Zinc stimulates the activity of the insulin- and nutrient-regulated protein kinase mTOR

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Lynch, Christopher J., Brian J. Patson, Stacy A. Goodman, Donald Trapolsi, and Scot R. Kimball. Zinc stimulates the activity of the insulin- and nutrient-regulated protein kinase mTOR. Am J Physiol Endocrinol Metab 281: E25–E34, 2001.—Recent studies indicate that zinc activates p70 S6 kinase (p70S6k) by a mechanism involving phosphatidylinositol 3-kinase (PI 3-kinase) and Akt (protein kinase B). Here it is shown that phenanthroline, a zinc and heavy metal chelator, inhibited both amino acid- and insulin-stimulated phosphorylation of p70S6k. Both amino acid and insulin activations of p70S6k involve a rapamycin-sensitive step that involves the mammalian target of rapamycin (mTOR), also known as FRAP and RAFT. However, in contrast to insulin, amino acids activate p70S6k by an unknown PI 3-kinase- and Akt-independent mechanism. Thus the effects of chelator on amino acid activation of p70S6k were surprising. For this reason, we tested the hypothesis that zinc directly regulates mTOR activity, independently of PI 3-kinase activation. In support of this, basal and amino acid stimulation of p70S6k phosphorylation was increased by zinc addition to the incubation media. Furthermore, the protein kinase activities of mTOR immunoprecipitated from rat brain lysates were stimulated two- to fivefold by 10–300 μM Zn2+ in the presence of an excess of either Mn2+ or Mg2+, whereas incubation with 1,10-phenanthroline had no effect. These findings indicate that Zn2+ regulates, but is not absolutely required for, mTOR protein kinase activity. Zinc also stimulated a recombinant human form of mTOR. The stimulatory effects of Zn2+ were maximal at ~100 μM but decreased and became inhibitory at higher physiologically irrelevant concentrations. Micromolar concentrations of other divalent cations, Ca2+, Fe2+, and Mn2+, had no effect on the protein kinase activity of mTOR in the presence of excess Mg2+. Our results and the results of others suggest that zinc acts at multiple steps in amino acid- and insulin cell-signaling pathways, including mTOR, and that the additive effects of Zn2+ on these steps may thereby promote insulin and nutritional signaling.

insulin; ribosomal protein S6 kinase; 1,10-phenanthroline; mammalian target of rapamycin; eukaryotic initiation factor 4E binding protein 1; protein synthesis

ZINC IS CONSIDERED TO BE an essential nutrient that is required for optimal growth and normal development of vertebrate organisms. For example, zinc-deficient cells are not able to divide. The essential nature of this mineral is due in part to the function of zinc in the catalytic mechanisms of a number of metalloenzymes such as metazincins and carbonic anhydrase. Zinc is also important for maintaining the structure of many proteins, so it should not be surprising that Zn2+ affects the activities of a number of different enzymes or steps in cell signaling pathways in either an inhibitory or a stimulatory manner. Indeed, it has been known for many years that zinc mimics the actions of insulin on glucose transport and acts synergistically on this process when added together with insulin (11, 25–27, 29, 36, 41, 46, 57, 58).

In the present study, we have examined the effect of zinc and the heavy metal chelator phenanthroline on the intracellular signal transduction pathway that includes the Ser/Thr protein kinase termed mammalian target of rapamycin (mTOR), also known as FRAP and RAFT. This signaling pathway is regulated by amino acids and growth factors, including insulin (8, 22, 23, 30, 40, 43, 48, 51, 56, 63). Targets downstream of mTOR include translational regulatory proteins such as eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and p70 S6 kinase (p70S6k) (15, 31, 38, 40, 62). In dividing cells, mTOR signaling is important for cell cycle progression, as is zinc (27, 34, 36). In postmitotic cells, such as adipocytes, mTOR has been posited to play an important role in tissue morphogenesis, matrix invasion, protein synthesis, and leptin secretion (for review, see Ref. 43).

Stimulation of the Ser/Thr protein kinase activity of mTOR stimulates protein synthesis through effects on the initiation step of mRNA translation. Two mechanisms have been implicated in this regulation. The first involves the phosphorylation of 4E-BP1, a translational repressor also known as PHAS-I (8, 42). Phosphorylation of 4E-BP1 on multiple Ser and Thr residues decreases the binding affinity of eIF4E for the protein, which in turn allows eIF4E to be incorporated into the eIF4F complex (7, 50). This association is required for translation of capped mRNAs, which represent the majority of cytoplasmic messages. In addi-
tion, it is believed to be important for increasing the efficiency of translation of certain mRNA species that contain significant secondary structure in their 5′-capped untranslated structures. 4E-BP1 is a direct substrate of mTOR in vitro kinase assays. Multisite phosphorylation and activation of p70\textsuperscript{S6k} are thought to be a second mechanism by which mTOR regulates translation initiation (for review, see Ref. 62). Activation of p70\textsuperscript{S6k} and subsequent phosphorylation of ribosomal protein S6 are associated with increased translation of mRNA species that contain a polypyrimidine tract in their 5′-untranslated regions.

In this communication, we show that phenanthroline, which chelates zinc and other heavy metals, inhibits insulin-stimulated multisite phosphorylation of p70\textsuperscript{S6k}. Phenanthroline also inhibits phosphorylation at site T389 of p70\textsuperscript{S6k}. Phosphorylation of this site is thought to be regulated by mTOR and is associated with increased p70\textsuperscript{S6k} activity (53, 66). This observation is consistent with previous observed effects of zinc on signal transduction involving mTOR (27, 36) and with a very recent study by Kim et al. (34), showing that adding extracellular zinc activates p70\textsuperscript{S6k}. The activation of p70\textsuperscript{S6k} required both PI 3-kinase and Akt, which are also required for insulin stimulation of p70\textsuperscript{S6k} activity (48, 52, 61, 63, 64).

In previous studies, we have found that amino acids promote p70\textsuperscript{S6k} phosphorylation in rat adipocytes (19, 51). Amino acids signal through mTOR independently of insulin in a number of cell types. In contrast to insulin, amino acids affect the mTOR-signaling pathway by a PI 3-kinase- and Akt-independent mechanism that does not involve protein tyrosine phosphorylation (51). Therefore, we were surprised that phenanthroline also inhibited the stimulation of p70\textsuperscript{S6k} phosphorylation caused by amino acids. Therefore, in studies described herein, we explored the possibility that mTOR might have a protein kinase activity that is regulated by zinc. The results are consistent with the hypothesis that zinc regulates mTOR and other strategic cell-signaling steps in a concerted fashion that promotes or mimics the actions of insulin and amino acids overall.

EXPERIMENTAL PROCEDURES

Isolation of adipocytes. Adipocytes were isolated from epididymal fat pads of 7- to 8-wk-old male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) by collagenase digestion as previously described (19, 20). The cells were washed three times in Krebs-Ringer-HEPES (KRH, in mM: 118 NaCl, 4.7 KCl, 1.2 MgSO\textsubscript{4}, 2.5 CaCl\textsubscript{2}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 25 HEPES, 1 EDTA, 2 glucose, and 200 mM adenosine) buffer containing 2% bovine serum albumin fraction V (BSA). Aliquots of cells (150 μL, 60–80% cytocrit) were generally added to 500 μL of BSA-free KRH with or without 500 μM 1,10-phenanthroline (Sigma, St Louis, MO) as indicated and allowed to incubate for 20–60 min at 37°C. After this incubation period, the underlying buffer was removed from beneath the cells with a syringe. This buffer was replaced with one of three different buffers either with or without 1,10-phenanthroline: BSA-free KRH ("control"), BSA-free KRH with 1 μg/ml insulin ("insulin"), or BSA-free KRH containing an amino acid mixture ("amino acids"). The concentration of amino acids in the latter buffer was as follows [in μM: 2,395 Ala, 312 Asn, 176 Asp, 651 Arg, 389 Cys, 2,278 Gln, 1,950 Gly, 385 His, 649 Ile, 1,040 Leu, 2,335 Lys, 259 Met, 326 Phe, 651 Pro, 1,302 Ser, 1,170 Thr, 454 Trp, 371 Tyr, 1,170 Val (these concentrations of amino acids become diluted when 500 μL are added to ~125 μL of cells suspended in KRH)]. Cells were allowed to incubate for 30–45 min at 37°C. After this incubation, the buffer was removed from beneath the cells, and the cells were frozen in liquid nitrogen. In experiments to examine the effects of the addition of 100 μM of either zinc sulfate or zinc acetate, it was observed that addition of zinc to KRH resulted in the slow formation of a zinc precipitate with one of the components of the buffer. Therefore in subsequent experiments to test the effects of adding zinc, a 100 μM final concentration of zinc was added from a stock solution right after the insulin or amino acids were added. Furthermore, the addition of zinc was repeated two additional times (every 10 min) over the 30-min incubation period.

Phosphorylation of 4E-BP1 and p70\textsuperscript{S6k}. Phosphorylation of 4E-BP1 and p70\textsuperscript{S6k} was determined by examining changes in electrophoretic mobility during SDS-PAGE of immunoprecipitates, as previously described (19, 20, 44, 51). In some experiments, phosphorylation of p70\textsuperscript{S6k} on T389 was examined in p70\textsuperscript{S6k} immunoprecipitates with the use of an antibody that recognizes p70\textsuperscript{S6k} only when it is phosphorylated at T389. The Western blots were performed according to the antibody manufacturer's protocol (Cell Signaling Technology, Beverly, MA).

Immunoprecipitation of mTOR from rat brain. Brain homogenates were prepared from the cerebral cortex of Sprague-Dawley rats. The tissue was homogenized on ice in homogenization buffer J (JHB), which contained 2 mM EGTA, 100 mM NaCl, 50 mM Tris•HCl, 2 mM β-mercaptoethanol, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 10 mM ethanol, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 10 μg/ml leupeptin, 10 μg/ml aprotinin, 110 μl/ml Microcystin LR, 10% glycerol (vol/vol), and 0.1% Tween-20 (vol/vol) by use of a Tissumizer (Tekmar, Cincinnati, OH) at 10,000 rpm. Lysates were centrifuged at 10,000 g for 30 min at 4°C. Supernatants were pooled, aliquoted, flash-frozen in liquid nitrogen, and stored at −84°C. Protein concentration was determined on a sample of the homogenate using a protein assay kit (Bio-Rad, Hercules, CA).

To affinity purify mTOR for the immunoprecipitation kinase assays, an anti-peptide antibody, MTAB4, was made in rabbits to the peptide PQWYRHTFEE, corresponding to amino acids 230–240 in the rat mTOR amino acid sequence. Antibody was attached to protein A agarose beads (Sigma) as follows. First, 5 mg of beads per sample were hydrated in JHB. The beads were then washed two times by centrifugation (15,000 g for 2 min) in JHB. Antibody (20 μl) was allowed to bind to the beads (10 μl in JHB) by rocking at 4°C for 2 h. Unbound protein was removed from the beads by two washes with Tris-buffered saline (TBS: 100 mM NaCl and 50 mM Tris-HCl, pH 7.4). Beads were then incubated with brain homogenate (1 mg protein/sample) and JHB (normalized to 500 μl/sample) at 4°C overnight. Next, beads were washed two times by centrifugation with each of the following solutions: buffer A (110 mM NaCl, 50 mM Tris•HCl, 1 mM dithiothreitol (DTT), 10% glycerol (vol/vol), and 0.1% Tween-20 (vol/vol; pH 7.4)), buffer B (in mM: 50 NaCl, 1 DTT, and 10 HEPES, pH 7.4), and either MnCl\textsubscript{2} or MgCl\textsubscript{2}-containing kinase assay buffer (in mM: 55.5 NaCl, 1.1 DTT, 11.1 HEPES, 55.5 β-glycerophosphate, 1 μMol Microcystin LR, and 11.1 mM of either MnCl\textsubscript{2} or MgCl\textsubscript{2}, as indicated). After the final washing, beads were resuspended in kinase assay...
buffer (100 μl/sample) and then aliquoted (~100 μl) into microcentrifuge tubes. The beads were prepared for kinase assay (see Protein kinase assays) by centrifugation at 15,000 g for 2 min and removal of the supernatant with vacuum.

Recombinant protein expression in Sf9 cells. A baculovirus transfer vector containing the FLAG-tagged FRAP sequence (FRAP/FLAG 1392) described previously (5, 6) was obtained as a generous gift from Dr. Stuart L. Schreiber's laboratory (Boston, MA). The wild-type FRAP construct was derived from the baculovirus expression vector pVL1392. The FLAG tag sequence encodes for the amino terminal epitope (MDYKDDDK). Sf9 cells were obtained from Pharmingen (San Diego, CA) and maintained according to the company's protocol in TNM-FH insect medium containing 10% fetal bovine serum at 27°C. FRAP/FLAG 1392 was transfected into Sf9 insect cells by means of a Baculogold kit (Pharmingen). The Sf9 cells, which had been infected for 3–5 days, were harvested by centrifugation at 300 g for 10 min at 4°C, and cell pellets were kept frozen at −84°C until use.

Purification of recombinant mTOR. FLAG-tagged protein was expressed in insect cells by means of a Baculogold kit (Pharmingen). The Sf9 cells, which had been infected for 3–5 days, were harvested by centrifugation at 100 g for 10 min at 4°C, and cell pellets were kept frozen at −84°C until use.

Protein kinase assays. The Mg2+ - or Mn2+-stimulated protein kinase activity of mTOR was determined by measuring the incorporation of 32P, from [γ-32P]ATP into 4E-BP1 with the use of mTOR immunoprecipitates or recombinant FLAG-tagged FRAP as a source of mTOR activity. A buffer incubation preceded the start of the kinase assays. Ten microliters of either the already described Mg2+ - or Mn2+-kinase assay buffers (as indicated in the figure legends with or without 1,10-phenanthroline or Zn2+) were added to microcentrifuge tubes containing mTOR immunoprecipitates or recombinant FLAG-tagged FRAP. The tubes were then incubated for 15 min at 30°C before the kinase assays were started by adding 100 μl of SDS-PAGE sample buffer and heating at 100°C for 5 min. Proteins in an aliquot of the sample buffer were resolved by electrophoresis on a 10–20% acrylamide gradient SDS-polyacrylamide gel. The gels were then stained with Coomassie blue and dried. Radioactivity incorporated into 4E-BP1 was determined using a phosphomager.

RESULTS

Effect of phenanthroline and zinc on p70S6k in vivo. Phosphorylation of p70S6k is associated with changes in mobility of the protein during SDS-PAGE. Thus changes in protein phosphorylation can be assessed by examining the distribution of p70S6k immunoreactivity in discrete mobility forms in Western blots. Figure 1 shows a representative Western blot in which the changes in the distribution of p70S6k among the different isoforms can be seen. Incubation of cells with insulin or amino acids caused a generalized increase in p70S6k phosphorylation as indicated by the increase in forms of p70S6k with a decreased mobility during SDS-PAGE. In cells treated with amino acids or insulin, four bands can be seen with decreased mobility relative to the one or two forms that are seen in the absence of these additives (Fig. 1).

Incubation of cells with the heavy metal chelator 1,10-phenanthroline prevented much of the increase in p70S6k multisite phosphorylation observed in cells incubated with amino acids or insulin but had little effect on the amount of p70S6k immunoprecipitated or on the mobility of p70S6k from control-treated cells (Fig. 1). Treatment of adipocytes with either amino acids or insulin resulted in enhanced phosphorylation of p70S6k at T389 as determined with a site-specific anti-phosphopeptide antibody (Fig. 2). Phosphorylation at this site closely correlates with changes in p70S6k activity (53, 66). Phospho-T389 immunoreactivity was rarely observed in control-treated cells. Others have shown that zinc promotes activation through the mTOR signaling pathway (27, 34, 36). Because phenanthroline chelates zinc (as well as other heavy metals), the effects that we observed might be due to the ability of phenanthroline to prevent zinc-dependent phosphorylation of p70S6k.
the result of zinc chelation. The effects that zinc has on p70S6k may occur, in part, by an Akt- and PI 3-kinase-dependent mechanism (34), possibly secondary to phosphotyrosine phosphatase (PTPase) inhibition (39, 41, 60). However, such effects would not be expected to have an impact on amino acid stimulation of p70S6k phosphorylation, because we have previously shown that amino acid signaling to p70S6k occurs independently of protein tyrosine phosphorylation, PI 3-kinase, or Akt activation (51). Therefore, to evaluate the idea that zinc might be working by directly regulating mTOR, we examined the effects of zinc addition on basal and amino acid-stimulated p70S6k phosphorylation. Figure 3 shows that zinc addition increased both basal and amino acid-stimulated phosphorylation of p70S6k. This finding supports, but does not prove, that zinc acts directly on mTOR.

Effect of phenanthroline and zinc on mTOR protein kinase activity. Insulin and amino acids stimulate p70S6k by different mechanisms; nevertheless, both require the activity of mTOR. Therefore, we explored the possibility that mTOR activity might be stimulated by micromolar concentrations of zinc. The kinase activity of mTOR was measured in mTOR immunoprecipitates from rat brain lysates prepared in the presence of 11 mM Mg2+ or Mn2+, with 4E-BP1 as the substrate (Figs. 4–6). As has been previously reported (67), mTOR kinase activity was typically about twofold higher within the same experiment when Mn2+ was used instead of Mg2+ as the divalent cation (data not shown). Intracellular concentrations of zinc in some cells have been estimated to be ~100 μM (1); therefore, we first examined the effect of 100 μM Zn2+ on mTOR kinase activity in the mTOR immunoprecipitation kinase assay. Figure 4 shows that 100 μM Zn2+ stimulated mTOR kinase activity with use of the Mg2+-containing kinase assay buffer. In a number of different experiments, the activity was variously stim-

Table 1. Effect of phenanthroline and zinc on p70S6k phosphorylation at T389.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Phospho-T389</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.10±0.02</td>
</tr>
<tr>
<td>Am Acids</td>
<td>0.20±0.03</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.30±0.04</td>
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Fig. 2. Effect of phenanthroline on phosphorylation of p70S6k at site T389. Anti-p70S6k immunoprecipitates were prepared as in Fig. 1. Protein in the immunoprecipitates was separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF). Top: membranes were probed, according to the manufacturer’s recommendations, with an antibody that recognizes only p70S6k that is phosphorylated on the site required for activation of the kinase, T389. A representative blot is shown. Bottom: the same membranes were stripped and reprobed with an antibody that recognizes both the phosphorylated and unphosphorylated forms (i.e., total) of p70S6k as in Fig. 1. The phosphospecific and total p70S6k blots were analyzed by densitometry, and the ratio of phospho-T389 to total p70S6k immunoreactivity was determined. Results are means ± SE and are representative of 2 experiments.

Fig. 3. Effect of zinc on phosphorylation of p70S6k at T389. Zinc was added to adipocytes incubated in Krebs-Ringer-HEPES (KRH) or amino acid-supplemented KRH for measurement of p70S6k phosphorylation, as described in EXPERIMENTAL PROCEDURES. Anti-p70S6k immunoprecipitates were prepared, and protein in the immunoprecipitates was separated by SDS-PAGE and transferred to PVDF. Top: membranes were probed with an antibody that recognizes only p70S6k phosphorylated on the site required for activation of the kinase, T389. A representative blot is shown. Bottom: the same membranes were stripped and reprobed with an antibody that recognizes both the phosphorylated and unphosphorylated forms (i.e., total) of p70S6k as in Fig. 1. The phosphospecific and total p70S6k blots were analyzed by densitometry, and the ratio of phospho-T389 to total p70S6k immunoreactivity was determined. The average response of amino acid-treated cells was arbitrarily set to 100%, and the other responses are expressed as a percentage of that value. Results are means ± SE from a representative experiment.
regulated between two- and fivefold by this concentration of Zn$^{2+}$. Addition of 500 mM 1,10-phenanthroline had no statistically significant effect on activity in this assay. This is not surprising, because the protocol for the immunoprecipitation includes a divalent cation chelator, EDTA.

We next investigated the effects of other biologically important divalent cations on mTOR protein kinase activity. The effects of two concentrations, 10 and 100 μM, of Zn$^{2+}$, Ca$^{2+}$, Fe$^{2+}$, and Mn$^{2+}$ were evaluated. Of these cations, only Zn$^{2+}$ had a statistically significant and concentration-dependent effect on kinase activity with the Mg$^{2+}$-containing kinase assay buffer (Fig. 5). Thus, although kinase activity was generally greater with the use of the Mn$^{2+}$-containing (11 mM MnCl$_2$) kinase assay buffer compared with the use of the Mg$^{2+}$-containing kinase assay buffer, lower concentrations of Mn$^{2+}$ had no significant effect on activity. Intracellular total Mn$^{2+}$ concentrations in mammalian cells have been determined to be ~1 μM; therefore, it seems unlikely that the Mn$^{2+}$-stimulated activity seen in vitro at millimolar concentrations is physiologically important.

Figure 6 shows that micromolar concentrations of Zn$^{2+}$ also stimulated the activity of the enzyme with the Mn$^{2+}$-containing kinase assay buffer. The activity increased to a maximum that peaked at either 100 or 300 μM Zn$^{2+}$ in different experiments and thereafter decreased (Fig. 6). This was the case for both the Mn$^{2+}$ (Fig. 6) and Mg$^{2+}$ (data not shown) -stimulated kinase activities. At concentrations >1,000 μM Zn$^{2+}$, both the...
Mn\(^{2+}\) - and Mg\(^{2+}\)-dependent kinase activities were further reduced (data not shown). At these higher, unphysiological concentrations, zinc may be interfering with the formation of the substrate complex ATP-Mn\(^{2+}\) or ATP-Mg\(^{2+}\).

Figure 7 shows that zinc also stimulated the activity of purified recombinant mTOR. The magnitude of the increase in 4E-BP1 phosphorylation in the presence of 100 \(\mu\)M Zn\(^{2+}\) was similar to that observed in the rat brain immunoprecipitates as a source of mTOR.

**DISCUSSION**

In this report, we have shown that phenanthroline inhibits the phosphorylation of adipocyte p70\(S6k\) induced by either insulin or amino acids. A model that depicts how zinc is posited to affect the activation of p70\(S6k\) by insulin or amino acids is shown in Fig. 8. It is proposed that zinc addition or chelation affects two or more components of the insulin signal transduction pathway but that activation of p70\(S6k\) by amino acids affects only one of these.

As mentioned earlier, given the number of proteins that may potentially bind zinc and the number of enzymes that use zinc as part of their catalytic mechanisms, it is not surprising that zinc might affect steps in different cell signaling pathways in either a stimulatory or an inhibitory fashion. Indeed, there is considerable literature on the effects of zinc and heavy metal/zinc chelation on various steps in the insulin-signaling pathway (10, 11, 25, 27, 29, 36, 37, 41, 46, 57). What is apparent from these studies, but possibly unappreciated, is that, at each step where zinc has been reported to have an effect, the effect would be expected a priori to ultimately promote insulin signaling overall. Indeed, in adipocytes, zinc both modifies the effects of insulin and has insulin-like effects. For example, zinc increases the uptake of glucose and de novo lipogenesis when added alone (10, 11) at concentrations from 10 to 2,000 \(\mu\)M.

Zinc promotes insulin action at many steps. First, zinc has been reported to exert positive effects on insulin synthesis and secretion and is required for the structural conformation of insulin (for review, see Ref. 9). Second, zinc stimulates insulin-specific binding in adipocytes by an unknown mechanism (21, 25). A third site affected by zinc in the insulin-signaling pathway is the membrane-associated PTPase activity that antagonizes the effects of the insulin receptor and other growth factor-associated tyrosine kinases (39, 41, 55). Zinc inhibits this membranal PTPase activity, which is at an early and critical juncture in insulin signaling (41). Several branch points are found among subsequent steps in the insulin-signaling pathway. One branch begins with activation of PI 3-kinase and ultimately leads to redistribution to the plasma membrane of cytoplasmic membrane vesicles containing GLUT-4. Certain isoforms of protein kinase C (PKC) appear to be required for this redistribution (2, 3, 32, 33, 59). Zinc stimulates both membrane localization and activity of PKC (13, 17, 18, 28, 54, 69). Thus PKC represents a fourth step at which zinc promotes insulin signaling.
Another branch of the insulin-signaling pathway that begins at the PI 3-kinase activation step is the mTOR-signaling pathway. One ultimate target of this pathway is ribosomal protein S6, a substrate of p70S6K. Moreover, zinc inhibits a Ser/Thr phosphatase that regulates the phosphorylation state of S6 (24), and this represents a fifth step in the insulin cell-signaling pathway that seems to be affected by zinc in a manner that is predicted to promote insulin signaling overall.

In contrast to insulin, amino acid signaling is mTOR dependent and occurs through a phosphoryrosine kinase-independent mechanism that does not rely on protein tyrosine phosphorylation, activation of PI3-kinase, or changes in Akt activity. This PI 3-kinase- and Akt-independent mechanism of mTOR activation by the amino acids has been reported both in adipocytes (51) and in other tissues (30, 35, 49). Therefore changes in PTPase activity in vivo (e.g., brought about by heavy metal chelation) would not be expected to significantly impact amino acid stimulation of p70S6K phosphorylation. Similarly, in adipocytes, amino acid signaling does not involve PKC (51). Therefore, any effects of heavy metal chelation or zinc on PKC in adipocytes should not impact mTOR signaling by amino acids. Instead, the data herein support the hypothesis that the observed effects of zinc addition or heavy metal chelation on p70S6K can be attributed, in part, to direct effects of zinc on mTOR activity. Zinc stimulated mTOR in in vitro immunoprecipitation kinase assays as well as in assays using purified recombinant FLAG-tagged FRAP, an epitope-tagged human version of mTOR. Thus mTOR represents a sixth step at which zinc may promote insulin signaling but the only shared step at which both amino acids and insulin may stimulate p70S6K phosphorylation in adipocytes.

Intracellular concentrations of Fe^{2+} and Mn^{2+} have been determined to be ~10 and 1 μM, respectively (1), whereas intracellular free Ca^{2+} concentrations are typically submicromolar (45). At either 10 or 100 μM, neither Fe^{2+}, Mn^{2+}, nor Ca^{2+} affected mTOR activity. It is unlikely, therefore, that these cations are physiologically important regulators of mTOR. A higher Mn^{2+} concentration (11 mM) did stimulate mTOR kinase activity toward 4E-BP1, as has previously been reported for mTOR autokinase activity (67). Because zinc also stimulated kinase activity measured using the Mn^{2+}-containing kinase assay buffer, it seems unlikely that zinc and the higher Mn^{2+} concentration stimulate mTOR activity by the same mechanism. This effect of higher Mn^{2+} concentrations is also probably not physiologically relevant, because in vivo, the concentration of Mn^{2+} is ~10 times lower than the lowest concentration that we tested here and found to have no effect on mTOR protein kinase activity (1).

Zinc stimulated mTOR kinase activity at concentrations >1 μM, peaking at 100–300 μM in different experiments. It is of interest to know whether there would ever be sufficient free intracellular zinc to interact with a protein, either persistently or in a regulated fashion (e.g., in response to diurnal or dietary changes in zinc) within this range of concentrations. Our experiments in adipocytes suggest that there is. Phenanthroline attenuated the effects of amino acids on p70S6K phosphorylation that were mediated by mTOR. If there were not sufficient zinc to interact with mTOR, this would not be expected. Similarly, adding zinc to freshly isolated adipocytes increased mTOR signaling by amino acids, as reflected in increased p70S6K phosphorylation. These observations raise the possibility that mTOR may be affected by diurnal changes in zinc metabolism, intake, or excretion. Further studies will be required to evaluate this idea; it is noteworthy, however, that diabetes mellitus is associated with hypozincemia and decreased insulin sensitivity (9, 14, 58).

Estimates of both extracellular and intracellular concentrations of zinc are available, and these also support the view that the range of zinc concentrations over which Zn^{2+} affected mTOR in vitro in our experiments is physiologically relevant. The circulating plasma concentration of zinc in Sprague-Dawley rats fed rat chow ad libitum is 25 μM (68); at least one-half of this is thought to be bound. This is consistent with estimates of interstitial zinc concentrations that range from 10 to 40 μM (65). In liver cells, total extractable zinc ranged from 264 to 415 μmol/kg wet wt in fasting and fed states (4), ~300–450 μM. Assuming that 70–80% of that intracellular zinc was tightly bound (i.e., chelator insensitive, see Ref. 18), that still leaves micromolar concentrations of zinc available to interact with mTOR. In Erlich and Yoshida cells, “labile” Zn^{2+} was determined to be ~100 μM (1). These values are within the range of concentrations we used in in vitro experiments. There is no widely accepted method for measuring free intracellular zinc that is as widely accepted as the methods for measuring intracellular free calcium. At least one limitation is the selectivity of fluorescent probes for zinc over other heavy metals. Caveats aside, a limited number of estimates of the intracellular free zinc concentration have been attempted. Resting intracellular Zn^{2+} concentration in human lymphocytes, rat hepatocytes, splenocytes, and rat thymocytes ranged from ~1 to >100 μM, with the highest and lowest values being observed in hepatocytes (4, 12, 70). The average value seems to be at or a little below the rat plasma zinc concentration, 25 μM.

In summary, it is conceivable that zinc may achieve sufficient intracellular concentrations in vivo to affect mTOR signaling and that the effects of zinc that we observed in in vitro experiments may be physiologically relevant. Thus treatment of adipocytes with 1,10-phenanthroline may impair amino acid-stimulated phosphorylation of p70S6K by removing the divalent zinc that is required for maximal mTOR activity. Additionally, because insulin activation of p70S6K is rapamycin sensitive (23, 47, 48, 63, 64, 66), some of the effects of heavy metal chelation on insulin-stimulated phosphorylation of p70S6K may be at the level of both mTOR and PTPase (Fig. 7).

Determination of the molecular site at which zinc stimulates mTOR will require further study. Because only a partial crystal structure of mTOR is available...
and because the protein kinase mechanism of this class of kinases has not been determined, it is difficult to speculate reasonably on possible locations where zinc may be exerting its stimulatory effect. Zinc may bridge any number of neighboring histidines in mTOR's tertiary structure that provide a configuration promoting optimal activity. In PKC, zinc is coordinated by cysteine-rich motifs (54) similar to those seen in GAL4, TFIIIA, and the glucocorticoid receptor; however, such motifs are not found in mTOR. On the other hand, it is noteworthy that both rat and human mTOR contains a discrete zinc-binding region signature consensus site (ILWHMEMWHEG) like that found in neutral zinc metalloproteases. This putative zinc-binding site occurs in the primary sequence at amino acids 2021–2030. Notably, this region is between the phosphatidylinositol-specific phospholipase X-box domain profile (amino acids 1921–1935) and the PI 3- and 4-kinase signature domains 1 and 2 (regions including amino acids 2186–2408). This is close to S2035, mutation of which can confer rapamycin resistance (8). Thus the putative zinc-binding region signature consensus site in mTOR is well placed to potentially regulate the protein kinase activity of mTOR and should probably be considered a potential candidate for investigations into the location of the zinc binding site that regulates mTOR activity. Finally, on the basis of the results of the present study, it can be recommended that micromolar zinc be added as an ingredient in future mTOR protein kinase assays.

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