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REVIEW

Autophagic proteolysis: control and specificity

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Summary

The rate of proteolysis is an important determinant of the intracellular protein content. Part of the degradation of intracellular proteins occurs in the lysosomes and is mediated by macroautophagy. In liver, macroautophagy is very active and almost completely accounts for starvation-induced proteolysis. Factors inhibiting this process include amino acids, cell swelling and insulin. In the mechanisms controlling macroautophagy, protein phosphorylation plays an important role. Activation of a signal transduction pathway, ultimately leading to phosphorylation of ribosomal protein S6, accompanies inhibition of macroautophagy. Components of this pathway may include a heterotrimeric G_{i3}-protein, phosphatidylinositol 3-kinase and p70S6 kinase. Recent evidence indicates that lysosomal protein degradation can be selective and occurs via ubiquitin-dependent and -independent pathways.

Introduction

The protein content of the cell is determined by the balance between protein synthesis and protein degradation. At constant intracellular protein concentration, i.e. at steady state, rates of protein synthesis and degradation are equal. Although turnover of protein results in energy dissipation, regulation at the level of protein degradation effectively controls protein levels (Paskin & Mayer, 1977; Mayer & Doherty, 1986).

Both intra- and extralysosomal pathways are responsible for the breakdown of cellular proteins. Extralysosomal proteolytic pathways include the 26S proteasome complex with multiple proteolytic activities, which is also part of the ATP- and ubiquitin-dependent proteolytic system (Hershko & Ciechanover, 1992; Jennissen, 1995; Hilt & Wolf, 1996), the calpains or Ca^{2+}-dependent proteases (Mellgren, 1987; Saido *et al*., 1994) and other, presumably ATP-independent, proteases. In the liver, these extralysosomal pathways are responsible for the degradation of a small pool of proteins with a high turnover rate, whereas the breakdown of long-lived protein occurs predominantly in the lysosomes by macroautophagy (Mortimore *et al*., 1989; Seglen & Bohley, 1992). In muscle, however, the relative contribution of extralysosomal proteolytic systems to overall protein degradation is larger, and accelerated breakdown of myofibrillar protein, e.g. during starvation, mainly proceeds via the ubiquitin system (Wing & Goldberg, 1993).

Lysosomal protein degradation

Intracellular proteins to be degraded in the lysosomes can get access to these organelles by the following processes: macroautophagy, microautophagy, crinophagy and selective, chaperonin-mediated, direct uptake of proteins. Each of these processes will be briefly described first. This is then followed by a detailed discussion of the mechanisms controlling macroautophagy, which is, quantitatively, the most important process responsible for the lysosomal degradation of intracellular proteins. In the past, excellent reviews on macroautophagy have appeared (Mortimore *et al*., 1989; Seglen & Bohley 1992; Dunn, 1994). Therefore, we will restrict ourselves mainly to very recent developments in this field.

Because most of the studies on macroautophagy have been carried out in liver, presumably because

of its quantitative importance in this organ, our discussion will mainly be based on results obtained with liver parenchymal cells. However, where appropriate, data obtained with other cell types will also be mentioned.

Macroautophagy

During macroautophagy, portions of the cytoplasm, sometimes even containing whole organelles, are surrounded by a sequestering membrane (Fig. 1). Autophagosomes thus formed possess a double membrane. The inner and outer membrane of autophagosomes are presumably formed by invagination of ribosome-free parts of the rough endoplasmic reticulum: membrane proteins of the rough endoplasmic reticulum are found in both limiting membranes of the autophagosomes, while secretory proteins and glucose 6-phosphatase, present in the lumen of the rough endoplasmic reticulum, are found in the space between the inner and outer membrane of some of the autophagosomes (Dunn, 1990a; Yokota, 1993). However, because a significant percentage of autophagosomes appears to lack proteins of the rough endoplasmic reticulum, it has been postulated that there is recycling or degradation of membrane proteins of the rough endoplasmic reticulum (Dunn, 1994). Another view is that autophagosomes do not originate from the endoplasmic reticulum, but rather from post-Golgi membranes containing complex type N- and/or O-linked oligosaccharide chains (Yamamoto *et al*., 1990a, b). Alternatively, there may be an additional mechanism of autophagosome formation from pre-existing membrane structures, phagophores, as proposed by Seglen and colleagues (Seglen, 1987; Fengsrud *et al*., 1995).

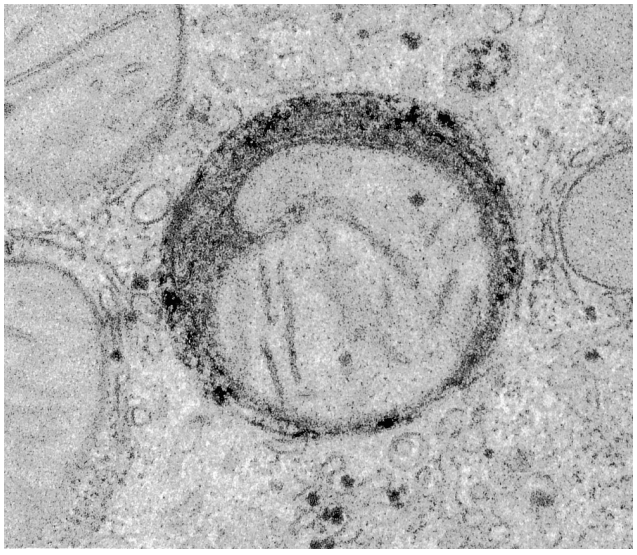


Fig. 1. Electron micrograph of an autophagolysosome in a rat hepatocyte containing a still recognizable mitochondrion on its pathway to degradation (352 000). The enclosed mitochondrion is surrounded by precipitate resulting from an incubation to demonstrate acid phosphatase activity (courtesy of Dr J.P.M. Schellens).

The newly formed, initial autophagosomes mature into degradative autophagosomes in a stepwise process: acquisition of lysosomal membrane proteins by fusion with vesicles deficient in hydrolytic enzymes, followed by acidification, fusion with existing lysosomes for acquisition of hydrolytic enzymes and degradation of protein and other macromolecular material in the autophagolysosomes (Dunn, 1990b, 1994). According to Seglen and colleagues (Gordon & Seglen, 1988; Gordon *et al*., 1992; Strømhaug & Seglen, 1993; Fengsrud *et al*., 1995), hepatic autophagosomes may become acidic by fusion with early endosomes to organelles that were called amphisomes. Convergence of initial autophagosomes and early endosomes has also been observed in the pancreas (Tooze *et al*., 1990). In hepatocytes from rats treated with a peroxisome proliferator, initial autophagosomes appear able to fuse with late, but not early, endosomes (Yokota *et al*., 1995). The view that autophagosomes fuse with vesicles deficient in hydrolytic enzymes is not generally accepted: Lawrence and Brown (1992) believe that lysosomal enzyme delivery to nascent autophagosomes occurs primarily by fusion with pre-existing mature lysosomes. However, it is possible that the exact mechanism varies with the cell type and with the methods used to induce autophagy (Lawrence & Brown, 1992; Dunn, 1994).

In liver, autophagosomes are synthesized and degraded with a half-life of 8 min (Pfeifer *et al*., 1978; Schworer & Mortimore, 1979). Because of their high turnover rate, the steady-state volume of autophagosomes in the cell is low, even when autophagic flux is high. Thus, in hepatocytes, the volume of autophagosomes varies between 0.2% in the fed state to 1–1.5% of the total cell volume in the fasted state (Schworer & Mortimore, 1979; Kovács *et al*., 1982; Pfeifer, 1987).

In many cell types, macroautophagy is responsible for the acceleration of proteolysis when the concentration of amino acids and insulin falls. In rat liver *in vivo*, for example, when rates of protein synthesis and degradation are equal, protein turnover (which is a mixture of both intra- and extralysosomal proteolysis) is 1.5–2% of total intracellular protein per hour (Mortimore *et al*., 1989). During fasting, this balance is disturbed primarily because of an increase in macroautophagy. In rats and mice, there is a net loss of 20–25% of liver cell protein after 24 h fasting (Mortimore *et al*., 1989; Cuervo *et al*., 1995), equivalent to a rate of 1% per hour. Under extreme experimental conditions, i.e. in the absence of amino acids in a perfused liver

system or in isolated hepatocytes, macroautophagy can reach values as high as 4–5% protein loss per hour (Mortimore *et al*., 1989; Seglen & Bohley, 1992).

Microautophagy

During microautophagy, portions of the cytoplasm are taken up directly by the lysosomes by invagination of their membranes (Ahlberg *et al*., 1982; Mortimore *et al*., 1983, 1988a; de Waal *et al*., 1986). Microautophagosomes are formed, but they are inside the lysosomes, where they are rapidly degraded. In contrast to macroautophagy, microautophagy is insensitive to inhibition by amino acids (Mortimore *et al*., 1983, 1988a). It is also assumed in the literature that microautophagy is ATP independent (Ahlberg *et al*., 1982). In our opinion, however, it is difficult to understand that this complicated process of membrane flow does not require an input of energy.

There are indications that microautophagy declines in long-term starvation, although the signal responsible for this is unknown (Mortimore *et al*., 1983, 1988a). An attractive idea, although never tested, is that ketone bodies, which can replace glucose as a fuel for the brain under those conditions, are able to control this process.

Crinophagy

Proteins destined for secretion from the cell can be delivered to the lysosomes by a process called crinophagy. In this process, mature secretory vesicles fuse with existing lysosomes, autophagosomes, endosomes or amphisomes (Seglen & Bohley, 1992). Although the significance of this process is not clear, it has been proposed that crinophagy may be a mechanism controlling the secretion rate of export proteins (Yi *et al*., 1993).

Selective targeting of cytosolic proteins to lysosomes. Under stress conditions, such as starvation or serum withdrawal from cell culture media, in many cell types (e.g. liver, kidney, heart, fibroblasts, but not in skeletal muscle; Wing *et al*., 1991), specific cytosolic proteins can be targeted directly to the lysosomes for degradation. Proteins following this route possess peptide sequences similar to Lys-Phe-Glu-Arg-Gln (KFERQ) (Dice, 1990; Cuervo *et al*., 1995). This peptide signal is recognized by the cytosolic heat shock cognate protein hsc73, which binds to the KFERQ region and helps in the recognition process by the lysosomes (Terlecki *et al*., 1992; Terlecki, 1994; Cuervo *et al*., 1995). After binding of the complex to a lysosomal receptor protein, the KFERQ-containing protein is translocated across the lysosomal membrane and subsequently degraded (Terlecki & Dice, 1993). Serum withdrawal from cultured fibroblasts (Terlecki & Dice 1993) or starvation of rat liver (Cuervo *et al*., 1995) results in an activation of the hsc73 protein and in increased association of this protein with the lysosomes (rather than in an increase in the amount of hsc73), so that the recognition of KFERQ-like peptide motifs by the lysosomes becomes more effective. The lysosomal membrane glycoprotein LGP96 was recently identified as a receptor for this selective import and degradation of proteins in the lysosomes (Cuervo & Dice, 1996).

Although cells rapidly respond to starvation (or amino acid depletion) by increasing the rate of macroautophagy, the selective pathway of protein degradation becomes activated only after prolonged starvation. In rat liver, for example, 25% of all intracellular proteins are degraded by macroautophagy in the first 24 h of starvation, and rapid depletion of cytosolic proteins would occur after prolonged starvation, if no mechanism existed to prevent this. The selective pathway, which in rat liver becomes activated after about 2 days of starvation, degrades almost all of the 30% of cytosolic proteins containing the KFERQ-like motifs (Cuervo *et al*., 1995). Proteins not containing this sequence are spared from degradation during long-term starvation, presumably because they are indispensable under these conditions.

Another example of specific targeting of a cytosolic protein to the lysosome was discovered in the yeast *Saccharomyces cerevisiae* (Chiang & Schekman, 1991). Shifting yeast from a medium containing ethanol to glucose as a carbon source resulted in the breakdown of the gluconeogenic enzyme fructose 1,6-diphosphatase in the yeast vacuole, which is the equivalent of the lysosome in mammalian cells. Mutants deficient in vacuolar proteases were unable to degrade fructose 1,6-diphosphatase. Presumably, glucose stimulates a receptor protein in the membrane of the vacuole. Whether the mechanism of vacuolar uptake involves microautophagy or a chaperonin-mediated direct translocation of fructose 1,6-diphosphatase into the vacuole, or a combination of both, is not entirely clear (Chiang *et al*., 1996). An alternative view is that fructose 1,6-phosphatase is not targeted to the vacuole but, instead, is degraded via the ubiquitin–proteasome pathway (Schork *et al*., 1995).

Control of macroautophagy

Energy requirement

Macroautophagy is ATP dependent (Plomp *et al*., 1988; Schellens & Meijer, 1991), with ATP being required for each of the steps involved in the process: sequestration, vesicle fusion and intralysosomal proteolysis (Plomp *et al*., 1989). The ATP dependence of the last step presumably reflects the

ATP requirement of the lysosomal H^+ pump. Because of the very low K_m of the lysosomal H^+ pump for ATP, it is unlikely that variation in the cytosolic concentration of ATP will affect the activity of this pump under normal conditions (Luiken *et al*., 1996). By contrast, hepatic autophagic sequestration, as measured by the sequestration of electro-injected cytosolic [^{14}C]sucrose, is very sensitive towards small changes in the intracellular concentration of ATP (Plomp *et al*., 1988). Although under normal conditions the intrahepatic ATP concentration is rather strongly buffered, it is postulated that under certain pathological conditions, e.g. in alcoholic liver disease, the decline in autophagy resulting from decreasing ATP concentrations contributes to the accumulation of fat and protein (Plomp *et al*., 1988).

Control by amino acids and hormones

Since one of the major functions of macroautophagy is to produce amino acids for the survival of the organism when nutrients fall short, it is not surprising that amino acids are effective product inhibitors of this process. The system is also hormonally controlled: insulin inhibits, while glucagon stimulates (Mortimore *et al*., 1989; Seglen & Bohley, 1992). The relevant parameter is presumably the glucagon–insulin ratio (Parrilla *et al*., 1974). *In vitro*, in the isolated perfused liver, the hormones are only effective as modulators of autophagy at intermediate amino acid concentrations. Under these experimental conditions, insulin and glucagon have no effect in the absence of amino acids when autophagic flux is maximal, nor do they act in the presence of high concentrations of amino acids when macroautophagy is maximally inhibited (Mortimore *et al*., 1989).

Electron microscopic studies (Schworer & Mortimore, 1979; Kovács *et al*., 1981) and data obtained with electro-injected cytosolic [^{14}C]sucrose and other sugars (Seglen & Gordon, 1984; Seglen & Bohley, 1992) have led to the conclusion that the inhibition of macroautophagy by amino acids primarily occurs at the sequestration step. High concentrations of amino acids do not, however, completely inhibit autophagic sequestration of [^{14}C]sucrose (Seglen & Gordon, 1984), even though they completely inhibit overall autophagic proteolytic flux. This indicates that amino acids also interfere with post-sequestrational steps. This is, indeed, the case. At high concentrations, the amino acid asparagine, for example, is also able to inhibit the fusion between autophagosomes and lysosomes (Høyvik *et al*., 1991). A direct effect of some amino acids on the lysosomes, resulting in a rise in the intralysosomal pH, can also contribute to the inhibition of proteolysis (Völkl *et al*., 1993; Luiken *et al*., 1996).

It has also become clear that not all amino acids are equally effective as inhibitors of macroautophagy. From studies with the perfused rat liver, Mortimore *et al*. (Pösö & Mortimore, 1984; Mortimore *et al*., 1988b, 1989, 1991) defined a regulatory group of eight amino acids consisting of leucine, phenylalanine, tyrosine, glutamine, proline, histidine, tryptophan and methionine with alanine, which by itself does not affect macroautophagy, as a synergistic co-regulator. A similar group of regulatory amino acids (except that asparagine replaced methionine) was defined by Seglen and colleagues in experiments carried out with isolated rat hepatocytes (Seglen *et al*., 1980). In perifused rat hepatocytes, under true steady-state conditions, combinations of near-physiological concentrations of leucine with either alanine, proline, glutamine or asparagine were particularly effective in inhibiting autophagic proteolysis (Leverve *et al*., 1987; Caro *et al*., 1989). This was also found in the perfused liver (Mortimore *et al*., 1988b). Glutamine is also effective as an inhibitor of hepatic proteolysis in the rat *in vivo* (Bergamini *et al*., 1994).

Information on the nature of amino acids controlling macroautophagy has also been obtained from the liver in the perinatal period. In this period, the liver grows rapidly, which in part results from suppression of the process (compared with the adult situation) by high plasma amino acid concentrations combined with the fact that, in this period, glucagon is not yet able to exert its catabolic effects (Blommaart *et al*., 1993). From a detailed study on the relationship between extracellular and intracellular amino acid concentrations in cultured hepatocytes obtained from rats at various stages of development, it was concluded that leucine, phenylalanine and tyrosine are important regulators of macroautophagy *in vivo* (Blommaart *et al*., 1993).

To summarize, the data obtained with liver suggest that leucine, phenylalanine and tyrosine, in combination with a few other amino acids such as alanine and glutamine, are the most important amino acids involved in the control of autophagic proteolysis.

The specificity of amino acid inhibition of autophagic proteolysis has also been studied in other tissues. In kidney, leucine and phenylalanine, but not tyrosine, are inhibitory amino acids (Rabkin *et al*., 1991), while in heart only leucine is effective (Chua, 1994). Leucine and glutamine have been mentioned as regulators of lysosomal proteolysis in skeletal muscle (see Meijer *et al*., 1990 for review). Under many conditions, both *in vitro* and *in vivo*, there is an inverse relationship between the intramuscular glutamine concentration and the rate of proteolysis (Rennie *et al*., 1986). Parenteral administration of the dipeptide alanylglutamine (glutamine is chemically more stable as a dipeptide) to patients after surgery appears to be able to correct, at least

in part, the negative nitrogen balance (Stehle *et al*., 1989; Neu *et al*., 1996). However, it has to be borne in mind that the situation in muscle is different from that in liver. While non-fibrillar proteins are degraded by autophagy in muscle, myofibrillar protein is degraded by the ubiquitin–proteasome pathway located in the cytosol. The latter process is quantitatively more important. In muscle from fasted rats, flux through both pathways is increased, with glucocorticoids being responsible for the rise in ubiquitin-dependent proteolysis (Wing & Goldberg, 1993). In many other conditions with increased muscle proteolysis (e.g. sepsis, trauma, cancer, acidosis, denervation atrophy), it is the ubiquitin–proteasome pathway rather than lysosomal proteolysis that is increased (Attaix *et al*., 1994; Llovera *et al*., 1995; Medina *et al*., 1995; Bailey *et al*., 1996; Tiao *et al*., 1996). It is likely that amino acids primarily inhibit lysosomal proteolysis in muscle (Wing & Goldberg, 1993). For this reason, the effectiveness of amino acids in preventing a negative nitrogen balance in catabolic situations may be less than one would wish.

An exciting new development has been the discovery that cell swelling has anabolic effects on many metabolic pathways, closely resembling the effects of insulin (Häussinger *et al*. 1990; Baquet *et al*. 1990; Häussinger & Lang, 1991). For example, in liver an increase in cell volume stimulates the synthesis of glycogen (Baquet *et al*., 1990), fat (Baquet *et al*, 1991a) and protein (Stoll *et al*., 1992) and has inhibitory effects on glycogenolysis (Lang *et al*., 1989) and autophagic protein degradation (Häussinger *et al*., 1990). Hypo-osmotic cell swelling, like insulin (Mortimore *et al*., 1987), increases the sensitivity of macroautophagic proteolysis to inhibition by low concentrations of amino acids (Meijer *et al*., 1993; Luiken *et al*., 1994). By contrast, Häussinger and colleagues have found that an increase in cell volume alone is able to inhibit proteolysis and does not require the presence of amino acids (Hallbrucker *et al*., 1991a; vom Dahl *et al*., 1995). However, amino acids are always present in a proteolytic environment and differences in results probably relate to differences in experimental conditions (Meijer, 1995; vom Dahl & Häussinger, 1995). It has even been proposed that the antiproteolytic effect of insulin proceeds via an increase in cell volume, caused by activation of the $Na^+K^+/2Cl^-$ co-transporter with massive influx of KCl and NaCl (Hallbrucker *et al*., 1991b).

An increase in cell volume can also be caused by an influx of amino acids via Na^+-coupled amino acid transport systems. Owing to intracellular amino acid accumulation, intracellular osmolarity rises and the cells swell. Alanine and glutamine are present in plasma at relatively high concentrations and both amino acids are transported via Na^+-coupled transporters. It is likely that these two amino acids exert their antiproteolytic effect via cell swelling (Häussinger & Lang, 1991; Hallbrucker *et al*., 1991a). Leucine, tyrosine and phenylalanine, on the other hand, are not transported via Na^+-coupled systems and these amino acids do not accumulate inside cells but rather equilibrate across the plasma membrane. The antiproteolytic effects of these amino acids are probably specifically exerted at the macroautophagic sequestration step (Mortimore *et al*., 1989; Seglen & Bohley, 1992) and are independent of cell swelling. Synergism between, for example, alanine, glutamine and leucine (Leverve *et al*., 1987; Mortimore *et al*., 1988b; Caro *et al*., 1989; Venerando *et al*., 1994), can now be explained by the fact that cell swelling caused by glutamine or alanine influx potentiates the inhibition of macroautophagic sequestration by leucine.

The importance of cell volume in the control of proteolysis *in vivo* is highlighted by the observation that in a large number of clinical patients the cellular hydration state of skeletal muscle was inversely correlated with the degree of nitrogen loss from this tissue (Häussinger *et al*., 1993; Finn *et al*., 1996). Significantly, cell swelling, like insulin, promotes transport of glutamine into muscle cells, whereas cell shrinkage has the opposite effect (Low *et al*., 1996). However, it is not clear whether the loss of muscle protein by cellular dehydration is caused by increased macroautophagy or by an increase in extralysosomal, ubiquitin-mediated proteolysis, which in muscle is quantitatively the most important proteolytic pathway, as discussed above.

Little is known about the mechanism by which amino acids and changes in cell volume affect autophagic sequestration. In fact, this question can only be properly answered if information becomes available about the molecular machinery involved in the process. In this respect, yeast cells may become extremely useful, because autophagy in yeast is essentially similar to that in mammalian cells (Veenhuis *et al*., 1983; Baba *et al*., 1994) and allows the application of molecular genetic procedures in order to identify gene products involved in this process. Complementation studies with yeast mutants deficient in autophagy, obtained by screening on loss of cell viability during nitrogen starvation, have shown the participation of at least 15 different protein components (Tsukada & Ohsumi, 1993), and many more will be identified (Thumm *et al*., 1994). However, the nature of these proteins is, as yet, unknown.

It is likely that the cytoskeleton is involved in the process of autophagy. For example, in kidney cells, cytochalasins B and D, compounds that cause depolymerization of microfilaments, inhibit auto-

phagosome formation, while nocodazole, which causes depolymerization of microtubuli, inhibits the fusion between autophagosomes and lysosomes (Aplin *et al*., 1992). Likewise, the microtubule inhibitors vinblastine and colchicine prevent the fusion between autophagosomes and lysosomes with no effect on autophagic sequestration, as was shown in freshly isolated hepatocytes (Høyvik *et al*., 1986, 1991; Fengsrud *et al*., 1995). Apparently, an intact microtubule network is required for fusion between autophagosomes and lysosomes but not for the initial formation of autophagosomes, which appears to require cytokeratin filaments instead (Blankson *et al*., 1995). A connection of lysosomes with microtubuli was observed by Collot *et al*. (1984). Lysosomal movement along the microtubule network was observed by Sakai *et al*. (1989) and is likely to be mediated by kinesins, a group of microtubule-associated mechanochemical motor proteins (Swanson *et al*., 1992).

Data from vom Dahl *et al*. (1995) indicated that the cytoskeleton is not only required for the autophagic machinery but, in addition, is involved in the control of autophagy by cell swelling. Thus, these authors were able to show that low concentrations of the microtubule inhibitors colchicine and colcemid prevent the antiproteolytic effect of cell swelling and thus accelerate proteolysis.

Another mechanism by which changes in cell volume affect autophagy may be by changes in intracellular ion concentrations. When cells are exposed to conditions that alter their volume, they will try to restore their original size. Thus, cell swelling is followed by a regulatory volume decrease (RVD), caused by KCl efflux in most cells, and cell shrinking by a regulatory volume increase (RVI), because of a net uptake of NaCl and KCl (Hoffmann & Simonsen, 1989; Häussinger & Lang, 1991; Häussinger, 1996). Neither RVD nor RVI completely restores the initial volume, leaving the cell in a slightly swollen or shrunken state respectively. However, even although the net change in cell volume is relatively small, intracellular ion concentrations can be drastically changed. For example, we have shown in hepatocytes that the decrease in intracellular chloride caused by RVD deinhibits glycogen synthase phosphatase (Meijer *et al*., 1992). That a change in chloride also affects macroautophagy is less likely because hypo-osmotic cell swelling itself, which decreases intracellular chloride by about 50% (Meijer *et al*., 1992), does not affect autophagic proteolysis in isolated hepatocytes (Meijer *et al*. 1993; Fosse *et al*., 1995). Intracellular Ca^{2+} has been shown to increase upon cell swelling (Hoffmann & Simonsen, 1989; Baquet *et al*., 1991b; contrast Schreiber *et al*., 1996, who found no change), but this cannot explain the antiproteolytic effect of cell swelling because an increase in Ca^{2+} results in activation rather than in inhibition of macroautophagy (Caro, 1989; Gordon *et al*., 1993). Recent work by Häussinger and colleagues (Schreiber & Häussinger, 1995; Schreiber *et al*., 1996) with fluorescein isothiocyanate (FITC)–dextran indicates that cell swelling causes alkalinization of an early endocytic compartment rather than alkalinization of the lysosomes, as was originally thought (Busch *et al*., 1994). The latter finding is in line with recent data of Luiken *et al*. (1996): in order to monitor changes in lysosomal pH in hepatocytes, we used the pH-dependent conversion of lysyl-alanyl-4-methoxy-2-naphthylamide to lysylalanine and fluorescent 4-methoxy-2-naphthylamine by the lysosomal enzyme dipeptidylpeptidase II, and also failed to observe changes in lysosomal pH after hypo-osmotic swelling of the cells. Häussinger speculates that alkalinization of early endosomes inhibits their fusion with initial autophagosomes (Schreiber & Häussinger, 1995; Häussinger, 1996). Whatever the mechanism by which cell swelling-induced alkalinization of vesicles interferes with the autophagic pathway, it is clear that this mechanism is not related to the specific inhibitory effects of amino acids, such as leucine, phenylalanine and tyrosine, on autophagic sequestration (discussed in the next section), because these amino acids do not induce cell swelling, since their transport is not coupled to the Na^+ gradient across the plasma membrane.

Autophagy and signal transduction

Experiments with isolated hepatocytes indicate strongly that protein phosphorylation is involved in the control of autophagy. Thus, okadaic acid, an inhibitor of protein phosphatase 2A, strongly inhibits autophagic sequestration and simultaneously causes disruption of the cytoskeleton without affecting cell viability (Holen *et al*., 1992, 1993). Inhibition of autophagic sequestration by okadaic acid could be prevented by naringin and several other flavanone and flavone glycosides, and it was proposed that these compounds may be useful as protectants against pathological hyperphosphorylations (Gordon *et al*., 1995). Inhibition of autophagic sequestration by okadaic acid was also counteracted by KN-62, a compound that specifically inhibits Ca^{2+}/calmodulin-dependent protein kinase II (Holen *et al*., 1992, 1993). Apparently, Ca^{2+}/calmodulin-dependent protein kinase II exerts negative control on autophagic sequestration.

A possible target for okadaic acid action may be a cytokeratin with a molecular mass of 52 kDa, because inhibitors of protein phosphatase 2A increase the phosphorylation of this protein (Falconer & Yeung, 1992) and because Ca^{2+}/calmodulin-

dependent protein kinase II is one of the kinases that phosphorylates this cytokeratin (Yano *et al*., 1991).

In contrast to Ca^{2+}/calmodulin-dependent protein kinase II, other protein kinases may exert positive control on autophagy in that their activity is required for autophagosome formation. This is indicated by the fact that several inhibitors of tyrosine protein kinases strongly inhibit autophagic sequestration (Holen *et al*., 1992, 1995).

Recent evidence obtained from our own laboratory also strongly supports the view that protein phosphorylation is involved in the control of autophagy. More specifically, these experiments have yielded results that throw light on the mechanism by which amino acids control autophagic sequestration. Under a variety of experimental conditions, in the absence of protein synthesis, we observed that the degree of inhibition of macroautophagic proteolysis by single amino acids or by amino acid combinations strongly correlated with the phosphorylation of a 31-kDa protein, which we have identified as ribosomal protein S6. Moreover, hypo-osmolarity, while having no effect on its own, not only increased the sensitivity of proteolysis to inhibition by amino acids, but also promoted the effect of amino acids on the S6 phosphorylation. Likewise, in the presence of low concentrations of amino acids, glucagon stimulated proteolysis and reduced S6 phosphorylation, whereas the opposite effect, inhibition of proteolysis and increased S6 phosphorylation, was obtained with insulin (Luiken *et al*., 1994; Blommaart *et al*., 1995). Significantly, a combination of leucine, phenylalanine and tyrosine under hypo-osmotic incubation conditions largely mimicked the effect of a complete, physiological mixture of amino acids on both proteolysis and S6 phosphorylation (Luiken *et al*., 1994; Blommaart *et al*., 1995).

Stimulation of S6 phosphorylation by amino acids was completely prevented by the macrolide rapamycin, a selective inhibitor of p70S6 kinase activation, whereas dephosphorylation of S6 in the presence of rapamycin was not affected by amino acids (Blommaart *et al*., 1995). These results demonstrate that the increase in S6 phosphorylation caused by amino acids results from activation of p70S6 kinase and is not caused by an inhibition of S6 phosphatase. Direct evidence in favour of a connection between S6 phosphorylation and the control of autophagy was obtained from the finding that rapamycin was able to counteract, albeit not completely, the inhibition by amino acids of autophagic sequestration (Blommaart *et al*. 1995).

S6 can be phosphorylated at five different serine residues (Wettenhall *et al*., 1982). Although the function of these phosphorylations is not certain (Sturgill & Wu, 1991; Kozma & Thomas, 1992), it is assumed that S6 phosphorylation enhances the efficiency of mRNA translation (Palen & Traugh, 1987; Morley & Traugh, 1993), the more so because S6 phosphorylation occurs under conditions that promote cell growth and cell division (Sturgill & Wu, 1991; Kozma & Thomas, 1992). Recent evidence indicates that phosphorylation of S6 promotes the translation of a family of mRNAs, encoding for ribosomal proteins and elongation factors of protein synthesis, which is characterized by a polypyrimidine tract immediately after the N7-methylguanosine cap (Jefferies *et al*., 1994; Terada *et al*., 1994). In our experiments, however, protein synthesis was blocked by low concentrations of cycloheximide so that the effect of S6 phosphorylation on proteolysis could not be caused by an increase in the rate of protein synthesis. Interestingly, rapamycin has been shown to induce a starvation response in yeast, which has been ascribed to impairment of protein synthesis (Hall, 1995). However, an alternative interpretation is that in this organism rapamycin also accelerates autophagy.

Of course, it is possible that the same signal, i.e. a protein kinase, increases the phosphorylation of S6 in order to increase protein synthesis and at the same time phosphorylates another, as yet unknown, protein to inhibit macroautophagic proteolysis simultaneously. However, an alternative view is that S6 phosphorylation is directly involved in the control of the autophagic process. The problem then is to understand the mechanism by which a ribosomal protein can control autophagy. The key to the answer of this problem is perhaps the observation that the autophagosomal membrane is derived from ribosome-free regions of the rough endoplasmic reticulum (Dunn, 1990a). A high degree of phosphorylation of S6 may promote the binding of ribosomes to such regions so that less membrane becomes available for autophagosome formation. In this way, the same signal, i.e. S6 phosphorylation, would then contribute to the control of both protein synthesis and protein degradation. In addition to the docking protein, the binding of ribosomes to the endoplasmic reticulum is promoted by membrane receptor proteins with molecular masses of 34 kDa (Ichimura *et al*., 1992) and 180 kDa (Savitz & Meyer, 1993), and by the Sec61p-complex (Kalies *et al*., 1994). It is possible that these proteins not only control the rate of protein synthesis, but also simultaneously the activity of the autophagic pathway. One can even envisage a scenario in which autophagosomes are continuously formed on the endoplasmic reticulum unless ribosomal attachment prevents this. In this context, it is of interest to note that ribosomal attachment to the endoplasmic reticulum is not only controlled by the signal

recognition particle but also by a heterodimeric nascent polypeptide-associated protein complex, which prevents the targeting to the endoplasmic reticulum of ribosomes synthesizing proteins lacking signal peptides (Lauring *et al.*, 1995). There are indications in the literature supporting the view that attachment of ribosomes to the endoplasmic reticulum affects autophagosome formation. In the pancreas, for example, with its highly developed rough endoplasmic reticulum, degranulation of the endoplasmic reticulum caused by administration of puromycin or other compounds *in vivo* is accompanied by enhanced autophagosome formation (Réz *et al.*, 1976). In cultured hepatocytes, synthesis of export proteins, synthesized on the endoplasmic reticulum, but not of house-keeping proteins, which are largely synthesized on free ribosomes in the cytosol, decreases with decreasing concentration of amino acids in the culture medium (Tanaka & Ichihara, 1983; Dahn *et al.*, 1993).

The previous discussion suggests that phosphorylation of S6 stimulates protein synthesis, increases ribosome binding to the endoplasmic reticulum and thereby inhibits macroautophagic sequestration. This would be an attractive mechanism to prevent futile cycling between protein synthesis and degradation.

The next question concerns the mechanism by which amino acids increase phosphorylation of S6. As indicated above, the effect of amino acids proceeds via activation of p70S6 kinase rather than through inhibition of S6 phosphatase. Recent experiments by Miotto *et al*. (1994) with isolated hepatocytes have provided direct evidence that the initial effect of amino acids may be at the level of the plasma membrane, in line with an earlier proposal (Miotto *et al*., 1992). They synthesized an isopeptide (H-Leu8-Lys4-Lys2-Lys-âAla-OH), consisting of a branched tree of seven lysines, to which eight leucines are coupled via their carboxyl group to the Æ- and å-groups of the terminal lysines. This peptide did not permeate the plasma membrane, nor was it hydrolysed. Nevertheless, the peptide effectively inhibited autophagic proteolysis at low concentration, analogous with the effect of free leucine. Upon replacement of the terminal leucines by isoleucine, which is a non-regulating amino acid, the antiproteolytic action of the peptide was lost. The simplest explanation is that the plasma membrane contains a receptor for amino acids, which, by analogy with hormone and growth factor receptors, is coupled to S6 via a protein phosphorylation cascade. According to this, as yet hypothetical, mechanism, the inhibitory effect of cell swelling on proteolysis can now be explained by assuming that stretching of the plasma membrane increases the affinity of the amino acid receptor for amino acids. Cross-linking experiments with an azide derivative of the above-mentioned isopeptide led to the identification of a plasma membrane protein with a molecular mass of 340 kDa. This protein may be the putative amino acid receptor (Mortimore *et al*., 1994).

It is likely that phosphatidylinositol 3-kinase (PtdIns 3-kinase) is a component of the signal transduction pathway responsible for amino acid stimulation of S6 phosphorylation. Experiments with wortmannin and LY294002 [2-(4-morpholinyl)-8-phenylchromone], selective inhibitors of PtdIns 3-kinase, and with genetically modified PtdIns 3-kinase in a number of different cell types have indicated that activity of this enzyme is required for the stimulation of p70S6 kinase by insulin, growth factors and cytokines (Cheatham *et al*., 1994; Chung *et al*., 1994; Monfar *et al*., 1995; Yamamoto-Honda *et al*., 1995). Furthermore, these studies have shown that PtdIns 3-kinase activity is required for many processes that are dependent on membrane traffic (Shepherd *et al*., 1996). These include translocation of GLUT1 and GLUT4 to the plasma membrane (Clarke *et al*., 1994; Evans *et al*., 1995; Quon *et al*., 1995), endocytosis (Clague *et al*., 1995; Li *et al*., 1995), endosome fusion (Jones & Clague, 1995), lysosomal protein sorting (Brown *et al*., 1995; Davidson, 1995) and transcytosis (Cardone & Mostov, 1995).

Because membrane traffic plays a crucial role in autophagy, in both the formation and the fusion of vesicles, we tested the possibility that PtdIns 3-kinase is also involved in this process by studying the effects of wortmannin and LY294002 (Blommaart & Meijer, 1996). We found that wortmannin and LY294002, at concentrations that inhibited PtdIns 3-kinase activity, indeed completely prevented autophagic sequestration and had a strong antiproteolytic effect in isolated rat hepatocytes. Strikingly, 3-methyladenine, the classical inhibitor of autophagic sequestration (Seglen & Gordon, 1982), turned out to be an inhibitor of PtdIns 3-kinase as well, thus providing a satisfactory explanation for its action as an autophagy inhibitor (Blommaart & Meijer, 1996; Blommaart *et al*., 1997).

In mammalian cells, the lipid kinase activity of PtdIns 3-kinase results in the formation of phosphatidylinositol 3-phosphate (PtdIns3P), phosphatidylinositol 3,4-phosphate (PtdIns3,4P_2) and phosphatidylinositol 3,4,5-phosphate (PtdIns3,4,5P_3). The first product is constitutively formed and the other two products are formed only after stimulation of the enzyme with hormones or growth factors (Auger *et al*., 1989; Kelly & Ruderman, 1993; Navé *et al*., 1996). The yeast homologue of the p110 catalytic subunit of the mammalian PtdIns 3-kinase, VPS34p, is a lipid kinase with substrate specificity towards PtdIns. It cannot form PtdIns3,4P_2 or

PtdIns3,4,5P_3, and VPS34 mutants are disturbed in membrane flow (Schu *et al*., 1993; Stack & Emr, 1994). Therefore, the important factor here seems to be PtdIns3P, since the two other lipid molecules are absent in yeast cells. It must be pointed out that, in our experiments, the effects of wortmannin and LY294002 were tested in hepatocytes that were incubated under conditions of maximal autophagic flux, i.e. in the absence of stimuli of signal transduction. Apparently, in hepatocytes, basal activity of PtdIns 3-kinase is essential and sufficient to allow maximal autophagic sequestration. Stimulation of signal transduction by, for example, insulin and amino acids, results in inhibition of autophagic sequestration (Blommaart *et al*., 1995). Clearly, this inhibition must be downstream of PtdIns 3-kinase. This is consistent with the fact, mentioned above, that rapamycin, a specific inhibitor of p70S6k, is able to counteract, at least in part, the inhibition of autophagic sequestration by amino acids. Indeed, activation of p70 S6-kinase in hepatocytes by amino acids has recently been shown to be sensitive to inhibition by wortmannin, indicating that, in hepatocytes, PtdIns 3-kinase is one of the kinases involved in the signal transduction pathway finally leading to S6 phosphorylation (Krause *et al*., 1996; Blommaart *et al*., 1997), as in other cell types (see above).

It is our hypothesis, then, that PtdIns3P is essential for autophagic sequestration to proceed, and that PtdIns3,4P_2 and/or PtdIns3,4,5P_3 are inhibitory to the process.

It is not known whether, in addition to PtdIns 3-kinase, other components also participate in the signal transduction pathway responsible for amino acid-induced activation of p70S6 kinase. The protein kinase C family (reviewed by Nishizuka, 1992) is often proposed as being involved in the induction of cell proliferation. Because phorbol-myristate-acetate did not affect S6 phosphorylation in hepatocytes (J.J.F.P. Luiken & A.J. Meijer, unpublished observations), 'classical' PKCs are not involved. A possible candidate could still be PKC-æ, the only known PKC isoform that cannot be activated by phorbol esters (Ways *et al*., 1992) and which is, together with PKC-Æ and PKC-ä, one of the three isoforms expressed in hepatocytes (Wetsel *et al*., 1992). Although the target(s) of PKC-æ have not yet been identified, there is some indirect evidence that PKC-æ is upstream of p70S6 kinase (Monfar *et al*., 1995). Also of interest in this context is that PKC-æ can be activated by PtdIns3,4,5P_3 under *in vitro* conditions (Nakanishi *et al*., 1993). This allows the speculation that PKC-æ can be placed between PtdIns 3-kinase and p70S6 kinase.

Another protein kinase that may be a component of the signal transduction cascade resulting in S6 phosphorylation upon amino acid stimulation is the 60-kDa serine/threonine protein kinase B (PKB), also known as Akt. PKB/Akt is activated by PtdIns 3-kinase and in turn mediates activation of p70S6-kinase (Bos, 1995; Chou & Blenis, 1995; Proud, 1996). However, direct evidence in favour of the involvement of both PKB/Akt and PKC-æ in the regulation of autophagy is not yet available.

An important new development in studies on the regulation of autophagy is the finding that hepatocytes permeabilized with *Staphylococcus aureus* Æ-toxin, which creates stable plasma membrane channels allowing only small molecules (, 1000 Da) to pass, are still capable of autophagy provided ATP is added (Kadowaki *et al*., 1994). Under these conditions, the process is sensitive to inhibition by the sequestration inhibitor, 3-methyladenine, but the inhibitory effect of amino acids is lost. The latter observation suggests that the amino acid signal transduction pathway, starting at the plasma membrane level (Fig. 2), is interrupted in this system. Also of interest is that autophagy in these permeabilized cells is inhibited by low concentrations of GTP^{a}s. This indicates that one or more GTP-binding proteins are involved in the formation of autophagosomes (Kadowaki *et al*., 1994). Perhaps these proteins belong to the GTP-binding proteins of the *rab* family (Olkkonen *et al*., 1993; Li *et al*., 1995).

Strong evidence in favour of the involvement of a heterotrimeric G_{i3}-protein in the control of autophagic sequestration was obtained by Ogier-Denis *et al*. (1995) in the undifferentiated human colon cancer cell line HT-29. In these cells, autophagy is responsible for the degradation of high-mannose glycoproteins (Houri *et al*., 1993, 1995) and of sphingo(glyco)lipids (Ghidoni *et al*., 1996), and thus interferes with their processing. In differentiated cells, the autophagic pathway is greatly reduced (Houri *et al*., 1993, 1995; Ghidoni *et al*., 1996). Pertussis toxin, which ADP-ribosylates heterotrimeric G_i-proteins and prevents the interaction of G_i-proteins with their receptors, strongly inhibited autophagic sequestration (Ogier-Denis *et al*., 1995, 1996). By contrast, cholera toxin, acting on G_s-proteins, did not affect the process. Moreover, overexpression of the $Æ_{i3}$-subunit increased the rate of autophagic sequestration in a manner roughly proportional to the degree of overexpression. Using site-directed mutagenesis and stable cell transfections, it was demonstrated that autophagic sequestration becomes activated when the G_{i3}-protein is bound to GDP, while the process is inhibited when the protein is bound to GTP. In order to initiate the autophagic process, the GDP-bound form of G_{i3} must attach to an intracellular membrane (Ogier-Denis *et al*., 1996). In mutants expressing a pertussis toxin-insensitive modification of the Æi3-subunit,

autophagic sequestration was insensitive to pertussis toxin but still sensitive to 3-methyladenine, demonstrating that the target of 3-methyladenine is different from that of pertussis toxin (Ogier-Denis *et al*., 1996). This is consistent with the fact that 3-methyladenine acts by inhibiting phosphatidylinositol 3-kinase (Blommaart & Meijer, 1996; Blommaart *et al*., 1997), as discussed above.

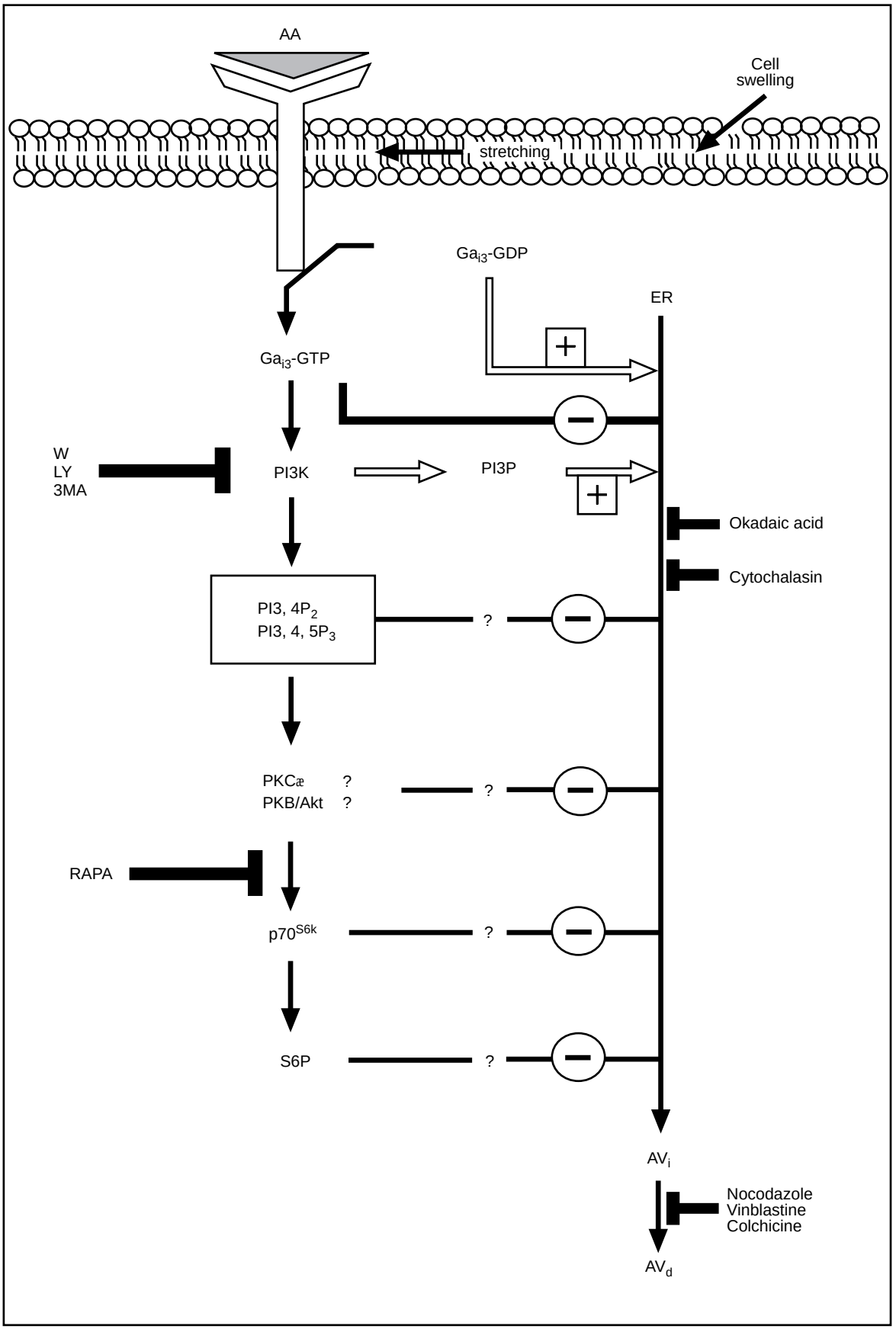
In Fig. 2, we have summarized the interaction of amino acids with the autophagic proteolytic pathway schematically. The scheme includes the amino acid receptor, the protein kinase pathway leading to S6 phosphorylation, including PtdIns 3-kinase, PKB/Akt, PKC-æ and p70S6 kinase. Because, at least in rat fibroblasts, p70S6 kinase is activated by G_i-coupled receptor agonists in a PtdIns 3-kinase-dependent and pertussis toxin-sensitive pathway (Wilson *et al*., 1996), we have placed the G_i-protein upstream of PtdIns 3-kinase. In the scheme, we have assumed that the ribosome-free region of the rough endoplasmic reticulum is the origin of the autophagosomal membrane. As discussed earlier, binding of ribosomes (after S6 phosphorylation) to the endoplasmic reticulum would provide an attractive mechanism for the control of autophagic sequestration. However, components upstream of S6 in the signal transduction pathway may also directly affect autophagosome formation, as indicated in Fig. 2. This would be equally well compatible with the possibility that the endoplasmic reticulum is not the origin of the autophagosomal membrane and that autophagosomes originate from, e.g. post-Golgi membranes or phagophores.

Specificity of macroautophagy

It has been assumed for a long time that macroautophagy is a non-selective process, in which macromolecules are randomly degraded in the same ratio as they occur in the cytoplasm (Kominami *et al*., 1983; Kopitz *et al*., 1990). However, recent observations strongly suggest that this may not always be the case, and that macroautophagy can be selective under some conditions. For example, in the perfused liver, although autophagic breakdown of protein and RNA (mainly ribosomal RNA) is sensitive to inhibition by amino acids and insulin, glucagon accelerates proteolysis but has no effect on RNA degradation (Lardeux & Mortimore, 1987). The mechanism by which ribosomes are excluded from autophagic degradation under these conditions is unknown. One may speculate that, in the hepatocyte during starvation, this is a means of preventing excessive degradation of ribosomes, since these structures are required for the synthesis of essential proteins.

Another example of selective autophagy is the degradation of superfluous peroxisomes in hepatocytes from clofibrate-treated rats. When hepatocytes from these rats, in which the number of peroxisomes is greatly increased, are incubated in the absence of amino acids to ensure maximal flux through the macroautophagic pathway, peroxisomes are degraded at a relative rate that exceeds that of any other component in the liver cell (Luiken *et al*., 1992). The accelerated degradation of peroxisomes was sensitive to inhibition by 3-methyladenine, a specific autophagic sequestration inhibitor. Interestingly, the accelerated removal of peroxisomes was prevented by long-chain but not short-chain fatty acids (Luiken *et al*., 1992). Since long-chain fatty acids are substrates for peroxisomal â-oxidation, this indicates that these organelles are removed by autophagy when they are functionally redundant. Our hypothesis is that acylation (palmitoylation?) of a peroxisomal membrane protein protects the peroxisome against autophagic sequestration. Indeed, incubation of rat hepatocytes with [1-^{14}C]palmitate results in acylation of a 69-kDa membrane protein, which is inducible by clofibrate and thus presumably of peroxisomal origin (Fig. 3). Unfortunately, however, a polyclonal antibody against the rat 69-kDa peroxisomal membrane protein did not immunoprecipitate the acylated

Fig. 2. Schematic overview of the involvement of signal transduction in the regulation of macroautophagic proteolysis by amino acids and cell swelling. Amino acids (AA) stimulate a protein kinase cascade via a plasma membrane receptor. Receptor activation results in activation of PtdIns 3-kinase (PI3K), possibly via a heterotrimeric $GÆ_{i3}$ protein. This is followed by activation of PKC-æ, PKB/Akt, p70S6 kinase ($p70^{S6k}$) and finally phosphorylation of ribosomal protein S6 (S6P). The GDP-bound form of $GÆ_{i3}$ is required for autophagic sequestration (open arrow), whereas the GTP-bound form is inhibitory. The constitutively formed phosphatidylinositol 3-phosphate (PI3P) (open arrow) is also required for autophagic sequestration. Therefore, inhibition of PtdIns 3-kinase activity by wortmannin (W), LY294002 (LY) or 3-methyladenine (3MA) prevents autophagic sequestration. Activation of PKC-æ and PKB/Akt is mediated by the 3,4- and 3,4,5-phosphate forms of phosphatidylinositol ($PI3,4P_2$ and $PI3,4,5P_3$) that are produced upon activation of PtdIns 3-kinase. As a result of this, the first step of the macroautophagic pathway is inhibited by components of the cascade that are downstream of PtdIns 3-kinase. Inhibition of this downstream cascade by rapamycin (RAPA) accelerates autophagic sequestration. Cell swelling potentiates the effect of amino acids via a change in the receptor owing to membrane stretch. Furthermore, the site of action of the different effectors of the cytoskeleton (okadaic acid, cytochalasin, nocodazole, vinblastin and colchicine) are indicated. AV_i, initial autophagic vacuole; AV_d, mature degradative autophagic vacuole, ER, endoplasmic reticulum.


AA
Cell swelling
stretching
Ga_{i3}-GDP
ER
Ga_{i3}-GTP
W
LY
3MA
PI3K
PI3P
Okadaic acid
Cytochalasin
PI3, $4P_2$
PI3, 4, $5P_3$
?
PKCæ ?
PKB/Akt ?
RAPA
$p70^{S6k}$
S6P
AV_i
Nocodazole
Vinblastine
Colchicine
AV_d

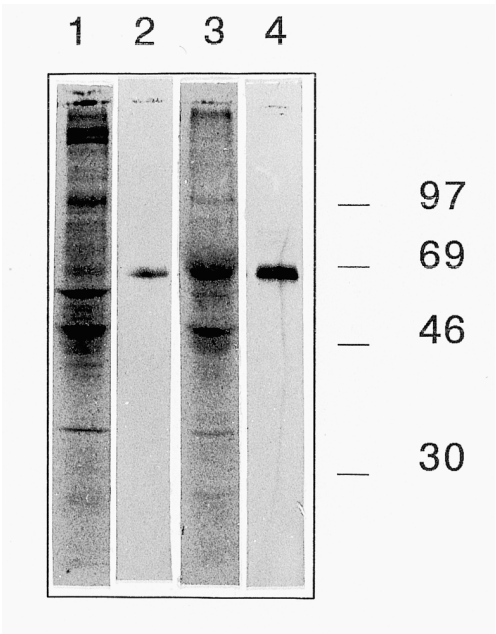


Fig. 3. Palmitoylation of membrane proteins in hepatocytes from a rat fed either a normal diet or clofibrate for 3 weeks. Hepatocytes from a normal-fed rat (lanes 1 and 2) or a clofibrate-treated rat were incubated for 60 min with 20 μCi of 0.4 mM [^{14}C]palmitate and membranes were isolated. Analysis of palmitoylated membrane proteins was performed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography (lanes 1 and 3). Lanes 2 and 4 are immunoblots of the 69-kDa peroxisomal membrane protein under the same experimental conditions, using a rat polyclonal antibody against this protein. Data were obtained from Luiken (1996).

protein, so that its definitive identification and characterization has to await further experimentation.

Selective degradation of hepatic peroxisomes has also been demonstrated *in vivo* in rats treated with di-(2-ethylhexyl)phthalate, another peroxisome proliferator, followed by the removal of this drug (Yokota, 1993; Yokota *et al.*, 1993, 1994, 1995). Remnants of peroxisomes have been found inside autophagolysosomal structures in fibroblasts from Zellweger patients (Heikoop *et al.*, 1992). In these patients, there is a defect in the import system for many peroxisomal proteins, so that the remnants of these organelles, often referred to as peroxisomal 'ghosts', are not functional and must be removed.

The selective degradation of peroxisomes was confirmed in methylotrophic yeasts. In these cells, methanol is a peroxisomal substrate. and growth on methanol results in induction of peroxisomes. Switching these cells from methanol to a glucose-containing growth medium results in a specific removal of these organelles by autophagy, while mitochondria, for example, are retained under these conditions (Tuttle *et al.*, 1993). Interestingly, the mechanism by which the peroxisomes are taken up by the vacuole is dependent on the nature of the nutrient switch. When ethanol replaces methanol, peroxisomes are first sequestered into autophagosomes by wrapping membranes, which subsequently fuse with the vacuole, a process that strongly resembles macroautophagy, and which did not seem to require protein synthesis because it was found to be cycloheximide insensitive. However, when glucose replaces methanol, peroxisomes are taken up directly by the vacuoles by a process resembling microautophagy, which is sensitive to inhibition by cycloheximide (Tuttle & Dunn, 1995). Similarly, when glucose-starved *S. cerevisiae* cells are replenished with glucose, peroxisomes are degraded by microautophagy (Chiang *et al.*, 1996).

Two yeast mutants defective in the selective macroautophagic degradation of peroxisomes have recently been isolated and characterized. One of these mutants is defective in autophagic sequestration, whereas the other is defective in the fusion between sequestered peroxisomes and the vacuole (Titorenko *et al.*, 1995).

Indications for the selective autophagic elimination of organelles other than peroxisomes are also present in the literature, albeit scarce. Selective hepatic elimination of smooth endoplasmic reticulum, after prior induction with phenobarbital, followed by removal of the drug, has been reported (Masaki *et al.*, 1987). Experiments with hepatocytes isolated from phenobarbital-treated rats showed that this is because of increased autophagy rather than decreased synthesis of smooth endoplasmic reticulum components (Luiken, 1996). Selective degradation, of damaged mitochondria in this case, was found in liver biopsies of patients with Reye's syndrome (Partin *et al.*, 1971) and in an influenza B virus model of Reye's syndrome in the mouse (Woodfin & Davis, 1986).

From all these observations, we conclude that under normal conditions macroautophagy may be largely unselective and serves, for example, to produce amino acids for gluconeogenesis and the synthesis of essential proteins in starvation. However, when cell structures are functionally redundant or when they become damaged, the autophagic system is able to recognize this and is able to degrade the structure concerned. As yet, nothing is known about the recognition signals. A possibility is that ubiquitination of membrane proteins is required to mark the structure to be degraded for autophagic sequestration (see also next section).

Ubiquitin may be involved in macroautophagy

According to recent evidence, ubiquitin not only contributes to extralysosomal proteolysis but is also

involved in autophagic protein degradation. Thus, in fibroblasts ubiquitin–protein conjugates can be found in the lysosomes, as shown by immunohistochemistry and immunogold electron microscopy (Doherty *et al*., 1989; Laszlo *et al*., 1990). Free ubiquitin can also be found inside lysosomes (Schwartz *et al*., 1988). Accumulations of ubiquitin–protein conjugates in filamentous, presumably lysosomal, structures are also found in a large number of neurodegenerative diseases (Mayer *et al*., 1991; Muller & Schwartz 1995). Mallory bodies in the liver of alcoholics also contain ubiquitin–protein conjugates (Mayer *et al*., 1991).

This presence of ubiquitin–protein conjugates in filamentous inclusions in neurons and other cells can be caused by a defect in the extralysosomal ubiquitin-dependent proteolytic pathway. However, it is also possible that these filamentous inclusions represent an attempt of the cell to get rid of unwanted material (proteins, organelles) via autophagy (Mayer *et al*., 1991).

Direct evidence that ubiquitin may be involved in the control of macroautophagy came from experiments with CHO cells with a temperature-sensitive mutation in the ubiquitin-activating enzyme E1 (Gropper *et al*., 1991). Wild-type cells increased their rate of proteolysis in response to stress (amino acid depletion, increased temperature). This was prevented by the acidotropic agent ammonia or by the autophagic sequestration inhibitor 3-methyladenine, indicating that the accelerated proteolysis occurred by autophagy. In the mutant cells, there was no such increase in proteolysis in response to stress at the restrictive temperature. Apparently, functional E1 is required for autophagic proteolysis. However, formation of initial autophagosomes was not affected in the mutant cells (Lenk *et al*., 1992; Schwartz *et al*., 1992). Perhaps these autophagosomes are not functional because ubiquitin–protein conjugates are not available as autophagic substrates. An alternative interpretation is that the ubiquitin system is required for maturation of autophagosomes and/or their fusion with lysosomes. Another possibility is that a short-lived protein in the cell specifically inhibits the fusion step and that ubiquitination and degradation of this protein is required for macroautophagy to proceed (Gropper *et al*., 1991).

Autophagy and carcinogenesis

In cancer development, cell growth is mainly induced by inhibition of protein degradation, since differences in the rate of protein synthesis between tumorigenic cells and their normal counterparts are rather small (Canuto *et al*., 1993). A striking example of how reduced autophagic proteolysis can contribute to cell growth can be found in the development of liver carcinogenesis. This decrease in autophagic flux results from a decrease in the rate of autophagic sequestration and is already detectable in the early preneoplastic stage (Schwarze & Seglen, 1985; Yucel *et al*., 1989; Kisen *et al*., 1993). Autophagic flux is then hardly inhibitable by amino acids nor is it inducible by catabolic stimuli (Schwarze & Seglen, 1985; Tessitore *et al*., 1988; Kisen *et al*., 1993) and declines in the more advanced stage of cancer development to a rate of less than 20% of that seen in normal hepatocytes (Kisen *et al*., 1993). The fact that the addition of 3-methyladenine to hepatocytes from normal rats increased hepatocyte viability to the same level as observed for the tumour cells (Schwarze & Seglen, 1985) strongly suggests that the fall in autophagic proteolysis contributes to the rapid growth rate of these cells and gives them a selective advantage over the normal hepatocytes.

However, the mechanism underlying the decreased rate of autophagic flux in liver tumour cells is by no means clear. It may be that the capacity for autophagy is diminished, but it is also possible that autocrine or paracrine factors, secreted by the tumour cells themselves, continuously suppress macroautophagy (Kisen *et al*., 1993). Additionally, since amino acids are not able to down-regulate proteolytic rates in tumorigenic cells, one may also speculate that in these cells the components of the S6 signal transduction pathway are under continuous pressure to remain in a highly phosphorylated state. This could be caused by an oncogenic transformation of an upstream component of this signal transduction route, which would then lead to uncoupling of the autophagic process from regulation by external factors, such as amino acids and hormones. It would, therefore, be important to assess the phosphorylation state of S6 under different metabolic conditions in hepatocytes at various stages of cancer development. Speculating even further, rapamycin may enhance autophagic rates in tumorigenic hepatocytes and may cause a decrease in cell survival in cultures of transformed hepatocytes. The possibility of using rapamycin as an anti-cancer agent has been investigated in rhabdomyosarcoma cells. In this cell line, rapamycin inhibits proliferation by the inhibition of signalling downstream of the insulin-like growth factor receptor (Dilling *et al*., 1994).

Conclusions

For a long time, autophagy has been a rather neglected area and, until recently, research in this field has been largely descriptive. Underlying control mechanisms, however, are gradually being unravelled. It is perhaps not surprising that protein phosphorylation and signal transduction are key

elements in these mechanisms. The discovery of an amino acid receptor in the plasma membrane of the hepatocyte with a signal transduction pathway coupled to it may have important repercussions, not only for the control of macroautophagy but also for the control of other pathways. In addition, it is likely that amino acid- and insulin-stimulated signal transduction converge at some stage of this complicated process and that in this way the two stimuli cooperate in their anabolic effects. If this view is correct, a defect in the amino acid receptor would have consequences as serious as a defect in the insulin receptor.

The genetic approach in the yeast system will be of great value in identifying individual protein components participating in the autophagic process. There is no doubt that proteins of the cytoskeleton and protein kinases/phosphatases will be among them. It remains to be seen, however, whether the details of the mechanisms controlling the process in yeast are similar to those in mammalian cells. For example, it is not known whether amino acids are able to control the process as they do in mammalian cells.

Another step forward has been the discovery that macroautophagy is not always a random process, but can be very specific under certain conditions. However, the recognition mechanisms responsible for the selective elimination of specific cell structures are unknown at present. Again, the yeast system may provide information on this. Even though these signals may differ from those in mammalian cells, yeast will certainly provide the biochemical principles of such mechanisms.

In this review, we hope to have made it clear that autophagy is indeed a fascinating cell biological process. Surprisingly, the process has received relatively little attention so far. Undoubtedly, this will change in the near future.

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