Regulation of metabolism by amino acid dependant signal transduction

Dubbelhuis, P.F.

Citation for published version (APA):
Chapter 1

Introduction
Chapter 1

The balance between protein synthesis and protein degradation controls cell homeostasis and cell growth. Most research has focused on the role of protein synthesis, but protein degradation has proved to be of equal importance, if not more so. In this thesis, studies on mechanisms involved in the regulation of autophagic protein degradation in mammalian cells are described.

Mammalian cells possess two major pathways for protein degradation: the proteasomal and the lysosomal system. Proteasomes are specialized in the selective breakdown of short-lived proteins [1], and lysosomes are responsible for the turnover of long-lived proteins [2]. Proteins destined for proteasome-mediated destruction are marked by covalent attachment of ubiquitin molecules [3] (Fig. 1). For an extensive discussion of proteasome-mediated protein degradation the reader is referred to Hershko and Ciechanover [1] and Ulrich [3].

![Ubiquitin-like reactions involved in yeast during autophagy.](image)

*Fig 1. Ubiquitin-like reactions involved in yeast during autophagy.* Apg8 and Apg12 are ubiquitin-like proteins. Apg7 is an E1-like enzyme whereas Apg3 and Apg10 are E2-like. No E3-like protein has been discovered yet in yeast. See text and table 1 for details. Modified from [4].
For the transport of cytoplasmic material to the lysosome, four different routes are known: macroautophagy, microautophagy, chaperone-mediated autophagy, and crinophagy [2]. In this chapter, the lysosomal pathways of protein degradation and the involvement of amino acids in the regulation of this process are discussed. Macroautophagy (hereafter referred to as "autophagy") which is, quantitatively, the most important process responsible for the lysosomal degradation of cytosolic material, is discussed in detail and microautophagy, chaperone-mediated autophagy and crinophagy are described briefly. Studies on autophagy have been carried out both in mammalian cells, and more recently, in yeast cells. These will be both discussed in this chapter.

**Autophagy**

During autophagy, a small part of the cytoplasm is sequestered by a double isolation membrane, leading to the formation of an autophagosome (from Greek: *auto*, self; *phagos*, to eat; *soma*, body) [5] (Fig. 2). An autophagosome fuses with a lysosome to form an autophagolysosome or it fuses with an endosome to form an amphisome, followed by fusion with a lysosome to form an autophagolysosome [6,7]. When fusion is complete, the outer membrane of the autophagosome is incorporated into the membrane of the lysosome, resulting in a single-membrane vesicle, the autophagic body, which is degraded inside the autophagolysosome [2,8-10].

---

*Fig 2. The autophagic lysosomal proteolytic pathway.*

Cytoplasmic material is sequestered by an isolation membrane. The origin of the isolation membrane could be ER or Golgi; another option is a pre-existing membrane, called the phagophore, or *de novo* synthesis. The autophagosome matures into an autophagolysosome either directly by fusion with a lysosome or via fusion with an amphisome followed by a fusion with a lysosome. The lysosome provides the acid hydrolases (AH) which are necessary for degradation of the engulfed material. Modified from [11].
Induction of autophagy

Autophagy is up-regulated during starvation and down-regulated by feeding. When nutrients are limited, autophagy is largely non-selective, i.e. the same concentrations of material found in the cytoplasm can be found in the newly formed autophagosomes [12,13]. In many cells, autophagy is inhibited by amino acids [8,14] (see section "Amino acid regulation of autophagy") and this inhibition can be reversed by rapamycin [15], a compound which blocks the activity of the protein kinase mTOR (mammalian target of rapamycin) [16] (see section mTOR/Tor). This effect was later confirmed in yeast where, under nutrient-rich conditions, rapamycin also induced autophagy via inhibition of Tor [17].

The biogenesis and formation of autophagosomes

As mentioned above, upon induction of autophagy, cytoplasmic material with or without organelles is sequestered by a double isolation membrane, which leads to the formation of autophagosomes. However, the origin of this isolation membrane is still under debate and for mammalian cells several options have been proposed. One of these was that the double isolation membrane originates from the ribosome-free parts of the rough endoplasmic reticulum (ER) [18]. Immunoblotting studies revealed that the autophagosomal membranes did indeed contain the proteins cytochrome P450 and NADPH-cytochrome c reductase which are both markers of the rough ER [19]. Other candidates for the origin of the double isolation membrane are the trans-Golgi network [20,21] and a pre-existing membrane called the phagophore [22].

A problem in studies on the origin of the double isolation membrane has been the lack of good marker molecules caused by the low numbers of transmembrane proteins and peripheral surface-membrane proteins, as demonstrated by freeze-fracture electron microscopy [23]. Fortunately, this problem can now be approached by using the large amount of information on the molecular mechanism of autophagy obtained from genetic studies with the yeast Saccharomyces cerevisiae. For example, yeast mutants deficient in autophagy were generated by selecting cells that were unable to survive under nutrient-deficient conditions. At least 16 genes involved in autophagosome formation were identified by two different groups: the APG (autophagy) genes by Tsukada et al. [24] and the AUT (autophagocytosis) genes by Thumm et al. [25] (Table 1). Light microscopy was used to identify the apg mutants, which were unable to accumulate autophagic bodies in a PMSF-containing nitrogen starvation medium (PMSF is a lysosomal protease inhibitor) [24]. An immunological approach was used to identify the aut mutants, which were in the first place unable to degrade fatty acid synthase in the vacuole (the yeast lysosome) during nutrient starvation and which were subsequently selected on the loss of accumulating autophagic bodies in the presence of PMSF [25].
### Table 1. Proteins involved in autophagy (modified from [26])

<table>
<thead>
<tr>
<th>Apg*</th>
<th>Mammalian homologue</th>
<th>Cvt</th>
<th>Cvt homologue</th>
<th>Function characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apg1</td>
<td>UIK1</td>
<td>Cvt10</td>
<td>Cvt10</td>
<td>Serine/threonine kinase interacting with Apg13, Apg7 and Cvt7, PAS localization</td>
</tr>
<tr>
<td>Apg2</td>
<td>Cvt2</td>
<td>Apg4</td>
<td>Cvt5</td>
<td>Peripheral membrane protein, PAS localization</td>
</tr>
<tr>
<td>Apg3</td>
<td>Cvt3</td>
<td>Apg6</td>
<td>Cvt6</td>
<td>ApG8-conjugating enzyme (EI-lik)</td>
</tr>
<tr>
<td>Apg4</td>
<td>Cvt4</td>
<td>Apg7</td>
<td>Cvt7</td>
<td>Cysteine protease</td>
</tr>
<tr>
<td>Apg5</td>
<td>Cvt5</td>
<td>Apg8</td>
<td>Cvt8</td>
<td>Component of Apg2-Apg5-Apg16 complex, required for degradation of isolation membrane</td>
</tr>
<tr>
<td>Apg6</td>
<td>Cvt6</td>
<td>Apg9</td>
<td>Cvt9</td>
<td>Ubiquitin-like protein, conjugates to PI-locates on PAS, autophagosomes and Cvt vesicles</td>
</tr>
<tr>
<td>Apg7</td>
<td>Cvt7</td>
<td>Apg10</td>
<td>Cvt10</td>
<td>Ubiquitin-like protein conjugates to Apg9</td>
</tr>
<tr>
<td>Apg8</td>
<td>Cvt8</td>
<td>Apg11</td>
<td>Cvt11</td>
<td>Transmembrane protein</td>
</tr>
<tr>
<td>Apg9</td>
<td>Cvt9</td>
<td>Apg12</td>
<td>Cvt12</td>
<td>Ubiquitin-like protein interacting with Apg13, deamidylated during starvation conditions</td>
</tr>
<tr>
<td>Apg10</td>
<td>Cvt10</td>
<td>Apg13</td>
<td>Cvt13</td>
<td>Lipase required for degradation of autophagic bodies</td>
</tr>
<tr>
<td>Apg11</td>
<td>Cvt11</td>
<td>Apg14</td>
<td>Cvt14</td>
<td>Lipase required for localization of Apg2</td>
</tr>
<tr>
<td>Apg12</td>
<td>Cvt12</td>
<td>Apg15</td>
<td>Cvt15</td>
<td>Involved in an early step of autophagy</td>
</tr>
<tr>
<td>Apg13</td>
<td>Cvt13</td>
<td>Apg16</td>
<td>Cvt16</td>
<td>Involved in an early step of autophagy</td>
</tr>
<tr>
<td>Apg14</td>
<td>Cvt14</td>
<td>Apg17</td>
<td>Cvt17</td>
<td>Involved in an early step of autophagy</td>
</tr>
<tr>
<td>Apg15</td>
<td>Cvt15</td>
<td>Apg18</td>
<td>Cvt18</td>
<td>Involved in an early step of autophagy</td>
</tr>
<tr>
<td>Apg16</td>
<td>Cvt16</td>
<td>Apg19</td>
<td>Cvt19</td>
<td>Involved in an early step of autophagy</td>
</tr>
<tr>
<td>Apg17</td>
<td>Cvt17</td>
<td>Apg20</td>
<td>Cvt20</td>
<td>Involved in an early step of autophagy</td>
</tr>
<tr>
<td>Apg18</td>
<td>Cvt18</td>
<td>Apg21</td>
<td>Cvt21</td>
<td>Involved in an early step of autophagy</td>
</tr>
<tr>
<td>Apg19</td>
<td>Cvt19</td>
<td>Apg22</td>
<td>Cvt22</td>
<td>Involved in an early step of autophagy</td>
</tr>
<tr>
<td>Apg20</td>
<td>Cvt20</td>
<td>Apg23</td>
<td>Cvt23</td>
<td>Involved in an early step of autophagy</td>
</tr>
<tr>
<td>Apg21</td>
<td>Cvt21</td>
<td>Apg24</td>
<td>Cvt24</td>
<td>Involved in an early step of autophagy</td>
</tr>
<tr>
<td>Apg22</td>
<td>Cvt22</td>
<td>Apg25</td>
<td>Cvt25</td>
<td>Involved in an early step of autophagy</td>
</tr>
</tbody>
</table>

*Unified nomenclature for autophagy related proteins [27]

*The standard name for this protein is Vps30
Mammalian counterparts were found for most of the APG/AUT genes (Table 1) and are now used to unravel the mechanisms involved in autophagy. For example, in mouse embryonic stem (ES) cells, Apg12 and Apg5 proteins were found on the initial isolation membrane [28]. These two proteins were known to be covalently attached to each other via a conjugating system, in which Apg7 (E1-like) and Apg10 (E2-like) proteins are involved, similar to the ubiquitin system involved in proteasome-dependent proteolysis [29] (Fig. 1). It was further found that the Apg12-Apg5 complex is essential for autophagy [28]. With the help of a green fluorescent protein-tagged Apg5 it was demonstrated that in mouse ES cells punctated structures are the precursors of the initial isolation membrane and that these small membranes are probably not derived from pre-existing membranes such as the ER [28]. Whether or not these isolation membranes are identical to the phagophores described by Seglen et al. [22] is not known and whether or not these isolation membranes are formed de novo in mammalian cells, like in yeast [30], is also still unknown.

Studies with mice showed that Apg12-Apg5 forms a complex with the mammalian homologue of yeast Apg16, named Apg16-like protein (Apg16L), a protein of approximately 800 kDa [31]. Apg16L is the functional counterpart of yeast Apg16, although it is much larger because its C-terminal region has additional WD repeats. WD repeats serve as a platform on which multiple proteins can interact with each other [32]. This suggests that Apg16L may interact with proteins other than Apg12 and Apg5. Most of the Apg16L is free in the cytosol but amino acid starvation leads to the formation of Apg16L dots. During elongation of the isolation membrane, Apg16L was found on the outside of cup-shaped isolation membranes [31], like the Apg12-Apg5 complex before [28]. Upon completion of autophagosome formation, the Apg12-Apg5-Apg16L complex dissociates from the membrane [26] (Fig. 3).

Fig 3. Representation of autophagosome formation in the mammalian cell. The Apg12-Apg5-Apg16L complex is localized on the isolation membrane during sequestration, just like LC3. Upon completion of the autophagosome, the Apg12-Apg5-Apg16L complex dissociates from the autophagosome membrane in contrast to LC3 which remains on the autophagosome. Modified from [33].
In yeast, in another ubiquitin-like system (UBL), Apg8 is involved in the regulation of autophagosome formation [34] (Fig. 1). In mammals, three Apg8 homologues exist: microtubule-associated protein 1 light chain 3 (LC3) [35], Golgi-associated ATPase Enhancer of 16kD (GATE-16) [36] and γ-aminobutyric acid (GABA)_A-receptor-associated protein (GABARAP) [37]. Of these three homologues only LC3 appears to be involved in the formation of autophagosomes [38]. Unlike the Apg12-Apg5-Apg16L complex, which can only be found temporarily on isolation membranes during elongation of the isolation membrane, LC3 can also be detected on the membranes of the autophagosome (Fig. 3) [28,33,38]. For example, in rat, LC3 is localized on autophagosomes and is processed after synthesis by cleavage of its C-terminal part [38]. This modification is similar to that of the yeast Apg8 [39]. The cleavage is catalysed by the mammalian Apg4 homologue and leads to the formation of LC3-I with a glycine residue at the C-terminal end [26]. Subsequently, Apg7 (E1-like) activates LC3-I so that it can be transferred to Apg3 [40] (E2-like). This is followed by the formation of an LC3-II conjugate with an unknown substrate, perhaps phosphatidylethanolamine (PE) as in yeast [39] (Fig. 1). After conjugation, LC3-II tightly binds to the autophagosomal membrane and behaves differently from LC3-I on SDS-PAGE [38].

The two UBL systems are connected to each other not only by sharing Apg7 but also by the fact that Apg5 is needed for the generation of LC3-II, which notion was confirmed by the finding that in ES apg5<sup>−/−</sup> cells LC3 is exclusively located in the cytosol [28].

Autophagic sequestration of cytoplasmic material is blocked by the classical autophagy inhibitor 3-methyladenine [41] via inhibition of phosphatidylinositol 3-kinase (PI 3-kinase) [42]. Two other structurally unrelated PI 3-kinase inhibitors, LY294002 and wortmannin, are also able to block autophagic sequestration [42], indicating that PI 3-kinase family members are involved in the sequestration step (see also section "Regulation of autophagy by signalling", part "Phosphatidylinositol 3-kinase"). This conclusion was supported by studies with a human colon cancer cell line, HT-29, in which autophagic sequestration could be enhanced either by feeding the cells the product of class III PI 3-kinase, phosphatidylinositol 3-phosphate (PtdIns(3)P), in a synthetic form (dipalmitoylphosphatidylinositol 3-phosphate) or by overexpressing the p150 adaptor of class III PI 3-kinase [43]. Additional proof came from experiments carried out with green fluorescent protein-tagged Apg5 in ES cells (see above), which showed that the formation of the precursors of the isolation membrane was prevented by the different inhibitors of PI 3-kinase [28]. In yeast cells, PI 3-kinase exists as a complex made up of Vps34 (a PI 3-kinase class III homologue), Vps15 (a p150 adaptor homologue), Apg6/Vps30 and Apg14 which are all needed for the formation of the pre-autophagosomal structure (PAS) [44]. This complex is localized near the yeast vacuole from which the autophagosome seems to be generated [45]. Whether a PAS-like structure also plays a role in autophagosome formation in mammalian cells is not known. Until now, the mammalian homologue of Apg14 is still missing, but Beclin-1 was identified as mammalian Apg6/Vps30 [46], so that almost all components for PAS formation are present in mammals. In HeLa cells, the Beclin-1/PI 3-kinase class III complex is localized at the trans-Golgi network and produces phosphatidylinositol 3-phosphate (PtdIns(3)P) [47]. Data from
Chapter 1

Nice et al. strongly suggest that PtdIns(3)P is required in the formation of the PAS in yeast [48]. Whether PtdIns(3)P is directly involved in supplying membrane components to the autophagic pathway and/or recruiting proteins from the cytosol for autophagosome formation is not known.

Although much information has been obtained about the mechanism of autophagosome formation, many questions still remain to be answered. These include the origin of the components for de novo synthesis of the autophagosomal membrane, the origin of the membrane if it is not synthesized de novo, and the exact mechanism of the two UBL systems, Apg12-Apg5 and Apg8, in connection with the elongation step of the isolation membrane.

Formation of the autophagolysosome

When cell material is engulfed by the autophagosome it has to be delivered to the lysosome for fusion. Nocodazole, a compound that destabilizes microtubules, blocks this fusion step [49]. Likewise, the microtubuli inhibitor vinblastin inhibits fusion [50]. These observations indicate that an intact cytoskeleton is needed for autophagolysosome formation. In yeast, the SNARE machinery is needed for fusion of autophagosomes with the vacuole [51] and one of the components of this machinery is Sec18 (N-ethylmaleimide-sensitive fusion protein, NSF). NSF is one of the most studied members of the AAA (ATPases associated with cellular activities)-type ATPases family and its involvement in autophagolysosome formation was confirmed in studies with temperature-sensitive yeast mutants [52]. In HeLa cells overexpressing a dominant negative mutant of mouse SKD1 (suppressor of potassium transport growth defect 1 protein) [53], which is another AAA-ATPase family member and a key regulator of endosome sorting, normal transport from endosome to autophagosome was disturbed, which led to impairment of autophagolysosome formation [54].

Formation of a complete autophagolysosome requires the contribution of elements that control intracellular trafficking from the ER (SNARE machinery) and from Golgi compartments (PI 3-kinase class III; see section The biogenesis and formation of autophagosomes), suggesting that different membrane pools converge to form a PAS. This would agree with the different origins reported in the literature for the autophagosomal membrane.

Final step in autophagy: degradation

When cargo protein is delivered to the lysosome, it will be degraded by different proteases, such as proteinase A and proteinase B, which need an acidic environment for proper activity (see, e.g. [55,56], with regard to the yeast system). However, before these proteases can get access to their substrates, lipase activity is required for breakdown of the membrane surrounding the autophagic bodies inside the autophagolysosome. One of the lipases involved might be encoded by the AUT5 gene found in yeast. Aut5 is a protein that contains an essential domain found in many lipases and by site-directed mutagenesis the lipase-active site in this
domain was shown to be crucial for degradation of the autophagic body [57,58]. Autophagic body accumulation, such as takes place in aut5 mutants, also occurred in aut4 mutants [59]. Aut4 is a mult spanning transmembrane protein and can be found on the vacuolar membrane. Not much is known about its function but AUT4-deleted yeast cells grow on nutrient-rich medium like wild-type yeast. Perhaps Aut4 functions as an efflux transporter due to its limited homology to permeases [59], but the substrate of Aut4 is not known. No mammalian Aut5 or Aut4 homologue has been found yet.

Connection of the autophagic pathway with the cytoplasm-to-vacuole targeting pathway

Surprisingly, in yeast, the autophagic machinery shows overlap with the cytoplasm-to-vacuole targeting (Cvt) pathway [60,61] in that cvt mutants partially overlap with apg/aut mutants (Table 1). Under nutrient-rich conditions, the Cvt pathway is needed for proper delivery to the vacuole of the hydrolases α-mannosidase (Ams1) [62] and aminopeptidase I (Ape1) [63]. Just like autophagosomes, Cvt vesicles have a double membrane and fuse with the vacuole. However, the Cvt-mediated Ape1 transport is a constitutive process, whereas autophagy is inducible. In addition, Cvt vesicles are much smaller than autophagosomes (140-160 nm versus 300-900 nm) [64]. It is not known whether the origin of the two vesicles is the same. A Cvt pathway has not yet been discovered in higher eukaryotic cells; although homologues of Ape1 and Ams1 have been detected, not much is known about their localization [65].

At present, more and more information is becoming available about the molecular machinery of autophagosome formation and the degradation of sequestered material in the autophagolysosome. In this thesis, the next step is described, i.e. using this information to unravel signal transduction pathways controlling the autophagic process and the involvement of amino acids in the regulation of the different steps in autophagy.

Amino acid regulation of autophagy

Since amino acids are the end products of autophagy, it is not surprising that they are effective inhibitors of this process. The system is also controlled by hormones, especially insulin (inhibiting) and glucagon (stimulating) [8,14]. In the liver, insulin and glucagon are only effective as modulators of autophagy at intermediate, but not at either very low or very high, amino acid concentrations when autophagic flux is maximal or minimal, respectively [8,14]. Amino acids exert their inhibitory effect by blocking the sequestration step [8,14], but they can also interfere with post-sequestrational steps, as was found in experiments with rat hepatocytes in which amino acids completely inhibited autophagic proteolysis while inhibition of electro-injected cytosolic [14C] sucrose sequestration was not complete [66].
Chapter 1

Much attention has been paid to the specificity of amino acid inhibition of autophagic sequestration. Studies performed with perfused rat liver showed that the amino acids leucine, phenylalanine, tyrosine, glutamine, proline, histidine, tryptophan and methionine form a group known in the literature as the "regulatory amino acids". This group of amino acids together with alanine, which by itself has no effect on autophagy but acts as a synergistic coregulator, was able to inhibit autophagy as strongly as a complete physiological mixture of amino acids [67-69]. An almost identical group of regulatory amino acids was identified in experiments in which sequestration of cytosolic [$^{14}$C] sucrose was measured in isolated hepatocytes, except that asparagine replaced methionine [70]. Studies with perfused hepatocytes showed that leucine in combination with alanine, glutamine, proline or asparagine, at near physiological concentrations, strongly inhibited autophagic proteolysis under steady-state conditions [71,72]. Leucine in combination with intracellularly produced glutamate and/or aspartate from other amino acids is probably sufficient to inhibit autophagy maximally [72]. Studies with cultured hepatocytes obtained from rats at various stages of development, with different autophagic capacities, revealed that leucine, phenylalanine, tyrosine and lysine were involved in the regulation of autophagy [73].

Considering all these data on the inhibition of autophagy by specific amino acids, it was concluded that leucine, phenylalanine and tyrosine in combination with a few other amino acids, such as alanine and glutamine, are the most likely candidates for regulation of autophagic proteolysis [2].

The same amino acids that inhibit autophagy in liver also inhibit autophagy in other tissues. In kidney, leucine and phenylalanine are most important [74] while in the heart only leucine is involved [75]. In skeletal muscle, leucine and glutamine are potent inhibitors of proteolysis [76]. In myotubes of the cell line C2C12, autophagic proteolysis was induced by leucine limitation and was 3-MA sensitive [77]. Rat-muscle proteolysis, measured by the production of 3-methylhistidine from myofibrillar proteins, was inhibited after oral feeding of proteins or of leucine alone [78,79]. Although it has always been assumed that myofibrillar proteolysis occurs by an extralysosomal pathway that is not controlled by amino acids [80,81], perhaps this idea needs to be reconsidered.

An important and exciting discovery, first made in rat liver, was the fact that cell swelling mimics many of the anabolic effects of insulin such as, stimulation of protein synthesis and glycogen synthesis, and inhibition of proteolysis and glycogenolysis [82]. Cell swelling can be induced by hypo-osmolarity of the extracellular environment or by an influx of amino acids via Na$^+$-coupled transporters. Production of the impermeant amino acids glutamate and aspartate may further enhance intracellular osmolarity and lead to even more cell swelling [83-85]. When the cell volume is increased, the cell will try to restore its original size by a mechanism known as "regulatory volume decrease" (RVD): KCl is released from the swollen cell [86], but the initial volume is not fully restored, which leaves the cell in a slightly swollen state. Although these changes in cell volume are not great, the changes in intracellular ion concentrations can be high
and can strongly influence enzyme activities. In rat hepatocytes this was shown for glycogen synthase phosphatase, which becomes activated when intracellular chloride falls [84].

In contrast to glycogen synthesis, cell swelling has no effect on autophagy by itself but, like insulin, it can potentiate the effect of low concentrations of amino acids [87-89]. Conversely, leucine inhibits autophagy but has no effect on glycogen synthesis [88,90,91]. Clearly, the mechanisms underlying the inhibition of autophagy and the stimulation of glycogen synthesis by amino acids are different.

Regulation of autophagy by signalling

*mTOR/Tor*

The first indications that mTOR might be involved in the regulation of autophagy came from studies with rat hepatocytes carried out by our own laboratory. In these studies, it was shown that addition of amino acids to rat hepatocytes stimulated the phosphorylation of a 31 kD protein which was identified as ribosomal protein S6 [15,88]. A linear relationship was found between the percentage of inhibition of autophagy and the degree of S6 phosphorylation when hepatocytes were incubated with different amino acid mixtures [15]. The effect of amino acids on S6 phosphorylation could be prevented by rapamycin, indicating the involvement of mTOR-dependent activation of p70S6 kinase, the protein kinase responsible for S6 phosphorylation. Surprisingly, rapamycin also partly reversed the inhibitory effect of amino acids on autophagy [15].

S6 phosphorylation is required for the translation of a specific family of mRNA molecules known as TOP (terminal oligopyrimidine) mRNAs, which encode proteins belonging to the protein-translation machinery [92]. An interesting hypothesis is that S6 phosphorylation promotes ribosome binding to the ER, so that less ribosome-free ER membrane is available for autophagosome formation and at the same time S6 phosphorylation stimulates ER-linked protein synthesis [15].

In yeast, autophagy can also be induced by rapamycin inhibition of Tor proteins [17]. In this organism, active Tor blocks the protein phosphatase PP2A via the protein Tap42 which, if phosphorylated via Tor, forms a complex with PP2A, which then becomes inhibited. This process can be reversed by rapamycin [93]. In hepatocytes, autophagy could be blocked by the PP2A inhibitor okadaic acid at the sequestration step [94], although S6 phosphorylation was not affected [15].

Another target of Tor in yeast that is related to autophagy is Apg13, which becomes hyperphosphorylated under nutrient-rich conditions. This prevents the interaction of Apg13 with the Ser/Thr protein kinase Apg1 [95,96] which is essential for autophagy and the Cvt pathway. Whether or not kinase activity of Apg1 is important for autophagy is under debate: one group
claimed that Apg1 kinase activity for autophosphorylation was inhibited during autophagy [97], while another group found that kinase activity was increased towards the artificial substrate myelin basic protein under autophagic conditions [95]. More information about Apg1 came from studies in which it was shown that inhibition of the Apg1 kinase activity did not interfere with autophagy, whereas kinase activity was needed for Ape1 trafficking through the Cvt pathway [98]. In the same study it was found that increased interaction of Apg13 with Apg1 upon rapamycin treatment or nutrient limitation resulted in a conformational change in Apg1 which is important for autophagy. Apg1 controlled by Tor seems to act as a switch between the Cvt pathway and the autophagy pathway. Two mammalian homologues of Apg1 exist [99] but their role in autophagy is still to be examined. No mammalian homologue has yet been found for Apg13. Whether or not an Apg1/Apg13 switch system between Cvt and autophagy similar to that in yeast is present in higher eukaryotes is not known. Apg8 (see above) is highly upregulated in yeast during starvation or rapamycin addition [100,101], again indicating that Tor is involved. The induction of Apg8 during starvation is not necessary for autophagy, as was demonstrated with protein synthesis inhibitors [96], but the size of the formed autophagosomes was much smaller under these conditions, which suggest that induction Apg8 is needed for proper vesicle expansion.

**Phosphatidylinositol 3-kinase**

In rat hepatocytes, the stimulation of S6 phosphorylation by amino acids could be prevented not only by rapamycin but also by PI 3-kinase inhibitors [42]. Unexpectedly, this did not accelerate autophagy in contrast to the effect of rapamycin. To explain this result, it was speculated that two classes of PI 3-kinase enzymes are involved in the regulation of autophagy [42]. It was proposed that class III PI 3-kinase, which constitutively produces PtdIns(3)P, is needed for autophagy (see section "The biogenesis of the autophagosome"). The basis for this proposal was the situation in yeast, where VPS34p, the homologue of the p110 catalytic subunit of mammalian PI 3-kinase which is involved in membrane flow [102], can only use PtdIns but not PtdIns(4)P or PtdIns(4,5)P2 as a substrate. Furthermore, it was speculated that the production of PtdIns(3,4)P2 and PtdIns(3,4,5)P3 by class I PI 3-kinase, which does not occur in yeast cells, might be involved in the control of S6 phosphorylation [42]. If the PI 3-kinase inhibitors could not discriminate between the two PI 3-kinase enzymes, that would explain why S6 phosphorylation and autophagy would be inhibited simultaneously. An elegant study with HT-29 cells proved the hypothesis to be correct [43]. Addition of PtdIns(3)P accelerated autophagic sequestration and proteolysis in the presence of 3-methyladenine, the classical autophagy inhibitor, which turned out to be an inhibitor of PI 3-kinase [42,43]. The addition of PtdIns(3,4)P2 and PtdIns(3,4,5)P3 or stimulation of class I PI 3-kinase activity by interleukin-13 inhibited autophagic sequestration and proteolysis in HT-29 cells [43]. Tassa et al. [103] recently showed that in C2C12 myotubes, autophagy induced by amino acids depletion was accompanied by an increase in Beclin1-associated PI 3-kinase class III activity. This demonstrates that production of PtdIns(3)P for autophagy is directly controlled by amino acids.
The products of class I PI 3-kinase can bind to the pleckstrin homology domains of protein kinase B (PKB) and phosphoinositide-dependent kinase-1 (PDK1) [104], components of the insulin-stimulated signal-transduction pathway (see chapter 7). Studies with HT-29 cells have shown that expression of a constitutively active construct of PKB [105] and of PDK1 (P.Codogno, personal communication) also inhibit autophagy. mTOR, located downstream of PI 3-kinase, also participate in this mechanism in HT-29 cells: autophagy in HT-29 cells harbouring a rapamycin insensitive mutant of mTOR, was completely inhibited by amino acids and could not be reactivated by rapamycin (P.Codogno, personal communication). Whether or not p70S6 kinase controls autophagy in HT-29 cells is not known yet.

Whether or not amino acids can directly stimulate PI 3-kinase class I activity, and thereby stimulate p70S6 kinase activation and inhibit autophagy, is still controversial. Some groups have found transient activation of PI 3-kinase by amino acids [106,107], while others have failed to show direct activation of PI 3-kinase class I by amino acids [108-110]. PKB, which is downstream of PI 3-kinase class I, is not stimulated by amino acids [111] (see also chapter 7).

**PTEN**

PTEN (phosphatase and tensin homologue), also known as MMAC1 (mutated in multiple advanced cancers) or TEP1 (TGFβ-regulated and epithelial cell–enriched phosphatase), is a dual protein/lipid phosphatase which removes the 3-phosphate from PtdIns(3,4,5)P3 [112,113]. PTEN is a tumor suppressor gene which is mutated in a wide variety of cancers. Decreasing the level of PtdIns(3,4,5)P3 in HT-29 cells by overexpression of the PTEN gene counteracted the inhibitory effect of IL-13 on autophagic sequestration and proteolysis [105] (see also chapter 2). Under these conditions, PI 3-kinase-mediated activation of PKB by IL-13 was blocked. These experiments provided additional experimental support of the importance of PI 3-kinase products in the control of autophagy.

**G-proteins**

The first indications for a possible involvement of heterotrimeric G-proteins (αβγ) in the control of autophagy came from studies with α-toxin-permeabilized rat hepatocytes, in which it was shown that GTPγS, a nonhydrolyzable GTP analogue, completely prevented autophagic vacuole formation [114]. Transfection studies with HT-29 cells provided evidence that autophagic sequestration is under the control of the trimeric G\(_3\) protein [115]. When the α-subunit of G\(_3\) (G\(_{α3}\)) is bound to GTP, sequestration is inhibited, while when it is bound to GDP, the process is stimulated [116]. Autophagy is also reduced in HT-29 cells transfected with the G203A mutant of G\(_{α3}\), which is impaired in the dissociation of the βγ dimer from the GTP-bound G\(_{α3}\)-subunit [116]. Transfection studies with chimaeric Gαi proteins showed that the G\(_{α3}\) protein must be localized near Golgi and ER membranes in order to be able to control
autophagic sequestration [117]. When \( G_{a3} \) is ectopically expressed at the plasma membrane, it no longer has an effect on autophagy [117].

The GTPase activity of the \( G_{a3} \) protein is increased by the G-alpha interacting protein (GAIP) [118] which belongs to the protein family of regulators of G protein signalling (RGS) [119]. Autophagic sequestration could be stimulated in HT-29 cells by overexpression of GAIP, but was inhibited by GAIP antisense transfection [118]. Interestingly, differentiated HT-29 cells have a lower autophagic capacity than undifferentiated HT-29 cells, which correlates with lower expression levels of GAIP in differentiated HT-29 cells [118]. GAIP activity towards \( G_{a3} \) is stimulated by phosphorylation of a conserved serine residue and this phosphorylation of GAIP is prevented by amino acids via inhibition of the MAP kinases ERK1/2 [120]. Further research showed that amino acids reduced the kinase activity of Raf-1 by stimulation of phosphorylation on Ser\(^{259}\), resulting in a loss of Raf-1 kinase activity towards the ERK1/2 kinase MEK1/2 [121]. It must be pointed out that inhibition of MAPK by amino acids was not found in other cell types, such as hepatocytes [11] or CHO-IR cells [108].

More proof in favour of the involvement of \( G_{a3} \) in autophagy came from studies carried out with AGS3, a protein which acts as a guanosine nucleotide dissociation inhibitor towards the \( G_{a3} \) protein [122]. AGS3 contains four GoLoco motifs in its carboxyl-terminal part and seven TPR repeats in its amino-terminal part. Via its GoLoco motifs, AGS3 selectively interacts with the GDP-bound form of \( G_{a3} \) proteins and inhibits the association of GDP-bound \( G_{a3} \) proteins with \( \beta \gamma \) dimers [123-125]. In HT-29 cells, autophagy is stimulated by AGS3 overexpression, which is in accordance with the requirement of the GDP-bound form of \( G_{a3} \) for autophagy [126]. In contrast, overexpression of the non-\( G_{a3} \)-interacting amino-terminal part or of the \( G_{a3} \)-interacting carboxyl-terminal part inhibited autophagy, probably by competing with endogenous AGS3 for binding with \( G_{a3} \). This suggests that in HT-29 cells the full-length form of AGS3 is needed for stimulation of autophagy. Immunofluorescence studies showed that a fraction of AGS3 co-localizes with \( G_{a3} \) and GAIP on ER and Golgi membranes [117,126], both of which are possible sources for the isolation membrane, as discussed earlier. AGS3 does not co-localize with autophagosomes and the intracellular distribution of AGS3, \( G_{a3} \) and GAIP is not changed during nutrient-deprivation-induced autophagy, which would suggest that these proteins are involved in an early step along the autophagic pathway [126].

**AMP-activated protein kinase (AMPK)**

Hepatic autophagy is inhibited by AMP and by the AMP analogue AICArriboside (AICAR), suggesting that AMPK negatively regulates autophagy [127,128]. Activation of AMPK results in the inhibition of ATP-consuming processes (such as protein synthesis, cholesterol synthesis and fatty acid synthesis) and in the stimulation of ATP producing processes (such as glycolysis and \( \beta \)-oxidation of fatty acids) [129,130]. Because autophagy is an ATP-dependent process [131,132], the physiological relevance of the inhibition by AMPK of autophagy might be to shut down this energy-requiring process when the ATP/AMP ratio declines [127].
Recently, we and others discovered that in hepatocytes and muscle cells stimulation of the mTOR/p70S6 kinase pathway by amino acids and insulin is inhibited by AMPK [133-136] (see also chapter 4). One would expect this to result in stimulation of autophagy. Why this is not the case in hepatocytes is not known, but in yeast the AMPK homologue Snf1 is, indeed, required for autophagy [137].

eIF2α (GCN2)

In yeast, amino acid starvation leads to activation of the eIF2α kinase GCN2 which phosphorylates the GTP-binding protein eIF2α on Ser51. This is followed by increased synthesis of the transcriptional activator of amino acid biosynthetic genes, GCN4 [138]. Microarray analysis in yeast has revealed that at least three APG genes (APG1, APG13 and APG14) are GCN4 targets, although GCN4-deleted yeast cells still produced autophagic bodies [139]. By contrast, Tallóczy et al. [140] showed inhibition of autophagic body formation by nitrogen starvation or rapamycin addition in GCN4-deleted yeast cells. Another study in yeast showed that phosphorylation of GCN2 at Ser577, blocking kinase activity, and blocking eIF2α phosphorylation are all three affected by rapamycin in a Tor/TAP42-dependent manner [141]. Unexpectedly, in GCN2-deleted yeast cells nitrogen starvation could not induce autophagy, whereas rapamycin was still able to do so [140]. Therefore, the issue of whether or not GCN2 is downstream of Tor remains to be clarified (see also chapter 7).

In mammals, four eIF2α kinases are known: GCN2, PKR, PERK and HRI, which are all capable of phosphorylating eIF2α on Ser51 although they are activated under different conditions [142]. In mammalian cells, PKR becomes activated after viral infection and is involved in both inhibition of translation and activation of autophagy. Furthermore, PKR can rescue autophagy in GCN2-deleted yeast cells during nitrogen starvation. These data indicate that autophagy is used as a defence mechanism against viral infections [140]. It must be stressed, however, that some viruses can also use the autophagic pathway to replicate [143].

Selectivity of autophagy

Autophagy is generally considered to be nonselective under conditions of starvation, but the process can be selective under other conditions.

In yeast, selective degradation of peroxisomes, named pexophagy, was found when cells were transferred from a medium containing methanol, a peroxisomal substrate, to a medium containing glucose [144]. Mitochondria are not affected under these conditions [145]. Pexophagy can occur by micropexophagy, during which peroxisomes are directly taken up by the vacuole, or by macropexophagy, when peroxisomes are first enclosed by a double sequestering membrane and then fuse with the vacuole [146]. Many genes involved in pexophagy are also required for autophagy and the Cvt pathway; this shows that these processes use the same machinery [65].
Pexophagy was also found in hepatocytes from rats treated with clofibrate, a peroxisome proliferator, after withdrawal of the compound [147]. In vitro, autophagy of peroxisomes in hepatocytes could be prevented by addition of long-chain, but not short-chain, fatty acids [147]. Because long-chain fatty acids, but not short-chain fatty acids are oxidised by peroxisomes, this indicates that peroxisomes are degraded by autophagy when they are functionally redundant [147]. In the presence of [1-14C] palmitate, a 69-kDa membrane protein was palmitoylated and it was suggested that this might protect the peroxisome against autophagic degradation [148].

In studies carried out with the perfused liver, glucagon accelerated autophagic protein breakdown but did not influence degradation of ribosomal RNA [149]. Selective degradation of smooth ER was found in rats pretreated with phenobarbital followed by removal of the drug [148]; the decrease in smooth ER components under these conditions was not due to decreased synthesis [148].

When mitochondria become depolarized by a process known as “mitochondrial permeability transition” (MPT) [150], mitochondrial autophagy is initiated and the depolarized mitochondria are sequestered by the autophagic system [151]. When apoptotic cells are treated with caspase inhibitors, mitochondria are also selectively taken up by the autophagic system [152]. In liver biopsies of patients with Reye’s syndrome, selective degradation of damaged mitochondria was found [153] as was also the case in liver of an Influenza B virus mouse model of Reye’s syndrome [154]. Interestingly, compounds which are thought to be involved in the pathogenesis of Reye’s syndrome (e.g. aspirin) are also involved in induction of MPT [155].

The data described above indicate that autophagy can selectively remove damaged organelles, which contributes to cellular homeostasis. In this context, it is of interest to note that the autophagic capacity declines during aging [156]. Restricting caloric intake in rats increased autophagic proteolysis and may contribute to increased longevity [156]. In contrast, overfeeding may inhibit autophagic proteolysis because plasma levels of amino acids and insulin will increase under this condition [157]. In the fat-specific insulin receptor knockout (FIRKO) mouse it was found that longevity increased [158]. It is tempting to speculate that this can be explained by a higher autophagic capacity in these mice. Also in the nematode Caenorhabditis elegans and in the fruit fly Drosophila melanogaster longevity was increased in mutants that reduce insulin signalling [159-163].

In conclusion, apparently, the autophagic system is able to recognize cell structures that have to be degraded because they are either functionally redundant or damaged. However, so far nothing is known about the underlying recognition signals.

Microautophagy

Microautophagy is defined as the process in which portions of the cytoplasm are directly engulfed by the lysosomal membrane [2]. In contrast to autophagy (see section "Amino acid regulation of autophagy"), microautophagy is not inhibited by amino acids. There are indications
that the process declines under conditions of long-term starvation and that it is probably important for the degradation of long-lived proteins [164,165]. In vitro, experiments with lysosomes suggested that microautophagy is ATP-independent [166], but further research showed that ATP is needed for the intra-lysosomal degradation of proteins taken up by microautophagy [167]. More proof came from experiments carried out with a cell-free microautophagic system derived from yeast, in which the authors showed that the vacuole invaginated fluorescent dyes in an ATP-dependent fashion [168]. A specialized structure called the autophagic tube is involved in the uptake during microautophagy and is formed via an inverse budding mechanism [169]. Yeast APG mutants had fewer autophagic tubes, and wild-type yeast cells had more autophagic tubes during starvation [169]. Components involved in homotypic vacuole fusion, such as e.g. Sec17, Sec18 and SNAREs, which are needed for autophagy, are not involved in microautophagy, which would suggest that the two processes are mechanistically distinct [168]. The function of microautophagy might be the regulation of vacuole membrane homeostasis and the regulation of the size of the vacuole [169]. Like autophagy, microautophagy is involved in the degradation of peroxisomes (see section "selectivity of autophagy"). A novel form of selective microautophagy was found in yeast, in which the process is involved in the degradation of nonessential parts of the nucleus; this process was called piecemeal microautophagy of the nucleus (Pmn) [170]. Pmn is active during logarithmic growth and probably responds to the gradual depletion of carbon and nitrogen [170]. Apg7 is not needed for Pmn, but it is essential for autophagy; however both processes are induced by rapamycin [170]. Almost nothing is as yet known about how Pmn is regulated and whether or not the process is active in mammalian cells.

**Chaperone-mediated autophagy**

Chaperone-mediated autophagy (Cma) involves direct targeting of specific cytosolic proteins to the lysosomes for degradation [171]. All substrate proteins possess peptide sequences related to KFERQ [172,173]. During Cma, the transported protein is in an unfolded state and the targeting peptide is recognized in an ATP-dependent manner by the molecular chaperone heat-shock cognate protein hsc73 [174,175]. The complex so formed associates with the lysosomal receptor lamp2a [176] and a lysosomal hsc73 protein is required to translocate the substrate into the lysosome [177,178]. Cma is activated after serum withdrawal from confluent fibroblasts [173] and in rat liver during prolonged starvation [179], but how the process is mechanistically activated is still unknown. Although inhibitors of PI-3kinase inhibit autophagy [42], they have no effect on Cma [180]. During prolonged starvation, ketone bodies accumulate in the circulation of mammals and the ketone body β-hydroxybutyrate activates Cma [180]. The physiological importance of Cma is not yet clear, but it may be that during prolonged starvation dispensable proteins or even specific harmful proteins must be degraded to keep homeostasis under a stressful situation[180]. Interestingly, during aging Cma activity declines, probably because of
lower lamp2a levels [181,182]. This is perhaps one of the reasons why more damaged proteins accumulate during aging.

**Crinophagy**

Direct fusion of lysosomes with secretory vesicles, a process known as crinophagy, results in the degradation of secretory proteins. During crinophagy, mature secretory vesicles fuse with lysosomes, autophagosomes, endosomes or amphisomes [14]. Studies performed with the cortex zona fasciculata of rats suggested that crinophagy is involved in the regulation of hormone secretion in the pituitary-adrenal gland axis [183]. Crinophagy also proved to be involved in the degradation of prolactin in mammatroph cells from ewes after lactation followed by 7 days of weaning [184].

**Scope of this thesis**

The aim of this dissertation was to gain more information on the signal transduction pathway initiated by amino acids and insulin and their influences on autophagic protein degradation and glycogen metabolism. Chapter 2 describes the effect of overexpression of the tumor suppressor PTEN on autophagy and signal transduction in the human colon cancer cell line HT-29. It was reported that PTEN negatively regulates the PI-3 kinase/PKB pathway and thereby stimulates autophagy. In chapter 3 it is shown that in rat hepatocytes inhibitors of PI-3 kinase interfere with the regulation of glycogen metabolism probably by their anti-proteolytic effect and by enhancing glycogenolysis. Further it was found that PI-3 kinase class I is not involved in amino acid-stimulated glycogen production. Chapter 4 shows that activation of the mTOR/p70S6 kinase pathway by amino acids is regulated by the energy status of the cell via the AMP-activated protein kinase. Insulin signalling is not under the control of the AMP-activated protein kinase. Chapter 5 describes the interaction between amino acids and insulin in stimulating the PI-3 kinase/PKB/mTOR/p70S6 kinase pathway in rat hepatocytes. In chapter 6 data is presented that the mTOR/p70S6 kinase pathway might be controlled by the degree of charged tRNA and evidence was found that chloride might play a role as second messenger that could explain some of the amino acid effects found in rat hepatocytes. Finally, in chapter 7 mechanisms by which amino acids can stimulate signalling are discussed.
References


Introduction


Chapter 1


Introduction


Chapter 1


Introduction


Chapter 1


Introduction


Chapter 1


