the appearance of membrane packages consisting of membrane multilayers associated with a thick deposit of osmiophilic material, probably lipid[133,134], bearing some resemblance to the phospholipid-rich 'multilamellar bodies' observed in many cells[82]. Autophagosome walls are usually of variable thickness: they can be multilayered or double-layered[109], and in electron micrographs fractures between the membranes often occur, producing a picture of autophagosomes as cytoplasmic areas surrounded by an optically clear cleft[110,136].

Whether phagophores are disassembled and assembled de novo for each round of sequestration, or are recycled from lysosomes or prelysosomal vacuoles as coherent structures is not known. The ability of autophagy to proceed for hours in the presence of protein synthesis inhibitors[99] at least indicates that de novo synthesis of phagophore proteins is not needed.

The amphisome

In the second autophagic step the contents of the autophagosomes are transferred to another autophagic vacuole, called an *amphisome*. The discovery of the amphisome was based on the ability of high concentrations of asparagine to inhibit autophagic-lysosomal fusion[56,166] without interfering with the endocytic pathway[169]. Autophagy of electroninjected lactose will normally cause lactose degradation in the lysosomes[85], but in the presence of asparagine lactose was found to accumulate in a prelysosomal compartment, where it was accessible to degradation by endocytosed β-galactosidase[63]. The ability of this compartment to receive material from endocytosis as well as from autophagy suggested that it did not simply represent the autophagosome, but also a subsequent autophagic vacuole with amphifunctional properties.

The endocytic influx to the amphisome can be blocked by microtubule inhibitors like vinblastine[63], whereas the autophagic influx cannot: if amphisomes are preloaded endocytically with the sucrose-degrading enzyme invertase, autophaged sucrose will be degraded continuously even in the presence of vinblastine[57]. The fact that amphisomes can be preloaded would seem to indicate that they are organelles of some permanence, not just the product of fusion between an autophagosome and an endosome. The effective intra-amphisomal degradation of lactose and sucrose by their respective low-pH-requiring enzymes suggests that the amphisome is an acidic compartment; proton pumps could conceivably be delivered from the endosomes. The intermediate autophagic vacuoles described by Dunn[50], distinguishable from autophagosomes by virtue of their acidity, probably correspond to amphisomes.

The transfer of material from autophagosomes to amphisomes can provisionally be regarded as a fusion process, although the mechanism is not really understood. Ultrastructural studies of autophagy in guinea pig pancreas indicate that several autophagosomes may enter the same amphisome[190]; multi-autophagic vacuoles may therefore tentatively be identified as amphisomes as long as there is no evidence for degradation of their contents. Autophagic vacuoles seem to acquire ER membrane markers very early[49], which suggests the possibility that there may be a pathway from ER to amphisomes. Certain incomplete protein complexes that are known to be degraded lysosomally without passing through the Golgi[107] could take this short-cut, probably passing through

the proteolytic, ER-associated 'salvage compartment' (see below). Amphisomes do not appear to contain lysosomal proteinase activity, as is indicated by the ability of autophaged cytosolic enzymes to accumulate in the presence of asparagine[84,95], but the possibility cannot be excluded that other proteolytic activities normally ascribed to the salvage compartment could be amphisomal. The presence of mannose 6-phosphate receptors (MPRs) in a substantial fraction of autophagic vacuoles in liver cells[50] might be taken as evidence for prelysosomal entry of lysosomal enzymes into the autophagic pathway, but it should be cautioned that the specificity of MPR as a marker of prelysosomal, late endosomes[68] is not universal: in guinea pig pancreas, lysosomes are MPR-positive whereas amphisomes are not[190]. The question of whether the MPR-positive autophagic vacuoles in hepatocytes correspond to lysosomes or to late endosomes, which might then be identical to amphisomes, is therefore in need of further study.

In addition to the endocytic-amphisomal pathway there appears to be a direct, autophagy-independent endocytic route to the lysosome, as indicated by the inability of asparagine[169] or 3MA[162] to inhibit the lysosomal degradation of endocytosed asialoglycoprotein[169]. The degradation of autophaged sucrose by endocytosed invertase despite the presence of these inhibitors[57,86] likewise suggests the existence of a direct pathway. Whether there is also a direct autophagic-lysosomal route, i.e. fusion between autophagosomes and lysosomes independently of endocytosis, is not known.

What determines whether an endocytosed protein proceeds directly to the lysosome or via the amphisome junction? Both ligand-receptor interactions and the autophagic flux rate may presumably be of importance. In hepatocytes, invertase can use both routes[57], and β-galactosidase can at least take the amphisomal route[63], whereas both EGF[49] and asialoglycoproteins[162,169] appear to travel by the direct route only. In the guinea pig pancreas, endocytosed horseradish peroxidase has been shown to take the amphisomal route[190]. In fibroblasts, starvation of serum and amino acids was found to induce not only enhanced degradation of endogenous proteins, but also enhanced degradation of endocytosed α_2-macroglobulin and EGF[78], both types of degradation being 3MA-sensitive. It is thus possible that certain aspects of endocytic protein degradation in fibroblasts are tightly coupled to autophagic degradation, possibly through the amphisome junction. Although most of these observations will require more extensive investigation, together they suggest that the amphisome may play a pivotal role in intracellular protein catabolism.

The end of the autophagic-lysosomal pathway: intralysosomal degradation

In the third autophagic step the amphisome fuses with a lysosome, either a previously inactive dense lysosome or a light lysosome already engaged in autophagy[177]. The fusion step can be inhibited specifically by asparagine[63,84] and non-specifically by vinblastine[85]. The fusion brings the autophaged material into contact with lysosomal hydrolases, which effect the fourth and final step in the autophagic pathway: intralysosomal degradation. There is no reason to think that the lysosomal degradation of autophaged material is any different from the degradation of endocytosed material. It has in fact been shown that all lysosomes participating in autophagy can simultaneously engage in endocytosis[86,168], which suggests that lysosomes are completely amphifunctional. Degradation or denaturation of autophaged material would not seem to be before it has reached its lysosomal destination: a number of cytosolic enzymes were shown to retain full enzymatic activity in prelysosomal vacuoles accumulating in the presence of fusion inhibitors (asparagine plus vinblastine)[95]. Even inside lysosomes, enzymatic activity could be preserved by the cysteine proteinase inhibitor leupeptin, implying that an initial endoproteolytic attack by a cysteine proteinase (cathepsin B, H, L or a related enzyme) may be obligatory for the degradation of endogenous cell proteins[95].

The properties of lysosomes and the details of intralysosomal protein degradation are reviewed in the preceding article.

Biological regulation of autophagic sequestration

Functional considerations suggest that biological regulation of autophagy should be exerted at the sequestration step. Although autophagic-lysosomal fusion can be inhibited by amino acids[170], it is difficult to see that regulation at this step can be of biological value: unless a feedback inhibition of sequestration is operating, a fusion blockade will merely cause accumulation of sequestered, non-functional cytoplasm. A similar argument would apply to the regulation of lysosomal activity: cytoplasmic material which has reached the lysosome will be functionally useless, and interference with its degradation will merely cause problems.

Autophagic sequestration can be quantitatively measured with high precision in isolated rat hepatocytes, using a bioassay based on the transfer of an inert radiolabelled probe (routinely[^{3}H]raffinose; in earlier studies also digiton-extractable[^{14}C]sucrose) from cytosol to sedimentable vacuoles[52,60,65,167,171]. Although the probe will eventually accumulate in all types of autophagic vacuoles, there is no escape route, and the rate of accumulation will therefore strictly reflect the rate of sequestration.

Temperature and energy. Autophagic sequestration is strongly temperature-sensitive, being virtually abolished below 20 °C[161]. This corresponds to the temperature-sensitivity estimated for autophagic protein degradation by the use of selective inhibitors[59]. The sequestration furthermore appears to be energy-dependent, as relative-

ly moderate reductions in cellular ATP content significantly depress the sequestration rate[138]. An energy requirement can also be demonstrated at the final lysosomal degradation step (presumably reflecting the activity of the ATP-dependent proton pump) and is indicated at some intermediate (fusion) step[137]. The overall quantitative energy requirement of the whole autophagic-lysosomal degradation pathway is in accordance with sequestration being the rate-limiting step under conditions of energy deprivation[137].

Several energy substrates, like fructose, glycerol, trioses and pyruvate are so efficiently phosphorylated in hepatocytes that they cause a depletion of cellular ATP. This results, secondarily, in an inhibition of hepatocytic autophagy[97,137,173]. It is therefore important to monitor the energy status of the cells in all studies of agents which inhibit autophagy or autophagic protein degradation.

Amino acids. Autophagic-lysosomal protein degradation is subject to physiological feedback inhibition by amino acids[98,122]. A complete, balanced amino acid mixture can be shown to exert its effect on isolated hepatocytes by inhibition at the autophagic sequestration step[65]. Although several individual amino acids can inhibit the autophagic-lysosomal fusion step as well[170], this inhibition is unlikely to represent physiological regulation for the reasons discussed above. A small group of amino acids accounts for most of the inhibitory effect of the mixture[163]. Among these, tryptophan has the strongest effect when given alone, and the tryptophan metabolite kynurenine, previously shown to inhibit hepatocytic protein degradation[69], is also an effective inhibitor of autophagy[165]. On the other hand, leucine is unique in being indispensable for a maximum effect of the complete mixture, and a combination of leucine and tryptophan along with high concentrations of proline or cysteine can effectively replace the complete amino acid mixture[173]. No signal transduction mechanism is yet known for the amino acids. They do not alter hepatocytic ATP levels, nor do they act via cyclic AMP, cyclic GMP, protein kinase C or the cyclic nucleotide-activated protein kinases[165].

Purines. Various purines (adenine, adenosine and several of their methylated and thiolated derivatives) have previously been shown to inhibit autophagic-lysosomal protein degradation in hepatocytes[31,61,99]. While many of the purines inhibit protein synthesis and some other processes as well, 3-methyladenine (3MA) has been found to be relatively specific, at least in hepatocytes, where it has no effects on protein synthesis, endocytic protein degradation or ATP levels[61,162]. In other cell types 3MA may be a more general inhibitor of the starvation-induced response[78], or it may even display some cytotoxicity[174]. 3MA exerts its effect at the autophagic sequestration step, which is completely blocked when the concentration of the purine is 10 mM[168,171]. Although 3MA has been reported to induce a moderate rise in hepatocytic cAMP levels[31], the change is usually insignificant (I. Holen and P. O. Seglen, unpublished results). The molecular mechanism of autophagy regulation by purines therefore remains unknown.

Hormones. Several hormones have been found to influence hepatocytic autophagy. Insulin inhibits autophagy at the sequestration step, in synergy with amino acids[58]. This effect would seem to account for the amino acid-dependent inhibition of autophagic protein degradation previously observed with insulin[121,139]. Glucagon, which is known to antagonize the amino acid inhibition of protein degradation[139,157], has a similar effect on autophagic sequestration, but surprisingly inhibits autophagy if amino acids are replaced by pyruvate. The latter effect could be shown to reflect a fall in hepatocytic ATP levels, apparently due to accelerated gluconeogenesis from pyruvate[97,173].

The β-adrenergic agonist isoproterenol has been shown to stimulate hepatic autophagy when applied to the liver in situ[135]. However, freshly isolated hepatocytes are deficient in β-adrenergic receptors[146], which may partially explain why a variety of adrenergic agonists (epinephrine, norepinephrine, isoproterenol and phenylephrine) were found to inhibit rather than stimulate autophagic sequestration in such cells[55]. Antagonist studies indicated that an α_1-adrenergic (prazosin-sensitive) mechanism was responsible for the inhibition. Since the phorbol ester TPA was without effect[55], mediation by protein kinase C would seem unlikely, leaving intracellular Ca^{2+} as the most likely transmitter of the autophagy-inhibitory adrenergic signal.

Cyclic nucleotides and protein phosphorylation. Addition of the permeant cAMP analog dibutyryl-cAMP to isolated hepatocytes caused a marked (40%) inhibition of autophagic sequestration, and the adenylate cyclase activator deacetylforskolin produced a similar effect[81]. Dibutyryl-cGMP also inhibited autophagic sequestration, but the guanylate cyclase activator atriopeptin had no effect, rendering the idea of physiological autophagy control by cGMP questionable. General inhibitors of cyclic nucleotide phosphodiesterases, such as theophylline or isobutylmethylxanthine, raised the levels of both cAMP and cGMP in hepatocytes, and suppressed autophagic sequestration virtually completely. Autophagy was also suppressed by milrinone, which elevated cAMP but no cGMP[81]. A role for cAMP in the regulation of hepatocytic autophagy would thus seem to be indicated. Protein kinase A inhibitors (e.g. staurosporine) did not prevent the autophagy-suppressive effect of dibutyryl-cAMP, which suggested that the cyclic nucleotide effect is not mediated by a protein kinase. Protein phosphorylation may nevertheless be involved in the regulation, since the protein phosphatase inhibitor okadaic acid, which causes a general hyperphosphorylation of hepatocytic proteins[74], was able to inhibit autophagic sequestration almost completely[165].

Functional aspects of autophagy

The bulk character of cytoplasm degradation by the autophagic pathway suggests a twofold functional significance: cellular macromolecules are converted to energy substrates and metabolic building materials at the expense of cell size, i.e. a role of autophagy both in metabolic homeostasis and in growth control would be expected. The contribution of autophagy to protein metabolism is particularly well documented, and represents a major function, considering that some 80% of the cytoplasmic dry weight is protein. When running at maximum capacity (about 4% of the cytoplasm degraded per h) autophagy may account for 70–80% of intracellular protein degradation. The autophagic protein degradation is subject to feedback inhibition by amino acids, and is strongly suppressed e.g. in the liver after a meal[90] or upon incubation of isolated livers or hepatocytes with high concentrations of amino acids[122,169]. Autophagy is also a major mechanism for degradation of RNA[100], suggesting that the inhibition of autophagic degradation by adenine, adenosine and some of their derivatives[31,61,99,162] may reflect the existence of a functional feedback mechanism analogous to that involving the amino acids. It should be noted that apart from serving as building blocks for resynthesis of proteins and nucleic acids, both amino acids and nucleosides can provide metabolic energy through gluconeogenesis in the liver.

The growth-regulatory aspect of autophagy has been less elaborated than its metabolic control function, but there is considerable indirect evidence for its importance. The rate of cell growth in culture is a direct function of the rate of protein accumulation, which in turn represents the arithmetical difference between the rates of protein synthesis and of protein degradation[18,151,153]. Both of the two latter processes have been shown to be subject to regulation by serum and growth factors, and by culture conditions like cell density and substratum anchorage[6,15,76,130,152]. In those cases where distinguishing criteria are available (sensitivity towards inhibitors of autophagic-lysosomal function; no preference for short-lived protein), they are in general compatible with the notion that the growth-relevant protein-degrading process is identical to autophagy. Since accumulation of cytoplasm is a major aspect of growth, a process effecting bulk degradation of cytoplasm would indeed be well suited as a mechanism of growth restriction. In nutritionally deprived hepatocytes, autophagic degradation of cell mass may occur to such an extent as to cause extensive cell death within 24 h unless autophagy inhibitors are added[156]. The reduction in autophagic responsiveness by neoplastic cells[9,156] may thus significantly improve their ability to survive and grow under restrictive conditions, thereby contributing to the malignant phenotype.

Microautophagy

Lysosomes of the highly condensed type (dense bodies) have a relatively high surface-to-volume ratio, thus tending to develop invaginations and assume cup-like shapes which may look like rings in electron micrographs[3]. These structural properties can give the impression that dense bodies are able to sequester cytosol by invagination of the lysosomal membrane, a possibility described by the term *microautophagy*[40]. While dense bodies seem to be passive in relation to ordinary (macro)autophagy[177], it has been postulated that they may account for the basal, non-autophagic (amino acid-insensitive) protein degradation[110,117]. This notion is mainly based on the good correlations observed between hepatic protein degradation rates in vivo, the proteolytic activity of lysosomal preparations in vitro, and the volume fraction of the lysosomal compartment[118,119,123,158]. However, it should be noted that the calculation of these parameters is in large measure based on a posteriori premises which are debatable, and that correlations do not necessarily imply a causal relationship. For example, the fact that neither the lysosomal compartment nor protein degradation rates can be suppressed to zero[158] does not suffice to identify the residual proteolysis as lysosomal. In fact, this basal degradation is resistant to all conceivable combinations of lysosome inhibitors at maximally effective concentrations[160,162,164], and is thus very unlikely to be lysosomal.

More direct support for microautophagic activity comes from experiments with isolated dense bodies in vitro. These lysosomes have been shown to be able to sequester colloidal silica particles by means of membrane invagination[108]. Isolated lysosome preparations can apparently also take up and degrade added proteins by a process sensitive to chloroquine and NH_4Cl[3,34,195]. More detailed studies of this and similar experimental systems should be performed to establish the relationship between in vitro and in vivo proteolysis. It is noteworthy that e.g. 3MA completely inhibits the sequestration of sugar probes or cytosolic enzymes in intact cells[95]; any physiologically relevant in vitro sequestration process should therefore display sensitivity towards this inhibitor.

Signal-dependent uptake and proteolysis in lysosomes

In certain fibroblastic cell lines, serum starvation approximately doubles the rate of degradation of long-lived proteins[8]. The majority of cytosolic proteins are subject to this accelerated degradation[182], provided they fulfil certain structural requirements, e.g. the possession of a KFERQ sequence[10,42]. The accelerated degradation is inhibited by NH_4Cl[113] and by 3MA[78], and would therefore seem to have properties consistent with an autophagic-lysosomal mechanism, albeit of a selective kind contrasting with the non-selective autophagy in hepatocytes[95].

However, experiments with permeabilized cells and cell-free lysosome preparations indicate that the accelerated

protein degradation in fibroblasts is not equivalent to autophagy, but rather represents a direct uptake of proteins across the membrane of the lysosome (or some related vacuole), chaperoned by a member of the 70-kDa heat shock protein family which recognizes KFERQ sequences [34]. Similarly chaperoned mechanisms of protein translocation are involved in mitochondrial protein import [73] and probably in the secretion of certain growth factors and other polypeptides that lack the signal sequence for translation-dependent translocation into the ER [124]. The fact that microinjected proteins containing the KFERQ recognition sequence, and also their degradation products, can be secreted from fibroblasts [87] may actually indicate that these proteins are taken up and degraded in a compartment which communicates with a secretory pathway. Since the degradation of both exogenous and endogenous protein is accelerated by serum starvation and inhibited by 3MA in fibroblasts [78], it would seem that endocytic, exocytic and proteolytic compartments in these cells are tightly coupled both in the physical and in the regulatory sense.

Endoplasmic reticulum and the 'salvage compartment'

The endoplasmic reticulum (ER) with its associated post-ER 'salvage compartment' is a proteolytically active organelle. Resident ER proteins are often short-lived; for example, glucosidase II has a half-life of 0.8 h [72]. Another short-lived ER enzyme, HMG-CoA reductase, is feedback-controlled by its end product cholesterol which induces phosphorylation and accelerated degradation of the enzyme [198], apparently by a non-lysosomal mechanism [131, 186]. In addition, the ER serves an important function in the cleavage of signal peptides [197] and in the degradation of newly synthesized proteins which are improperly folded or assembled [91].

Secretory and membrane proteins carry an N-terminal signal sequence that targets them to the ER and ensures their transfer through the ER membrane during translation [144]. The signal-carrying N-terminus is cleaved off by a signal peptidase, a serine proteinase located in the ER membrane with its catalytic site in the ER lumen [197]. The signal peptidase is unaffected by leupeptin, but inhibited by chymostatin [184], possibly contributing to the large effect of the latter inhibitor on non-lysosomal protein degradation [70]. Subsequent proteolysis of signal peptides to amino acids would be expected to represent a substantial contribution to the overall degradation of short-lived protein, but unfortunately the proteolytic fate of the signal peptides is unknown. The amino acid-stimulated, non-lysosomal protein degradation specifically observed with[^{35}S]methionine-labelled peptides (which would be enriched in N-termini)[155] is one possible candidate process for signal peptide proteolysis.

Most, if not all, ER-associated protein degradation appears to take place in a post-ER compartment known as the 'salvage compartment'. Newly synthesized secretory and membrane proteins seem to be transferred in bulk from the ER to this compartment along with resident ER proteins. The resident proteins return to the ER by a signal-dependent mechanism (a luminal KDEL sequence), while the remaining proteins are either degraded in the salvage compartment or passed on to the Golgi apparatus for secretion [91, 104, 132]. In the case of the T-cell receptor, a protein complex with seven structurally different subunits, it was shown that unassembled chains and dimers were degraded in the salvage compartment, while incomplete oligomeric receptors were transported through the Golgi and eventually degraded in lysosomes [33, 103]. Degradation in the salvage compartment was found to be insensitive to pH-disruption agents and various inhibitors of autophagy and lysosomal function; even energy inhibitors had little effect. However, the degradation was temperature-sensitive, and was abolished by alkalinization of the cytosol to pH 8.0 [103]. Unassembled asialoglycoprotein receptor subunits [4], variant α_1-antitrypsin [102] and immunoglobulin single chains [91, 180] showed similar characteristics of degradation in an ER-associated compartment. In the case of unassembled acetylcholinesterase chains in muscle cells, degradation seemed to take place after passage to, and exit from, the cis/medial Golgi cisternae [150], suggesting the existence of a pathway from the Golgi to the salvage compartment.

It has been suggested that peptides of the major histocompatibility complex (MHC) class are degraded to some extent in lysosomes prior to their processing in the Golgi [107]. The short-lived ER enzyme HMG-CoA reductase similarly appears to be degraded in part by a process with lysosomal characteristics [131]. The possibility should therefore be considered that there may be a short-cut pathway from the salvage compartment to lysosomes, possibly via amphisomes. The rapid acquisition of ER marker proteins by autophagic vacuoles [49] could be interpreted as an additional indication of relatively direct traffic between these two compartments.

Protein degradation in endosomes

The subcellular fractionation studies by T. Berg and P. Stahl [19, 46] provided the first indications that endosomes might be proteolytically active. Subsequent investigations have shown that protein degradation can occur in a series of sequential endocytic compartments, in which there is progressive proteolytic modification of endocytosed ligands [66]. A part from the sequential heterogeneity of endosomes, there is considerable heterogeneity with respect to ligand entry, with different receptors internalizing to different endosomes [66, 196]. The proteolytic endosomes can apparently receive material both from fluid-phase and receptor-mediated endocytosis [25, 35]); in addition there is a separate receptor-mediated pathway to non-proteolytic endosomes, as shown in the case of asialoglycoprotein endocytosis [35]. Conver-

gence of separate endocytic pathways for fluid and adsorbed markers has been demonstrated at the level of late endosomes[25], but it is not clear whether the proteolytic and non-proteolytic pathways similarly converge.

Early endosomes are moderately acidic (pH 6.0–6.5), the intermediate pH being maintained by the antagonistic effects of an electrogenic proton pump and an electrogenic Na^+,K^+-ATPase[29,52]. Later endosomes and lysosomes lack the Na^+,K^+-ATPase and are therefore more acidic (pH 5.0–5.5). All of these acidic vacuoles provide a suitable environment for hydrolytic enzyme activity, but their pH differences may ensure different enzymatic activity profiles even if the vacuoles contain similar complements of enzymes.

Early endosomes contain a number of lysosomal enzymes, like cathepsin B, cathepsin D, acid phosphatase and esterases[22,25]. These enzymes appear to have permanent residence in endosomes, but their amount is very low compared to the amount found in lysosomes[25]. Some of the enzymes, like acid phosphatase, are brought to early endosomes as transmembrane proteins, passing through the Golgi apparatus, the *trans*-Golgi network (TGN) and the early endosomes on their way to the lysosomes[188]. In some cells (but not all[187]), several rounds of enzyme recycling between the plasma membrane and the endosome may occur[26]. Other enzymes, like several of the lysosomal cathepsins, are picked up in the TGN and transported to early endosomes by a small (46 kDa) mannose 6-phosphate receptor (MPR)[32]; the enzymes are thus present in endosomes in their immature proenzyme form, but nevertheless display some proteolytic activity. It is not clear how the enzymes are retained in endosomes after delivery; possibly a binding protein in the endosomal membrane is involved[44].

The small MPR receptor competes in the TGN with a large (300 kDa) MPR; the latter picks up the majority of the mannose 6-phosphate-containing lysosomal enzymes and delivers them to a late endosome, where dissociation occurs and the receptor returns to the TGN while the enzymes enter the lysosomes[37,68]. If the latter route is interrupted by elevation of the pH in the TGN e.g. with monensin or acidotropic amines (resulting in dissociation of enzymes from the MPRs), by changes in receptor affinities, overexpression of the small relative to the large receptor, or overexpression of the enzymes, a major fraction of the lysosomal enzymes may actually be secreted[32]. Such changes may take place in cancer cells[48], where secretion of proteinases like procathepsin L ('major excreted protein') or other procathepsins is thought to contribute to the invasive and metastatic properties of malignant cells[1,27,111].

Several nonlysosomal proteinases have also been found in early endosomes. An endosomal enzyme capable of degrading protein-polylysine conjugates appears to be a serine proteinase, inhibitable by the Bowman-Birk trypsin inhibitor[178]. The endosomal insulin-degrading proteinase is an enzyme which requires a low pH[12,41], but it is clearly different from the lysosomal cathepsins, as is indicated by its insensitivity towards inhibitors like leupeptin and pepstatin. The enzyme is sensitive to bacitracin and to the metalloproteinase inhibitor phenanthroline[47,89]. The insulin-degrading metalloproteinase appears to be essential for myoblast differentiation, possibly by generating a secondary effector from insulin[89]. This regulatory function could be relevant to the proposed role of endosomal metalloproteinases in the control of endosomal fusion and recycling processes[185]. Another leupeptin-resistant enzyme, which degrades the LDL receptor under certain conditions, is localized in a post-Golgi compartment which may correspond to early endosomes[67,179]. Interestingly, a pH-independent degradation of acetylated LDL in macrophages could be inhibited by interferon without causing any intracellular LDL accumulation, suggesting endocytic-exocytic recycling of undegraded ligand through endosomes under these conditions[51].

Calpains, the calcium-requiring proteinases, are at least partially localized in endosomal membranes (accessible to inactivation by endocytosed calpastatin), but their enzymatic activity may be exerted on the cytoplasmic side of the membrane, and particularly after translocation to the plasma membrane[140].

Proteolytic events related to antibody presentation

Antibodies bound to surface Fc receptors on macrophages first enter a proteinase-free endocytic vesicle, then an early endocytic compartment containing all the molecular machinery believed to be required for antigen processing and presentation, including proteolytic enzymes like cathepsin B and cathepsin D[71,112]. Antibody degradation has been shown to require a low pH[143]. Since endosomes do not contain a complete complement of lysosomal enzymes, peptidic cleavage products are not degraded to the level of amino acids in these vacuoles. Some peptides are secreted (exocytosed) as soluble fragments[45], whereas others may be picked up by antigen receptors (major histocompatibility complex class II molecules) and subsequently presented at the cell surface[28]. The MCH II molecules are brought to endosomes (along the secretory pathway) in a complex with an inhibitor, the invariant chain, which also serves to target the complex to endosomes (by means of a signal in its cytoplasmic tail). The invariant chain detaches from the MHC II molecule in the acidic endosomal environment, thereby leaving the latter active for binding of antigens. The invariant chain itself is apparently degraded in the endosomes[13,28,107]. If the invariant chain is expressed alone, i.e. in the absence of MHC molecules, it leaves the secretory pathway already in the post-ER (salvage) compartment and goes to the lysosomes to be degraded[28].

Proteolytic processing in the Golgi apparatus and in secretory granules

Both the *trans*-Golgi network and the condensing secretory vacuoles (and some, but not all secretory granules) are acidic compartments[7,129,192], and pH-elevating agents have accordingly been shown to suppress the secretion[128,176] as well as the proteolytic processing of secretory protein[83,106,126,127]. These agents furthermore disrupt the *trans*-Golgi sorting of secretory proteins to specific secretory granules and instead cause a generalized secretion through the non-regulated (constitutive) pathway[116,191,192].

Proteolytic processing of secretory proteins takes place shortly before secretion[88], apparently in the *trans*-Golgi network[80] by a calcium-dependent proteolytic process[39,88]. In liver Golgi fractions, the conversion of proalbumin to albumin is catalyzed by calcium-dependent endoproteinase. This membrane-bound enzyme is weakly inhibited by serine proteinase inhibitors, but it is resistant to chymostatin and to the thiol proteinase inhibitor E-64, suggesting a similarity to subtilisin-like serine proteinases[115]. Related protein-processing proteinases can be found in secretory granules[38], and in some cancer cells secretion of calcium-dependent serine proteinases has been described[154].

The degradation of some secretory proteins may be relatively pH-independent: normal collagen degradation is only slightly affected by NH_4Cl and other lysosome inhibitors, in contrast to the degradation of abnormal collagen, which appears to occur in lysosomes[16].

An endosomal switch between exocytosis/endocytosis and lysosomal degradation?

The endosomal compartment is a meeting place where pathways enter from endocytosis and the *trans*-Golgi network, and exit to exocytosis/secretion and lysosomal degradation. Flux through the latter pathway seems to be highly regulated at the endosomal level. It has thus been shown that amino acids exert negative control over the fraction of endocytosed fluid-phase markers channeled in the lysosomal direction[20]; the ability of insulin to stimulate exocytic recycling[189] may be another aspect of the same mechanism. Conversely, if fibroblasts are starved of serum and amino acids, the lysosomal degradation of endocytosed α_2-macroglobulin and EGF is strongly accelerated, while there is relatively little effect on the rates of endocytosis[78]. Interestingly, the autophagy inhibitor 3MA suppressed the starvation-induced (but not the basal) degradation of these endocytosed ligands[78], which suggests either a common control mechanism for starvation-induced processes, or a functional coupling between autophagy and a regulated endocytic-lysosomal pathway.

Net rates of protein secretion are controlled in part by intracellular, lysosomal degradation of secretory protein[21,125], i.e. a pathway switch between secretion and lysosomal degradation must exist. The effect of this switch can be substantial; in the case of lipoprotein lipase synthesis in chicken adipocytes, heparin could reduce the fraction degraded from 76 to 21%, thus trebling the net rate of secretion[36]. The suppression of casein secretion by extracellular milk proteins seems to depend on the ability of the latter to accelerate the intracellular degradation of secretory milk proteins in mammary gland cells[194]. In mammary explants from pregnant rabbits as much as 50–90% of the newly synthesized casein may be degraded intracellularly, apparently in lysosomes[145]. Abnormal collagen chains which fail to form normal oligomeric complexes (triple helices) are degraded lysosomally[17]; these are probably routed to lysosomes (after passage through the Golgi) along the same pathway as incompletely assembled receptors[33]. The ability of hormones and growth factors to stimulate lysosomal enzyme secretion in some cancer cells[30,53,142,193] may be due to the 'ectopic' presence of these enzymes in the compartment regulated by the secretion/degradation switch. It is not clear whether secretory proteins exit to lysosomal degradation from the *trans*-Golgi network or from endosomes, but the possibility should be considered that the secretion/degradation switch may be identical to the endosomal exocytosis/degradation switch.

Crinophagy

Ultrastructural studies suggest that even mature secretory granules may deliver their contents to lysosomes for degradation, by fusing with lysosomes or prelysosomal vacuoles[110], or with endosomes[82]. The relationship between this process, termed *crinophagy*[183], and the mechanisms for degradation of secretory protein discussed above, is not clear. Crinophagy could be the morphological equivalent of the pathway for secretory protein from *trans*-Golgi/endosomes to lysosomes, or it could be an additional, separate degradation mechanism. Secretory granules can also be delivered to amphisomes and lysosomes by autophagy, and the relative roles of these different mechanisms can only be assessed by more extensive experimentation.

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Genetic analysis of ubiquitin-dependent protein degradation

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Abstract. Selective degradation of cellular proteins serves to eliminate abnormal proteins and to mediate the turnover of certain short-lived proteins, many of which have regulatory functions. In eukaryotes a major pathway for selective protein degradation is ATP-dependent and is mediated by the ubiquitin system. This pathway involves substrate recognition by components of a ubiquitin-protein ligase system, covalent attachment of ubiquitin moieties to proteolytic substrates, and subsequent degradation of these conjugates by a multicatalytic protease complex. Recent genetic evidence suggests that the remarkable selectivity of this process is largely controlled at the level of substrate recognition by the ubiquitin ligase system. In *Saccharomyces cerevisiae*, ubiquitin-conjugating enzymes UBC1, UBC4 and UBC5 have been identified as key components of this highly conserved degradation pathway. Genetic analysis indicates that ubiquitin-dependent proteolysis is essential for cell viability and that UBC4 and UBC5 enzymes are essential components of the eukaryotic stress response.
Key words. Yeast; protein degradation; ubiquitin conjugating enzymes; signals for proteolysis; stress response.

Introduction

Protein levels in living cells are controlled both by synthesis and degradation. In every cell, proteins with relatively long half-lives coexist with proteins with short half-lives. This remarkable difference in protein stability requires mechanisms which precisely control the selectivity of the degradation process. Selective degradation is involved both in the turnover of short-lived proteins such as crucial cellular regulators, and in the selective elimination of abnormal, mislocalized and misassembled proteins.

Eukaryotes have evolved different degradative pathways. Protein degradation via lysosomes is strongly induced under starvation conditions and appears to be rather unselective. However, recent evidence suggests that a specific sequence motif (e.g. Lys-Phe-Glu-Arg-Gln) [7] may target some long-lived proteins for lysosomal degradation. A member of the hsp70 stress protein family was found to bind a protein bearing this sequence motif, and may function in the translocation of proteins across lysosomal membranes. Recently, a novel degradation pathway located in the endoplasmic reticulum has been described [21]. This pathway appears to be responsible for the elimination of newly-synthesized membrane proteins passing through the secretory pathway when these have failed to fold correctly or to assemble into the requisite oligomeric complexes.

A major pathway for the selective degradation of abnormal and short-lived proteins in the cytosol depends both on ATP and ubiquitin [24]. Biochemical and genetic evidence indicates that ubiquitin-protein conjugates are essential intermediates for this proteolysis pathway [16]. Degradation by the ubiquitin system serves essential functions of the cellular stress response. Moreover, this pathway is involved in the control of the half-lives of important cellular regulators. The ubiquitin system mediates the selective degradation of the far-red light absorbing form of phytochrome (Pfr), a regulatory photoreceptor of higher plants [30]. Recent in vitro studies indicate that the short half-life of p53, a negative regulator of cell proliferation, can be controlled by the ubiquitin system [26]. In addition, a key step governing the exit from mitosis, degradation of the cell cycle regulator cyclin, is mediated by the ubiquitin pathway [11].