Branched-chain Amino Acids Are Essential in the Regulation of PHAS-I and p70 S6 Kinase by Pancreatic β-Cells

A POSSIBLE ROLE IN PROTEIN TRANSLATION AND MITOGENIC SIGNALING*

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Amino acids have been identified as important signaling molecules involved in pancreatic β-cell proliferation, although the cellular mechanism responsible for this effect is not well defined. We previously reported that amino acids are required for glucose or exogenous insulin to stimulate phosphorylation of PHAS-I (phosphorylated heat- and acid-stable protein regulated by insulin), a recently discovered regulator of translation initiation during cell mitogenesis. Here we demonstrate that essential amino acids, in particular branched-chain amino acids (leucine, valine, and isoleucine), are largely responsible for mediating this effect. The transamination product of leucine, α-ketoisocaproic acid, also stimulates PHAS-I phosphorylation although the transamination products of isoleucine and valine are ineffective. Since amino acids are secretagogues for insulin secretion by β-cells, we investigated whether endogenous insulin secreted by β-cells is involved. Interestingly, branched-chain amino acids stimulate phosphorylation of PHAS-I independent of endogenous insulin secretion since genistein (10 μM) and herbimycin A (1 μM), two tyrosine kinase inhibitors in the insulin signaling pathway, exert no effect on amino acid-induced phosphorylation of PHAS-I. Furthermore, branched-chain amino acids retain their ability to induce phosphorylation of PHAS-I under conditions that block insulin secretion from β-cells. In exploring the signaling pathway responsible for these effects, we find that rapamycin (25 nM) inhibits the ability of branched-chain amino acids to stimulate the phosphorylation of PHAS-I and p70S6 kinase, suggesting that the mammalian target of rapamycin signaling pathway is involved. The branched-chain amino acid, leucine, also exerts similar effects on PHAS-I phosphorylation in isolated pancreatic islets. In addition, we find that amino acids are necessary for insulin-like growth factor (IGF-I) to stimulate the phosphorylation of PHAS-I indicating that a requirement for amino acids may be essential for other β-cell growth factors in addition to insulin and IGF-I to activate this signaling pathway. We propose that amino acids, in particular branched-chain amino acids, may promote β-cell proliferation either by stimulating phosphorylation of PHAS-I and p70S6k via the mammalian target of rapamycin pathway and/or by facilitating the proliferative effect mediated by growth factors such as insulin and IGF-I.

Nutrients, in particular, amino acids have been recognized as important signaling mediators in pancreatic β-cell function. In addition to modulating insulin secretion by β-cells and glucagon secretion by α-cells, amino acids have been identified as important signaling agents in the initiation and modulation of β-cell proliferation. Previous studies by Swenne (1, 2) have demonstrated that increases in essential amino acids significantly enhance [3H]thymidine incorporation into DNA by pancreatic islets. Although amino acids alone and in combination with insulin, insulin-like growth factor (IGF-I), and other growth factors are well documented to exert a mitogenic effect on both adult and fetal islets, the cellular mechanism responsible for this effect is not well defined.

Insulin and other growth factors induce increases in protein synthesis, in part, by stimulating mRNA translation. The regulation of protein synthesis is believed to be central to cell replication since re-entry of cells into the cell cycle in response to growth factors and nutrients is accompanied by an approximate doubling of protein synthesis before the onset of DNA synthesis and cell division (3, 4). This obligatory role for an increase in the rate of protein synthesis for cells to enter and progress through the cell cycle has suggested that activation of protein translation is an important initial event in the mitogenic response.

The initiation phase of mRNA translation is generally rate-limiting for protein synthesis (see Refs. 3–6 for reviews). Initiation is mediated in part by the eIF-4F complex, which is composed of three subunits, eIF-4γ, eIF-4A, and eIF-4E (6). eIF-4γ is a large subunit (Mr = 220,000) that binds eIF-4A (Mr = 45,000) and eIF-4E (Mr = 25,000). eIF-4A is an ATP-dependent helicase, and eIF-4E is the mRNA cap-binding protein. The initiation factor, eIF-4E, is the least abundant of the eIF-4F subunits, and it is generally believed that the amount of eIF-4E is limiting for translation initiation. eIF-4E is involved in recognition of the mRNA cap, a structure of m7GpppN (N represents any nucleotide) located at the 5'-end of almost all eukaryotic mRNA translation products of leucine, valine, and isoleucine, are largely responsible for mediating this effect.

The abbreviations used are: IGF-I, insulin-like growth factor-I; eIF, eukaryotic initiation factor; PI 3-kinase, phosphoinositide 3-kinase; TOR, target of rapamycin; mTOR, mammalian target of rapamycin; KBB, Krebs-Ringer bicarbonate buffer; KIC, α-ketoisocaproic acid; KIV, α-ketoisovalerate; KMV, α-keto-β-methylvalerate; MEM, minimal essential medium; IRS, insulin receptor substrate; PHAS-I, phosphorylated heat- and acid-stable protein regulated by insulin.
otic messages. The availability of eIF-4E is regulated by PHAS-I (also designated as 4E-BP1), a heat- and acid-stable eIF-4E-binding protein first identified in rat adipocytes (7, 8). PHAS-I inhibits the binding of eIF-4E to eIF-4G (also designated as eIF-4γ), thus preventing formation of a complex necessary for efficient binding and proper positioning of the 40S ribosomal subunit of mRNA. When phosphorylated in the appropriate sites after exposure of responsive cells to insulin, PHAS-I dissociates from eIF-4E, thus allowing eIF-4E to participate in translation initiation.

Recent studies have implicated a signaling pathway involving the mammalian target of rapamycin (mTOR) as an up-stream regulator of PHAS-I phosphorylation. Rapamycin is a potent immunosuppressant and antiproliferative agent that mediates its effects through formation of an active complex with its intracellular receptor, FK506-binding protein of Mr = 12,000 (9). This rapamycin complex then binds to members of the TOR (target of rapamycin) family which include TOR1p and TOR2p in yeast and a mammalian homologue, mTOR, also designated as FRAP or RAFT1 (10, 11). In yeast and mammalian cells, activation of TOR is required for G1 progression and cell proliferation (12). Recent studies indicate that mTOR directly phosphorylates PHAS-I, although how mTOR is regulated is not yet defined (13–15). In insulin-sensitive cells, activation of the insulin receptor is believed to result in phosphorylation of insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2) which then activates phosphoinositide (PI) 3-kinase (16). New evidence supports the conclusion that insulin-stimulated phosphorylation and activation of mTOR is mediated by the protein kinase B signaling pathway (17). PHAS-I and p70S6K are located downstream in this pathway and are proposed to be regulated by mTOR in a parallel manner (11, 18, 19). This signaling pathway can be blocked by inhibiting PI 3-kinase with wortmannin, although higher concentrations of wortmannin also inhibit mTOR. Phosphorylation and activation of p70S6K mediates ribosomal protein S6 phosphorylation and may be essential during the G1 phase of the cell cycle (18, 19). Overall, these studies suggest that PHAS-I and p70S6K exert important roles in growth-regulated protein translation in a variety of cells.

Our studies have recently shown that incubation of pancreatic islets with elevated glucose levels results in rapid and concentration-dependent phosphorylation of PHAS-I (20). This effect is due, in part, to insulin released into the incubation medium, since exogenous insulin increases whereas conditions that block insulin exocytosis from the β-cell inhibit phosphorylation of PHAS-I. In addition, conditions that stimulate insulin secretion from the β-cell lines, βTC6-F7 and βTC3, increase phosphorylation of PHAS-I, suggesting that β-cells of the islet, in part, mediate this effect. These findings indicate that glucose stimulates PHAS-I phosphorylation via insulin interacting with its own receptor on the β-cell which may serve as an important mechanism for autoregulation of protein synthesis by translation. During the course of these studies, we discovered that amino acids are required for glucose or exogenous insulin to stimulate the phosphorylation of PHAS-I. Furthermore, amino acids alone dose-dependently stimulate phosphorylation of PHAS-I in a rapamycin-sensitive manner which is further enhanced by insulin. We have therefore examined in the present study the signal transduction pathways whereby amino acids, in particular branched-chain amino acids, alone and in synergy with insulin and other growth factors activate the mTOR signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Male Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Collagenase type P was obtained from Boehringer Mannheim. CMRL-1066 and RPMI 1640 tissue culture media, penicillin, streptomycin, Hanks’ balanced salt solution, t-glutamine, MEM amino acids solution, and MEM non-essential amino acids solution were obtained from Life Technologies, Inc. Fetal bovine serum was from HyClone (Logan, UT). Rapamycin was from Biomol (Plymouth Meeting, PA). Ficoll was from HyClone. PAN was prepared in rabbit with recombinant His-tagged rat PHAS-I (7). The secondary antibody was peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). The antibody for p70S6K was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were from commercially available sources.

**Amino Acid Composition**—Krebs-Ringer bicarbonate buffer (KRBB): 25 mM HEPES, 115 mM NaCl, 25 mM NaHCO3, 5 mM KCl, 2.5 mM CaCl2, and 1 mM MgCl2 (pH 7.4, 0.1% bovine serum albumin) was supplemented with MEM amino acids solution, MEM non-essential amino acids solution, and t-glutamine. For these experiments, the 1X concentration of amino acids was defined as the following in mM: l-arginine 0.73, l-lysine 2.0, l-histidine/HCl/H2O 0.2, l-isoleucine 0.4, l-leucine 0.4, l-lysine HCl 0.5, l-phenylalanine 0.2, l-threonine 0.4, 1-tryptophan 0.05, l-tyrosine 0.2, l-valine 0.4, l-alanine 0.1, l-asparagine 0.1, l-aspartic acid 0.1, l-glutamic acid 0.1, glycine 0.1, l-proline 0.1, l-serine 0.1. The osmolarity of each solution was measured using a Wescor Vapor Pressure Osmometer (Logan, UT) and found to be between 300 and 310 mOsm/kg.

**Pancreatic β-Cell Lines—**RINm5F cells, an insulin-secreting-cell line (21, 22), were cultured by the Washington University Tissue Culture Support Center in RPMI 1640 containing 10% (v/v) heat-inactivated fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Islets were isolated from 200–250 g by collagenase digestion as described previously (23). Briefly, pancreata were inflated with Hanks’ balanced salt solution, and the tissue was isolated, minced, and digested with 7 mg of collagenase/pancreas for 7 min at 39 °C. Islets were separated on a Ficoll step density gradient and then selected with a stereomicroscope to exclude any contaminating tissues. Islets were cultured overnight in 36 °C culture medium containing 5% CO2 in a humid atmosphere of 95% air, 5% CO2 for 24 h before initiating experiments. Where “CMRL” is stated, this culture media does not contain fetal bovine serum.

**Results**

**PHAS-I and p70S6K Assays—**To achieve a quiescent state, RINm5F cells (1×106 cells/3 ml) or islets (200/1 ml) were washed free of culture media and fetal bovine serum and preincubated for 2 h at 24 or 37 °C in KRBB in the absence of glucose and amino acids. KRBB was replaced as described in the figure legends. Following experimental treatments, cells or islets were washed with phosphate-buffered saline and solubilized in 300 or 30 μl, respectively, of Laemmli sample buffer, heated at 100 °C for 5 min, and centrifuged at 10,000 × g for 15 min to remove insoluble materials. The supernatants were processed for SDS-polyacrylamide gel electrophoresis and Western blotting of PHAS-I or p70S6K

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**RESULTS**

**Essential Amino Acids Induce Phosphorylation of PHAS-I in RINm5F Cells—**Previous studies have demonstrated that phosphorylation of PHAS-I, a necessary step for the release of the rate-limiting initiation factor, eIF-4E, results in a decrease in electrophoretic mobility of this protein when separated on SDS-polyacrylamide gels (24). Thus, the hyperphosphorylated α-form of PHAS-I migrates most slowly, followed by the intermediary β-form and the nonphosphorylated α-form of PHAS-I. Under some experimental conditions in the present study, PHAS-I is detected which represents a more hyperphosphorylated form of PHAS-I than PHAS-1γ.

Our recent studies demonstrated that a complete comple-
PHAS-I in RINm5F cells. RINm5F cells were preincubated in KRBB (1x), alanine (1x), valine (1x), and glutamine (1x) as indicated for 30 min. Cells were processed for immunoblotting of PHAS-I as described under “Experimental Procedures.” Results are representative of three separate experiments.

of amino acids normally present in tissue culture medium stimulated in a concentration-dependent manner phosphorylation of PHAS-I with a maximal effect observed at 1x amino acids (20). To determine which amino acids are responsible for this effect, RINm5F cells were treated with Krebs-Ringer bicarbonate buffer (KRBB) supplemented with either a complete complement of amino acids (73%), essential amino acids (1x), or glutamine (1x) for 30 min following a 2-h preincubation in KRBB (amino acid- and glucose-free). As shown in Fig. 1, in the absence of amino acids (lane 1), only 28% of PHAS-I is phosphorylated to the level of γ. Essential amino acids (1x, lane 3) were as effective in mediating phosphorylation of PHAS-Iγ (69%) compared with a complete complement of amino acids (73%, lane 2). It is noted in Fig. 1 that the nonphosphorylated form of PHAS-I, PHAS-Iα, is not detected. PHAS-Iα does occur in RINm5F cells and intact islets, although occasionally it is not detected dependent on the degree of quiescence of the cells. Although non-essential amino acids (lane 4) and glutamine (lane 5) did cause some PHAS-Iγ phosphorylation, 30 and 40% respectively, the effect was not nearly as significant as that produced by either essential or a complete complement of amino acids. Therefore, our present studies have focused specifically in defining the role of essential amino acids in mitogenic signaling.

Since essential amino acids consist of a mixture of 12 different amino acids, we determined next if single amino acids could mediate the phosphorylation of PHAS-I. To this end, RINm5F cells were exposed to KRBB containing each essential amino acid (1 mM) for 30 min following a 2-h preincubation in KRBB (amino acid- and glucose-free). As shown in Fig. 2 (lane 1), in the absence of amino acids 30% of PHAS-I was in the γ and δ-form. A complete mixture of amino acids (1x, lane 2) resulted in 72% of PHAS-I to be in the γ and δ-form. All 12 of the minimal essential amino acids caused some increase in the percent of PHAS-Iγ and δ compared with the absence of amino acids. The branched-chain amino acids, leucine (1x, 72%), isoleucine (Ile, 65%), and valine (Val, 77%) were among the most effective in stimulating formation of PHAS-Iγ and δ (lanes 3–5), compared with the absence of amino acids (lane 1) and a complete complement of amino acids (lane 2). It is noted that valine (10 mM) also results in a significant appearance of the hyperphosphorylated form of PHAS-I, PHAS-Iδ (lane 5). Of the remaining essential amino acids (methionine, phenylala-

nine, tryptophan, threonine, tyrosine, arginine, histidine, and lysine), only tyrosine (65%) was as effective as the branched-chain amino acids (leucine, isoleucine, and valine) to induce the formation of PHAS-Iγ (lanes 6–14). We therefore decided to limit the remainder of this study to the branched-chain amino acids, although these data do not negate a role for the other amino acids in the regulation of protein translation.

To explore further the mechanism by which essential amino acids stimulate PHAS-I phosphorylation, the effects of branched-chain amino acids and their metabolites were evaluated. As shown in Fig. 3 (A and B), leucine and its transamination product, α-ketoisocaproic acid (KIC), dose-dependently mediated the increased formation of PHAS-Iγ. Leucine at concentrations of 2 mM and greater resulted in enhanced phosphorylation of PHAS-I, whereas this same effect was observed at a 10-fold lower concentration for KIC. The effects of valine, isoleucine, and their metabolites, α-ketoisovalerate (KIV) and α-keto-β-methylvalerate (K MV), respectively, were also examined on PHAS-I phosphorylation. Although both valine and isoleucine dose-dependently induced PHAS-I phosphorylation (Fig. 4, A and B), the transamination products of valine, KIV, and isoleucine, KMV, were ineffective in stimulating the phosphorylation of PHAS-I (Fig. 4C, lanes 4 and 6). These latter findings suggest that unlike the metabolite of leucine, KIC, either the transport of KIV and KMV into β-cells is impaired and/or their metabolism is not sufficient to induce phosphorylation of PHAS-I.

Branched-chain Amino Acids Stimulate Phosphorylation of PHAS-I Independent of Insulin Secreted by β-Cells—Since amino acids are secretagogues for insulin secretion by β-cells, we next determined whether endogenous insulin secreted by RINm5F cells is involved in amino acid-induced phosphorylation of PHAS-I. In the first approach, RINm5F cells were treated with KRBB-containing branched-chain amino acids (leucine, isoleucine, and valine at 3.3 mM for each amino acid) ± genistein (10 μM) or herbimycin A (1 μM), two tyrosine kinase inhibitors, with different mechanisms of action, in the upstream insulin signaling pathway (28, 29). As shown in Fig. 5A, branched-chain amino acids stimulated phosphorylation of PHAS-I (lane 3), in comparison to the absence of amino acids (lane 1) and a complete complement of amino acids (lane 2). This effect was not prevented by either genistein or herbimycin A (lanes 4 and 5). Similar results were obtained with genistein at concentrations of 1 and 100 μM (data not shown). Genistein and herbimycin A are effective tyrosine kinase inhibitors in islets and RINm5F cells. Previously we have shown that these inhibitors block interleukin-1-stimulated tyrosine kinase activation, resulting in the inhibition of NFκB translocation to the nucleus and blockade of subsequent transcription and translation of the inducible nitric oxide synthase gene by rat islets and RINm5F cells (30, 31). These data suggest that branched-chain amino acids induce phosphorylation of PHAS-I independent of the upstream insulin signaling pathway by β-cells.

To support further this possibility, RINm5F cells were incubated at 24 °C, a condition that blocks almost completely secretagogue-induced insulin exocytosis. Under conditions of reduced temperature, ion fluxes and metabolism of amino acids are minimally affected (32, 33). As shown in Fig. 5B, blocking endogenous insulin secretion from β-cells by reducing the incubation temperature from 37 °C (lanes 1–3) to 24 °C (lanes 4–6) did not attenuate the ability of branched-chain amino acids to induce PHAS-Iγ formation (compare lanes 5 and 6 with lanes 2 and 3). In these studies (Fig. 5B), insulin secretion levels in the absence of amino acids were 3.2 ± 0.1 nm at 37 °C (lane 1) and 2.1 ± 0.2 nm at 24 °C (lane 4). At a concentration of 1x amino acids, insulin levels were 6.1 ± 0.9 nm at 37 °C
Insulin secretion levels with branched-chain amino acids (10 mM) were 8.6 ± 2.2 nM at 37 °C (lane 3) and 2.5 ± 0.3 nM at 24 °C (lane 6). These results suggest that endogenous insulin secretion exerted no effect on amino acid-induced phosphorylation of PHAS-I. Taken together, these findings support the concept that phosphorylation of PHAS-I stimulated by amino acids is independent of endogenous insulin secretion by β-cells.

**Branched-chain Amino Acids Induce Phosphorylation of PHAS-I via the mTOR Pathway**—Although several studies suggest that PHAS-I is phosphorylated in cell-free preparations by a variety of kinases such as casein kinase II, protein kinase C, and mitogen-activated protein kinase (24), recent studies suggest that mTOR (mammalian target of rapamycin) is the predominant PHAS-I kinase (13–15). In addition, mTOR appears to regulate phosphorylation of PHAS-I and p70s6k in a parallel manner (11, 18, 19). In exploring the signaling pathway involved in amino acid-induced phosphorylation of PHAS-I, RINm5F cells were exposed to branched-chain amino acids (leucine, isoleucine, and valine) in the presence or absence of the mTOR inhibitor, rapamycin (25 mM), and the PI-3 kinase inhibitor, wortmannin (100 nM), for 30 min. As shown in Fig. 6, branched-chain amino acids induced the formation of PHAS-Iγ (lane 3), in comparison to the absence of amino acids (lane 1) and a complete complement of amino acids (lane 2). PHAS-1γ is also observed in lane 3 which may be attributed to the presence of valine in the branched-chain amino acid mixture. This increased phosphorylation of PHAS-I by branched-chain amino acids was blocked by both rapamycin and wortmannin (lanes 4 and 5).

The ability of amino acids to also enhance phosphorylation of p70s6k was determined by gel mobility shift assays in Fig. 6B. Both a complete complement of amino acids (1X) and a combination of the branched-chain amino acids, leucine, isoleucine, and valine, resulted in increased phosphorylation (lanes 2 and 3) of p70s6k resulting in decreased mobility. Rapamycin (25 nM) and wortmannin (100 nM) completely blocked the shift of the p70s6k band to a more slowly migrating species (lanes 4 and 5). In addition, leucine (10 mM) alone caused increased phosphorylation of p70s6k as effective as a complete complement of amino acids (1X), whereas isoleucine (10 mM) or valine (10 mM) resulted in a partial shift in mobility of p70s6k (data not shown).

**Amino Acid-induced PHAS-I and p70s6k Phosphorylation by β-Cells**

Amino Acid-induced Phosphorylation of PHAS-I in Pancreatic Islets—Studies were performed next to determine if amino acid-induced phosphorylation of PHAS-I in isolated pancreatic islets was similar to that described in the β-cell line, RINm5F. This experimental protocol was performed over an incubation period of 3 h as shown in Fig. 7A to allow for more complete uptake and oxidation of leucine by isolated islets (25, 26).
FIG. 5. Branched-chain amino acids (leucine, isoleucine, and valine) stimulate phosphorylation of PHAS-I in RINm5F cells independent of endogenous insulin. A, RINm5F cells were preincubated in 3 ml of KRBB in the absence of glucose and amino acids for 2 h. During the last hour of preincubation, genistein (10 μM) or herbimycin A (1 μM) were added to the cells as indicated. Following preincubation, buffer was replaced with KRBB containing branched-chain amino acids (leucine, isoleucine, and valine at 3.3 mM for each amino acid) plus inhibitors as indicated for 30 min. B, RINm5F cells were incubated in KRBB in the absence of glucose and amino acids at 37 °C (lanes 1–3) or 24 °C (lanes 4–6) for 2 h. Following preincubation, buffer was replaced with KRBB containing amino acids at 37 °C (lanes 1–3) or 24 °C (lanes 4–6) as indicated for 30 min. Supernatants were saved for insulin radioimmunoassay. In both experimental designs, RINm5F cells were processed for immunoblotting of PHAS-I as described under “Experimental Procedures.” Results are representative of three separate experiments.

Under these conditions, an increase in glucose concentration from 3 to 20 mM (Fig. 7A, lanes 1 and 2) in the absence of amino acids resulted in a shift of PHAS-I from α to β and to some extent formation of PHAS-Iγ. This small increase in the formation of PHAS-Iγ in the absence of added amino acids is believed to result from proteolysis and release of amino acids intracellularly due to the total incubation period of 5 h without exogenous amino acids. In all previous studies conducted for 30 min following a 2-h incubation without amino acids, the formation of PHAS-Iγ was not observed by islets or β-cells. The presence of 10 mM leucine in combination with 20 mM glucose facilitated a significant shift of PHAS-I to the γ-form which was also accompanied by enhanced insulin secretion (Fig. 7, A and B, lanes 5). These findings indicate that amino acids synergize with insulin similar to RINm5F cells to fully activate this signal transduction pathway. Furthermore, leucine alone stimulates some PHAS-Iγ formation under conditions of only basal insulin secretion (Fig. 7, A and B, lanes 4), suggesting that leucine alone can also stimulate phosphorylation of PHAS-I similar to that observed with RINm5F cells.

Under conditions of reduced temperature (24 °C) which significantly blocks glucose-induced exocytosis, leucine (10 mM) resulted in enhanced formation of PHAS-Iβ and loss of PHAS-Iα (Fig. 7A, lanes 6 and 7), confirming the above findings that leucine alone is independent of insulin secretion can stimulate PHAS-I phosphorylation in islets. Interestingly, leucine in the presence of 20 mM glucose at 24 °C (Fig. 7A, lane 8), conditions where enhanced basal insulin secretion is observed (Fig. 7B, lane 8), resulted in significant formation of PHAS-Iγ. The explanation for the ability of leucine to convert PHAS-I to fully phosphorylated PHAS-Iγ under these conditions is believed to be due to the presence of a threshold level of insulin accumulated, approximately 3-fold over basal levels, over this 3-h incubation period which synergizes with amino acids to shift PHAS-Iβ to PHAS-Iγ. These findings indicate the following: 1) both isolated islets and the β-cell line, RINm5F, require amino acids to facilitate glucose-stimulated phosphorylation of PHAS-I; 2) amino acids mediate PHAS-I phosphorylation independent of endogenous insulin secretion; and 3) amino acids also synergize with insulin to further enhance PHAS-I phosphorylation.

FIG. 6. Effects of rapamycin and wortmannin on branched-chain amino acid-induced phosphorylation of PHAS-I and p70s6k in RINm5F cells. RINm5F cells were preincubated in KRBB in the absence of glucose and amino acids for 2 h. During the last hour of preincubation, rapamycin (25 nM) and wortmannin (100 nM) were added to cells. Following the preincubation, buffer was replaced with KRBB containing branched-chain amino acids (leucine, isoleucine, and valine at 3.3 mM for each amino acid) plus inhibitors as indicated for 30 min. RINm5F cells were processed for immunoblotting of PHAS-I (A) and p70s6k (B) as described under “Experimental Procedures.” Results are representative of three separate experiments.

IGF-I Requires Amino Acids to Stimulate Phosphorylation of PHAS-I—Our previous studies indicated that amino acids are essential for insulin to induce phosphorylation of PHAS-I, suggesting a critical role for amino acids in facilitating this effect (20). To define further whether the requirement for amino acids to facilitate PHAS-I phosphorylation may represent a general phenomenon for other growth factors, we evaluated the effect of insulin-like growth factor I (IGF-I) on PHAS-I phosphorylation. In this design, RINm5F cells were incubated with KRBB ± IGF-I (1–100 nM) supplemented with amino acids (0. 1×) for 30 min. As shown in Fig. 8, IGF-I (100 nM) alone is ineffective in inducing phosphorylation of PHAS-I (lane 3) in comparison to the absence of amino acids (lane 1) and a complete complement of amino acids (lane 2). In this case, the absence of amino acids results in PHAS-Iα and some β being detected. The amount of PHAS-Iα detected, as stated earlier (see Fig. 1), can vary dependent on the level of quiescence of the cells. However, IGF-I (1–100 nM) plus amino acids (0. 1×) caused a dose-dependent enhancing effect on formation of PHAS-Iγ (lanes 5–8). These results indicate that a requirement for amino acids in PHAS-I phosphorylation may be essential for other β-cell growth factors in addition to insulin and IGF-I to activate this signaling pathway. These results further raise the possibility that amino acids facilitate the proliferative effect of growth factors such as insulin and IGF-I by inducing the phosphorylation of PHAS-I.
Amino acids (leucine, valine, and isoleucine), as a group, were the most effective in stimulating the phosphorylation of PHAS-I. To define the mechanism by which branched-chain amino acids stimulated the phosphorylation of PHAS-I, the effects of their metabolites on PHAS-I phosphorylation were evaluated. These studies demonstrated that the transamination product of leucine, α-ketosacaproic acid (KIC), dose-dependently mediated the increased phosphorylation of PHAS-I. This suggested that the transport and metabolism of leucine is required although the possibility exists that leucine independent of its metabolism may also contribute to the phosphorylation of PHAS-I. However, unlike KIC, the transamination products of valine, α-ketoisovalerate (KIV), and isoleucine, α-keto-β-methylvalerate (KMV), were ineffective in stimulating the phosphorylation of PHAS-I, indicating that either the transport of KIV and KMV into β-cells and/or their metabolism is insufficient to induce phosphorylation of PHAS-I. Even though it is likely that if isoleucine and valine were transported into β-cells, these branched-chain amino acids would be converted to their α-keto transamination products in a manner similar to leucine. In support of this possibility, previous reports have indicated that a single enzyme, branched-chain aminotransferase, catalyzes the reversible transamination of all the branched-chain amino acids; leucine, isoleucine, and valine to their respective α-keto acids (35, 36). Since valine and to a lesser extent isoleucine stimulated PHAS-I phosphorylation as shown in Fig. 4, it is reasonable to assume that exogenous KIV and KMV probably did not gain adequate entry into β-cells in this in vitro experimental design.

Amino acids are potent stimuli for insulin secretion from β-cells. For example, leucine as previously discussed serves as an insulin secretagogue under some conditions and also potentiates glucose-stimulated insulin secretion from β-cells (37, 38). Although the amino acids arginine, lysine, and isoleucine alone are not insulin secretagogues, these amino acids potentiate glucose-stimulated insulin secretion by β-cells (39). Amino acids also stimulate glucagon secretion from α-cells which may in turn trigger insulin secretion from β-cells (40). Multiple approaches were employed in the present study to assess whether amino acid-induced phosphorylation of PHAS-I was due to endogenous insulin secretion by β-cells. Both inhibitors of tyrosine kinase activity and reduction of the incubation temperature from 37 to 24 °C, conditions which block almost completely secretagogue-induced insulin exocytosis without significantly inhibiting β-cell metabolism, failed to attenuate the ability of branched-chain amino acids to induce PHAS-I phosphorylation. These findings support the concept that amino acid-induced phosphorylation of PHAS-I is independent of insulin secretion by β-cells. Moreover, a recent study by Patti et al. (41) has also demonstrated that high physiologic concentrations of amino acids stimulate PHAS-I phosphorylation in cultured hepatoma cells.

In insulin-sensitive cells, insulin and other growth factors including IGF-I mediate PHAS-I phosphorylation via a signaling pathway involving activation of IRS-1 and IRS-2 and also PI 3-kinase (16). Both PHAS-I and p70^6k are located downstream in this insulin signaling pathway and are regulated by mTOR in a parallel manner. More recently, studies have indicated that the serine/threonine kinase, Akt (protein kinase B), is required for insulin and growth factors to mediate the phosphorylation of PHAS-I (42, 43). Akt is proposed to be located downstream of PI 3-kinase and upstream of mTOR and functions in concert with mTOR to phosphorylate PHAS-I (17). In the present study, the signaling pathway involved in amino acid-induced phosphorylation of PHAS-I was evaluated by treating RINm5F cells with branched-chain amino acids in the

**DISCUSSION**

Our previous studies indicated that a complete complement of amino acids normally present in tissue culture medium stimulated the phosphorylation of PHAS-I in β-cells (20). An initial focus of the present study indicated that essential amino acids in comparison to non-essential amino acids were most effective in inducing the phosphorylation of PHAS-I. However, these findings do not necessarily preclude the possibility that non-essential amino acids also mediate to a lesser extent the phosphorylation of PHAS-I. Since some shift of PHAS-I to more phosphorylated forms was observed in RINm5F cells treated with non-essential amino acids in comparison to the absence of amino acids. This possibility is in agreement with a recent report indicating that non-essential amino acids (alanine and glycine) significantly increased cell proliferation in bovine embryos cultured in a chemically defined medium (34).

Among all essential amino acids tested, the branched-chain amino acids (leucine, valine, and isoleucine), as a group, were the most effective in stimulating the phosphorylation of PHAS-I. To define the mechanism by which branched-chain amino acids stimulated the phosphorylation of PHAS-I, the effects of their metabolites on PHAS-I phosphorylation were evaluated. These studies demonstrated that the transamination product of leucine, α-ketosacaproic acid (KIC), dose-dependently mediated the increased phosphorylation of PHAS-I. This suggested that the transport and metabolism of leucine is required although the possibility exists that leucine independent of its metabolism may also contribute to the phosphorylation of PHAS-I. However, unlike KIC, the transamination products of valine, α-ketoisovalerate (KIV), and isoleucine, α-keto-β-methylvalerate (KMV), were ineffective in stimulating the phosphorylation of PHAS-I, indicating that either the transport of KIV and KMV into β-cells and/or their metabolism is insufficient to induce phosphorylation of PHAS-I. Even though it is likely that if isoleucine and valine were transported into β-cells, these branched-chain amino acids would be converted to their α-keto transamination products in a manner similar to leucine. In support of this possibility, previous reports have indicated that a single enzyme, branched-chain aminotransferase, catalyzes the reversible transamination of all the branched-chain amino acids; leucine, isoleucine, and valine to their respective α-keto acids (35, 36). Since valine and to a lesser extent isoleucine stimulated PHAS-I phosphorylation as shown in Fig. 4, it is reasonable to assume that exogenous KIV and KMV probably did not gain adequate entry into β-cells in this in vitro experimental design.

Amino acids are potent stimuli for insulin secretion from β-cells. For example, leucine as previously discussed serves as an insulin secretagogue under some conditions and also potentiates glucose-stimulated insulin secretion from β-cells (37, 38). Although the amino acids arginine, lysine, and isoleucine alone are not insulin secretagogues, these amino acids potentiate glucose-stimulated insulin secretion by β-cells (39). Amino acids also stimulate glucagon secretion from α-cells which may in turn trigger insulin secretion from β-cells (40). Multiple approaches were employed in the present study to assess whether amino acid-induced phosphorylation of PHAS-I was due to endogenous insulin secretion by β-cells. Both inhibitors of tyrosine kinase activity and reduction of the incubation temperature from 37 to 24 °C, conditions which block almost completely secretagogue-induced insulin exocytosis without significantly inhibiting β-cell metabolism, failed to attenuate the ability of branched-chain amino acids to induce PHAS-I phosphorylation. These findings support the concept that amino acid-induced phosphorylation of PHAS-I is independent of insulin secretion by β-cells. Moreover, a recent study by Patti et al. (41) has also demonstrated that high physiologic concentrations of amino acids stimulate PHAS-I phosphorylation in cultured hepatoma cells.

In insulin-sensitive cells, insulin and other growth factors including IGF-I mediate PHAS-I phosphorylation via a signaling pathway involving activation of IRS-1 and IRS-2 and also PI 3-kinase (16). Both PHAS-I and p70^6k are located downstream in this insulin signaling pathway and are regulated by mTOR in a parallel manner. More recently, studies have indicated that the serine/threonine kinase, Akt (protein kinase B), is required for insulin and growth factors to mediate the phosphorylation of PHAS-I (42, 43). Akt is proposed to be located downstream of PI 3-kinase and upstream of mTOR and functions in concert with mTOR to phosphorylate PHAS-I (17). In the present study, the signaling pathway involved in amino acid-induced phosphorylation of PHAS-I was evaluated by treating RINm5F cells with branched-chain amino acids in the...
absence or presence of the mTOR inhibitor, rapamycin (25 nM), and PI 3-kinase inhibitor, Wortmannin (100 nM). These studies indicated that rapamycin (25 nM) effectively blocked phosphorylation of both PHAS-I and p70^S6K^ based on gel mobility shift assays following exposure to branched-chain amino acids. In a similar manner, the PI 3-kinase inhibitor, Wortmannin (100 nM), also prevented amino acid-induced phosphorylation of PHAS-I and p70^S6K^.

Although the ability of rapamycin to block amino acid-induced phosphorylation of PHAS-I and p70^S6K^ is predicted by its inhibitory effect on mTOR, an explanation for similar inhibitory effects produced by the PI 3-kinase inhibitor, Wortmannin, is more complex. Although it is possible that Wortmannin also inhibits mTOR under these conditions, the concentration of Wortmannin (100 nM) used in the present studies is significantly lower than that required for inhibition of mTOR activity (12). Alternatively, Patti et al. (41) recently reported that the PI 3-kinase inhibitor, Wortmannin, also abolished amino acid activation of p70^S6K^ in hepatoma cells at concentrations well below that required for inhibition of mTOR. These authors concluded that both mTOR and other Wortmannin-sensitive kinases including PI 3-kinase are involved in amino acid-induced phosphorylation of PHAS-I in this hepatoma cell line. These latter findings are also consistent with our results indicating that branched-chain amino acids induce PHAS-I phosphorylation in a rapamycin- and Wortmannin-sensitive manner in β-cells. Although neither the addition of amino acids with cultured hepatoma cells (41) nor their removal with CHO-IR cells (44) altered protein kinase B/Akt activity, the effects of branched-chain amino acids on Akt activity in β-cells has not yet been evaluated.

Since amino acids have been shown to exert a mitogenic effect on β-cells (1, 2) and to also increase the release of insulin and insulin-like growth factors in vivo, we evaluated the ability of insulin-like growth factor I (IGF-I) to mediate the phosphorylation of PHAS-I. These studies demonstrated that IGF-I similar to insulin stimulated PHAS-I phosphorylation in an amino acid-dependent manner. This ability of IGF-I to stimulate PHAS-I phosphorylation is consistent with a recent study indicating that IGF-I-induced DNA synthesis in RINm5F cells is associated with the phosphorylation of IGF-I receptors and IRS-2 (45). The specific mechanism whereby amino acids facilitate both insulin and IGF-I-induced phosphorylation of PHAS-I in β-cells is not presently known. Our studies also indicated that IGF-I-like insulin synergizes with amino acids to enhance further PHAS-I phosphorylation in β-cells. It is possible that this synergistic effect of insulin and IGF-I in combination with amino acids is due to an increase in the transport and/or metabolism of amino acids by β-cells.

These findings clearly emphasize the importance of branched-chain amino acids alone and in combination with insulin, IGF-I, and possibly other growth factors to mediate protein synthesis, β-cell growth, and proliferation under different nutritional and disease states, including diabetes mellitus (35). These results may explain, in part, the ability of branched-chain amino acids, in particular leucine to increase protein turnover in muscle (46, 47). A novel aspect of the present study is the demonstration that both insulin-secreting β-cells and classical insulin target cells (skeletal muscle, adipocytes, and liver) utilize similar if not identical signaling pathways proposed to up-regulate protein translation, cell cycle progression, and cell proliferation. A more fundamental understanding of the striking similarities in these critical signal transduction pathways in β-cells and insulin target cells may aid in defining common signaling defects responsible for impaired insulin secretion and the development of insulin resistance associated with diabetes mellitus.

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