Interplay between insulin and nutrients in the regulation of translation factors
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Abstract
Protein synthesis in mammalian cells is regulated through alterations in the states of phosphorylation of eukaryotic initiation factors and elongation factors (eIFs and eEFs respectively) and of other regulatory proteins. This modulates their activities or their abilities to interact with one another. Insulin activates several of these proteins including the following: the guanine-nucleotide exchange factor eIF2B; the eIF4F complex, which (through eIF4E) interacts with the cap of the mRNA; p70 S6 kinase; and elongation factor eEF2, which mediates the translocation step of elongation. Control of the last three of these is linked to mTOR (mammalian target of rapamycin). In Chinese hamster ovary cells, regulation

References

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of all these proteins by insulin is modulated by the presence of amino acids and/or glucose in the medium. For example, p70 S6 kinase activity declines in the absence of amino acids and cannot be stimulated by insulin under this condition. The readdition of amino acids, especially leucine, restores activity and sensitivity to insulin. With eIF2B and eEF2, both amino acids and glucose must be provided for insulin to regulate their activities. In contrast, insulin-stimulation of the formation of eIF4F complexes requires glucose but not amino acids. Glucose metabolism is required for this permissive effect. Our recent studies have also identified the mechanism by which mTOR signalling regulates the phosphorylation of eEF2. eEF2 kinase is phosphorylated by p70 S6 kinase at Ser-366; this results in the inactivation of eEF2 kinase, especially at low (micromolar) Ca++ concentrations.

Introduction
The control of mRNA translation in mammalian cells involves changes in the states of phosphorylation and function of multiple components of the translational machinery. These components include initiation and elongation factors (eIFs and eEFs respectively) and ribosomal proteins. Protein synthesis is activated by hormones such as insulin and by growth factors and is inhibited under stressful conditions. Its stimulation by insulin has been the subject of considerable investigation and several steps in the process of mRNA translation are now known to be regulated by this hormone [1]. More recently it has become clear that the ability of insulin to modulate the activities and/or states of phosphorylation of these proteins is affected by the nutrient status of the cell, for example the availability of glucose or amino acids [2]. Here we summarize the current understanding of the impact of nutrient conditions on the control of translation, focusing on the interplay between nutrients and the regulation of these proteins by insulin, an area in which this group has been active. A excellent short review of this subject was published recently [2].

Several regulatory components of the translational machinery are controlled through the rapamycin-sensitive mTOR (mammalian target of rapamycin) signalling pathway. mTOR is a large multidomain protein of 290 kDa including a region that shows similarity to lipid kinases of the phosphoinositide kinase family [3]. Rapamycin [when bound to FKBP-12 (FK506-binding protein)] inhibits the activity of mTOR, so the sensitivity of a given event to rapamycin indicates a role for mTOR. Three regulatory steps in mRNA translation are known to depend on mTOR. First, the activation of the 70 kDa protein kinase that phosphorylates ribosomal protein S6 (p70 S6 kinase) is blocked by rapamycin. S6 phosphorylation is believed to be important in up-regulation (e.g. by insulin or serum) of the translation of the set of mRNA species termed 5'-TOP (tract of pyrimidine) mRNA species, which encode ribosomal proteins and elongation factors. Up-regulation of their translation, which is also blocked by rapamycin, thus offers a mechanism by which the synthesis of these components of the translational machinery can be quickly increased in response to agents that turn on protein synthesis [4,5].

Secondly, the phosphorylation of the eIF 4E-binding proteins 1 and 2 (4E-BP1 and 2) is also blocked by rapamycin (reviewed in [6,7]). By interacting with eIF4E, these proteins block its ability to interact with the scaffolding protein eIF4G and form eIF4F complexes competent to bind 40 S subunits and participate in scanning to locate the start codon on the mRNA. eIF4E has a key role here because it binds to the 5' cap (7-methylguanosine) of the mRNA and thus recruits eIF4F to the 5' end of the mRNA. 4E-BP1 undergoes phosphorylation in response, for example, to insulin at multiple sites [6,7]. Evidence has been provided that at least some of these sites are direct substrates for mTOR's protein kinase activity [8,9].

Thirdly, the phosphorylation of eEF2 is under the control of signalling via mTOR [10,11]. Phosphorylation of eEF2 (at Thr-56) inhibits its activity. Insulin induces the dephosphorylation of this site and this involves a decreased activity of eEF2 kinase [10-12], a Ca++/calmodulin-dependent enzyme that does not belong to the main protein kinase superfamily [13]. Both the inactivation of eEF2 kinase and the dephosphorylation of eEF2 are blocked by rapamycin [10,14], demonstrating that these effects require mTOR.

Insulin also activates the guanine-nucleotide exchange factor eIF2B, which is important in the overall control of translation initiation. This seems to involve a decreased phosphorylation of eIF2B's catalytic (ε) subunit at an inhibitory phosphorylation site that is a target for glycogen synthase kinase 3 (GSK3) [15]. Insulin inactivates GSK3 via signalling through phosphoinositide 3-kinase and protein kinase B (PKB); activation of eIF2B
Nutrient withdrawal does not affect the ability of insulin to activate PKB or inactivate GSK3 but prevents the activation of eIF2B

CHO cells were transferred to the indicated medium for 60 min and then treated with insulin (10 nM) for 15 min before the extraction and assay of the indicated components. Further details can be found in [17], from where these data were taken. Numbers in parentheses indicate the numbers of independent experiments performed. Results are means±S.E.M. Abbreviation: n.d., not determined.

<table>
<thead>
<tr>
<th>Condition</th>
<th>PKB activity (% of control)</th>
<th>GSK3 activity (% of control)</th>
<th>eIF2B activity (% of control)</th>
<th>p70 S6 kinase activity (% of control)</th>
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<tr>
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<td>100 (3)</td>
<td>100 (7)</td>
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<tr>
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<td>100 (3)</td>
<td>94±28 (7)</td>
<td>33±8 (8)</td>
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<td>38±3 (3)</td>
<td>88±27 (7)</td>
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<td>46±2 (3)</td>
<td>261±22 (6)</td>
<td>243±42 (5)</td>
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Regulation of 4E-BP1 and p70 S6 kinase by insulin

When CHO cells are shifted into medium lacking amino acids, 4E-BP1 rapidly undergoes dephosphorylation, as manifested by its altered mobility on SDS/PAGE: the dephosphorylated form(s) run faster than the more phosphorylated ones [18]. As expected, this net dephosphorylation of 4E-BP1 results in its increased binding to eIF4E and loss of eIF4F complexes [less eIF4G was associated with eIF4E in amino-acid-starved cells (Figure 1A)].

This suggested that amino acids themselves exert an effect, directly or indirectly, on the signalling events that modulate the phosphorylation of 4E-BP1. Consistent with this, readdition of a mixture of amino acids led to increased phosphorylation of 4E-BP1, its dissociation from eIF4E and increased levels of eIF4F complexes (Figure 1A). The formation of eIF4F complexes was not increased further by insulin, showing that amino acids themselves suffice to drive the formation of these complexes in CHO cells. No single amino acid alone was able to elicit this effect at the concentration found in the mixture, although high concentrations of leucine did bring about a partial phosphorylation of 4E-
This permissive effect of leucine has also been reported by others (reviewed in [2]). It is important to note that the administration of leucine increases protein synthesis and eIF4F in muscle in vivo [19] and that amino acids can themselves promote muscle protein synthesis in the absence of insulin [20].

Because the phosphorylation of 4E-BP1 depends on mTOR, it was possible that the effects of amino acids on 4E-BP1 phosphorylation were mediated through modulation of the mTOR pathway. To study this, we examined the effects of amino acid withdrawal on the phosphorylation of another target of mTOR signalling, p70 S6 kinase. Withdrawal of amino acids caused the inactivation of p70 S6 kinase [17]. These studies also revealed that amino acids are required for the regulation of p70 S6 kinase by insulin: in control cells, maintained in normal medium, insulin activated p70 S6 kinase approx. 2-fold in CHO cells (Table 1). However, insulin was unable to increase p70 S6 kinase activity significantly above basal in cells deprived of amino acids [17] (Table 1). Insulin can stimulate p70 S6 kinase (to differing extents) in cells given the amino acid mixture or leucine [17].

These results show that amino acids exert a dual effect on the control of p70 S6 kinase: maintaining its basal activity and also acting to permit its activation by insulin. Analogous results were also reported by others [21–23]. For 4E-BP1 the situation is different, as described above. Amino acids alone seem to suffice for the full formation of eIF4F complexes in these cells but not for the full activation of p70 S6 kinase, which also requires an additional input, for example insulin.

**Figure 1**

Modulation of 4E-BP1/eIF4F by nutrients

(A) CHO. K1 cells were maintained in medium (control; C; Ham's F12) or transferred to amino acid-free medium (containing 5 mM glucose) for the durations indicated. In some cases, after 45 min without amino acids, amino acids were then added back for the indicated durations. All plates were extracted as described in [17,18] and extracts were subjected to chromatography on m7GTP-Sepharose before analysis of the bound proteins by SDS/PAGE and immunoblotting with antisera against eIF4G, eIF4E or 4E-BP1, whose positions are indicated. eIF4F is a complex containing eIF4E, eIF4G and certain other proteins. (B) CHO. K1 cells were kept in medium (Ham's F12, control) or transferred to D-PBS (with 5 mM D-glucose or a mixture of amino acids [18] where indicated). After 1 h, cells were in some cases (+) treated with 20 nM insulin for 15 min. Cells were extracted and lysates were processed as above.

Glucose also modulates the responsiveness of 4E-BP1 phosphorylation to insulin

Glucose also alters the regulation of 4E-BP1 by insulin. In cells deprived of glucose and amino acids, insulin is unable to induce any detectable change in the mobility of 4E-BP1. The use of phosphospecific antisera revealed that under this condition, insulin could bring about the phosphorylation of Thr-69 but not Thr-36/45 or Ser-64 (J. V. Patel, X. Wang and C. G. Proud, unpublished work). In contrast, for cells in medium containing D-glucose (5 mM), insulin brought about a substantial shift of 4E-BP1 into the most highly phosphorylated γ form. Analysis with the use of the phosphospecific antisera revealed that this was associated with increased phosphorylation of Thr-36/45 and Ser-64. Thus D-glucose exerts a permissive effect with regard to insulin-induced phosphorylation of 4E-BP1. Glucose analogues that can be transported but not fully metabolized did not support insulin-induced phosphorylation of 4E-BP1 (J. V. Patel, X. Wang and C. G. Proud, unpublished work). However, two other hexoses, D-mannose and D-glucosamine, did exert a similar permissive effect to that of insulin. Thus it seems that hexose metabolism is necessary for this effect, which is not specific for glucose. One possibility is that a metabolite of glucose provides this ‘signal’; it is not clear at this point whether this acts allosterically to modulate a component of the signalling pathways regulating mTOR and/or 4E-BP1 phosphorylation. This picture differs from the situation for p70 S6 kinase, in which glucose
Insulin Action

alone did not act permissively with respect to the activation of p70 S6 kinase by insulin [17].

**Phosphorylation of eEF2 is also modulated by nutrients**

The ability of insulin to bring about the dephosphorylation of eEF2 is blocked by rapamycin [10]. In experiments similar to those described above for eIF2B and 4E-BP1, both glucose and a mixture of amino acids were required as components of the medium for insulin to bring about significant dephosphorylation of eEF2 [17]. This strongly supports the emerging picture that amino acids regulate mTOR-coupled signalling pathways in mammalian cells.

Recent work in this laboratory has revealed new insights into the regulation of eEF2. First, these studies have delineated a mechanism by which insulin, through mTOR, brings about the inactivation of eEF2 kinase (eEF2k) and the dephosphorylation of eEF2. eEF2k is efficiently phosphorylated by p70 S6 kinase 1 (and a newly discovered isoform, S6 kinase 2). This occurs at a single, conserved serine residue (Ser-366 of human eEF2k) and results in the inactivation of eEF2k especially at low (1–5 μM) Ca²⁺ concentrations. Mutation of Ser-366 to Ala prevents phosphorylation, whereas mutation to Glu results in an enzyme with a low basal activity at low [Ca²⁺], indicating that it mimics the effect of phosphorylation. This identifies eEF2k as a new substrate for p70 S6 kinase and explains how insulin activates peptide chain elongation (Scheme 1).

Recent work in this laboratory has shown that treatment of CHO cells with 5-amino-4-imidazolecarboxamide riboside (AICAriboside) causes a substantial increase in the level of phosphorylation of eEF2. After entry into the cell, AICA-riboside is converted to an analogue of 5'AMP that activates the AMP-activated protein kinase (AMP-k). This enzyme, whose counterparts have key roles in nutrient regulation in yeast and plants, phosphorylates and regulates several enzymes and other proteins to switch off energy-consuming processes and activate energy-generating ones [24]. Thus, when ATP levels are low (and AMP is high), for example after glucose deprivation, the cell can cut down on energy expenditure and turn on ATP production. Because protein synthesis is the second most energy-consuming process in the cell, it would be important to decrease the rate of mRNA translation to save ATP for the most essential processes. Our results suggest that activation of AMP-k leads to inhibition of peptide-chain elongation, which would have exactly this desirable effect. Preliminary results show that AMP-k phosphorylates eEF2k at two or more sites; studies are under way to identify them and to study their roles in regulating eEF2k activity. It is possible that the permissive effect of glucose on the ability of insulin to bring about the dephosphorylation of eEF2 is connected to the regulation of eEF2k by AMP-k.

**Summary and perspectives**

It is barely 3 years since the appearance of the first reports of regulation of p70 S6 kinase and other targets of mTOR in mammalian cells. It is now clear that the control of several translation factors requires ‘inputs’ from nutrients (Scheme 2). Because mRNA translation consumes both energy and amino acids, it makes very good physiological

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**Scheme 1**

**Regulation of translation factors by nutrients and insulin**

The regulation of eEF2 phosphorylation involves the inactivation of eEF2 kinase by phosphorylation of this enzyme at Ser-366 by p70 S6 kinase. This contributes to the dephosphorylation (dephosph) of eEF2. p70 S6 kinase is activated by insulin through signalling mechanisms that involve mTOR (see Scheme 2) and require the presence of amino acids in the medium (in many cell types). This might reflect the regulation of mTOR function, by unknown mechanisms, in response to amino acid availability. Question marks indicate steps or links that are poorly understood.

- **Insulin**  
  - ??  
  - Amino acids  
  - ??  
  - mTOR  
  - p70 S6 kinase (on)  
  - eEF2 kinase (S366, less active)  
  - eEF2 (dephosph, activation)  
  - Accelerated translation elongation

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Scheme 2
Role of mTOR in the regulation of translation

The regulation of several proteins that control mRNA translation is linked to mTOR: 4E-BPI controls the formation of elf4F complexes, whereas the phosphorylation of S6 is thought to regulate the translation of the 5'-TOP (tract of pyrimidine) mRNA species in a positive manner. As shown in Scheme 1, the control of eEF2 is also mediated via mTOR and this affects global protein synthesis rather than the translation of specific mRNA species. Signalling to all three proteins is affected by amino acid supply, and for eEF2 and 4E-BPI, glucose acts permissively with regard to their control by insulin. The activity of elf2B is regulated through a different pathway involving the phosphorylation and inactivation of GSK3 by PKB, leading to the dephosphorylation and activation of elf2B. The activation of elf2B is also modulated by glucose and amino acids but this seems to require (as yet unknown) inputs distinct from the PKB/GSK3 pathway.

Glucose, Glucose Amino acids
Receptor, e.g., insulin

Glucose, Amino acids

I
??
??
??

II
??
??
??

PI 3-kinase
PKB
GSK-3 (off)

mTOR

4E-BPI
p70 S6k
elf4F complex

 elf2B

ACtivATIOn OF TRANSLATION OF SPECIFIC mRNAs

ACtivATIOn OF OVERALL mRNA TRANSLATION

'sense' that its activation should be dependent on both amino acids and a source of metabolic energy. However, the signalling/regulatory events involved in these processes remain obscure. The control of mTOR and its downstream signalling components are very poorly understood, and much future work will focus on these issues. Different regulatory inputs must be involved in the control of elf2B, because its activation is not blocked by rapamycin [16].

It is also not known how cells 'sense' nutrient levels. The requirement for metabolizable glucose analogues tends to suggest that glucose metabolism rather than a glucose receptor (such as yeast Rgt2 or Snf3 [25]) is involved. Yeast also possesses an amino acid sensor [26] and it is conceivable that such a system also operates in mammalian cells, especially given the rapidity of the response to amino acid withdrawal or addition (on the scale of a few minutes). However, some results suggest that amino acid metabolism might be involved; the picture is very far from clear [2,27].

Deviations from the sensing and signalling events involved in nutrient regulation in mammals is not only of fundamental importance for our understanding of mammalian cell physiology but also of substantial clinical significance in relation, for example, to human (mal)nutrition and other conditions.

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References
Abstract

Effects of insulin on the expression of liver-specific genes are part of the adaptive mechanisms aimed at maintaining energy homeostasis in mammals. When the diet is rich in carbohydrates, secreted insulin stimulates the expression of genes for enzymes involved in glucose utilization (glucokinase, L-type pyruvate kinase and lipogenic enzymes) and inhibits genes for enzymes involved in glucose production (phosphoenolpyruvate carboxykinase). The mechanisms by which insulin controls the expression of these genes have been poorly understood. Recently, the transcription factor sterol-regulatory-element-binding protein 1c has been proposed as a key mediator of insulin transcriptional effects. Here we review the evidence that has led to this proposal and the consequences for our understanding of insulin effects in physiological or pathological conditions.

Introduction

Glucose homeostasis is a major requirement for survival in mammals because this substrate is used continuously at a high rate by organs such as the liver, SREBP-1c, glucokinase, glucose homeostasis. Key words: liver, SREBP-1c, glucokinase, glucose homeostasis. Abbreviations used: ACC, acetyl-CoA carboxylase; FAS, fatty-acid synthase; GK, glucokinase; L-PK, L-type pyruvate kinase; MAP, mitogen-activated protein; PEPCK, phosphoenolpyruvate carboxykinase; PKB, protein kinase B; SRE, sterol response element; SREBP, sterol-regulatory-element-binding protein.

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Sterol-regulatory-element-binding protein 1c mediates insulin action on hepatic gene expression

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