Regulation of metabolism by amino acid dependant signal transduction

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Chapter 7

General Discussion:
Amino acids, signalling and the regulation of metabolism
Introduction

Although amino acids are important as substrates for many metabolic pathways such as protein synthesis, gluconeogenesis, synthesis of urea and other N-containing compounds, they can also serve as regulators of metabolism. For example, in the liver, the central amino acid catabolite glutamate promotes the activity of the ornithine cycle via synthesis of N-acetylglutamate, the essential activator of carbamoyl-phosphate synthase [1]. Glutamate and aspartate are essential components of the malate/aspartate shuttle responsible for the mitochondrial oxidation of cytosolic NADH during aerobic glycolysis in many tissues and during ethanol oxidation in the liver, and they kinetically control the rate of these processes [2]. Other pathways that are stimulated by amino acids are glucose-driven glycogen synthesis [3] (see also chapter 3) and the inhibition of autophagy by amino acids [4-6] (see also chapter 1 and 3). The latter two properties of amino acids represent typical insulin-like actions.

Stimulation of glycogen synthesis by certain amino acids (glutamine, proline, alanine) is caused by cell swelling due to concentrative, Na\(^+\)-dependent, amino acid transport across the plasma membrane and by the intracellular production of impermeant catabolites such as glutamate and aspartate [3,7] (see also chapter 6). In response to the initial swelling, cells undergo "regulatory volume decrease" when they try to restore their original volume by releasing KCl. The decrease in intracellular chloride concentration can be considerable and activates glycogen synthase phosphatase because chloride inhibits this enzyme at concentrations usually found in cells [8].

By contrast, autophagy is inhibited by amino acids such as leucine, tyrosine and phenylalanine that are transported by H\(^+\)-dependent mechanisms and are not concentrated to a major extent inside the cells [6]. Autophagic protein degradation in freshly isolated hepatocytes is not affected by cell swelling per se, but an increase in cell volume promotes the ability of leucine, tyrosine and phenylalanine to inhibit proteolysis [9].

Clearly, the reciprocal regulation of glycogen synthesis and of autophagic proteolysis by amino acids share a common element, cell swelling, but differences also exist. For instance, leucine, tyrosine and phenylalanine do not affect glycogen synthesis [3,10] (see also chapter 3).

The potency of leucine as an inhibitor of autophagic proteolysis ([6], for review; see also chapter 1) was of interest because for a long time leucine had also been known as an effective stimulator of protein synthesis not only in the liver but also in other cell types [11]. Our laboratory was the first to show that the addition to isolated hepatocytes of a complete mixture of amino acids at physiological concentration strongly and rapidly stimulated the phosphorylation of a protein of 31 kDa that was identified as ribosomal protein S6 [9,10]. S6 has 5 phosphorylation sites, is a component of the 40S ribosomal subunit and its phosphorylation is required for the translation of the terminal oligopyrimidine ("TOP") family of mRNA molecules containing an oligopyrimidine tract upstream of their transcription-initiation site [12]; these mRNA molecules encode proteins belonging to the protein-translation machinery. Amino acid-
stimulated phosphorylation of S6 was completely eliminated by rapamycin, indicating that the serine/threonine protein kinase mTOR (mammalian target of rapamycin) was on the pathway of amino acid-stimulated S6 phosphorylation. The stimulation of S6 phosphorylation by amino acids resembled that by insulin ([13], for review). It must be stressed that in these experiments with amino acids, insulin was not present. Moreover, when insulin was added in the absence of amino acids, no effect on S6 phosphorylation was observed [9]. However, although ineffective on its own, insulin did increase the effectiveness of amino acids to induce S6 phosphorylation in that lower concentrations of amino acids were needed to induce maximal S6 phosphorylation. The reason for the effectiveness of insulin in stimulating S6 phosphorylation in other cell types, as reported in the earlier literature was the presence of amino acids in the culture media. Another important observation was that hypo-osmotically-induced cell swelling, like insulin, also increased the effectiveness of amino acids such as leucine, tyrosine and phenylalanine to promote S6 phosphorylation. Furthermore, under a wide variety of conditions a linear relationship was found between the degree of S6 phosphorylation and the percentage of inhibition of proteolysis, as measured in the presence of cycloheximide to inhibit simultaneous protein synthesis [9]. Of great significance was that rapamycin addition could partially, albeit not completely, prevent the inhibition of autophagic proteolysis by amino acids under these conditions. In the absence of cycloheximide, protein synthesis was also partially inhibited by rapamycin. It was concluded that the same mechanism is perhaps involved in the reciprocal control of protein synthesis and degradation which would be extremely efficient from the point of view of metabolic regulation [9]. Interestingly, a few years later it was reported that rapamycin addition to yeast cells also stimulated autophagy [14], indicating conservation of this control mechanism in evolution.

Amino acid-induced S6 phosphorylation was not only inhibited by rapamycin but also by wortmannin and LY294002, two structurally unrelated inhibitors of phospatidylinositol 3-kinase (PI 3-kinase); it was concluded that PI 3-kinase is another component of the amino acid-dependent signalling pathway [15]. It should be noted that the PI 3-kinase that is activated by insulin and other growth factors is, in fact, PI 3-kinase class I, which produces PtdIns(3,4)P2 and PtdIns(3,4,5)P3 [16]. By contrast, PI 3-kinase class III is probably not affected by insulin but is under the control of amino acids [17] (see section 1.5 below), and produces PtdIns(3)P. It has recently been shown that PtdIns(3)P is essential for autophagy while PtdIns(3,4)P2 and PtdIns(3,4,5)P3 are inhibitory [18,19] (see also chapters 1 and 2).

It thus appears that amino acids are able to activate a signal transduction pathway that shares components of a signalling pathway that is also stimulated by insulin. The mechanism by which amino acids induce cell signalling is still largely unknown. However, the last few years much information has become available in this rapidly expanding field which may shed some light on this mechanism. In this final chapter, I will discuss the interaction between amino acids, the mTOR/p70S6 kinase pathway and autophagy, as well as the interaction between amino acids and proteins that are part of the mTOR/p70S6 kinase pathway: these include protein kinases, protein phosphatases, adapter-like proteins and scaffold proteins. I will then propose a few possible mechanisms by which amino acids might stimulate signal transduction. Finally, I will discuss the
possible role of amino acid-dependent signal transduction in the initiation and progression of some diseases. Amino acid-dependent signaling is also important in the regulation of gene expression but this is beyond the scope of this thesis. For a review on this subject the reader is referred to Jousse et al. [20].

1 Amino acid-dependent signalling

1.1 Amino acids and p70S6 kinase activation

The existence of amino acid signalling and its interaction with insulin signalling, initially observed in hepatocytes [9,10], have been confirmed or, in some cases, rediscovered in many cell types. These include muscle cells, adipocytes, hepatoma cells, CHO cells and pancreatic β-cells [21-26]. In most of these studies, the phosphorylation was measured of p70S6 kinase and of 4E-BP1, other downstream targets of mTOR, and the data were discussed in relation to the regulation of protein synthesis rather than that of autophagic proteolysis. Apart from the fact that amino acid-stimulated phosphorylation of 4E-BP1, p70S6 kinase and of S6 was rapamycin-sensitive, the involvement of mTOR in the amino acid response was also supported by other experiments. For instance, in human rhabdomyosarcoma Rh30 cells harbouring a rapamycin-resistant mutant of mTOR, amino acids stimulated p70S6 kinase activity in a rapamycin-insensitive manner [27]. In CHO-IR cells, a rapamycin-resistant mutant of p70S6 kinase could be phosphorylated at Thr^{412} (critical for enzyme activity) in the presence of insulin in a wortmannin-sensitive manner, irrespective of the presence of amino acids [21].

As in hepatocytes, in many other cell types amino acids and insulin were found to act in synergy [21,22,26,28,29] (see also chapter 5). Insulin alone did not induce p70S6 kinase activation; in cases where it did stimulate on its own this could be ascribed to amino acids produced by autophagy [30]. Also, as in hepatocytes, leucine proved to be the most effective among the various amino acids in many cell types [21,22,24,26,31-34] (see also chapter 6). The data also showed that leucine alone could not completely mimic the effect of a mixture of all amino acids. Apparently, other amino acids act in concert with leucine to elicit full activation of p70S6 kinase. As discussed in chapter 6, a possible explanation may be that some amino acids which are transported together with Na^{+} increase cell volume and decrease chloride levels which can positively influence mTOR activation by leucine.

1.2 Amino acid stimulation of 4E-BP1 phosphorylation

Another substrate of mTOR, apart from p70S6 kinase, is 4E-BP1 (also known as PHAS-1) [35,36]. 4E-BP1 has several phosphorylation sites that are targets of different protein kinases, and phosphorylation of the protein results in dissociation of the eIF4E.4E-BP1 complex; eIF4E then becomes available for initiation of cap-dependent mRNA translation [37]. Like p70S6
kinase, in various cell types phosphorylation of 4E-BP1 was greatly stimulated by amino acids in a rapamycin-sensitive manner [21,22,24-27,30,38] (see also chapter 6), with leucine again being the most effective [21,25,26]. Insulin was not required for this effect. In the absence of amino acids, insulin was unable to stimulate 4E-BP1 phosphorylation [21,38].

1.3 Amino acid stimulation of eEF2 kinase

Eukaryotic elongation factor 2 (eEF2), which mediates the translocation step of elongation, becomes inactive when it is phosphorylated at Thr56. Phosphorylation at this position is controlled by eEF2 kinase. This kinase becomes inhibited when phosphorylated at Ser363 by p70S6 kinase, so that the same factors (e.g. amino acids) that control p70S6 kinase activity also control eEF2 kinase phosphorylation, and thus the activity of eEF2 [39]. eEF2 kinase can also be phosphorylated by p90RSK, in which case eEF2 phosphorylation is insensitive to rapamycin but sensitive to inhibitors of the MEK/Erk signalling pathway [39].

1.4 Amino acid stimulation of eIF2α

Another factor controlling protein synthesis is the eukaryotic initiation factor eIF2. In the absence of amino acids, this factor becomes inactivated when Ser51 of the α subunit of eIF2 is phosphorylated. In the presence of amino acids, when Ser51 is dephosphorylated, eIF2 becomes active and recruits charged initiator tRNA to the 40S ribosomal subunit [40]. In the activation process by amino acid-induced dephosphorylation, leucine in particular is effective [31].

In yeast, a single kinase, GCN2 (general control non-depressible), is responsible for eIF2α phosphorylation. This kinase is activated by uncharged tRNAs because its C-terminus structurally resembles histidinyl-tRNA synthetase and other amino acyl-tRNA synthetases [41]. This is then followed by increased synthesis of the transcription factor GCN4 which, in turn, is responsible for increased transcription of a large number of genes involved in amino acid synthesis and other metabolic processes needed under these conditions, including genes encoding proteins required for autophagy [41,42]. Although this has been disputed, uncharged tRNA may be a sensor of amino acid starvation in mammalian cells (see chapter 6 and section 3). In this context, it is of importance to note that eIF2α kinase is not only important in the regulation of protein synthesis, but is also essential in starvation-induced autophagy in both yeast and mammalian cells [43] (see also chapter 1).

1.5 PI3-kinase class III/Beclin1

An important step forward in our understanding of the relationship between amino acid signalling and autophagy was recently obtained in experiments performed with C2C12 myotubes. It was found that autophagy, induced by amino acid depletion, was accompanied by an increase in Beclin1-associated PI 3-kinase class III activity [17]. This is the first demonstration that the
production of PI(3)P for autophagy is controlled by amino acids and is stimulated when amino acid levels fall. Although the specificity of the amino acid effect was not investigated, it is, in analogy with the mTOR/TSC1/2 complex (cf. Discussion of chapter 5), tempting to speculate that amino acid-induced cell swelling is responsible for dissociation of the Beclin1/PI 3-kinase class III complex, and thus contributes to the inhibition of autophagy. The observation in hepatocytes that inhibition of autophagy by amino acids cannot be completely reversed by rapamycin (see introduction) may now be explained by the fact that mTOR does not affect the Beclin1/PI 3-kinase class III complex. Another interesting observation is that in C2C12 myotubes amino acid addition did not result in S6 phosphorylation; however, rapamycin addition to these cells did increase autophagy [17]. Data obtained with myeloblastic cells [44] and with Drosophila [45] also seem to exclude a role for p70S6 kinase, and thus for S6, in the control of autophagy. This would refute our initial hypothesis (see section 3) based on data with hepatocytes, that S6 phosphorylation itself may control autophagy, for example by changing the degree of occupancy of the endoplasmic reticulum with ribosomes, the ribosome-free endoplasmic reticulum being a putative source of membrane for autophagosome formation [9]. It cannot be excluded, however, that differences in mechanisms may exist between cell types.

1.6 Amino acid interaction with Erk1/2 kinases

In HT-29 cells, in addition to the PI 3-kinase/mTOR pathway, another amino acid signalling pathway can control autophagy (see chapter 1). In these cells, autophagy is also controlled by the trimeric G\textsubscript{a3} protein: when it is bound to GDP, it stimulates autophagy, but when it is bound to GTP it inhibits autophagy [46]. The GTP hydrolysis rate, and thus the rate of autophagy, is controlled by the activity of the Gx-interacting protein GAIP [47]. Activity of GAIP is enhanced by Erk1/2-mediated phosphorylation. In HT-29 cells, phosphorylation, and activity of Erk1/2, mediated by Raf-1 and Mek1/2, could be decreased by amino acid addition [48,49]. Amino acids proved to do so by stimulation of phosphorylation of Ser\textsuperscript{259}, and inactivation of Raf-1 [49]. Because Ca\textsuperscript{2+} is required for autophagy [50], it was suggested that the G\textsubscript{a3} protein may recruit Ca\textsuperscript{2+}-binding proteins to the Golgi or endoplasmic reticulum where autophagosome formation may begin [51]. Whether amino acid signalling to Raf-1 is mTOR-dependent is not yet known.

By contrast, in freshly prepared hepatocytes [52,53] and in C2C12 myotubes [17] inhibition of autophagy by amino acids was not accompanied by changes in Erk1/2 phosphorylation. Apparently, differences in amino acid signalling mechanisms and in the control of autophagy may exist, depending on the cell type and perhaps depending on the degree of differentiation [17].
1.7 p38MAPK

According to Häässinger and colleagues, amino acid-induced cell swelling in the isolated perfused rat liver and in cultured hepatocytes inhibits autophagy because it activates p38MAPK; inhibition of this stress kinase prevents the inhibition of autophagy [54]. Integrins appear to be involved in the osmosensing mechanism [55]. In C2C12 myotubes, inhibition of autophagy was independent of p38MAPK [17]. Because in freshly isolated hepatocytes cell swelling per se had no effect on autophagy but potentiated the inhibition of autophagy by leucine (and/or phenylalanine and tyrosine) via mTOR-dependent signalling (see introduction and also chapter 1), it was suggested that, apparently, multiple osmosensing mechanisms in hepatocytes may exist which, depending on the conditions, can be differentially linked to intracellular signalling pathways [56].

1.8 Participation of PI 3-kinase and protein kinase B in amino acid-dependent signalling?
Amino acid/insulin synergy

A simple explanation for the synergy between insulin and amino acids in stimulating mTOR downstream targets would be that insulin promotes amino acid transport across the plasma membrane (see chapter 5). This is not very likely, however, because stimulation of protein synthesis in muscle by insulin was accompanied by decreased rather than increased intracellular amino acid concentrations [23].

As discussed in sections 1.1 and 1.2, in the absence of amino acids, insulin alone did not affect phosphorylation of mTOR downstream targets. However, insulin alone did stimulate the activity of PI 3-kinase and protein kinase B (PKB) [21,22,24,28,30,57,58] (see chapter 5), which are both signalling components located upstream of mTOR [37]. Stimulation of phosphorylation of p70S6 kinase, of S6 and of 4E-BP1 by amino acids alone in the absence of insulin could be prevented by inhibitors of PI 3-kinase [15,21-25,32] (see chapter 5). This suggests that PI 3-kinase is located upstream of p70S6 kinase in the signalling pathway. As already discussed in chapter 1, it is not entirely clear whether or not amino acids are able to stimulate PI 3-kinase activity. There is general agreement, however, that amino acids do not affect PKB activity [21,22,24,27,28,57,59] (see also chapter 5).

Because amino acids may not directly activate PI 3-kinase, it is possible that PI 3-kinase is on a pathway parallel to that of amino acids, and that both the activation of PI 3-kinase (by insulin) and of mTOR or another kinase (by amino acids) are required for full activation of p70S6 kinase [37,39]. It has been suggested that the phosphatidylinositol lipids are required for membrane anchoring of one or more kinases, the activity of which is regulated by amino acids [60]. Another possibility arose from studies showing that amino acid-dependent p70S6 kinase activation was abrogated in PDK1 -/- cells; however, amino acids were still able to increase phosphorylation of 4E-BP1 in a rapamycin-sensitive manner. This indicated that activation of mTOR function in the presence of amino acids was maintained in these PDK1-deficient cells [39]. Using p70S6 kinase phospho-specific antibodies, Wang et al. [39] concluded that activation
of p70S6 kinase requires two separate inputs: one through PDK1, which results in phosphorylation of Thr\textsuperscript{229}, and another through mTOR (independently of PI 3-kinase and PDK1), which results in phosphorylation of Thr\textsuperscript{389} (Note: Thr\textsuperscript{229} and Thr\textsuperscript{389} are equivalent to Thr\textsuperscript{2522} and Thr\textsuperscript{412} of the long splice variant of p70S6 kinase) (see chapter 5).

In order to account for the ability of high concentrations of amino acids to activate p70S6 kinase in the absence of insulin by a mechanism that is sensitive to wortmannin or LY294002, however, one has to assume that either basal activity of PI 3-kinase or only a slight stimulation of PI 3-kinase by amino acids (or possibly inhibition of PTEN) may be sufficient for Thr\textsuperscript{389} phosphorylation in p70S6 kinase (see chapter 5 and [61]).

In our opinion, Fig. 1 satisfactorily accounts for the synergy between amino acids and insulin with regard to p70S6 kinase activation. Cell swelling may mimic the effect of insulin on PI 3-kinase [52,62], so that the effect of high concentrations of amino acids on p70S6 kinase consists of two components: one due to amino acids that increase cell volume resulting in PI 3-kinase and PDK1 activation, and another component (e.g. leucine) required for mTOR activation (see also chapter 5 and section 3). This would explain why high concentrations of amino acids are able to activate p70S6 kinase in the absence of insulin.

1.9 Amino acids and the activation of mTOR

The mechanism by which amino acids activate mTOR is still unclear and results are controversial. In vitro, mTOR kinase activity towards 4E-BP1, with mTOR being immunoprecipitated from either rapamycin-treated or amino acid-depleted CHO-IR or PC12 cells, was not different from mTOR immunoprecipitated from control cells [21,63]. This suggests that mTOR activity changes were lost during isolation and may perhaps not be due to phosphorylation of mTOR but rather be due to an allosteric effect on mTOR itself. Other studies, however, did show stable changes in mTOR activity. For instance, mTOR isolated from amino acid-stimulated Jurkat cells could phosphorylate the protein phosphatase PP2A in vitro [64]. Addition of amino acids to HEK293 cells in the absence of insulin increased phosphorylation of Ser\textsuperscript{2448} of mTOR; moreover, in vitro, mTOR could be phosphorylated by PKB, but only when mTOR was immunopurified from cells incubated with amino acids [65]. Changes in Ser\textsuperscript{2448} phosphorylation also ran in parallel with mTOR-dependent signalling and rates of protein synthesis in rat skeletal muscle in vivo [66]. Although these experiments do show that mTOR can undergo stable changes in phosphorylation in response to amino acid addition, it is not always clear whether Ser\textsuperscript{2448} phosphorylation is essential for mTOR activity [37,58]. Very recently it was reported that mTOR becomes phosphorylated at Thr\textsuperscript{2466} under conditions of nutrient deprivation, probably via activation of AMPK, and this phosphorylation negatively regulates mTOR activity [67]. Interestingly, phosphorylation of Thr\textsuperscript{2466} limited phosphorylation of Ser\textsuperscript{2448} and it was suggested that these two sites might act as a switch controlling protein synthesis [67]. It would be highly interesting to know whether phosphorylation of Thr\textsuperscript{2466} would stimulate autophagy; however, this was not investigated.
Fig 1. Amino acids and signalling. The scheme is a composition of data discussed in this chapter.

Abbreviations: IR, insulin receptor; IRS-1/2, insulin receptor substrate 1/2; PI3K, phosphatidylinositol 3-kinase class I; Class III PI3K, phosphatidylinositol 3-kinase class III; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PDK1, phosphoinositol-dependent kinase 1; PKB, protein kinase B; LY, LY294002; Wort, wortmannin; 3MA, 3-methyladenine; PTEN, phosphatase and tensin homologue; TSC1/2, tuberous sclerosis complex; Rheb, ras homologue enriched in brain; mTOR, mammalian target of rapamycin; PP2A, protein phosphatase 2A; p70S6K, p70 ribosomal S6 protein kinase; S6, a ribosomal protein; 4E-BP1, 4E binding protein 1; Gβδ, G-protein beta subunit like protein; Raptor, regulatory associated protein of mTOR; AA, amino acids; AMPK, AMP-activated protein kinase; GAPP, glutamate-activated protein phosphatase; Beclin1, coiled-coil myosin-like BCL2 interacting protein.

1.10 Amino acids and protein phosphatases

It has been suggested that amino acids act as inhibitors of a protein phosphatase. In yeast, for example, the rapamycin-sensitive TOR proteins affect PP2A activity, by modulating the association of PP2A with the Tap42 protein: in the presence of nutrients Tap42 became phosphorylated and associated with PP2A which then became inhibited, while nutrient deprivation or rapamycin addition reversed these events [37].

The involvement of PP2A in amino acid signalling in mammalian cells is controversial. For instance, in brain cells and in Jurkat cells, p70S6 kinase appeared to be tightly associated with
protein phosphatase 2A [64,68]. In cells carrying the p70S6 kinase mutant that is resistant to rapamycin and to amino acid depletion, the association with PP2A was lost [64]. Moreover, in some cell types, phosphorylation and activation of p70S6 kinase, S6 and of 4E-BP1 could be induced by PP2A inhibitors in the presence of rapamycin or in the absence of amino acids [60,64,69]. Similarly, dexamethasone-induced dephosphorylation of p70S6 kinase and of 4E-BP1 in L6 myoblasts in the presence of amino acids could be corrected by PP2A inhibitors [70]. In other cell types, however, PP2A inhibitors had no effect [21,68]. In rat hepatocytes, the PP2A inhibitor calyculin, but not okadaic acid, induced rapamycin-insensitive hyperphosphorylation of p70S6 kinase, which was additive to the rapamycin-sensitive phosphorylation induced by amino acid addition [71] (see chapter 5). However, the calyculin-induced hyperphosphorylation did not affect p70S6 kinase activity. In permeabilized hepatocytes, okadaic acid inhibited dephosphorylation of p70S6 kinase (chapter 6). Phosphatase inhibitors also did not affect amino acid-induced phosphorylation of S6 [9]. According to Krause et al.[72], there are two protein phosphatases in amino acid signalling: one protein phosphatase is located upstream of mTOR which is perhaps activated by glutamate (and activates both glycogen synthase and acetylCoA carboxylase), and a second protein phosphatase that dephosphorylates p70S6 kinase and becomes inhibited when mTOR is activated (cf. Fig. 1 and chapter 6). The existence of two protein phosphatases would explain discrepancies in results obtained with protein phosphatase inhibitors because the outcome of such experiments would have depended on whether the protein phosphatase inhibitor had been added before or after exposure of the cells to amino acids.

1.11 Negative feedback by amino acid signalling on insulin signalling

Although insulin and amino acids synergize with regard to their effects on mTOR-mediated signalling, there are now also several reports showing that in muscle cells, adipocytes and hepatoma cells (but not in hepatocytes[57]), amino acids cause a time-dependent down-regulation of insulin-mediated activation of PI 3-kinase, PKB and glucose transport in a rapamycin-sensitive fashion [22,29,73-75]. The presence of amino acids resulted in increased ser/thr phosphorylation of IRS-1, and in decreased binding of the p85 regulatory subunit of PI 3-kinase to IRS-1 followed by increased, presumably proteasomal, degradation of IRS-1 [29,73]. It has been proposed that this mechanism may underlie diminished glucose tolerance during high-protein feeding [22,29]. Consumption of fish protein may be favourable in this regard because of the relatively low plasma levels of leucine, tyrosine and some other amino acids under these conditions [29].

Apparently, and paradoxically, amino acids are required for insulin-mediated activation of mTOR and its downstream targets, but they inhibit the initial part of the insulin signalling pathway. The paradox lies in the fact that PI 3-kinase activity is essential for amino acid-induced activation of mTOR and its downstream targets (see section 1.8). Down-regulation of PI 3-kinase activation by amino acids would be counter-productive, therefore, and would eventually lead to diminished protein synthesis and increased autophagic protein breakdown. This is highly unlikely.
Possibly, part of the activation of PI 3-kinase by insulin proceeds independently of IRS-1. There is, indeed, evidence that the pathway via IRS-2 may escape feedback inhibition by amino acids [73]. This residual, IRS-2-mediated, activation of PI 3-kinase would in that case be sufficient for amino acid-induced activation of mTOR and its downstream targets. mTOR may thus be considered as a metabolic switch that integrates both nutrition-mediated and insulin-mediated signals [73]. Amino acids would then simultaneously decrease transport and utilisation of glucose by insulin-sensitive tissues, and at the same time increase protein synthesis and decrease autophagic proteolysis, thus contributing to stimulation of cell growth. The fact that an increase in cAMP, a catabolic signal, decreases mTOR activity [76] further supports a role of mTOR as a nutritional sensor and nicely accounts for the glucagon/insulin antagonism we previously observed with regard to S6 phosphorylation in hepatocytes [9].

1.12 mTOR-dependent signalling is antagonized by AMPK

In a recent study with HEK293 cells yet another function of mTOR was proposed in that this protein kinase may not only act as a sensor of amino acids but also of intracellular ATP [77]. It was noted that, in contrast to that of several other protein kinases, the $K_m$ of ATP for mTOR was high, about 1 mM, and within the physiological range of ATP concentrations. Moreover, by inhibiting either glycolytic or mitochondrial ATP production a correlation was found between the intracellular ATP concentration and the degree of phosphorylation of p70S6 kinase or 4E-BP1, as indicators of mTOR activity in situ [77]. Because inhibition of ATP production also increases intracellular AMP levels via adenylate kinase, we considered that AMP-activated protein kinase (AMPK) may also contribute to mTOR inhibition when energy production is compromised. Indeed, we and others found that under various conditions activation of AMPK strongly inhibited amino acid-dependent signalling (see chapter 4) [66,72,78,79] and protein synthesis [66,78,80]. Activation of AMPK inhibited protein synthesis even more than rapamycin, suggesting that AMPK inhibits protein synthesis not only through inhibition of mTOR-mediated signalling but also at other steps controlling the protein-synthesizing machinery [78]. AMPK can also phosphorylate (and activate) eEF2 kinase independently of mTOR [80,81]. As discussed in section 1.9, AMPK can directly phosphorylate mTOR and inhibit its activity [67]. AMPK is also able to phosphorylate TSC2 which results in further inhibition of mTOR signalling (see section 3).

The association of mTOR with the mitochondrial outer membrane [82] is of interest because adenylate kinase is located in the mitochondrial intermembrane space. mTOR is thus in a perfect position to sense changes in the AMP/ATP ratio [78].

It has been reported that glucose helped to stimulate mTOR-dependent signalling in CHO cells [83]. Although this possibility could not have been considered at the time, in retrospect this may have been due to a decrease in AMPK activity. Likewise, the activation by hypoxia of the endoplasmic reticulum protein kinase PERK [84], a mammalian eIF2a kinase, which results in inhibition of protein synthesis, may have been caused by AMPK activation.
In view of the inhibitory effect of AMPK activation on protein synthesis via interference with mTOR-dependent signalling, one would expect stimulation of autophagy. Indeed, in yeast, the AMPK homologue snf1p proved to be required for autophagy [85]. However, in hepatocytes, activation of AMPK by AICAR resulted in inhibition of autophagy [86]. Apparently, in hepatocytes, AMPK, in addition to its ability to inhibit mTOR signalling also inactivates other protein components involved in the regulation of autophagy. Alternatively, there is the possibility that AICAR may not be entirely specific. Preliminary transfection experiments in HT-29 cells in collaboration with P. Codogno et al. (Paris; personal communication), showed that overexpression of AMPK does stimulate autophagy.

2 Amino acid-dependent signalling: physiological implications

2.1 Amino acid signalling in pancreatic β-cells

Amino acid signalling also occurs in pancreatic β-cells and constitutes a fascinating feedback loop in the regulation of whole-body nitrogen metabolism. In these cells, too, amino acids stimulated p70S6 kinase and 4E-BP1 phosphorylation in a rapamycin-sensitive and wortmannin-sensitive fashion. Strikingly, β-cells from p70S6 kinase-deficient mice underproduced insulin [87]. As in other cells, insulin alone, whether produced by the β-cells themselves (after glucose addition) or added externally, was not effective unless amino acids were also present [26,38]. Among the various amino acids, leucine was again most effective [26,34]. In addition to the ability of amino acids to promote β-cell proliferation via increased signalling, it has been proposed that cytosolic glutamate in β-cells can directly stimulate exocytosis of insulin presumably by causing swelling of the insulin-containing granules [88]. Although attractive, this idea was refuted by data showing that with glutamine present, the intracellular glutamate concentration was extremely high, yet insulin release remained low [89]. Interestingly, glutamine and leucine (in the absence of glucose) acted synergistically, and in the presence of these two amino acids alone, insulin production was as high as observed in the presence of glucose alone. According to the traditional view, allosteric activation of glutamate dehydrogenase by leucine provides α-oxoglutarate for the citric acid cycle [89]. The combination of glutamine plus leucine was also particularly effective in stimulating p70S6 kinase phosphorylation [34,90] (see also chapter 6). Experiments with transaminase inhibitors and with leucine analogues indicated that both the metabolism of leucine and its ability to stimulate glutamate dehydrogenase were required to stimulate p70S6 kinase phosphorylation [34,90]. Inhibition of the mitochondrial respiratory chain, when glycolysis was the only source of ATP production, eliminated the ability of glutamine plus leucine to stimulate p70S6 kinase. It was concluded that the same mitochondrial events that generate signals for leucine-stimulated exocytosis of insulin are required to activate the amino acid signalling pathway, and that activation of protein synthesis by amino acid signalling contributes to enhanced β-cell function [34]. Although overall ATP levels did not change, it is
likely that AMP, and thus AMPK activity, was increased. If, as discussed in section 1.12, AMPK inhibits mTOR-dependent signaling, a mechanistic explanation is established for the coupling between mitochondrial function and insulin release, in addition to the increase in cytosolic Ca++ concentration following closure of the plasma membrane K+ATP channel by a high cytosolic ATP/ADP ratio [90]. It is possible that part of the stimulation of insulin production by glucose is caused by a decrease in the activity of AMPK [91,92]. Similarly, proper functioning of the malate-aspartate shuttle in β-cells [93,94] may be required to keep AMPK activity low. Antidiabetic agents such as metformin and thiazolidinediones can directly activate AMPK independently of changes in AMP concentration [95]. Although this decreases plasma glucose concentrations because glucose transport into the muscle becomes less insulin-dependent under these conditions [96], there may also be negative effects because AMPK activation in the β-cells will decrease insulin production [97,98], possibly by interference with amino acid signalling.

2.2 Amino acid signalling in vivo

Although studies on amino acid-dependent signal transduction have mainly been carried out in isolated cells there is now ample evidence that amino acid signalling also plays an important role in vivo. For example, it has been known for a long time that amino acid infusion increases the insulin sensitivity of muscle protein synthesis in vivo [99]. We now know that this is likely to be caused by amino acid signalling. Indeed, after a protein meal, the protein anabolic response in man, rats and mice was accompanied by increased phosphorylation of 4E-BP1 and p70S6 kinase in muscle and liver [23,100,101]. In the rat, inhibition of insulin production by diazoxide eliminated the effect of amino acids, suggesting that, as in isolated cells, insulin and amino acids are also both required in vivo to induce a positive nitrogen balance [101]. Likewise, in man, leucine and insulin synergized with respect to their ability to stimulate p70S6 kinase phosphorylation in muscle, while insulin but not leucine increased PKB phosphorylation [102]. Other data also suggested that in vivo the leucine-induced enhancement of protein synthesis and the phosphorylation states of 4E-BP1 and p70S6 kinase are facilitated by increases in serum insulin [103].

Regarding the effect of amino acids on glucose metabolism the situation is less clear. In vivo, the increase in amino acid concentrations in the portal vein during the fasted-fed transition was sufficient to increase liver-cell volume and to stimulate glycogen synthesis [104]. The increase in glycogen synthesis in astrocytes during hyperammonemia is likely to be caused by cell swelling when intracellular synthesis of glutamine exceeds the efflux of glutamine from the cells. This swelling of astrocytes presumably accounts for the increase in intracranial pressure in hepatic encephalopathy [105].

On the basis of the ability of amino acids to promote glycogen synthesis one may, therefore, predict that amino acid-induced cell swelling and signalling also promotes glucose consumption. The available evidence does not fully support this prediction, however. Notwithstanding the fact that amino acids, leucine in particular, stimulate insulin production in
vivo [106], amino acid infusion during an euglycaemic hyperinsulinemic clamp in fasted humans decreased rather than increased glucose disposal [107,108]. Although these data may be explained by substrate competition, i.e. the amino acids were oxidised instead of glucose, there are indications that amino acids, in fact, cause time-dependent, rapamycin-sensitive, down-regulation of the activation of PKB and of glucose transport by insulin (Fig. 1) [29,73,75]. As discussed in section 1.11, amino acids stimulated ser/thr phosphorylation of IRS-1, resulting in decreased binding of the p85 regulatory subunit of PI 3-kinase to IRS-1, followed by proteasomal degradation of IRS-1 [29,73]. Because, as we have seen, PI 3-kinase class I is required for mTOR-downstream signalling, perhaps this feedback system is part of a homeostatic mechanism which is required to prevent mTOR from being overactivated by amino acids.

Another example suggesting that amino acids may cause insulin resistance is that of glutamine. Although this amino acid is a potent stimulator of glycogen synthesis, it is also a substrate for the hexosamine pathway which has been shown to be involved in insulin resistance [109].

Although these findings suggest that amino acids decrease insulin sensitivity with regard to glucose consumption, there are also contrasting reports indicating that amino acids increase insulin sensitivity. For example, infusion of glutamine during a 5-h euglycaemic-hyperinsulinemic clamp significantly stimulated hepatic and muscle glucose consumption [110]. Oral supplementation of amino acids to type 2 diabetic patients for 2 months significantly decreased postprandial plasma glucose without changing insulin levels [111]. Leucine increased glucose transport in isolated rat skeletal muscle by a PI 3-kinase dependent, rapamycin-independent mechanism [112].

On the basis of these, apparently conflicting, findings it is difficult to draw definite conclusions. Clearly, more research needs to be performed before the question can be answered whether or not amino acids and, in relation to this, the protein content of the diet do affect insulin sensitivity in vivo, and in what direction. In this context, it is important to stress that an increase in protein content of the diet does not necessarily imply increased plasma amino acid concentrations because induction of amino acid catabolism may result in decreased rather than increased steady-state intracellular and plasma amino acid concentrations [113]. It must also be pointed out that a high concentration of leucine in plasma, i.e. in the mM range, may have deleterious effects in the brain because it decreases brain concentrations of several essential amino acids, including phenylalanine, as was found in patients with maple syrup urine disease. This compromises synthesis of protein and of neurotransmitters in the brain and may underlie the neurological dysfunction in patients with this disorder [114].

The importance of amino acid signalling in the coordination of whole-body metabolism is also indicated by its involvement in the regulation of leptin production by adipocytes [115]. This mechanism may account for the known decreased food uptake in animals fed diets containing an excess of leucine [106]. Adipogenesis itself is linked to amino acid signalling because of mTOR-mediated phosphorylation of lipin [116], an adipocyte-derived protein which is required for
adipocyte development. Mutations in its gene result in failure to develop normal adipose tissue, in hyperlipidemia, and in glucose intolerance.

2.3 Amino acid signalling as a function of age

In both liver and muscle cells, rapamycin-sensitive p70S6 kinase phosphorylation and activation decreased with age [117,118]. In muscle, this was caused by a decrease in the affinity for leucine, and not by a decrease in p70S6 kinase protein [118]. It is thought that this decrease in signalling contributes to the net protein loss with age. Although protein synthesis declines with age, proteasome-catalysed proteolysis [119] and autophagic proteolysis [120,121] also decrease. The two proteolytic mechanisms can be considered to be anti-aging repair mechanisms because they remove aberrant proteins and defective cell structures (see chapter 1). Significantly, caloric restriction not only increases proteasome-catalysed proteolysis [119] but also increases autophagy, a phenomenon which may contribute to increased longevity [120]. The increase in autophagy by caloric restriction may be related to decreased plasma insulin concentrations [121]. Autophagy as a lifespan-extending mechanism is apparently well conserved in evolution because it is also found in C. elegans [122]. Interestingly, extended lifespan was also observed in fat-tissue specific insulin receptor knock-out mice which are defective in insulin signalling [123]. These mice have reduced fat mass at all ages but normal food intake. The increased longevity was tentatively ascribed to decreased generation of oxygen free radicals. The possibility that autophagy might have increased was not considered. By extrapolation, it may be speculated that development of insulin resistance in elderly people is an adaptive mechanism to increase autophagy, which helps to increase the capacity to remove damaged cellular structures (e.g. mitochondria [124]), and thus helps to prolong life.

2.4 Amino acid signalling and cancer

The PI 3-kinase/PKB/mTOR-signalling pathway is frequently overactivated in cancer [125,126]. For example, the catalytic subunit of Class I PI 3-kinase is overexpressed in ovarian cancers [127]; mammary carcinogenesis is enhanced in certain mouse models by constitutively active PKB [128]; PKB genes are amplified or overexpressed in a number of human cancers, including gastric, ovarian, breast, pancreatic, and prostate cancer [125]. The tumor suppressor PTEN which downregulates the PI 3-kinase/PKB pathway by dephosphorylation of the 3-phosphate from PtdIns(3,4,5)P3 [129] is mutated in a wide variety of cancers [130]. Although mutations in mTOR and p70S6 kinase have not been identified yet, it is most likely that they are important in oncogenesis. The involvement of mTOR in oncogenesis, mediated by PI 3-kinase/PKB signalling, was shown by a study in chicken embryo fibroblast cultures where rapamycin could inhibit oncogenic transformation induced by constitutively active PI 3-kinase or PKB [131]. In contrast, oncogenic transformation induced by 11 other oncoproteins was not inhibited by rapamycin. With the help of microarray analysis, the involvement of p70S6 kinase in
human cancer was suggested by the frequent up-regulation of mRNAs for ribosomal proteins in expression profiles from diverse tumors [132] and by the amplification and overexpression of p70S6 kinase in breast cancers [133].

Thus, the importance of amino acid signalling in cancer is evident. Although in the past the role of increased protein synthesis in oncogenesis has always been emphasised, the contribution of suppressed (autophagic) proteolysis cannot be ignored [51,134] because, as we have seen, the same signalling pathway controls protein synthesis and autophagic protein degradation. As discussed in chapter 1, the importance of Class I PI 3-kinase in the control of autophagy was shown in HT-29 cells by feeding synthetic lipids or by IL-13 activation of the PI 3-kinase pathway [18]. As described in chapter 2, overexpression of wild type PTEN, but not a phosphoinositide phosphatase-deficient mutant of PTEN, counteracted the down regulation of autophagy in HT-29 cells by IL-13, suggesting that PTEN counteracts the inhibition of Class I PI 3-kinase on autophagy. In addition, a dominant negative mutant of PKB resulted in increased autophagy and a constitutively active form of PKB decreased autophagy, suggesting a critical role for the PKB oncogene in the negative regulation of autophagy. It can now be speculated that genetic disruption of autophagy control can stimulate oncogenesis. This is supported by observations that Beclin1, the mammalian homologue of yeast Apg6 (see chapter 1), could stimulate autophagy in MCF7 breast carcinoma cells and inhibit tumorigenicity [135]. The beclin 1 gene was mono-allelically deleted in 40-75% of breast and ovarian cancers [136], suggesting that it acts as a tumor suppressor. In MCF7 cells the anti-breast cancer drug tamoxifen induced Beclin 1 expression and autophagy [137,138]. Further experiments performed with MCF7 cells, in which the levels of ceramide were manipulated by pharmacological means, suggested that the stimulatory effect of tamoxifen on Beclin 1 and autophagy was dependent on ceramide [138]. Ceramide is a sphingolipid signalling molecule with an essential role in cell proliferation, differentiation and cell death [139,140]; these are situations where autophagy may be important [141]. In HT-29 cells ceramide stimulated autophagy probably by downregulation of the PI 3-kinase/PKB pathway and by increasing Beclin 1 [138]. Interestingly, in human colon cancer the levels of ceramide are lower than those of normal colon mucosa [142]. Beclin1 can be found in a complex with class III PI 3-kinase and Beclin 1-associated PI 3-kinase activity increased under conditions of amino acid deprivation that simultaneously induced autophagy [17]. All these data together suggest that downregulation of autophagy may contribute to development and/or progression of cancer. To combat cancer a good approach may be to stimulate autophagy by inhibition of the PI 3-kinase/PKB/mTOR pathway or by upregulation of autophagy genes like beclin1. At this moment two rapamycin ester analogues, CCI-779 and RAD001, are undergoing phase I and phases II/III clinical trials in patients with renal cell carcinoma, prostate cancer, breast cancer and pancreatic cancer [143].
3. MECHANISMS

From all these studies the picture emerges that amino acids somehow directly activate mTOR activity. Whether PI 3-kinase is necessary for this activation is not known; however, for stimulation of phosphorylation of downstream targets of mTOR (e.g. p70S6 kinase), PI 3-kinase activity is required. Very recent studies have shed further light on the mechanism by which mTOR mediated signalling may be controlled. mTOR-dependent signalling in normal cells appears to be restrained by the tumour suppressor proteins TSC1 (hamartin) and TSC2 (tuberin) (cf. also Discussion of chapter 5). Mutations in TSC1 and or TSC2 result in tuberous sclerosis complex (TSC), with development of benign tumours in various organs [144]. The loss of function of these proteins results in constitutive activation of p70S6 kinase/4E-BP1 [145-147]. There is debate as to whether TSC1/2 inhibit mTOR [145] or whether they directly affect the activity of p70S6 kinase/4E-BP1 [146]. Interestingly, in normal cells, activation of PI 3-kinase suppressed the function of TSC1/2 [146] and PKB-mediated phosphorylation of TSC2 inhibited TSC2 activity [145,146]. This mechanism is in agreement with the notion, discussed in section 1.8, that PI 3-kinase and amino acid-dependent signalling are, indeed, on parallel pathways leading to activation of mTOR and/or of mTOR downstream targets. It also nicely explains why amino acid-dependent signalling is sensitive to inhibition by PI 3-kinase inhibitors (Fig. 1). Moreover, it explains, at least in part, the synergy between amino acids and insulin.

Studies with HEK293 cells showed that phosphorylation of TSC2 by AMPK stimulated TSC2 activity to inhibit p70S6 kinase and 4E-BP1 phosphorylation [148]. AMPK was also involved in direct phosphorylation (and inhibition) of mTOR on Thr2446 (see section 1.9). This suggests that the energy level of the cell is sensed by mTOR via AMPK; in this way, important pathways such as protein synthesis and degradation are controlled. AMPK may not only act as an energy sensor but also as an amino acid sensor because AMPK activity was influenced by chloride, the concentration of which falls in response to amino acid-induced cell swelling (chapter 6). In this view, the fall in intracellular chloride levels following amino acid-induced cell swelling keeps the activity of AMPK low (chapter 6), perhaps favours phosphorylation of mTOR on Ser2488 instead of Thr2446 and releases mTOR from inhibition by TSC2; the final result is then stimulation of mTOR activity (cf. Fig. 1).

The direct target of TSC1/2 is probably not mTOR itself but rather Rheb, a GTP-binding protein of the Ras family which is downstream of TSC1/2 and upstream of mTOR and which, like mTOR, is required for cell growth [149].

Raptor is another protein that is associated with mTOR and functions as a scaffold for the mTOR-catalysed phosphorylation of its substrates 4E-BP1 and p70S6 kinase ([126], for review). The recently discovered protein GßL may also be part of this protein complex [150]. Whether or not amino acids directly affect the activities of TSC1/2, Rheb, raptor, GßL or mTOR itself, is not known. Direct activation of mTOR kinase activity by amino acids in vitro is considered unlikely [37,126]. Because changes in cell volume can affect molecular crowding [104],

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an attractive possibility would be that amino acid-induced cell swelling loosens the association of Rheb and TSC1/2 so that mTOR becomes activated. This would account for the synergy between cell swelling and activation of mTOR-dependent signalling by leucine.

The question still to be answered is the mechanism by which leucine, which does not affect cell volume, can activate mTOR. Apart from the possibility that it may stimulate a protein kinase acting on mTOR as substrate, an attractive mechanism is that it inhibits a protein phosphatase as discussed in section 1.9. A possible mechanism would be one in which, in analogy with yeast, mTOR in mammalian cells would be a direct substrate for PP2A, although this remains to be proven [65]. Association of PP2A with mTOR downstream targets would also be possible (cf. Fig. 1).

Whatever the nature of the protein(s) that are activated (protein kinases, raptor) or inhibited (protein phosphatases, TSC1-2) by amino acids, the amino acids can do so by direct, allosteric effect on these proteins, or as discussed above, by altering the degree of association of these proteins. However, it is also possible that the plasma membrane contains a specific amino acid receptor. The existence of an amino acid receptor was proposed on the basis of the specific binding to the hepatocyte plasma membrane of Leu₄-Map, a small cell-impermeant globular peptide with 8 leucine residues on the outside of the molecule. Moreover, the peptide effectively inhibited autophagy and replacement of the leucine residues by isoleucine rendered the peptide inactive [151]. However, Leu₄-Map did not affect amino acid signalling [33,152,153]. A disturbing factor in these studies may be its effect on autophagy due to the degradation of the peptide to free leucine [152]. Additional evidence supporting the existence of a plasma membrane amino acid receptor has been provided by a recent report showing that the rate of protein synthesis in skeletal muscle in man in vivo responds to changes in the extracellular, but not intracellular, concentration of amino acids [154]. An amino acid sensor in the plasma membrane would be in line with a similar, leucine-sensitive, sensor molecule, Ssy1p, which is part of the three-component SPS amino acid sensor complex in the yeast plasma membrane [155].

Evidence in support of an intracellular amino acid receptor came from studies with Xenopus oocytes. In these cells, leucine stimulated signalling only after the cell membrane had been made permeable to leucine by transfection with a leucine transporter [156]. This suggests that, at least in these cells, the amino acid sensor is intracellular rather than extracellular. In CHO cells, inhibition of protein synthesis increased mTOR signalling in amino acid-deprived cells [157]. It was concluded that, apparently, intracellular amino acids (obtained from proteolysis) rather than extracellular amino acids stimulate signalling and that the amino acid sensor must be intracellular rather than extracellular [157]. However, the possibility that proteolytically derived amino acids were transported to the extracellular fluid was not considered.

A combination of both intra- and extracellular sensing of amino acids would be in line with the situation in yeast. This organism senses intracellular amino acids through GCN2 while the extracellular amino acid concentration is sensed by the SPS complex [155]. Clearly, more experiments need to be performed before the issue of the existence of an intra- and/or extracellular amino acid receptor in mammalian cells is settled.
An, as yet, hypothetical mechanism is one in which the cell responds to changes in the charging of tRNAs. This hypothesis is based on data in yeast (see section 1.4) showing that upon amino acid starvation free, uncharged, tRNA strongly binds to the protein kinase GCN2, which then becomes activated and phosphorylates eIF2α. Whether or not free tRNA, indeed, controls amino acid signalling in mammalian cells is controversial. For instance, in a study with T-lymphoblastoid Jurkat cells, inhibition of amino acid-tRNA synthetase with amino alcohols did indeed prevent amino acid-induced activation of p70S6 kinase [27] and this was confirmed in freshly isolated hepatocytes (chapter 6). However, this could not be confirmed in studies with freshly isolated rat adipocytes [33] or with CHO cells [83]. In HEK-293 cells, amino acid deprivation did not affect aa-tRNA levels [77], which suggested that intracellular amino acid pools rather than the degree of aa-tRNA charging control amino acid signalling. Although these differences in results may be ascribed to difference in experimental systems, it is not likely that the amino acid-sensing mechanism would be cell type-dependent, especially because of the similarity in amino acid specificity of amino acid signalling in the various cell types. Again, here too further studies are clearly required to resolve this issue.

However, if, indeed tRNA is always fully charged with amino acids [77], the conclusion must be that protein synthesis is never substrate-limited, even under amino acid-deprived conditions, and that the rate of protein synthesis is determined by the amino acid concentration-dependence of amino acid signalling only. If, on the other hand, tRNA acts as an amino sensor (like in yeast) and tRNA charging determines amino acid-dependent signalling, a possible mechanism underlying the ability of cell swelling to potentiate this process can be provided in addition to the one proposed above. For instance, during regulatory volume decrease when intracellular chloride falls, amino acid-tRNA synthetases may become activated because chloride ions inhibit these enzymes, in analogy with the situation in certain bacteria (cf. Fig. 1) [152] (see also chapter 6).

Recently, it was shown that, in addition to amino acids, the mitogenic second messenger phosphatidylic acid was also able to activate mTOR-mediated signalling, but only when amino acids were present in sufficient amounts [158]. This indicates that phosphatidylic acid governs signalling in parallel to amino acids [159], similar to PI 3-kinase and amino acids (cf. Fig. 1). Whether amino acids are able to affect phosphatidylic acid concentrations (or vice versa) is not known.

Inorganic polyphosphate (polyP), linear polymers of hundreds of Pi residues which are present in all cell types, has been proposed as another component that may be involved in amino acid-dependent and mTOR-mediated signalling [160]. At physiological concentrations, this compound greatly stimulated mTOR kinase activity in vitro and entirely mimicked the stimulatory effect of adding amino acids plus insulin on mTOR kinase activity in intact cells. Moreover, expression of a yeast exopolyphosphatase gene in a human breast carcinoma cell line inhibited the ability of insulin and amino acids to stimulate mTOR-mediated signalling with no effect on insulin-stimulated PKB phosphorylation, and inhibited cell growth [160]. Whether autophagy was stimulated under these conditions was not investigated.
By contrast, in *E. coli*, polyP appears to inhibit protein synthesis and to promote protein degradation. In these cells, the level of polyP increased after amino acid deprivation, because of guanosinetetraphosphate (ppGpp)-mediated inhibition of exopolyporphatase, and polyP stimulated ribosomal protein degradation by binding to the ATP-dependent Lon protease. In this way the generation of amino acids for cell survival under these conditions is ensured [161].

An intriguing possibility that has not been mentioned in the literature so far is that diadenosine polyphosphates (ApnA) are involved in mTOR stimulation. Indirect evidence comes from two independent studies: in one study, leucine stimulated Ap4A production by the mitochondria in pancreatic β-cells [162], while in another study leucine stimulated mTOR activity through increased mitochondrial oxidative metabolism [34]. Because mTOR may be associated, at least in part, with mitochondria [82], it is tempting to speculate that Ap4A is a signal that connects mitochondrial metabolism to mTOR activity. In this context, it is important to note that Ap4A is a strong inhibitor of AMP-activated protein kinase [163]. As discussed before, inhibition of AMP-activated protein kinase stimulates mTOR-dependent signalling.

Previously it was postulated by Blommaart *et al.* [9] that amino acid signalling, ultimately leading to S6 phosphorylation, provides an efficient mechanism by which both autophagic protein degradation and protein synthesis could be oppositely controlled (cf. Introduction of this chapter). A mechanism by which S6 phosphorylation may contribute to the reciprocal control of protein synthesis and degradation was also proposed: S6 phosphorylation may promote binding of ribosomes to the endoplasmic reticulum (ER) and enhance ER-linked protein synthesis [9]. In this context, it is of importance that in hepatocytes, synthesis of export protein, but not of housekeeping protein, declines after amino acid deprivation [164]. Ribosome binding to the ER would reduce the availability of ribosome-free regions of the ER which are the source of the autophagosomal membrane [6] (cf. also section 1.5). Thus, a common mechanism would stimulate ER-linked protein synthesis while at the same time inhibiting proteolysis. Removal of ribosomes by autophagy [165] is thus prevented. I still think such a mechanism is possible, at least in hepatocytes (see section 1.5). As discussed before, activation of PI 3-kinase class I also simultaneously stimulates protein synthesis and inhibits autophagic protein degradation. This provides a second mechanism for the opposite control of protein synthesis and degradation.

4. Final remark

As discussed in this final chapter there is now overwhelming evidence that amino acids are important as signalling molecules, with insulin-like actions with regard to protein synthesis and (autophagic) protein degradation. The ability of amino acids to stimulate signalling in β-cells and insulin production further adds to their protein-anabolic properties. It is fascinating that the same signalling pathway appears to control both protein synthesis and degradation.

In amino acid-dependent signalling, mTOR occupies a central role both as a sensor of intracellular amino acid concentrations and, via AMPkinase, as a sensor of the cellular energy
state. It may be speculated that mTOR also senses amino acid-induced increases in cell volume or in connection with this, perhaps the intracellular chloride concentration. Amino acid induced cell swelling perhaps affects the association between mTOR, raptor, TSC1/2 and Rheb, and that between Beclin1 and PI 3-kinase class III. The nature of the protein(s) involved in the autophagic machinery that is (are) the target for phosphorylation by mTOR (or downstream kinases) is also as yet unknown.

A summary of the possible interactions of amino acids with signalling and autophagy is given in Fig. 1.

A role of mTOR in the control of autophagic protein degradation, first indicated by experiments in our laboratory [9], is now generally accepted, as indicated by a number of recent reviews [166-170]. The importance of (amino acid-dependent) mTOR-mediated signalling in cancer becomes more and more evident [37,125,126]. Interventions used to combat cancer growth that interfere with amino acid-dependent signalling, such as the treatment with rapamycin and rapamycin analogues [171], not only inhibit protein synthesis but also at the same time accelerate autophagic protein degradation, and thus act as a two-edged sword.
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