Insulin binding results in rapid phosphorylation of insulin receptor substrate-1 to activate p21ras and mitogen-activated protein kinase. Insulin also activates the ribosomal protein S6 kinase (pp70 S6 kinase) independently of the Ras pathway. Chronic (18 h) treatment of L6 muscle cells with insulin increases glucose transport activity severalfold due to biosynthetic elevation of the GLUT1 and GLUT3 but not the GLUT4 glucose transporters. Here we investigate the roles of p21ras and pp70 S6 kinase in the insulin-mediated increases in GLUT1 and GLUT3 expression. L6 cells were transfected with the dominant negative Ras(S17N) under the control of a dexamethasone-inducible promoter. Induction of Ras(S17N) failed to block the insulin-mediated increase in GLUT1 glucose transporter protein and mRNA; however, it abrogated the insulin-mediated increase in GLUT3 glucose transporter protein and mRNA. Inhibition of pp70 S6 kinase by rapamycin, on the other hand, eliminated the insulin-mediated increase in GLUT1 but had no effect on that of GLUT3 in both parental and Ras(S17N) transfected L6 cells. These results suggest that the biosynthetic regulation of glucose transporters is differentially determined, with pp70 S6 kinase and p21ras playing active roles in the insulin-stimulated increases in GLUT1 and GLUT3, respectively.

Insulin mediates a wide spectrum of biological responses including stimulation of glucose influx and metabolism in muscle and adipocytes, transport of amino acids, transcription of specific genes and mitogenesis (1, 2). These are determined by signals initiated by insulin binding, leading to rapid autophosphorylation of receptor tyrosine residues (3) and tyrosine phophorylation of Shc (4) and IRS-1. IRS-1 serves as a docking protein for Src homology 2 (SH2) domain proteins including phosphatidylinositol 3-kinase, and GRB2, an adaptor protein linked to Sos, a guanine nucleotide exchange factor (3, 5). Association of IRS-1 or Shc with GRB2-Sos results in the release of GDP from p21ras and a consequent increase in the amount of GTP-p21ras (5). Like all GTP-binding proteins, p21ras cycles between inactive GDP-bound and active GTP-bound conformations (6). Ras binds directly to the serine/threonine kinase Raf-1, which in turn phosphorylates and activates MAPK/ERK kinase to phosphorylate and activate MAPK (ERK) (7–10). Insulin also stimulates the ribosomal protein S6 kinases pp70 S6 kinase and pp90 S6 kinase (3, 11). The two S6 kinases, originally identified by their ability to phosphorylate the 40 S ribosomal protein S6, are regulated by distinct mechanisms, as MAPK directly phosphorylates and activates pp90 S6 kinase but has no effect on pp70 S6 kinase (3, 5), whereas phosphatidylinositol 3-kinase is required for activation of pp70 S6 kinase but not pp90 S6 kinase or MAPK (12).

The L6 cell line is derived from neonatal rat thigh skeletal muscle and retains several properties of skeletal muscle (13, 14). During all stages of their development, L6 cells express the GLUT1 glucose transporter, a ubiquitous isoform that is widely distributed and is believed to provide cells with basal glucose requirements (15). They also express the GLUT3 glucose transporter, which is expressed in fetal (16) and regenerating muscle (17) and in neuronal cells of the brain (18). In contrast, the fat/muscle-specific GLUT4 glucose transporter is not expressed until alignment and onset of cell fusion into myotubes (15, 19, 20). Our laboratory has previously demonstrated that in response to chronic treatment with insulin (for several hours), glucose transport activity increases severalfold due to an increase in GLUT1 mRNA and protein levels (2, 21). We also observed that sustained insulin-like growth factor-1 treatment leads to an increase in GLUT3 mRNA and protein levels (22). By contrast, prolonged insulin treatment was associated with a small decrease in the levels of GLUT4 protein and mRNA (21). Similar observations on GLUT1 and GLUT4 have been made in 3T3 F442A adipocytes (23).

Despite long standing recognition of the signaling cascades that link the insulin receptor to the nucleus, it remains unknown whether they participate in the insulin-induced regulation of expression of specific genes with the exception of those encoding for transcription factors (24). Therefore, the objective of this study was to investigate the role of the signaling cascade, in particular the role of p21ras and pp70 S6 kinase, in the insulin-induced increases in GLUT1 and GLUT3 expression after prolonged (18 h) treatment with the hormone. Using a constructed L6 cell line transfected with a dominant negative Ras, Ras(S17N) under the control of a dexamethasone-inducible promoter (25), and using rapamycin, a specific inhibitor of pp70 S6 kinase (11, 26), we investigated the roles of p21ras and pp70 S6 kinase in the insulin-mediated regulation of expression of GLUT1 and GLUT3 protein and mRNA.

The abbreviations used are: IRS-1, insulin receptor substrate-1; GRB2, growth receptor-bound protein 2; MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulated kinase; pp70 S6 kinase, 70-kDa ribosomal protein S6 kinase; pp90 S6 kinase, 90-kDa ribosomal protein S6 kinase; GLUT, glucose transporter; CHO, Chinese hamster ovary.

The Insulin-dependent Biosynthesis of GLUT1 and GLUT3 Glucose Transporters in L6 Muscle Cells Is Mediated by Distinct Pathways

ROLES OF p21ras AND pp70 S6 KINASE*

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Insulin binding results in rapid phosphorylation of insulin receptor substrate-1 to activate p21ras and mitogen-activated protein kinase. Insulin also activates the ribosomal protein S6 kinase (pp70 S6 kinase) independently of the Ras pathway. Chronic (18 h) treatment of L6 muscle cells with insulin increases glucose transport activity severalfold due to biosynthetic elevation of the GLUT1 and GLUT3 but not the GLUT4 glucose transporters. Here we investigate the roles of p21ras and pp70 S6 kinase in the insulin-mediated increases in GLUT1 and GLUT3 expression. L6 cells were transfected with the dominant negative Ras(S17N) under the control of a dexamethasone-inducible promoter. Induction of Ras(S17N) failed to block the insulin-mediated increase in GLUT1 glucose transporter protein and mRNA; however, it abrogated the insulin-mediated increase in GLUT3 glucose transporter protein and mRNA. Inhibition of pp70 S6 kinase by rapamycin, on the other hand, eliminated the insulin-mediated increase in GLUT1 but had no effect on that of GLUT3 in both parental and Ras(S17N) transfected L6 cells. These results suggest that the biosynthetic regulation of glucose transporters is differentially determined, with pp70 S6 kinase and p21ras playing active roles in the insulin-stimulated increases in GLUT1 and GLUT3, respectively.

Insulin mediates a wide spectrum of biological responses including stimulation of glucose influx and metabolism in muscle and adipocytes, transport of amino acids, transcription of specific genes and mitogenesis (1, 2). These are determined by signals initiated by insulin binding, leading to rapid autophosphorylation of receptor tyrosine residues (3) and tyrosine phos.
**EXPERIMENTAL PROCEDURES**

Materials—Porcine insulin was obtained from Sigma. Rapamycin was purchased from Calbiochem. Polyclonal anti-GLUT1 (Rα-GLUTTRANS) antibody was purchased from East Acres Biologicals (Southbridge, MA). Polyclonal anti-mouse GLUT3 antibody was a kind gift from Dr. I. Simpson (National Institutes of Health). The monoclonal antibody 6H to the α1 subunit of the Na^+−K^+−ATPase was a kind gift from Dr. M. Caplan (Department of Cellular and Molecular Physiology, Yale University). Monoclonal anti-phosphotyrosine antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Plasmids containing full-length cDNAs for GLUT1 (pGT4–12) and GLUT3 (pmGLUT3–6) were kindly provided, respectively, by Dr. M. Birnbaum (Department of Cell Biology, Harvard Medical School) and Dr. C. F. Burant (Department of Medicine, University of Chicago).

Cell Culture and Incubations—Construction of the rat L6 skeletal muscle cell line transfected with a plasmid containing Ras(S17N) under a mouse mammary tumor virus promoter inducible by dexamethasone was described previously (25). Parental cells and cells transfected with Ras(S17N) were maintained in myoblast monolayer culture in α-minimal essential medium containing 2% (v/v) fetal bovine serum and 1% (v/v) antibiotic/antimycotic solution (10,000 units/ml penicillin G, 10 μg/ml streptomycin, 25 μg/ml amphotericin B) in 80-cm² flasks in an atmosphere of 5% CO₂ at 37 °C. Cells were maintained in continuous passages (~8) by trypsinization of subconfluent cultures using 0.25% trypsin. For total membrane preparation and RNA isolation, myoblasts were seeded in 10-cm diameter dishes at approximately 4 × 10⁵ cells/cm². The cells were fed fresh medium every 48 h. Ras(S17N) was induced with 1.5 μM dexamethasone for 24 h before stimulation with 100 nM insulin for 18 h.

Total Membrane Preparation and Immunoblotting—Total membranes of myoblasts were isolated as described previously (20). Protein content was determined by the Bio-Rad Bradford procedure (27). Membranes of myoblasts were isolated as described previously (20). Protein content was determined by the Bio-Rad Bradford procedure (27). Membranes of myoblasts were isolated as described previously (20). Protein content was determined by the Bio-Rad Bradford procedure (27).

Detection of MAPK Phosphorylation—Parental and Ras(S17N) transfected L6 cells were treated with or without 1.5 μM dexamethasone for 24 h, then stimulated with or without 100 nM insulin for 5 min, and lysed essentially according to Lamphere and Lienhard (29). Briefly, cells were lysed in a solution containing 4% SDS, 10 mM dithiothreitol, 115 mM Tris/HCl (pH 6.8), 10% glycerol, 0.25 mg/ml bromphenol blue, protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 10 μM E-64, 1 μM pepstatin, and 1 μM leupeptin), and phosphatase inhibitors (40 mM sodium fluoride, 7.5 mM sodium pyrophosphate, and 1.5 mM sodium orthovanadate). Lysates were passed five times through a 27-gauge needle to shear the DNA and boiled for 3 min. A 30-μg sample of total protein from each condition was subjected to SDS-7.5% polyacrylamide gels. MAPK (ERK) phosphorylation was detected by immunoblotting using anti-phosphotyrosine monoclonal antibody (1:5000 dilution) followed by sheep anti-mouse immunoglobulin conjugated to horseradish peroxidase (1:5000 dilution) and visualized by the enhanced chemiluminescence method.

Statistical Analysis—Autoradiograms were quantified by laser scanning densitometry using a PD1 model DNA 35 scanner with version 1.3 of the discovery series one-dimensional gel analysis software. Statistical analysis was performed using the analysis of variance test (Fisher, multiple comparisons).

**RESULTS**

Insulin Stimulation of GLUT1 Expression Is Mediated by pp70 S6 Kinase but Not by p21ras—L6 cells transfected with dominant negative Ras(S17N) under the control of a dexamethasone-inducible promoter were treated with or without dexamethasone for 24 h prior to chronic (18 h) exposure to insulin. As shown in Fig. 1A and quantified in Fig. 1B, in the absence of dexamethasone insulin caused a 103% increase in total content of GLUT1 protein above basal levels. Although dexamethasone increased the basal level of GLUT1 protein, insulin still caused a 66% increase in GLUT1 protein above the value in the presence of dexamethasone. The induction of GLUT1 protein in basal cells in the absence of dexamethasone was assigned a value of 1.0, and other values were expressed in relative units. Values represent means ± S.E. of four independent experiments. ***, significance at 95% compared with basal cells in the absence of dexamethasone, **, significance at 95% compared with basal cells in the absence of dexamethasone,**, significance at 95% compared with basal cells in the absence of dexamethasone.

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**FIG. 1. Inhibition of insulin-induced elevation in GLUT1 protein by rapamycin but not by expression of Ras dominant negative mutant.** A, L6 cells overexpressing Ras(S17N) were treated with or without 1.5 μM dexamethasone (DEX) for 24 h prior to treatment without (basal (B)) or with 100 nM insulin (I) in the presence or absence of 30 nmol rapamycin (R) for 18 h. Total membranes were prepared and immunoblotted as described under “Experimental Procedures.” The content of the immunoreactive α1 Na^+−K^+−ATPase subunit is illustrated for assessment of equality of protein loading. This is a representative blot of four independent experiments. B, the results of four independent experiments were densitometrically scanned. The content of GLUT1 protein in basal cells in the absence of dexamethasone was assigned a value of 1.0, and other values were expressed in relative units. Values represent means ± S.E. of four independent experiments. ***, significance at 95% compared with basal cells in the absence of dexamethasone, **, significance at 95% compared with basal cells in the absence of dexamethasone.

On the other hand, rapamycin, a specific inhibitor of pp70 S6 kinase, almost completely eliminated the increase in GLUT1 glucose transporter protein in response to insulin (shown in Fig. 1A and quantified in Fig. 1B). This was observed under control conditions (in the absence of dexamethasone) as well as when the dominant negative p21ras was induced (in the presence of dexamethasone). Therefore, the insulin-stimulated increase in GLUT1 protein is a pp70 S6 kinase-dependent event.

Parallel results were obtained with GLUT1 mRNA; chronic treatment with insulin resulted in a 101% increase in GLUT1 mRNA above the basal value (mean of four independent experiments). The induction of dominant negative Ras by dexamethasone failed to block the insulin-mediated increase in GLUT1 mRNA. Insulin still caused a 128% increase in GLUT1 mRNA above the value in the presence of dexamethasone (mean of four independent experiments). These results indicate that insulin action on GLUT1 gene expression occurs independently of p21ras.

Insulin Stimulation of GLUT3 Expression Is Mediated by p21ras but Not by pp70 S6 Kinase—As demonstrated in Fig. 2A and quantified in Fig. 2B, insulin caused a 204% elevation in the total content of GLUT3 protein above basal levels. By contrast, when Ras(S17N) was induced by dexamethasone, the ability of insulin to increase GLUT3 protein was almost completely abrogated, suggesting that the insulin-stimulated increase in GLUT3 protein is p21ras-dependent. Moreover, unlike
In order to verify that the inhibition of the insulin-induced increase in GLUT1 by rapamycin seen in cells transfected with Ras(S17N) (Fig. 1) was not an artifactual effect due to the process of transfection, a similar set of experiments was performed in parental L6 cells. As shown in Fig. 4A, rapamycin abolished the insulin-induced increase in GLUT1 protein (compare lanes 2 and 4 of panel A). Interestingly, the elevation in GLUT1 by dexamethasone alone was also abolished by rapamycin (compare lanes 5 and 7 of panel A), an observation also seen in Ras(S17N)-transfected cells. Hence, both insulin- and dexamethasone-induced elevations in GLUT1 protein are pp70 S6 kinase-dependent events.

To confirm that the inhibition of the insulin-mediated increase in GLUT3 seen earlier after induction of Ras(S17N) by dexamethasone (Fig. 2) is not due to an inhibitory effect of dexamethasone itself, the elevation in GLUT3 in response to insulin was examined in the presence and absence of dexamethasone in parental L6 cells. As shown in Fig. 4B, dexamethasone had no effect on the insulin-mediated increase in GLUT3 (compare lanes 2 and 6 of panel B). Furthermore, rapamycin did not affect the increase in GLUT3 in response to insulin (lane 4 of panel B). Hence, insulin action on GLUT3 is indeed a p21ras-dependent and pp70 S6 kinase-independent phenomenon.

**DISCUSSION**

Exposure of L6 muscle cells to insulin for several hours increases total levels of both GLUT1 and GLUT3 glucose transporters but has no effect on GLUT4 expression (2, 21). Here we examined the roles of p21ras and pp70 S6 kinase as mediators in the pathway(s) by which insulin elevates GLUT1 and GLUT3 expression, since both p21ras and pp70 S6 kinase appear to mediate communication between the insulin receptor and nuclear events.

Ras(S17N) expressed in L6 muscle cells has reduced affinity for GTP and inhibits the activity of endogenous p21ras by interfering with Sos needed for Ras activation (25). Induction of Ras(S17N) by dexamethasone was also shown to diminish the ability of insulin to stimulate the tyrosine phosphorylation of MAPK (Ref. 25 and Fig. 3B). Moreover, dexamethasone itself had no inhibitory effect on the insulin-induced MAPK phosphorylation in parental L6 cells (Fig. 3A). Hence, L6 cells expressing Ras(S17N) provide a useful system for studying the role of p21ras in insulin signaling.

In agreement with the work of others (30), the use of the glucocorticoid dexamethasone was associated with an increase in total GLUT1 protein in parental (Fig. 4A) and in Ras(S17N)-transfected L6 cells (Fig. 1). However, induction of Ras(S17N) by dexamethasone failed to eliminate the insulin-mediated increase in total GLUT1 protein or mRNA, suggesting that insulin action on GLUT1 gene expression occurs independently of p21ras. The failure of Ras(S17N) to block the insulin-mediated increase in GLUT1 is not a result of a dysfunctional mutation since the same cells GLUT3 expression was prevented (Fig.
protein was similar in parental 3T3-L1 cells and in those ex-
2) and MAPK phosphorylation was diminished (38). In
another study, microinjection of dominant inhibitory forms of
p21ras interfered with the expression of Ras(S17N) by
dexamethasone (DEX) for 24 h prior to treatment without (basal (B)) or with
100 nm insulin (I) in the presence or absence of 30 ng/ml rapamycin (R)
for 18 h. Total membranes were prepared and subjected to immunoblot
analysis using specific anti-GLUT1 (A) or anti-GLUT3 (B) antibodies as
described under “Experimental Procedures.”

In conclusion, signaling for the insulin-dependent synthesis
of GLUT1 and GLUT3 glucose transporters occurs through different
pathways; expression of the former is governed by
pp70 S6 kinase dependent. The inhibition of insulin action on
GLUT3 after induction of Ras(S17N) by
dexamethasone was not due to any inhibitory effects of dexamethasone itself.
Dexamethasone did not affect the insulin-induced increase in
GLUT3 in parental L6 (Fig. 4B). In addition, rapamycin did
with the cell surface. The disparity between the results of
Hausdorff et al. (31) and those reported here could also be due to
the differences between a stable expression and microinjec-
tion or to cell type-specific regulation of GLUT1. Indeed, dom-
inant inhibitory mutants of p21ras interfere with the insulin-
mediated increases in GLUT1 protein and mRNA were found to be
p21ras-dependent. The inhibition of insulin action on
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due to any inhibitory effects of dexamethasone itself. Dexam-
ethasone did not affect the insulin-induced increase in
GLUT3 in parental L6 (Fig. 4B). In addition, rapamycin did
not interfere with the ability of insulin to stimulate GLUT3 protein
expression in parental (Fig. 4B) or transfected cells (Fig. 2), thus
ruling out a role for pp70 S6 kinase in the mechanism
by which GLUT3 glucose transporter protein is induced by
insulin.

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The Insulin-dependent Biosynthesis of GLUT1 and GLUT3 Glucose Transporters in L6 Muscle Cells Is Mediated by Distinct Pathways: ROLES OF p21 AND pp70 S6 KINASE

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