THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 2, Issue of January 12, pp. 952–956, 2001
Printed in U.S.A.
© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

Regulation of Glycogen Synthesis by Amino Acids in Cultured Human Muscle Cells*

Received for publication, June 5, 2000, and in revised form, September 6, 2000
Published, JBC Papers in Press, September 29, 2000, DOI 10.1074/jbc.M004812200

Jane L. Armstrong‡, Sylvie M. Bonavaud, Barry J. Toole, and Stephen J. Yeaman§

From the School of Biochemistry and Genetics, Medical School, University of Newcastle, Newcastle Upon Tyne NE2 4HH, United Kingdom

Insulin and a number of metabolic factors stimulate glycogen synthesis and the enzyme glycogen synthase. Using human muscle cells we find that glycogen synthesis is stimulated by treatment of the cells with lithium ions, which inhibit glycogen synthase kinase 3. Insulin further stimulates glycogen synthesis in the presence of lithium ions, an effect abolished by wortmannin and rapamycin. We report also that amino acids stimulate glycogen synthesis and glycogen synthase, these effects also being blocked by rapamycin and wortmannin. Amino acids stimulate p70^{s6k} and transiently inhibit glycogen synthase kinase 3 without effects on the activity of protein kinase B or the mitogen-activated protein kinase pathway. Thus, the work reported here demonstrates that amino acid availability can regulate glycogen synthesis. Furthermore, it demonstrates that glycogen synthase kinase 3 can be inactivated within cells independent of activation of protein kinase B and p90^{rsk}.

One of the key actions of insulin in the lowering of blood glucose is the stimulation of glycogen synthesis in skeletal muscle, involving activation of the enzyme glycogen synthase (GS)[1] (1). Phosphorylation decreases GS activity, and the extent of this inactivation depends on the sites phosphorylated and on the concentrations of allosteric effector molecules such as glucose-6-phosphate. Activation of GS by insulin results from dephosphorylation principally at a cluster of 3 serine residues collectively termed "site 3" located at the carboxyl terminus of GS (2). Further evidence indicates that the phosphorylation state of site 2 (or 2a or both) is also decreased in response to insulin (3). Phosphorylation at site 3 is catalyzed principally by glycogen synthase kinase 3 (GSK-3), and dephosphorylation is catalyzed by the glycogen-bound form of protein phosphatase 1 (PP1; Refs. 1, 4). Both PP1 and PP2A can dephosphorylate sites 2 and 3 *in vitro* (2), and there is evidence for a mechanism of the glycogen-bound form of PP1 activation by insulin that is mediated by p90^{rsk} (5, 6). This provides an attractive theory for insulin exerting this stimulatory effect on GS by activating the glycogen-bound form of PP1. Recent evidence, however, has questioned this theory. Of particular significance is the finding that PD98059, which blocks activation of p90^{rsk} (7), has no effect on insulin stimulation of glycogen synthesis and GS in various cell types (8–10).

Attention has since focused on the effects of insulin on GSK-3. GSK-3 is inactivated in response to insulin or growth factors by phosphorylation at a single serine residue close to its amino terminus (11, 12). Three insulin-stimulated kinases, namely p70^{s6k}, p90^{rsk}, and protein kinase B (PKB), have been shown to phosphorylate and inactivate GSK-3 *in vitro* (12, 13). However, agents that prevent the activation of both p70^{s6k} and p90^{rsk} by insulin do not block the inactivation of GSK-3, strongly suggesting that the kinase acting under these conditions is PKB (14).

Despite accumulating evidence for a key role of PKB and GSK-3 in controlling GS, there is evidence for one or more alternative pathways contributing to the effect of insulin. First, GSK-3 does not phosphorylate GS at site 2, which is also dephosphorylated in response to insulin (3). Second, use of the immunosuppressive drug rapamycin, which prevents the activation of p70^{s6k} (15, 16), has provided evidence for contribution of a rapamycin-sensitive pathway to the activation of glycogen synthesis and GS by insulin in skeletal muscle, adipocytes, and hepatoma cells (10, 17–19). In human muscle cells in culture, the activation of glycogen synthesis and GS by both insulin and epidermal growth factor is partly blocked by preincubation of the cells with rapamycin, which, however, is without effect on the inactivation of GSK-3 by these hormones (10, 20). This suggests that two pathways are contributing to the activation of glycogen synthesis in this system, the phosphoinositide-dependent kinase 1-PKB-GSK-3 pathway and a rapamycin-sensitive pathway, which can act downstream of, or independent of, GSK-3.

To investigate this further, one approach is to activate the two pathways independently. Recent work has established that activation of p70^{s6k} can be regulated by the availability of amino acids. Amino acid starvation causes inactivation of p70^{s6k}, which can be reversed by the readdition of amino acids, a process that is rapamycin sensitive and that does not involve activation of PKB or p90^{rsk} (21–23). Using this approach, we report here that the rate of glycogen synthesis and the activity of GS are increased in response to amino acids, indicating that the p70^{s6k} pathway can control these processes independent of other signaling pathways.

EXPERIMENTAL PROCEDURES

Materials—All tissue culture trays were from Costar (Cambridge, MA). Culture media, penicillin-streptomycin, and trypsin-EDTA were from Life Technologies (Paisley, UK). Chick embryo extract was obtained from ICN (Costa Mesa, CA). D-[U-^{14}C]Glucose (300 mCi/mmol)

* This work was supported by the British Diabetic Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Holds a studentship from the Biotechnology and Biological Sciences Research Council, United Kingdom.

§ To whom correspondence should be addressed: School of Biochemistry and Genetics, Medical School, University of Newcastle, Framlington Place, Newcastle Upon Tyne NE2 4HH, UK. Tel: 44-191-222-7433; Fax: 44-191-222-7424; E-mail: s.j.yeaman@ncl.ac.uk.

[1] The abbreviations used are: GS, glycogen synthase; GSK-3, glycogen synthase kinase 3; PP, protein phosphatase; PKB, protein kinase B; MAP, mitogen-activated protein; mTOR, mammalian target of rapamycin; MEM^S, serum-free minimum Eagle's medium salt solution containing 5.5 mM glucose; AA, amino acid.

This paper is available on line at http://www.jbc.org

This is an Open Access article under the CC BY license.

and uridine diphospho-D-[6-^{3}H]glucose (814 GBq/mmol) were from Amersham Pharmacia Biotech (Buckinghamshire, UK), and [γ-^{32}P]ATP (148 TBq/mmol) was obtained from ICN. The GSK-3 substrate phospho-eukaryotic initiation factor 2B peptide (RRAAEELDSRAGS(p)PQL; Ref. 24) was a kind gift from Prof. C. G. Proud (University of Dundee, Dundee, UK). The PKB peptide substrate Crosstide (GRPRTSSFAEG) and protein kinase A inhibitor (TTYADFIASGRTGRRNAIHD) were synthesized in the University of Newcastle Upon Tyne Facility for Molecular Biology. Wortmannin and rapamycin were from Sigma (Poole, UK). Actrapid insulin was from Novo Nordisc (Copenhagen, Denmark).

Anti-p70^{s6k}, anti-phospho (Thr^{389}) p70^{s6k}, and anti-phospho (Ser^{21} and Ser^{9}) GSK-3α and -β antibodies were obtained from New England Biolabs (Beverly, MA). Anti-GSK-3α and anti-PKBα-(PH domain) antibodies were obtained from Upstate Biotechnology (Lake Placid, NY).

Cell Culture—Human myoblasts were grown from needle biopsy samples taken from the gastrocnemius muscle of healthy subjects with no family history of type 2 diabetes and with normal glucose tolerance and normal insulin sensitivity as assessed using the short insulin tolerance test. Myoblasts were maintained in growth medium consisting of Ham's F-10 nutrient mixture containing 20% fetal calf serum, 1% chick embryo extract, 100 units/ml penicillin, and 100 μg/ml streptomycin. All experiments were performed using cells between the 5th and 15th passage at >90% confluence. Before hormone treatment, cells were incubated for at least 2 h in serum-free Ham's F-10 medium or serum-free minimum Eagle's medium (MEM) salt solution containing 5.5 mM glucose (MEM^S). For amino acid readdition experiments MEM^S was replaced with MEM^S plus amino acid (AA) medium containing 1× or 2× concentrations of amino acids. The concentrations of amino acids designated 1× are as follows (in mg/liter): L-Arg, 126.4; L-Cys, 24.02; L-Glu, 292; L-Ile, 52.46; L-Leu, 52.46; L-Lys, 73.06; L-Met, 14.92; L-Phe, 33.02; L-Thr, 47.64; L-Trp, 10.2; L-Tyr, 36.22; L-Val, 46.86; L-Ala, 8.9; L-Asn, 13.2; L-Asp, 13.3; L-Gln, 14.7; L-Gly, 7.5; L-Pro, 11.5; and L-Ser, 10.5.

Estimation of Glycogen Synthesis—Glycogen synthesis was determined as [^{14}C]glucose incorporation into glycogen over 1 h, as described previously (20). Results are expressed as picomoles of glucose incorporated into glycogen per minute per milligram of protein.

Assay of Glycogen Synthase—After the indicated treatments, cells were rapidly washed three times with ice-cold phosphate-buffered saline and collected, by scraping, into GS extraction buffer (10 mM Tris-HCl, pH 7.8, 150 mM KF, 15 mM EDTA, 60 mM sucrose, 1 mM 2-mercaptoethanol, 10 μg/ml leupeptin, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride). Cells were then disrupted by briefly sonicating using a Soniprep 150. Glycogen synthase activity was determined in whole lysates as the incorporation of [^{3}H]glucose from uridine-5′-diphosphate [U-^{3}H]glucose into glycogen, as described previously (25). Samples were incubated with reaction mixture (50 mM Tris-HCl, pH 7.8, 20 mM EDTA, 25 mM KF, 1% glycogen, 0.4 mM UDP-[^{3}H]glucose (specific activity, 3000 dpm/nmol), containing either 0.1 mM (active) or 10 mM (total) glucose-6-phosphate) for 30 min at 30 °C. Results are expressed as fractional activities (active/total).

Preparation of Myoblast Extracts—After incubation with appropriate hormones, growth factors, or other agents, cells were washed three times with ice-cold phosphate-buffered saline, and excess liquid was removed. Cells were then scraped into extraction buffer (100 mM Tris-HCl, pH 7.4, 100 mM KCl, 2 mM EDTA, 25 mM KF, 0.1% (v/v) Triton X-100, 1 mM benzamidine, 0.1 mM Na_3VO_4, 1 μg/ml pepstatin, 1 μg/ml antipain, and 1 μg/ml leupeptin), transferred to 1.5-ml Eppendorf tubes, and immediately frozen in liquid nitrogen. Before analysis, samples were thawed and dispersed by sonication for 1 min (Sonibath; Dawe). The protein concentration was determined by a dye binding method (26).

Western Blot Analysis—Cell extracts were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes for 2.5 h at 250 mA in 10 mM 3-[cyclohexlamino]-1-propanesulphonic acid (pH 11.0) containing 10% methanol using a Hoefer minigel transfer unit. Membranes were probed with anti-GSK-3α and -β or anti-p70^{s6k} phospho-specific antibodies (1:1000) or with anti-GSKα (0.5 μg/ml) antibodies. Membranes were then incubated with anti-rabbit or anti-sheep peroxidase-labeled secondary antibodies (1:2000) and visualized using enhanced chemiluminescence.

Determination of GSK-3 and PKB Activities—GSK-3 activity was determined as described previously (27) with modifications. Samples were assayed in a final volume of 50 μl containing 10 μl of total cell extract (~10 μg of protein) and 40 μl of assay mixture (5 μM protein kinase A inhibitor, 0.1 mM EGTA, 0.1 mM [γ-^{32} P]ATP (~500 cpm/pmol), 10 mM MgAc, 50 mM Tris-HCl, pH 7.5, 0.1% 2-mercaptoethanol or 0.2 mg/ml GSK phospho-eIF2B peptide, 50 mM β-glycerophosphate, pH 7.5, in the presence or absence of 50 mM LiCl). After a 15-min incubation at 30 °C, 40 μl were spotted onto Whatman P81 phosphocellulose paper squares. After washing in 175 mM phosphoric acid with four changes, the papers were dried, and phosphate incorporation was determined by liquid scintillation counting. Enzyme activity was defined as that which catalyzes the incorporation of 1 nmol of phosphate into peptide substrate in 1 min. GSK-3 activity is that in the absence of Li^+ minus that in its presence. PKBα was assayed after immunoprecipitation as described previously (28).

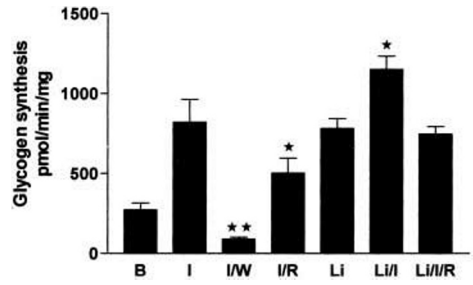

FIG. 1. **Effect of inhibitors on the regulation of glycogen synthesis by insulin and lithium.** Myoblasts were incubated in serum-free Ham's F-10 medium for 2 h before incubation with radioactive [U-^{14}C]glucose for 1 h in the absence (*B*) or presence of 100 nM insulin (*I*) or 50 mM lithium (*Li*) with no inhibitors present or including 100 nM wortmannin (*W*) or 100 nM rapamycin (*R*). Extracts were prepared, and the quantity of radioactive glucose incorporated into glycogen was determined. Results are mean ± S.E., at least $n = 4$, from at least three subjects. Values are expressed as picomoles per minute per milligram of protein. Statistical significance compared with the value in the presence of insulin is indicated by *one star* ($p < 0.05$) or *two stars* ($p < 0.01$).

RESULTS

In initial experiments, the effect of the inhibitors wortmannin, rapamycin, and lithium on insulin-stimulated glycogen synthesis was investigated in cultured human myoblasts (Fig. 1). Insulin stimulated glycogen synthesis from extracellular glucose by ~3-fold. This activation was completely blocked by wortmannin, a selective inhibitor of phosphatidylinositol 3-kinase, whereas only 40% inhibition was observed with rapamycin, which prevents the activation of p70^{s6k}. Rapamycin alone had little effect on basal glycogen synthesis, decreasing it by ~10% (data not shown). Wortmannin alone, however, induced a decrease of ~70%, implying that a wortmannin-sensitive mechanism is involved in basal glycogen synthesis. This is consistent with previous work from this laboratory (10, 20).

Work from several laboratories has indicated that the inactivation of GSK-3 is primarily responsible for dephosphorylation and activation of GS in response to insulin (10–12, 29, 30). Lithium is an allosteric inhibitor of GSK-3, mimicking the effect of insulin without affecting the activity of the mitogen-activated protein (MAP) kinase pathway (31). Treatment of cells with lithium chloride for 1 h caused a 3-fold increase in basal glycogen synthesis (Fig. 1), whereas no significant difference was observed in cells treated with an equal concentration of sodium chloride to correct for osmolarity changes (data not shown). This increase was not reduced significantly by wortmannin or by rapamycin (data not shown). In the presence of lithium, insulin stimulated glycogen synthesis a further 1.5-fold, an effect completely blocked by rapamycin (Fig. 1) or wortmannin (data not shown). Taken together, these data provide further support for a key role of GSK-3 and the presence of a rapamycin-sensitive, insulin-stimulated pathway in the control of glycogen synthesis.

To investigate the role of this rapamycin-sensitive pathway further, amino acids were used to activate p70^{s6k} selectively (21–23), and the activity of GS and glycogen synthesis was measured (Fig. 2). Myoblasts starved of amino acids for 5 h were then incubated in MEM^S containing 2× concentrations of

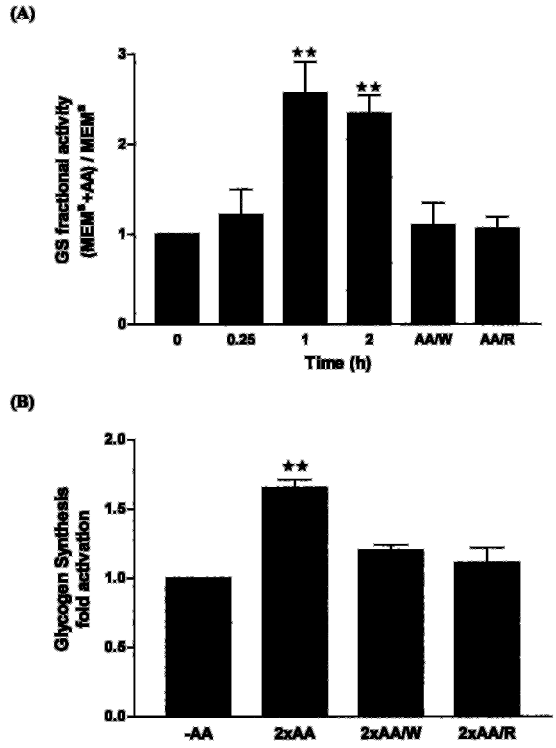

FIG. 2. **Effect of inhibitors on the stimulation of glycogen synthase and glycogen synthesis by amino acids.** Myoblasts were incubated in the MEM^S for 5 h before replacement of media with MEM^S (*−AA*) or MEM^S plus 2× AA (*2×AA*) or after preincubation with 100 nM wortmannin (*2×AA/W*), or 100 nM rapamycin (*2×AA/R*). The effects of inhibitors on GS were determined after 1 h, and those on glycogen synthesis were determined after 2 h. *A*, cell extracts were prepared, and GS activity was determined as incorporation of [^{14}C]glucose from UDP-[U-^{14}C]glucose into glycogen in the presence of high and low glucose-6-phosphate to give active and total activities. Fractional activity at time zero is ~0.015. *B*, radioactive [U-^{14}C]glucose was incorporated for the final hour of this incubation. Extracts were prepared, and the quantity of radioactive glucose incorporated into glycogen was calculated. Results are mean ± S.E., $n = 8$, from at least three subjects. Values are expressed as -fold activation over basal (*−AA*). Basal values were ~400 pmol · min^{-1} · mg^{-1}. Statistical significance compared with the value in the absence of amino acids is indicated by *two stars* ($p < 0.01$).

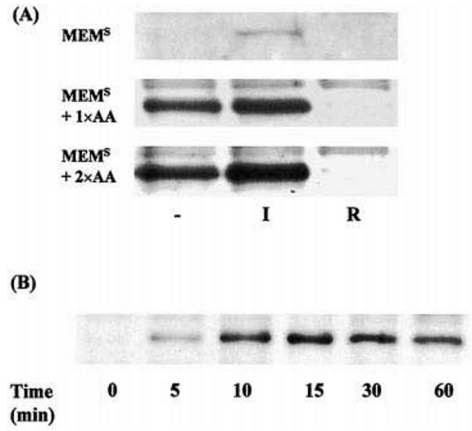

FIG. 3. **Effects of insulin and rapamycin on p70^{s6k} phosphorylation in cells cultured in the absence or presence of amino acids.** Myoblasts were incubated in MEM^S for 5 h before replacement of media with MEM^S or MEM^S plus 1× AA or MEM^S plus 2× AA for 2 h in the absence or presence of 100 nM rapamycin (*R*) or insulin (*I*; *A*) or MEM^S plus 2× AA for the times indicated (*B*). Total cell extracts were standardized for protein concentration and were separated in a 10% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and blotted with anti-phospho-p70^{s6k} (Thr^{389}) antibody. Similar results were obtained in four independent experiments from at least three subjects.

amino acids for various times. GS activity was measured, and the values are shown as a ratio of the GS fractional activity in the presence over the absence of amino acids in the culture medium. GS activity increased 2.5- and 2.0-fold in response to 2× concentrations of amino acids for 1 and 2 h, respectively. Glycogen synthesis increased ~1.5-fold in response to 2× concentrations of amino acids for 2 h. Both of these responses were completely inhibited by wortmannin and rapamycin. PD98059 had no significant effect on the amino acid stimulation, inhibiting basal and stimulated values to similar extents (data not shown).

To evaluate a possible role for p70^{s6k} in the stimulation of glycogen synthesis, the phosphorylation of this protein was measured under identical conditions. Fig. 3*A* shows that after 5 h of amino acid deprivation, readdition of 1× or 2× concentrations of amino acids resulted in the phosphorylation of p70^{s6k} at Thr^{389}, the phosphorylation of which is most closely related to the activation of p70^{s6k} *in vivo* (32). This phosphorylation was further increased in response to insulin. This phosphorylation in response to amino acids was completely blocked by rapamycin, indicating that the amino acid activation of p70^{s6k} involves the mammalian target of rapamycin (mTOR). A threshold level of amino acids is also required for the insulin-stimulated phosphorylation of p70^{s6k}, because the increase in phosphorylation in response to insulin was reduced by ~72% in cells incubated in the absence of amino acids compared with cells in the presence of amino acids (Fig. 3*A*), consistent with earlier findings (23, 33). Phosphorylation of p70^{s6k} in response to amino acids is time dependent, with a significant increase in phosphorylation appearing after 10 min and persisting for at least 2 h (Fig. 3*B*). In all experiments endogenous levels of p70^{s6k} protein did not change, as determined by immunoblotting (data not shown).

To investigate whether amino acid deprivation and readdition were affecting GSK-3, its activity and phosphorylation status were established under identical conditions. Fig. 4 shows that GSK-3 was inactivated by ~30% in response to amino acids, an effect seen within 10 min of amino acid addition and which was reversed after 30 min. Following a recent publication showing the inactivation of GSK3 in response to exercise without corresponding phosphorylation (34), the phosphorylation status of GSK-3 was also measured. Corresponding Western blots show a direct correlation of inactivation with phosphorylation (Fig. 4). During this experiment there was no significant change in GSK-3 protein expression. Although these effects are consistent with p70^{s6k} causing inactivation of GSK-3 and activation of GS, it is important to exclude the involvement of PKB or the MAP kinase pathway. Importantly, there was no significant change in PKB activity under matching conditions (Fig. 5), and the phosphorylation status of MAP kinase did not alter after amino acid withdrawal and readdition (data not shown). Wortmannin completely blocked the amino acid-induced inactivation and phosphorylation (Fig. 6) of GSK-3, whereas PD98059 was without effect (data not shown). Rapamycin essentially blocks the amino acid-induced phosphorylation and inactivation of GSK-3 (Fig. 6), indicating that the mTOR-p70^{s6k} pathway is principally responsible for the effects on GSK-3.

DISCUSSION

Insulin stimulates glycogen synthesis by both increasing glucose uptake and activating GS. Stimulation of GS by insulin involves the net dephosphorylation of specific serine residues, termed sites 2 and 3 (35). Inactivation of GSK-3 has been shown to be principally responsible for dephosphorylation of site 3, but the factor regulating site 2 dephosphorylation re-

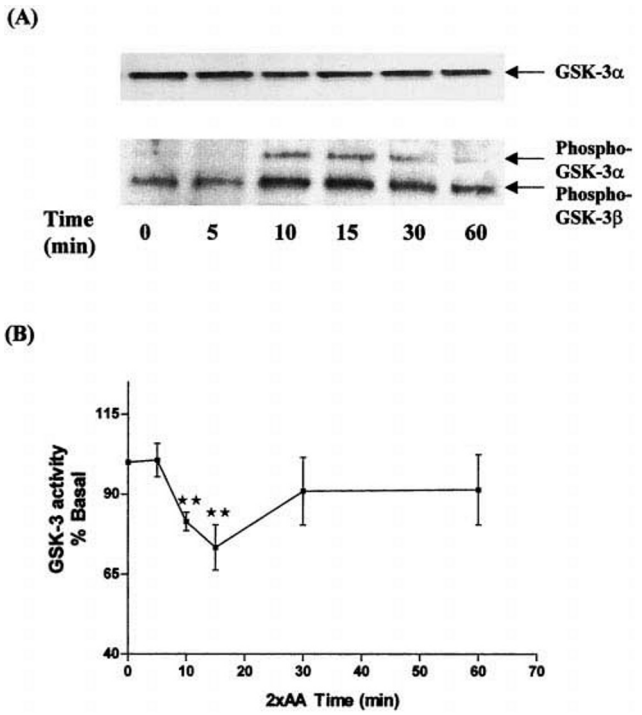

FIG. 4. **Effect of amino acids on GSK-3 activity.** Myoblasts were incubated in MEM^S for 5 h before replacement of media with MEM^S plus 2× AA for the times indicated. *A*, total cell extracts were standardized for protein concentration and were separated in a 10% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and blotted with anti-GSK-3α or anti-phospho GSK-3α and -β (Ser^{21} and Ser^9) antibodies. Similar results were obtained in four independent experiments from at least three subjects. *B*, total cell extracts were assayed for GSK-3 activity. Results are mean ± S.E., $n = 5$, from at least three subjects. Values are expressed as percent basal activity (time = 0). Basal values were ~180 pmol · min^{-1} · mg^{-1}. Statistical significance compared with the basal value is indicated by *two stars* ($p < 0.01$).

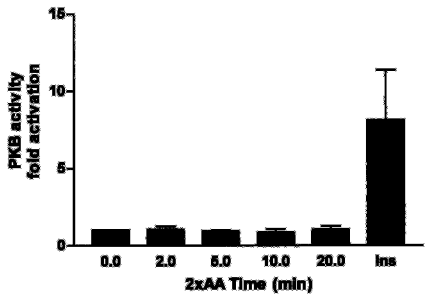

FIG. 5. **Effect of amino acids on PKB activity.** Myoblasts were incubated in MEM^S for 5 h before replacement of media with MEM^S plus 2× AA for the times indicated or treated with 100 nM insulin for 10 min (*I*). Total cell extracts were subject to immunoprecipitation with antibodies to PKBα and assayed for PKB activity. Results are mean ± S.E., $n = 3$, from two subjects. Values are expressed as percent basal activity (time = 0), which was ~1 pmol · min^{-1} · mg^{-1}.

mains unidentified. Glycogen synthesis can also be stimulated in an insulin-independent manner, for example, after exercise, a process associated with glycogen depletion (36). After glycogen depletion in cultured human muscle cells, readdition of glucose leads to the stimulation of GS by an as yet unknown mechanism but one that apparently does not involve phosphorylation and inactivation of GSK-3.[2] Therefore, the role of GSK-3 in controlling glycogen synthesis was further investigated using its allosteric inhibitor lithium ions. Incubation of

[2] J. L. Armstrong, S. M. Bonavaud, R. Halse, and S. J. Yeaman, unpublished data.

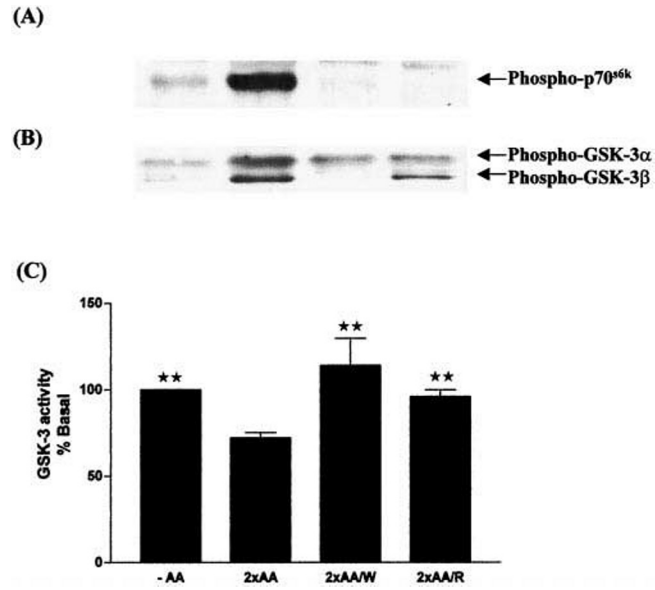

FIG. 6. **Effect of selective inhibitors on the activity and phosphorylation of GSK-3 and $p70^{s6k}$ by amino acids.** Myoblasts were incubated in MEM^S for 5 h before further incubation for 15 min in MEM^S (−*AA*) or MEM^S plus 2× AA (2×*AA*). Alternatively, cells were treated with 100 nM wortmannin (2×*AA/W*) or 100 nM rapamycin (2×*AA/R*) for 15 min before the addition of MEM^S plus 2× AA containing these inhibitors for 15 min. Total cell extracts were standardized for protein concentration and were separated in a 10% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and blotted with anti-phospho-$p70^{s6k}$ (Thr^{389}) antibodies (*A*) or anti-phospho GSK-3α and -β (Ser^{21} and Ser^9) antibodies (*B*). Similar results were obtained in four independent experiments from at least three subjects. Extracts were assayed for GSK-3 activity (*C*). Results are mean ± S.E., $n = 10$, from three subjects. Values are expressed as percent basal activity (time = 0). Statistical significance compared with the value in the presence of 2× AA is indicated by *two stars* ($p < 0.01$).

human myoblasts with lithium resulted in a significant increase in glycogen synthesis in these cells, which is consistent with a major role for GSK-3 in controlling glycogen synthesis. However, the addition of insulin resulted in a further, rapamycin-sensitive increase in glycogen synthesis, indicating a contribution by an alternative insulin- and rapamycin-sensitive pathway. The obvious candidate is the $p70^{s6k}$ signaling pathway, which fulfills both these criteria and for which we and others have obtained evidence previously (10, 17–19). To investigate this further we have used the findings that it is possible to activate $p70^{s6k}$ in a rapamycin-sensitive manner using amino acids, without the parallel activation of PKB or MAP kinase that occurs with insulin stimulation (21–23). We show here that amino acid depletion and subsequent readdition also lead to the stimulation of GS and glycogen synthesis (Fig. 2, *A* and *B*), effects that are completely blocked by both wortmannin and rapamycin. A key question is whether this activation involves inhibition of GSK-3. As shown in Fig. 4, GSK-3 is phosphorylated and inactivated in response to amino acids.

Three kinases, namely $p70^{s6k}$, $p90^{rsk}$, and PKB, have been shown to phosphorylate and inactivate GSK-3 *in vitro* (12, 13). Importantly, the present work and previous data (21–23) show that amino acids failed to activate PKB or the MAP kinase pathway. However, $p70^{s6k}$ is phosphorylated and presumably activated by amino acids (Fig. 3). Furthermore, GSK-3 inhibition is completely blocked by both wortmannin and rapamycin, implying that the $p70^{s6k}$ pathway is primarily responsible for the inactivation of GSK-3 in response to amino acids. Very recent work on amino acid transport in rat L6 myotubes has shown that leucine inactivates GSK-3 in a rapamycin-sensitive manner without activation of either PKB or the MAP kinase pathway (37).

The transient nature of the inhibition of GSK-3 (maximal after 15 min) does not coincide with the prolonged activation of GS and $p70^{s6k}$, which persists up to 2 h (Fig. 2*A*). It is possible that transient inactivation of GSK-3 triggers the activation of GS, which could then be maintained by a further rapamycin-sensitive pathway, perhaps acting on GS without involvement of GSK-3. This is supported by the data in Fig. 1, which indicate that when GSK-3 is inhibited by lithium ions, insulin can further stimulate glycogen synthesis in a rapamycin-sensitive manner. This would be consistent also with earlier work on insulin effects in human muscle cells (10, 20) and with work (38) that showed that rapamycin has no effect on the activation of GS in skeletal muscle up to 5 min after stimulation by insulin but has a significant inhibitory effect at longer time points.

The amino acid-stimulated activation of $p70^{s6k}$ observed in the human muscle cell lines is wortmannin and rapamycin sensitive. Rapamycin acts by associating with a cellular protein, FKBP12, and this complex then binds to mTOR, thus inhibiting its kinase activity. This would therefore indicate that mTOR is required for the response to amino acids, either directly or through the regulation of a downstream element, such as $p70^{s6k}$ or a protein phosphatase. Although mTOR has been shown to phosphorylate $p70^{s6k}$ directly *in vitro* (39), recent evidence points to the existence of a phosphatase, which is activated by rapamycin, via the inhibition of mTOR (23, 40). Phosphatidylinositol 3-kinase is also implicated because of its wortmannin sensitivity, but it has previously been reported that wortmannin can selectively inhibit mTOR directly by binding to the catalytic domain at concentrations of 0.1–1 μM (41, 42). Therefore, it is possible that phosphatidylinositol 3-kinase does not play a role in this mechanism despite the wortmannin-sensitive nature of the process. It has also recently been reported that phosphoinositide-dependent kinase 1 can phosphorylate $p70^{s6k}$ *in vivo* (43), but there is no evidence to date to suggest that phosphinositide-dependent kinase 1 kinase activity is increased in response to amino acids, especially because PKB activity does not increase (Fig. 5).

Because of the sustained amino acid-stimulated activation of $p70^{s6k}$ and the sensitivity to both wortmannin and rapamycin, this leads to the reasoning that this enzyme is part of the signal mechanism involved in the amino acid-stimulated increase in GS and glycogen synthesis activities. It is important to point out that $p70^{s6k}$ itself may not play a role, and events may stem directly from mTOR. It has recently been discovered that mTOR is involved in the regulation of PP2A (40), a phosphatase known to act on GSK-3 *in vitro*. This could provide evidence to support a theory whereby GSK-3 is regulated by this phosphatase in response to amino acid availability. PP2A also has the ability to dephosphorylate GS at sites 2 and 3, leading to its activation; therefore, this phosphatase may play a coordinating role in the overall process.

In summary, the work reported here demonstrates that amino acid availability can regulate glycogen synthesis. Furthermore, it demonstrates that GSK-3 can be inactivated within cells independent of activation of PKB and $p90^{rsk}$.

Acknowledgments—We thank Dorothy Fittes for excellent technical assistance and Dr. Mark Walker and Prof. Doug Turnbull for performing the muscle biopsies.

REFERENCES

1. Cohen, P. (1993) *Biochem. Soc. Trans.* **21,** 555–567
2. Parker, P. J., Caudwell, F. B., and Cohen, P. (1983) *Eur. J. Biochem.* **130,** 227–234
3. Skurat, A. V., Dietrich, A. D., and Roach, P. J. (2000) *Diabetes* **49,** 1096–1105
4. Lawrence, J. C., and Roach, P. J. (1997) *Diabetes* **46,** 541–547
5. Dent, P., Lavoinne, A., Nakielny, S., Caudwell, F. B., Watt, P., and Cohen, P. (1990) *Nature* **248,** 302–308
6. Sutherland, C., Campbell, D. G., and Cohen, P. (1993) *Eur. J. Biochem.* **212,** 581–588
7. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) *J. Biol. Chem.* **270,** 27489–27494
8. Lazar, D. F., Wiese, R. J., Brady, M. J., Mastick, C. C., Waters, S. B., Yamauchi, K., Pessin, J. E., Cuatrecasas, P., and Saltiel, A. R. (1995) *J. Biol. Chem.* **270,** 20801–20807
9. Azpiazu, I., Saltiel, A. R., DePaoli-Roach, A. A., and Lawrence, J. C. (1996) *J. Biol. Chem.* **271,** 5033–5039
10. Hurel, S. J., Rochford, J. J., Borthwick, A. C., Wells, A. M., Vandenheede, J. R., Turnbull, D. M., and Yeaman, S. J. (1996) *Biochem. J.* **320,** 871–877
11. Stambolic, V., and Woodgett, J. R. (1994) *Biochem. J.* **303,** 701–704
12. Sutherland, C., and Cohen, P. (1994) *FEBS Lett.* **338,** 37–42
13. Sutherland, C., Leighton, I. A., and Cohen, P. (1993) *Biochem. J.* **296,** 15–19
14. Cross, D. A. E., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) *Nature* **378,** 785–789
15. Chung, J., Kuo, C. J., Crabtree, G. R., and Blenis, J. (1992) *Cell* **69,** 1227–1236
16. Brown, E. J., Beal, P. A., Keith, C. T., Chen, J., Shin, T. B., and Schreiber, S. L. (1995) *Nature* **377,** 441–446
17. Shepherd, P. R., Nave, B. T., and Siddle, K. (1995) *Biochem. J.* **305,** 25–28
18. Sung, C. K., Choi, W. S., and Scalia, P. (1998) *J. Recept. Signal. Transduct. Res.* **18,** 243–263
19. Sanchez-Margalet, V. (2000) *J. Cell. Physiol.* **182,** 182–188
20. Halse, R., Rochford, J. J., McCormack, J. G., Vandenheede, J. R., Hemmings, B. A., and Yeaman, S. J. (1999) *J. Biol. Chem.* **274,** 776–780
21. Iiboshi, Y., Papst, P. J., Kawasome, H., Hosoi, H., Abraham, R. T., Houghton, P. J., and Terada, N. (1999) *J. Biol. Chem.* **274,** 1092–1099
22. Wang, X., Campbell, L. E., Miller, C. M., and Proud, C. G. (1998) *Biochem. J.* **334,** 261–267
23. Hara, K., Yonezawa, K., Weng, Q.-P., Kozlowski, M. T., Belham, C., and Avruch, J. (1998) *J. Biol. Chem.* **273,** 14484–14494
24. Welsh, G. I., Patel, J. C., and Proud, C. G. (1997) *Anal. Biochem.* **244,** 16–21
25. Guinovart, J. J., Salavert, A., Massague, J., Ciudad, C. J., Salsas, E., and Itarte, E. (1979) *FEBS Lett.* **106,** 284–288
26. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
27. Ryves, W. J., Fryer, L., Dale, T., and Harwood, A. J. (1998) *Anal. Biochem.* **264,** 124–127
28. Walker, K. S., Deak, M., Paterson, A., Hudson, K., Cohen, P., and Alessi, D. R. (1998) *Biochem. J.* **331,** 299–308
29. Eldar-Finkelman, H., Argast, G. M., Foord, O., Fischer, E. H., and Krebs, E. G. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 10228–10233
30. Skurat, A. V., and Roach, P. J. (1995) *J. Biol. Chem.* **270,** 12491–12497
31. Klein, P. S., and Melton, D. A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 8455–8459
32. Weng, Q.-P., Kozlowski, M., Belham, C., Zhang, A., Comb, M. J., and Avruch, J. (1998) *J. Biol. Chem.* **273,** 16621–16629
33. Campbell, L. E., Wang, X., and Proud, C. G. (1999) *Biochem. J.* **344,** 433–441
34. Markuns, J. F., Wojtaszewski, J. F. P., and Goodyear, L. J. (1999) *J. Biol. Chem.* **274,** 24896–24900
35. Skurat, A. V., Wang, Y., and Roach, P. J. (1994) *J. Biol. Chem.* **269,** 25534–25542
36. Price, T. B., Rothman, D. L., Taylor, R., Avison, M. J., Shulman, G. I., and Shulman, R. G. (1994) *J. Appl. Physiol.* **76,** 104–111
37. Peyrollier, K., Hajduch, E., Blair, A. S., Hyde, R., and Hundal, H. S. (2000) *Biochem. J.* **350,** 361–368
38. Cross, D. A., Watt, P. W., Shaw, M., van der Kaay, J., Downes, C. P., Holder, J. C., and Cohen, P. (1997) *FEBS Lett.* **406,** 211–215
39. Isotani, S., Hara, K., Tokunaga, C., Inoue, H., Avruch, J., and Yonezawa, K. (1999) *J. Biol. Chem.* **274,** 34493–34498
40. Peterson, R. T., Desai, B. N., Hardwick, J. S., and Schreiber, S. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4438–4442
41. Brunn, G. J., Williams, J., Sabers, C., Wiederrecht, G., Lawrence, J. C., and Abraham, R. T. (1996) *EMBO J.* **15,** 5256–5267
42. Withers, D. J., Ouwens, D. M., Nave, B. T., van der Zon, G. C. M., Alarcon, C. M., Cardenas, M. E., Heitman, J., Maassen, J. A., and Shepherd, P. R. (1997) *Biochem. Biophys. Res. Commun.* **241,** 704–709
43. Balendran, A., Currie, R., Armstrong, C. G., Avruch, J., and Alessi, D. R. (1999) *J. Biol. Chem.* **274,** 37400–37406