Glucose exerts a permissive effect on the regulation of the initiation factor 4E binding protein 4E-BP1

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The eukaryotic initiation factor 4E (eIF4E) binding protein (4E-BP1) interacts directly with eIF4E and prevents it from forming initiation factor (eIF4F) complexes required for the initiation of cap-dependent mRNA translation. Insulin and other agents induce the phosphorylation of 4E-BP1 at multiple sites, resulting in its release from eIF4E, and this involves signalling through the mammalian target of rapamycin (mTOR). Here we show that D-glucose promotes the ability of insulin to bring about the phosphorylation of 4E-BP1 and the formation of eIF4F complexes. This appears to involve facilitation of the phosphorylation of at least three phosphorylation sites on 4E-BP1, i.e. Thr-36, Thr-45 and Thr-69. Non-metabolizable glucose analogues cannot substitute for D-glucose, but other hexoses can. This suggests

that a product of hexose metabolism mediates the permissive effect of glucose. The effect of glucose was concentration-dependent within the range 1–5 mM. In contrast with the situation for 4E-BP1, glucose does not allow full activation of the 70 kDa ribosomal protein S6 kinase (p70 S6k; another target of mTOR signalling) or phosphorylation, *in vivo*, of its substrate, ribosomal protein S6. Taken together with earlier data showing that amino acids regulate 4E-BP1 and p70 S6k, the present findings show that 4E-BP1 in particular is regulated in response to the availability of both amino acids and sugars.

Key words: mRNA translation, mTOR, protein synthesis, translation factor.

INTRODUCTION

Recent studies in a number of laboratories ([1–12]; reviewed in [13]) have revealed that, in mammalian cells, amino acids exert a marked effect upon the regulation of several proteins involved in the control of protein synthesis (mRNA translation), but no studies have so far examined the effect of another important class of nutrient, sugars, on the control of these proteins. This paper reports, for the first time, that hexoses also provide an important permissive input into their control in mammalian cells.

Amino acids, especially leucine, exert positive regulatory effects on several proteins involved in the initiation phase of mRNA translation. Most studies have focused on two such proteins, namely the eukaryotic initiation factor 4E (eIF4E) binding protein (4E-BP1) and the 70 kDa protein kinase that phosphorylates ribosomal protein S6 (p70 S6k). eIF4E binds the 5'-cap (7-methylguanosine) of eukaryotic mRNAs and also interacts with a scaffold protein, eIF4G, which brings together other translation-initiation factors to form the eIF4F complex, which is required for cap-dependent translation and probably plays a role in ribosomal scanning [14–16]. 4E-BP1 (also termed PHAS-I [17]) binds to eIF4E at a site that overlaps that for eIF4G, and binding of 4E-BP1 to eIF4E thus prevents the formation of functional eIF4F complexes. Stimuli such as insulin bring about the phosphorylation of 4E-BP1 at several sites, resulting in its release from eIF4E, permitting eIF4E to bind eIF4G (reviewed in [14,17]). The insulin-induced release of 4E-BP1 is blocked by treatment of cells with rapamycin, showing that it requires mammalian target of rapamycin (mTOR). mTOR itself is a large polypeptide that shows similarity to lipid kinases, but whose true function is still poorly understood [18,19].

p70 S6k is activated by insulin and growth factors through phosphorylation of a number of sites in the p70 S6k polypeptide. Activation of p70 S6k is dependent on mTOR, since it is blocked by a specific inhibitor of mTOR, rapamycin [20]. Mutation of one phosphorylation site in p70 S6k to an acidic residue (Thr-389 → Glu) confers resistance to rapamycin, suggesting that this site plays a key role in the control of p70 S6k by mTOR [21]. It is thought that activation of p70 S6k may be important for the activation of the subset of mRNAs encoding ribosomal proteins and elongation factors; indeed, growth factors or insulin elicit up-regulation of the translation of these mRNAs in a rapamycin-sensitive manner (reviewed in [22]). Members of this set of mRNAs possess a 5'-tract of pyrimidines and hence are known as 5'-TOP mRNAs.

As mentioned above, several investigators have shown that amino acids play an important role in the regulation of p70 S6k and 4E-BP1. Firstly, amino acids (especially the branched-chain amino acid leucine) bring about phosphorylation of p70 S6k and 4E-BP1 and, secondly, they exert a permissive effect with respect to insulin [9,10] or growth factors [8], which, in a number of cell types, can only elicit phosphorylation/activation of p70 S6k or phosphorylation of 4E-BP1 if amino acids are present.

Here we extend studies of the effects of nutrients on the regulation of these proteins to examine the influence of sugars upon their phosphorylation. We show that hexoses such as D-glucose exert a marked permissive effect on the regulation by insulin of the phosphorylation of 4E-BP1. This effect requires hexose metabolism. Glucose also exerts a permissive effect on the ability of insulin to promote phosphorylation of p70 S6k, but only to a limited extent, and this does not result in phosphorylation of S6 itself, suggesting that additional inputs are required here.

Abbreviations used: eIF, eukaryotic initiation factor; 4E-BP1, eIF4E binding protein 1; CHO, Chinese-hamster ovary; D-PBS, Dulbecco's PBS; GCN2, general control of amino acid biosynthesis, non-derepressing 2 (a protein kinase acting on eIF2 α); mTOR, mammalian target of rapamycin; PKB, protein kinase B; p70 S6k, the 70 kDa ribosomal protein S6 kinase; S6, ribosomal protein S6; 5'-TOP mRNA, mRNA possessing a 5'-tract of pyrimidines.

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EXPERIMENTAL

Chemicals and biochemicals

Unless indicated otherwise, all reagents were obtained from Sigma or Merck. The phosphospecific antisera against p70 S6k were obtained from New England Biolabs (now Cell Signaling Technology). Antisera against eIF2 α [23] and phosphorylated eIF2 α [24] have been described previously, except for anti-phospho-S6 (Ser-235) antibody, which was generously donated by Dr Dario Alessi, University of Dundee. It was raised against a peptide corresponding to the region of S6 around Ser-235 (peptide sequence KRRRLSpSLRASTs, where Sp represents phosphoserine), one of the major sites of phosphorylation in S6 [25]. The phosphospecific antisera against 4E-BP1 were kindly provided by Dr John C. Lawrence (University of Virginia, Charlottesville, VA, U.S.A.), and their characterization has been described in detail in recent publications from his laboratory [26,27]. The anti-PKB antibody was generously provided by Dr Dario Alessi [28], while a phosphospecific antibody for protein kinase B (PKB) phosphorylated on Ser-473 was purchased from New England Biolabs.

Maintenance of cells

Chinese-hamster ovary (CHO) cells (K1 strain) were maintained as described previously [29]. When cells were about 80% confluent, some were transferred to Dulbecco's PBS (D-PBS), while others were transferred to the same medium supplemented with 5.5 mM D-glucose or other additions as indicated in the text. In some cases, cells were treated with insulin (10 nM).

Gel electrophoresis and Western blotting

These procedures were performed as described previously [30] using Immobilon[®]-P membrane (Millipore). For the analysis of 4E-BP1, gels contained 13.5% (w/v) acrylamide and 0.36% methylenebisacrylamide. For analysis of other proteins, gels containing 0.1% methylenebisacrylamide and either 10% or 12.5% acrylamide (as indicated) were used.

Measurement of the rate of protein synthesis

This was performed as described previously [8], with the modifications described in the Figure legends.

RESULTS

Glucose acts permissively to promote the regulation of targets of mTOR signalling

We noted previously in our studies on the effects of nutrients on the assembly of the eIF4F complex that the presence of glucose allowed insulin to induce formation of this complex even in the absence of amino acids [10]. In order to study this further, we first examined the effects of glucose and insulin on the phosphorylation of 4E-BP1.

CHO.K1 cells were incubated in the absence of any nutrients (in D-PBS) or in D-PBS plus glucose (at 5.5 mM unless indicated otherwise) for various times. In some cases, insulin was then added for 20 min. In control cells, i.e. those in Ham's F12 medium, 4E-BP1 was present as the more phosphorylated β and γ species, with none of the least phosphorylated α form being detected. (It should be noted that, since 4E-BP1 can be phosphorylated at five or more sites, the three bands seen on SDS/PAGE probably each represent a mixture of differently phosphorylated species.) As reported previously [9], withdrawal of nutrients caused dephosphorylation of 4E-BP1, as manifested by a shift on

Figure 1 Glucose promotes the insulin-stimulated phosphorylation of 4E-BP1

(A) CHO.K1 cells were maintained in Ham's F12 medium (control), or transferred to D-PBS alone or to D-PBS supplemented with 5.5 mM D-glucose. After 100 min, some plates were treated with insulin (20 nM) for 20 min. Cells were then extracted. Samples were analysed by SDS/PAGE and Western blotting using an antibody that detects 4E-BP1 irrespective of its state of phosphorylation. The gel system used resolves three species of 4E-BP1 (α , β and γ), as indicated. Similar findings were obtained in 14 separate experiments. (B) As for (A), but using a 10% (w/v) acrylamide gel, and blots were probed with antibodies to PKB that detect either the α isoform irrespective of its state of phosphorylation (upper panel) or the α/β isoforms only when phosphorylated at Ser-473 (lower panel). (C) As for (A), but an antiserum against 4E-BP1 phosphorylated at Thr-69 (T69) was used. Only the β and γ forms of 4E-BP1 are shown. The position of the α isoform is also indicated for orientation. (D) As in (A), but an antiserum for 4E-BP1 phosphorylated at Thr-36/45 was used. Only the β and γ forms of 4E-BP1 are shown, since the α form does not appear on such immunoblots, presumably because it is not phosphorylated at any of these three sites. For (B)–(D), similar data were obtained in three separate experiments.

SDS/PAGE from the β and γ species towards the more rapidly migrating, hypophosphorylated, α form. In particular, the γ species was absent from cells deprived of nutrients. Insulin did not elicit a discernible change in the migration of 4E-BP1 in cells deprived of glucose and amino acids (Figure 1A). In cells supplemented with glucose, a slightly higher proportion of the total 4E-BP1 ran as the β species than in nutrient-deprived cells. More strikingly, in the presence of glucose, insulin was able to bring about phosphorylation of 4E-BP1, with the majority of the protein shifting into the γ form, almost to the extent seen in cells maintained in Ham's F12 medium (Figure 1A). In agreement with our earlier findings with CHO.T cells [10], we found that that glucose exerted a permissive effect on the formation of eIF4F complexes in response to insulin in CHO.K1 cells (results not shown). Glucose had no effect on the basal level of eIF4F complexes, again as noted previously [10].

It did not appear likely that the effects of nutrients on the regulation of 4E-BP1 were due to a gross defect in insulin signalling in nutrient-deprived cells, since we have shown previously that, in nutrient-starved cells, insulin is able to activate PKB to at least the same extent as it does in cells maintained in normal culture medium or in glucose [10]. To verify again that

Figure 2 Glucose does not modulate the phosphorylation of key regulatory sites in p70 S6k or S6

(A) CHO.K1 cells were treated as described for Figure 1. Samples were analysed by SDS/PAGE (10% acrylamide gel) and immunoblotting using an antibody specific for p70 S6k that detects the protein irrespective of its state of phosphorylation. Multiple arrowheads mark the different forms of p70 S6k resolved by this method, which differ in their states of phosphorylation (the least phosphorylated one migrating most rapidly). (B, C) As for (A), but immunoblots were performed using antibodies that recognize p70 S6k only when it is phosphorylated at Thr-389 (B) or Ser-421/424 (C). (D) As for (A), but samples were analysed on a 12.5% acrylamide gel and immunoblots were developed with an antibody against phosphorylated S6. Similar data were obtained in at least three separate experiments.

insulin signalling was not defective in cells deprived of glucose, we studied the phosphorylation of PKB using a phosphospecific antibody that detects the protein only when phosphorylated at the C-terminal site, Ser-473. As shown in Figure 1(B), insulin was able to induce phosphorylation of this site (which is required for activation of PKB [31]) to the same extent in nutrient-deprived cells as in control cells kept in Ham's F12 medium. This corroborates our earlier data for PKB activity and underlines the fact that insulin signalling to this pathway, which is considered to be important in the regulation of 4E-BP1 phosphorylation [32,33], is still functional in nutrient-deprived cells. The defect(s) underlying the reduced basal phosphorylation and insulin responsiveness of 4E-BP1 in nutrient-deprived cells must therefore be in signalling components other than, or downstream of, PKB.

To study further the basis of the permissive effect of glucose on the phosphorylation of 4E-BP1, we made use of recently developed antisera specific for phosphorylated forms of 4E-BP1 [26,27]. This analysis revealed that Thr-69 was phosphorylated to at least some extent under all conditions, although its phosphorylation was decreased in the unstimulated nutrient-starved cells (Figure 1C). Phosphorylation of this residue was increased by the addition of glucose or insulin to the nutrient-starved cells. As

Figure 3 Glucose facilitates the activation of protein synthesis by insulin

CHO.K1 cells were incubated in D-PBS alone or in D-PBS plus 5.5 mM D-glucose for 90 min, and insulin (Ins; 10 nM) and ^{35}S methionine were then added for a further 30 min before samples were analysed for incorporation of radiolabel into trichloroacetic acid-precipitable material. Data are means $\pm$ S.E.M., and were derived from four separate experiments. Within each experiment, samples were processed in duplicate.

sometimes two, but on other occasions only one, band is recognized by this antiserum, it is difficult to be certain whether insulin regulates Thr-69 phosphorylation in control cells or cells given glucose; the data do suggest that insulin may increase Thr-69 phosphorylation in the latter situation. The sites Thr-36 and Thr-45 clearly underwent marked dephosphorylation in the absence of nutrients (Figure 1D). Insulin could not restore their phosphorylation in the absence of glucose. Addition of glucose had no effect on the phosphorylation of these sites, but glucose did allow insulin to increase their phosphorylation to the levels seen in control cells. In contrast, insulin had no obvious effect upon the phosphorylation of these sites in the control cells. A very similar pattern was observed for Ser-64: it was phosphorylated in control cells in the presence or absence of insulin, underwent dephosphorylation when nutrients were withdrawn, was not phosphorylated in response to insulin in starved cells, but was modified after insulin treatment of cells given glucose (results not shown). These data suggest that glucose facilitates the insulin-induced phosphorylation of at least three sites in 4E-BP1 (Thr-36, Thr-45 and Ser-64), but does not itself exert an effect on these sites. In the case of Thr-69, insulin may have a modest positive effect on the phosphorylation of this site.

Glucose exerts limited modulatory effects on the control of phosphorylation of p70 S6k

We also examined a second target of mTOR signalling, p70 S6k [20]. Insulin induced phosphorylation of p70 S6k in control cells, as manifested by the widely observed shift in migration that accompanies the phosphorylation of p70 S6k, but had no effect in cells deprived of nutrients (Figure 2A). Under these latter conditions, p70 S6k ran as a single, rapidly migrating, major species on SDS/PAGE, which is characteristic of dephosphorylated forms of the enzyme. Glucose did allow insulin to induce a modest change in the phosphorylation state of p70 S6k, as assessed by the mobility-shift assay. To study this further, we also tested the phosphorylation state of Thr-389 and Ser-421/424, which are key phosphorylation sites for the regulation of p70 S6k [21,34], using appropriate phosphospecific antibodies. The same pattern of events was seen using both antisera: these sites were not significantly phosphorylated in control cells, but were phosphorylated after insulin treatment (Figures 2B and 2C). No significant signal was observed for samples from nutrient-deprived cells or cells given glucose, even in the presence of

insulin. These data agree with the above findings showing that the insulin-induced phosphorylation of p70 S6k was only partially facilitated by glucose: it appears that glucose does not permit the phosphorylation of sites thought to be of key importance for activation of p70 S6k.

To study this further, we made use of an antibody that detects S6 (an *in vivo* substrate of p70 S6k) when phosphorylated at Ser-235. Insulin promoted the phosphorylation of S6 in the presence of growth medium, consistent with the marked shift in migration of p70 S6k that accompanies its phosphorylation and activation, and with our earlier data showing that p70 S6k is activated under these conditions [10]. In contrast, no increase in S6 phosphorylation was seen after insulin treatment of cells maintained without nutrients or in the presence of glucose (Figure 2D). This agrees with the above data for the phosphorylation of Thr-389 and Ser-421/424, and with our earlier data on the activity of p70 S6k [10]. Taken together, these data clearly differentiate the regulation of 4E-BP1 from that of p70 S6k, since glucose permits full regulation by insulin of the former, but not of the latter.

Glucose facilitates the stimulation of protein synthesis by insulin

A key question is the extent to which glucose deprivation affects the rate of protein synthesis and its control in CHO cells. We therefore measured rates of protein synthesis by assaying the incorporation of [³⁵S]methionine into trichloroacetic acid-insoluble material. In cells deprived of glucose and amino acids, the basal rate of protein synthesis was low, and was unaffected by the addition of insulin (Figure 3). In contrast, cells maintained in medium containing glucose showed a much higher (8-fold) basal rate of protein synthesis, and this was markedly increased by insulin (Figure 3).

Sugar metabolism is required for the permissive effect

To gain some insight into the mechanism by which glucose exerts its permissive effect, we explored whether different glucose analogues could substitute for glucose in permitting insulin to bring about phosphorylation of 4E-BP1 (Figure 4A). The non-physiological stereoisomer L-glucose was unable to do so; this was expected, as this isomer can be neither transported nor metabolized. In contrast, the D-glucose analogues 2-deoxyglucose and 3-O-methylglucose can be transported, but cannot be metabolized fully. These compounds were also unable to exert a permissive effect with respect to the ability of insulin to stimulate the phosphorylation of 4E-BP1. This strongly suggests (i) that glucose transport itself does not form part of the mechanism by which glucose exerts its effect, and (ii) that metabolism of glucose beyond glucose 6-phosphate is required. Thus it seemed likely that a metabolite of glucose, or perhaps energy generated as a consequence of glucose metabolism, might be the basis of this effect.

In order to test the effect of glucose over a range of concentrations, CHO.K1 cells were incubated in D-PBS containing 1–5 mM D-glucose. In some cases, insulin was added for the final 15 min. The most sensitive and informative way of studying the regulation of 4E-BP1 is to examine its association with eIF4E, i.e. its function. This is the most physiologically meaningful assay, as release of 4E-BP1 will allow eIF4E to bind eIF4G, thus promoting assembly of initiation-factor complexes. Samples of cell lysate were therefore subjected to chromatography on m⁷GTP-Sepharose, a resin that binds eIF4E and, with it, any associated 4E-BP1 (m⁷ is 7-methyl). Samples of the bound material were analysed by SDS/PAGE and Western blotting using anti-4E-BP1 serum. As shown in Figure 4(C), very little

Figure 4 Effects of glucose analogues and other sugars on insulin-stimulated phosphorylation of 4E-BP1

(A, B) CHO.K1 cells were incubated under the indicated conditions (where control is serum-free Ham's F12 medium) for 2 h. In some cases, insulin was added for the final 15 min (+). Where indicated, D-PBS was supplemented with L-glucose (L-Glc), 2-deoxyglucose (2-DOG), 3-O-methylglucose (3-OMG), D-glucose (D-Glc), D-glucosamine [D-Glc(NH₂)], L-lactate or D-mannose (D-Man), each at 5.5 mM. Samples were analysed by SDS/PAGE and Western blotting using anti-4E-BP1 antiserum. Similar data were obtained in three to six separate experiments. (C, D) As for (A) and (B), except that in some cases different D-glucose concentrations were employed (as indicated), or lactate (Lact; 5 mM) was added. In addition, samples of cell extract (150 µg of protein) were applied to m⁷GTP-Sepharose resin and, after washing the beads to remove unbound proteins, the bound material was analysed by SDS/PAGE and Western blotting using anti-eIF4G or anti-4E-BP1 antibodies, as indicated. Equal recovery of eIF4E was confirmed by re-probing the membranes with anti-eIF4E (results not shown). Similar data were obtained in three separate experiments.

4E-BP1 was bound to eIF4E in control cells (in Ham's F12 medium), and this small amount of 4E-BP1 was released in response to insulin. For cells in D-PBS, much more 4E-BP1 was bound to eIF4E, in accordance with its dephosphorylation under this condition (Figure 1A) and with an earlier data [10]. Insulin failed to bring about release of 4E-BP1 in these cells (Figure 4C), consistent with the modest change in 4E-BP1 phosphorylation seen under this condition (Figure 1). Little release was also observed in response to insulin in cells maintained in D-PBS with 0.5 mM D-glucose; however, as the glucose concentration was raised further, insulin was able to cause partial (1 mM) or almost complete (2.5, 5 mM) release of 4E-BP1, consistent with its ability to bring about extensive phosphorylation of 4E-BP1 at a concentration of 5 mM (Figure 1). Concomitant with the insulin-induced release of 4E-BP1 observed at 2.5 and 5 mM glucose, the binding of eIF4G to eIF4E was increased by insulin at these glucose concentrations.

When higher glucose concentrations were tested (up to 20 mM), we observed no further enhancement of the regulation of 4E-BP1 (over and above that seen in 5 mM glucose). This suggests that the effects of glucose occur primarily at subphysiological concentrations (hypoglycaemic range).

In order to test whether the effect was specific for glucose itself or whether other metabolizable sugars might also have a permissive influence on mTOR signalling, we tested the effects of other hexoses. D-Mannose exerted similar effects to those seen for D-glucose, as did D-glucosamine (Figure 4B). These data suggest that the effect is not specific for glucose and, consistent with the data described above for glucose analogues, that sugar metabolism generates energy (ATP?) or another signal (metabolite) that supports or mediates the permissive effect on mTOR-dependent signalling.

To examine whether the observed effect was likely to be connected to cellular energy supply, we tested whether an alternative metabolic fuel, lactate, could also act permissively with respect to insulin action. As shown in Figure 4(B), addition of lactate did not affect the basal level of 4E-BP1 phosphorylation or exert a marked permissive effect with respect to insulin, although some shift from the α to the β isoform was seen consistently in response to insulin. However, insulin was able to bring about marked release of 4E-BP1 in cells incubated in lactate (Figure 4D). Lactate also allowed insulin to enhance the binding of eIF4G to eIF4E, to about the same extent as did D-glucose (Figure 4C; see also [10]). These findings were unexpected, in view of the modest change in the electrophoretic mobility of 4E-BP1 seen under this condition (Figure 4B). This indicates that conversion of 4E-BP1 into the γ form is not essential for its release from eIF4E, in agreement with the observation that very little 4E-BP1 remains associated with eIF4E even under conditions where a significant proportion of the total 4E-BP1 remains in the β isoform (e.g. in unstimulated control cells, or in cells given glucose and stimulated by insulin; see Figures 1 and 4C and earlier data [10]). The band migrating as the β isoform clearly comprises a mixture of differently phosphorylated species, some of which bind to eIF4E and some of which do not. The existence of multiple species within each band is entirely expected, since there are at least five phosphorylation sites in 4E-BP1, but electrophoresis only resolves three forms (discussed in [10]; see also [35]).

Possible mechanisms by which hexoses permit regulation of mTOR-dependent phosphorylation events

It has been shown recently in yeast (*Saccharomyces cerevisiae*) that glucose starvation causes increased phosphorylation of the α subunit of eIF2 (eIF2 α) by activating the protein kinase Gcn2p [36]. Furthermore, Terada and co-workers [11] have suggested that starvation of mammalian cells of another nutrient, amino acids, may regulate mTOR signalling by activation of the mammalian homologue GCN2 (general control of amino acid biosynthesis, non-derepressing 2; a protein kinase acting on eIF2 α), which then feeds into mTOR signalling by an unknown mechanism. We therefore tested whether glucose starvation caused increased phosphorylation of eIF2 α . However, as we reported previously for CHO.T cells (a closely related cell line which overexpresses the human insulin receptor [9,10]), no consistent difference in eIF2 α phosphorylation was observed between CHO.K1 cells incubated with and without nutrients. No significant change in the level of phosphorylation of eIF2 α upon nutrient withdrawal was observed in four separate experiments carried out in the present study or in our earlier work on the effects on nutrients in CHO cells [10] (value in nutrient-deprived

Figure 5 Nutrient starvation does not affect the phosphorylation of eIF2 α at Ser-51

CHO.K1 cells were incubated under the indicated conditions (where control is serum-free Ham's F12 medium) for 2 h. In some cases, insulin was added for the final 15 min (+). Cells were extracted and samples of cell lysate (30 μ g of protein) were subjected to SDS/PAGE and Western blotting using antisera against eIF2 α or eIF2 α phosphorylated at Ser-51 (as indicated). Similar data were obtained in four separate experiments.

cells = $105 \pm 4\%$ of that in control cells when normalized for the signal for eIF2 α itself). Re-addition of glucose also had no significant effect on eIF2 α phosphorylation (Figure 5 and results not shown; see also [10]).

DISCUSSION

The data presented here demonstrate that glucose exerts a modulatory effect upon the regulation of proteins linked to mTOR signalling in mammalian cells. The absence of glucose is not, of course, a normal physiological situation for mammalian cells, but can arise during ischaemia. Examples of such situations are restriction of blood flow to the cerebellum (as following a stroke) or the myocardium (following infarction). The primary effect of glucose, at concentrations close to the physiological range, is to permit insulin to bring about the release of 4E-BP1 from eIF4E. In particular, glucose allowed insulin to bring about the phosphorylation of Thr-36, Thr-45 and Ser-64 on 4E-BP1, but did not itself enhance the level of phosphorylation of these sites. Glucose may thus allow insulin to activate the (poorly understood) kinase(s) and/or inhibit the phosphatase(s) that act on these phosphorylation sites. Glucose itself promoted the phosphorylation of Thr-69 in 4E-BP1. As we reported previously [10], glucose also promoted the ability of insulin to bring about the release of 4E-BP1 from eIF4E and hence the formation of the eIF4F complexes required for cap-dependent translation initiation.

In contrast, glucose was insufficient to allow insulin to bring about the activation of a second target of mTOR signalling, p70 S6k, as judged by the low level of phosphorylation of its substrate, S6, in cells maintained in glucose and treated with insulin. This matches our earlier finding that insulin caused no significant activation of p70 S6k when assessed in immunoprecipitates from cells maintained in glucose [10]. One surprising finding was that, in the presence of glucose, insulin did bring about some degree of phosphorylation of p70 S6k (as judged by its mobility on SDS/PAGE). Clearly phosphorylation of the sites modified in response to insulin under this condition is insufficient to activate p70 S6k. Consistent with this, our data show that, in the presence of glucose, insulin elicits no significant change in the phosphorylation of Thr-389 or Ser-421/424, key regulatory sites involved in the activation of p70 S6k (reviewed in [34]).

We have shown previously [10] that, in CHO cells, amino acids can themselves promote the formation of eIF4F complexes even in the absence of glucose or, more importantly, insulin. Amino

acids presumably, therefore, provide a qualitatively different input to the regulation of 4E-BP1 than does glucose, which primarily acts permissively. Amino acids also brought about a greater degree of activation of p70 S6k than glucose, and were much more effective than glucose in promoting insulin-stimulated activation of this enzyme [10]. Amino acids again appear to provide a more effective input into this second target of mTOR signalling. We have also shown previously that both glucose and amino acids are needed for the regulation of eIF2B (which is independent of mTOR [36]) and eEF2 by insulin [10].

How does glucose provide an input into the regulation of 4E-BP1? Our data clearly show that non-metabolizable analogues of glucose cannot substitute for glucose itself, but that other hexoses can do so. It is possible that glucose metabolism generates a product that exerts the permissive effect. It is also formally possible that mammalian cells possess glucose (hexose) sensors akin to those in yeast [40], and that these mediate the effects observed here. If this were the case, one would have to propose that such sensors did not detect the non-metabolizable sugar analogues used in the present study. The observation that lactate also promoted the insulin-induced release of 4E-BP1 from eIF4E lends support to the notion that these effects are related to metabolism.

The observation that phosphorylation of eIF2 α was not affected by nutrient starvation over the time periods used here suggests that glucose starvation does not activate the eIF2 kinase mGCN2 [37,38] (whereas its yeast homologue, Gcn2p, does appear to be activated by glucose starvation in that organism [39]). Another possible mediator of the effects of glucose withdrawal, via possible changes in the cellular adenine nucleotide 'charge', is the AMP-activated kinase [41]. To attempt to gain insight into the possible role of this enzyme in the regulation of 4E-BP1 phosphorylation, we made use of an artificial activator of the AMP-activated kinase, AICARiboside (5-amino-4-imidazolecarboxamide riboside) [41]. However, treatment of cells with this compound had no effect upon basal or insulin-stimulated 4E-BP1 phosphorylation, thus apparently ruling out a role for the AMP-activated protein kinase in regulating 4E-BP1.

How can these data be rationalized in terms of the physiological control of protein synthesis? Protein synthesis is a major consumer of energy (approx. 20 % of cellular energy is used for this process), and thus it makes excellent sense for cells to couple the availability of a metabolic fuel such as glucose to the regulation of translation factors. 4E-BP1 (and thus eIF4F) may be of especial importance in this context, since the latter is required for all cap-dependent translation-initiation events [14]. Regulation of this protein complex will thus impinge directly on the control of overall protein synthesis. It is therefore notable that provision of glucose allows insulin to activate overall protein synthesis. Glucose also increased the basal rate of protein synthesis; since this sugar did not itself promote the formation of eIF4F complexes, this effect must involve other, so far unidentified, effects on the translational machinery.

Our data demonstrate that other inputs in addition to glucose and insulin are required for the phosphorylation of S6 and for the full activation of p70 S6k. This extra input can be provided by amino acids (reviewed in [13]). As a corollary of this, it is probable that the translation of 5'-TOP mRNAs (which is coupled to S6 phosphorylation), and thus ribosome biogenesis, are only promoted in the presence of amino acids. Since ribosome synthesis itself consumes a large amount of amino acids, and since increased ribosome number is a long-term adaptation to allow higher rates of translation, it again makes physiological sense for amino acid availability to provide a key input into this process.

The present data point to novel connections between nutrient availability and the regulation of mRNA translation, in particular the control of phosphoproteins linked to mTOR. Unravelling the molecular mechanisms by which glucose and amino acids exert their effects in mammalian cells is a major goal for our understanding of nutrient signalling.

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REFERENCES

- Hara, K., Yonezawa, K., Weng, Q.-P., Kozlowski, M. T., Belham, C. and Avruch, J. (1998) Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF4E BP1 through a common effector mechanism. *J. Biol. Chem.* **273**, 14484–14494
- Patti, M.-E., Brambilla, E., Luzi, L., Landaker, E. J. and Kahn, C. R. (1998) Bidirectional modulation of insulin action by amino acids. *J. Clin. Invest.* **101**, 1519–1529
- Fox, H. L., Kimball, S. R., Jefferson, L. S. and Lynch, C. J. (1998) Amino acids stimulate phosphorylation of p70(S6k) and organization of rat adipocytes into multicellular clusters. *Am. J. Physiol.* **274**, C206–C213
- Fox, H. L., Pham, P. T., Kimball, S. R., Jefferson, L. S. and Lynch, C. J. (1998) Amino acid effects on translational repressor 4E-BP1 are mediated primarily by L-leucine in isolated adipocytes. *Am. J. Physiol.* **275**, C1232–C1238
- Shah, O. J., Antonetti, D. A., Kimball, S. R. and Jefferson, L. S. (1999) Leucine, glutamine and tyrosine reciprocally modulate the translation initiation factors eIF4F and eIF2B in perfused rat liver. *J. Biol. Chem.* **274**, 36168–36175
- Xu, G., Marshall, C. A., Lin, T.-A., Kwon, G., Munivenkatappa, R. B., Hill, J. R., Lawrence, J. C. and McDaniel, M. L. (1998) Insulin stimulates glucose-stimulated phosphorylation of PHAS-I by pancreatic beta cells: An insulin-receptor mechanism for autoregulation of protein synthesis by translation. *J. Biol. Chem.* **273**, 4485–4491
- Lynch, C. J., Fox, H. L., Vary, T. C., Jefferson, L. S. and Kimball, S. R. (2000) Regulation of amino acid-sensitive TOR signaling by leucine analogues in adipocytes. *J. Cell. Biochem.* **77**, 234–251
- Kleijn, M. and Proud, C. G. (2000) Glucose and amino acids modulate translation factor activation by growth factors in PC12 cells. *Biochem. J.* **347**, 399–406
- Wang, X., Campbell, L. E., Miller, C. M. and Proud, C. G. (1998) Amino acid availability regulates p70 S6 kinase and multiple translation factors. *Biochem. J.* **334**, 261–267
- Campbell, L. E., Wang, X. and Proud, C. G. (1999) Nutrients differentially modulate multiple translation factors and their control by insulin. *Biochem. J.* **344**, 433–441
- Iiboshi, Y., Papst, P. J., Kawasome, H., Hosoi, H., Abraham, R. T., Houghton, P. J. and Terada, N. (1999) Amino acid-dependent control of p70^{S6k}: involvement of tRNA amino acid in the regulation. *J. Biol. Chem.* **274**, 1092–1099
- Shigemitsu, K., Tsujishita, Y., Hara, K., Nanahoshi, M., Avruch, J. and Yonezawa, K. (1999) Regulation of translational effectors by amino acid and mammalian target of rapamycin signaling pathways: possible involvement of autophagy in cultured hepatoma cells. *J. Biol. Chem.* **274**, 1058–1065
- Kimball, S. R. and Jefferson, L. S. (2000) Regulation of translation initiation in mammalian cells by amino acids. In *Translational Control of Gene Expression* (Sonenberg, N., Hershey, J. W. B. and Mathews, M. B., eds.), pp. 561–579, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Gingras, A.-C., Raught, B. and Sonenberg, N. (1999) eIF4 translation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu. Rev. Biochem.* **68**, 913–963
- Raught, B., Gingras, A.-C. and Sonenberg, N. (2000) Regulation of ribosome recruitment in eukaryotes. In *Translational Control of Gene Expression* (Sonenberg, N., Hershey, J. W. B. and Mathews, M. B., eds.), pp. 245–293, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Proud, C. G. and Denton, R. M. (1997) Molecular mechanisms for the activation of protein synthesis by insulin. *Biochem. J.* **328**, 329–341
- Lawrence, J. C. and Abraham, R. T. (1997) PHAS/4E-BPs as regulators of mRNA translation and cell proliferation. *Trends Biochem. Sci.* **22**, 345–349
- Dennis, P. B., Fumagalli, S. and Thomas, G. (1999) Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation. *Curr. Opin. Genet. Dev.* **9**, 49–54

- 19 Abraham, R. T. (1998) Mammalian target of rapamycin: immunosuppressive drugs uncover a novel pathway of cytokine receptor signaling. *Curr. Opin. Immunol.* **10**, 330–336
- 20 Fumagalli, S. and Thomas, G. (2000) S6 phosphorylation and signal transduction. In *Translational Control of Gene Expression* (Sonenberg, N., Hershey, J. W. B. and Mathews, M. B., eds.), pp. 695–717, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 21 Pearson, R. B., Dennis, P. B., Han, J. W., Williamson, N. A., Kozma, S. C., Wettenhall, R. E. H. and Thomas, G. (1995) The principal target of rapamycin-induced p70^{S6k} inactivation is novel phosphorylation site within a conserved hydrophobic domain. *EMBO J.* **14**, 5279–5287
- 22 Meyuhas, O. and Hornstein, E. (2000) Translational control of TOP mRNAs. In *Translational Control of Gene Expression* (Sonenberg, N., Hershey, J. W. B. and Mathews, M. B., eds.), pp. 671–693, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 23 Scorsone, K. A., Panniers, R., Rowlands, A. G. and Henshaw, E. C. (1987) Phosphorylation of eukaryotic initiation factor 2 during physiological stresses which affect protein synthesis. *J. Biol. Chem.* **262**, 14538–14543
- 24 DeGracia, D. J., Sullivan, J. M., Neumar, R. W., Alousi, S. S., Hikade, K. R., Pittman, J. E., White, B. C., Rafols, J. A. and Krause, G. S. (1997) Effect of brain ischemia and reperfusion on the localization of phosphorylated eukaryotic initiation factor 2 α . *J. Cereb. Blood Flow Metab.* **17**, 1291–1302
- 25 Bandi, H. R., Ferrari, S., Krieg, J., Meyer, H. E. and Thomas, G. (1993) Identification of 40S ribosomal protein S6 phosphorylation sites in Swiss mouse 3T3 fibroblasts stimulated with serum. *J. Biol. Chem.* **268**, 4530–4533
- 26 Mothe-Satney, I., Yang, D., Fadden, P., Haystead, T. A. J. and Lawrence, J. C. (2000) Multiple mechanisms control phosphorylation of PHAS-I in five (S/T)P sites that govern translational repression. *Mol. Cell. Biol.* **20**, 3558–3567
- 27 Mothe-Satney, I., Brunn, G. J., McMahon, L. P., Capaldo, C. T., Abraham, R. T. and Lawrence, J. C. (2000) Mammalian target of rapamycin-dependent phosphorylation of PHAS-1 in four (S/T)P sites detected by phospho-specific antibodies. *J. Biol. Chem.* **275**, 33836–33843
- 28 Walker, K. S., Deak, M., Paterson, A., Hudson, K., Cohen, P. and Alessi, D. R. (1998) Activation of protein kinase B β and γ isoforms by insulin *in vivo* and by 3-phosphoinositide-dependent protein kinase-1 *in vitro*: comparison with protein kinase B α . *Biochem. J.* **331**, 299–308
- 29 Dickens, M., Chin, J. E., Roth, R. A., Ellis, L., Denton, R. M. and Tavaré, J. M. (1992) Characterization of insulin-stimulated protein serine/threonine kinases in CHO cells expressing human insulin receptors with point and deletion mutations. *Biochem. J.* **287**, 201–209
- 30 Price, N. T., Nakielnny, S. F., Clark, S. J. and Proud, C. G. (1989) The two forms of the β -subunit of initiation factor-2 from reticulocyte lysates arise from proteolytic degradation. *Biochim. Biophys. Acta* **1008**, 177–182
- 31 Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. and Hemmings, B. (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* **15**, 6541–6551
- 32 Gingras, A.-C., Kennedy, S. G., O'Leary, M. A., Sonenberg, N. and Hay, N. (1998) 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signalling pathway. *Genes Dev.* **12**, 502–513
- 33 Dufner, A., Andjelkovic, M., Burgering, B. M., Hemmings, B. A. and Thomas, G. (1999) Protein kinase B localization and activation differentially affect S6 kinase 1 activity and eukaryotic translation initiation factor 4E-binding protein 1 phosphorylation. *Mol. Cell. Biol.* **19**, 4525–4534
- 34 Pullen, N. and Thomas, G. (1997) The modular phosphorylation and activation of p70s6k. *FEBS Lett.* **410**, 78–82
- 35 Duncan, R. F. and Song, H. J. (1999) Striking multiplicity of eIF4E-BP1 phosphorylated isoforms identified by 2D gel electrophoresis: regulation by heat shock. *Eur. J. Biochem.* **265**, 728–743
- 36 Welsh, G. I., Stokes, C. M., Wang, X., Sakaue, H., Ogawa, W., Kasuga, M. and Proud, C. G. (1997) Activation of translation initiation factor eIF2B by insulin requires phosphatidylinositol 3-kinase. *FEBS Lett.* **410**, 418–422
- 37 Sood, R., Porter, A. C., Olsen, D., Cavener, D. R. and Wek, R. C. (2000) A mammalian homologue of GCN2 protein kinase important for translational control by phosphorylation of eukaryotic initiation factor 2 α . *Genetics* **154**, 787–801
- 38 Berlanga, J. J., Santoyo, J. and de Haro, C. (1999) Characterization of a mammalian homolog of the GCN2 eukaryotic initiation factor 2 α kinase. *Eur. J. Biochem.* **265**, 754–762
- 39 Yang, R., Wek, S. A. and Wek, R. C. (2000) Glucose limitation induces *GCN4* translation by activation of Gcn2 protein kinase. *Mol. Cell. Biol.* **20**, 2706–2717
- 40 Ozcan, S., Dover, J. and Johnston, M. (1998) Glucose sensing and signaling by two glucose receptors in the yeast *Saccharomyces cerevisiae*. *EMBO J.* **17**, 2566–2573
- 41 Hardie, D. G., Carling, D. and Carlson, M. (1998) The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu. Rev. Biochem.* **67**, 821–855