Actin Filaments Facilitate Insulin Activation of the Src and Collagen Homologous/Mitogen-activated Protein Kinase Pathway Leading to DNA Synthesis and c-fos Expression*

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The exact mechanism of the spatial organization of the insulin signaling pathway leading to nuclear events remains unknown. Here, we investigated the involvement of the actin cytoskeleton in propagation of insulin signaling events leading to DNA synthesis and expression of the immediate early genes c-fos and c-jun in L6 muscle cells. Insulin reorganized the cellular actin network and increased the rate of mRNA synthesis and the levels of c-fos mRNA, but not those of c-jun mRNA, in undifferentiated L6 myoblasts. Similarly, insulin markedly elevated the levels of c-fos mRNA but not of c-jun mRNA in differentiated L6 myotubes. Disassembly of the actin filaments by cytochalasin D, latrunculin B, or butulinum C2 toxin significantly inhibited insulin-mediated DNA synthesis in myoblasts and abolished stimulation of c-fos expression by the hormone in myoblasts and myotubes. Actin disassembly abolished insulin-induced phosphorylation and activation of extracellular signal-regulated kinases, activation of a 65-kDa member of the p21-activated kinases, and phosphorylation of p38 mitogen-activated protein kinases but did not prevent activation of phosphatidylinositol 3-kinase and p70s6k. Under these conditions, insulin-induced Ras activation was also abolished, and Grb2 association with the Src and collagen homologous (Shc) molecule was inhibited without inhibition of the tyrosine phosphorylation of Shc. We conclude that the actin filament network plays an essential role in insulin regulation of Shc-dependent signaling events governing gene expression by facilitating the interaction of Shc with Grb2.

The signaling pathways initiated by insulin are complex and involve a cascade of adaptor molecules, as well as protein- and lipid-regulated kinases and phosphatases that mediate the nuclear effects of the hormone (1–3). However, little is currently known regarding the nature of the organization of the signaling cascades regulated by insulin or of the physical interactions of these pathways with intracellular structures, which may facilitate communication between molecules. Although the actin filament network is a particularly attractive candidate to participate in the spatial organization of signal transduction, its role in the regulation of the insulin signaling cascade remains to be elucidated.

Binding of insulin to its receptor leads to tyrosine phosphorylation of two classes of adaptor molecules, the insulin receptor substrates (IRS)1 and the Src and collagen homologous proteins (Shc) (1–3). Phosphorylated tyrosine residues on these molecules act as docking sites where proteins containing Src homology-2 domains bind and thereby become activated. Tyrosine-phosphorylated IRS molecules bind and activate phosphatidylinositol 3-kinase (PI 3-kinase), leading in turn to activation of downstream molecules such as the ribosomal p70 S6 kinase (p70s6k) (1–3).

Tyrosine phosphorylation of Shc by the insulin receptor initiates a major branch of the insulin signaling cascade; phosphorylated Shc binds Grb2, a small Src homology-2-containing adaptor molecule, leading to GDP/GTP exchange and activation of Ras through the guanine nucleotide exchange factor Sos (4). Activated Ras is then able to recruit the serine/threonine kinase Raf-1 to the plasma membrane, leading in turn to activation of mitogen-activated protein kinase (MAPK) kinase and the MAPKs Erk 1 and Erk 2 (1, 5, 6). In addition, recent evidence suggests that Ras is also located upstream of Rac, a small GTPase of the Rho subfamily, which also regulates protein kinases, including the p21-activated kinases (PAKs) and two other members of the MAPK family, Jun N-terminal kinase and p38 MAPK (7–11). Recently, we reported that insulin can activate not only Erks but also PAK65 and p38 MAPK in muscle cells (12). Erks, Jun N-terminal kinase, and p38, all of which are members of the MAPK family, have been shown to participate in nuclear events, such as regulation of cell cycle and cell proliferation (13–15). Erks have been found to participate in the induction of the immediate early gene c-fos in response to insulin (16).

The purpose of the present study was to examine the participation of the actin cytoskeleton in the transmission of the

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The abbreviations used are: IRS, insulin receptor substrate; Shc, Src and collagen homologous; PI, phosphatidylinositol; p70s6k, ribosomal p70 S6 kinase; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PAK, p21-activated kinase; PAK65, 65-kDa PAK; Grb2, growth factor-receptor-binding protein 2; PBS, phosphate-buffered saline; C2 toxin, butulinum C2 toxin; GAPDH, glyceraldehyde-phosphate dehydrogenase; GST, glutathione S-transferase; RBD, Ras binding domain; PAGE, polyacrylamide gel electrophoresis.

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nuclear effects of insulin, including regulation of DNA synthesis and immediate early gene expression. We observed that the actin network is required for the insulin-induced DNA synthesis and c-fos expression in muscle cells. Intact actin microfilaments were found to be necessary for the propagation of Shc/Ras-dependent signaling. The first step requiring the cytoskeleton appears to be the interaction of Grb2 with Shc. We propose that an interaction of Shc with the actin network is important for downstream signal transduction in response to insulin.

EXPERIMENTAL PROCEDURES

Materials—a-Minimum essential medium, fetal bovine serum, antibiotic/antimycotic solution, and the Trizol reagent for RNA extraction were from Life Technologies, Inc. Human insulin was obtained from Lilly. Myelin basic protein, cytochalasin D, and all chemicals for SDS-PAGE and immunoblotting were obtained from Sigma. Latrunculin B was obtained from Biomol (Meeting Place, PA). Phosphatidylinositol was purchased from Polar Lipids Inc. (Newark, DE) [γ-32P]ATP and the ECL reagents were obtained from Amersham Pharmacal Biotech. Polyclonal antibodies to IRS-1 and to the 85kDa regulatory subunit (p85) of PI 3-kinase and the monoclonal anti-phosphotyrosine antibody were purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-Grb2 antibodies and agarose-conjugated protein A plus protein G were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal anti-phospho-Erk antibody was obtained from New England Biolabs (Beverly, MA), and the monoclonal anti-Ras antibody was obtained from Transduction Laboratories (Lexington, KY). Affinity-purified polyclonal anti-Shc antibody was a kind gift from Dr. Jane McGlade (AMGEM Institute, Toronto, Ontario, Canada). Rhodamine-phalloidin was purchased from Molecular Probes (Eugene, OR). Botulinum C2 toxin was isolated and purified as described previously (17).

Cell Culture, Incubations, and Treatments—Monolayers of L6 muscle cells were grown to the stages of myoblasts (day 3) or myotubes (day 7) as described previously (18). Cells were grown in a minimum medium containing 2% (v/v) fetal bovine serum and 1% (v/v) antibiotic/antimycotic solution (final concentrations: 100 units/ml penicillin, 0.1 mg/ml streptomycin, 0.25 μg/ml amphotericin B) at 37 °C in an atmosphere of 5% CO2 and 95% air. The cells were grown in 12-well plates (diameter of well, 2.5 cm) for lysate preparations or in 10-cm-diameter dishes for immunoprecipitation and subcellular fractionation experiments. Cells were serum-depleted for at least 5 h prior to experimental manipulations. To induce actin disassembly, the cells were incubated with or without cytochalasin D (1 μM), latrunculin B (1 μM), or botulinum C2 toxin (200 ng/ml) for 1 h prior to incubation with insulin (100 nM) for the indicated times. The actin-disrupting agents remained with the cells during the insulin treatment period. At the end of all treatments, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and were either lysed for whole cell lysate preparations or protein precipitation or subjected to a subcellular fractionation procedure as described below.

Fluorescence Microscopy—L6 muscle cells grown on glass coverslips were differentiated into myotubes and then serum-deprived and incubated with or without actin-disassembling agents or insulin. The cells were then washed twice with PBS, fixed with 4% paraformaldehyde for 20 min, incubated with 100 μM glycine in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 15 min, and washed an additional three times with PBS. Cells were incubated for 1 h at room temperature with rhodamine-labeled phalloidin (5 units/ml), washed three times with PBS, mounted on slides, and examined using a Zeiss confocal microscope.

Thymidine Incorporation—Freshly seeded myoblasts were first incubated for 24 h with normal growth medium (2% fetal bovine serum containing a-minimum essential medium) and then serum-deprived for 24 h. Cells were then treated for 1 h with or without 1 μM cytochalasin D followed by incubation with or without 100 nM insulin for an additional 8 h. At the end of this period, 1 μCi/ml of [3H]thymidine was added for an additional 8 h. Following these treatments, cells were washed and lysed, and the radioactivity that was incorporated into DNA and precipitated by trichloroacetic acid was counted by scintillation counting. All treatment conditions began with equal numbers of myoblasts, and the total amount of radioactivity incorporated into DNA in each treatment was used in all calculations.

RNA Isolation and Northern Blotting—Following the indicated treatments, myotubes or myoblasts were washed with PBS, and total RNA was extracted with the Trizol reagent (Life Technologies, Inc.) according to manufacturer’s instructions. An additional cycle of RNA extraction with chloroform/methanol was introduced following the initial Trizol/chloroform extraction in order to improve the purity of extracted RNA. Fifteen μg of total RNA from each condition were separated on denaturing 1% agarose/formaldehyde gels, transferred to nylon membranes, and cross-linked with a UV cross-linker (Stratagene UV Stat-alinker 2400). c-fos, c-jun, and GAPDH mRNAs were detected by hybridization of membranes with specific [32P]-labeled cDNA probes (kindly provided by Dr. T. Curran and Dr. J. P. Fujo). cDNA probes for c-fos, c-jun, and GAPDH were amplified in E. coli, purified, and labeled with [32P]dCTP. Membranes were first hybridized with the 32P-labeled c-fos cDNA probe and subjected to autoradiography. Subsequently, the blots were stripped and reprobed with cDNA probes for c-jun and GAPDH.

Immunoblotting of Whole Cell Lysates—Following the indicated treatments, cells were lysed with 4 × Laemmli sample buffer. Fifty μg of cell lysates were subjected to SDS-PAGE and immunoblotted with anti-phospho-Erk specific antibodies as described previously (19).

GTP-Ras Precipitation—Ras activation was estimated by evaluation of GTP-bound Ras levels purified by precipitation from treated and control myotubes. A glutathione S-transferase (GST) fusion protein of the Ras binding domain of c-Raf (RBD-Raf) was amplified in E. coli and used for GTP-Ras precipitation according to the method of Taylor and Shalloway (20).

Immunoprecipitation—Following treatment, cells were washed and lysed in 1 ml of HEPES-buffered saline containing 10% glycerol, 1% Nonidet P-40, and freshly added phosphatase and protease inhibitors as described earlier (21). Lysates were subject to centrifugation at 3500 × g, and the supernatant was incubated with either anti-IRS-1, anti-phospho-Erk, anti-phosphotyrosine, or anti-Shc antibodies overnight, rotating at 4 °C. Agarose-conjugated protein A plus protein G was used for 4 h at 4 °C to precipitate the immunocomplexes. The latter were subject to analysis by either SDS-PAGE and immunoblotting or by PI 3-kinase and p70S6K activity assays as described below.

In Vitro PI 3-Kinase and p70(S6K) Activity Assays—PI 3-kinase activity was estimated as described earlier (21). Briefly, immunocomplexes of anti-IRS-1 and anti-p85 immunoprecipitates were washed and incubated for 10 min with [γ-32P]ATP, cold ATP, and MgCl2 and phosphatidylinositol as substrate. The products of the reaction were extracted with chloroform/methanol, separated by thin layer chromatography, and quantitated by phosphorimaging. The p70S6K activity was assayed in anti-p70 immunoprecipitates essentially as described earlier (22, 23). Briefly, myotubes were washed twice with ice-cold PBS and lysed in 1 ml of Buffer B (50 mM HEPES, 7.5, 150 mM NaCl, 10 mM sodium pyrophosphate, 100 μM NaF, 1 mM Na3VO4, and 1% (v/v) Nonidet P-40) containing protease inhibitors. The supernatants of a 15,000 × g (15 min) spin were subject to immunoprecipitation with a specific polyclonal anti- p70S6K antibody and protein A plus protein G. Immunocomplexes were washed three times with Buffer A and twice with Buffer B (20 mM 4-morpholinepropanesulfonic acid, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 2 mM EDTA, 20 mM MgCl2, 2 mM Na3VO4, and 1 mM dithiothreitol). The assay was performed in a final volume of 50 μl of Buffer B containing 1 μM protein kinase A and 2 μM protein kinase C inhibitory peptides, 0.2 mM S6 peptide, and 0.25 mM [γ-32P]ATP at 30 °C for 10 min. Aliquots (30 μl) were spotted onto Whatman papers and washed with phosphoric acid. 32P incorporated onto the S6 peptide was measured by liquid scintillation counting.

Renaturable Kinase Assay—Renaturable kinase activities were examined as described earlier (12, 24). Myelin basic protein or histone VI-S was incorporated into the polyacrylamide gel solution (at 0.5 mg/ml) before polymerization. Fifty μg of protein from cell lysates were subjected to SDS-PAGE, and following denaturation and renaturation, kinase activities were estimated by incubation of the protein/Buffer containing [γ-32P]ATP (25 μCi/g), cold ATP, MgCl2, and dithiothreitol for 1 h. Following completion of the assay, gels were washed, dried, and analyzed by phosphorimaging.

Subcellular Fractionation—Isolation of cytoskeletal and noncytoskeletal fractions was performed according to the method of Torti et al. (25). Serum-deprived myotubes were lysed in a Triton X-100-containing buffer and subjected to differential centrifugation, which allowed isolation of cytosol, Triton X-100-soluble membranes, and Triton X-100-insoluble membrane skeleton and cytoskeleton, the latter two of which are rich in actin (see Fig. 9).
RESULTS

Effect of Insulin and Actin Filament-disrupting Agents on the Organization of the Actin Cytoskeleton

The purpose of this study was to investigate the role of the actin cytoskeleton in insulin-induced stimulation of DNA synthesis and gene expression. Accordingly, we initiated these studies by investigating the effects of insulin on the organization of the actin network of L6 myoblasts and myotubes (Fig. 1). As we (19) and others (26, 27) have observed previously, treatment of cells with insulin induced a rapid reorganization of the actin cytoskeleton, including formation of aggregates of F-actin at the plasma membrane. The actin reorganization and membrane ruffling was evident in both undifferentiated myoblasts and differentiated myotubes (Fig. 1). These events developed as early as 20 s after addition of the hormone and were maintained for at least 1 h in the continued presence of the hormone (not shown). Analysis by serial sectioning with confocal and phase contrast microscopy demonstrated that these aggregates colocalized with membrane ruffles (results not shown). Pretreatment of cells with the actin filament-disrupting agent cytochalasin D induced a dramatic disassembly of the actin cytoskeleton and abrogated insulin-induced membrane ruffling and actin reorganization in both myoblasts and myotubes. Additionally, preincubation of myotubes with either of two structurally dissimilar actin-disrupting agents, latrunculin B and botulinum C2 toxin (C2 toxin), induced a comparable or greater degree of actin disassembly and completely prevented insulin-induced actin reorganization as determined by fluorescence confocal microscopy (Fig. 1). These results confirm the responsiveness of the actin cytoskeleton of both myoblasts and myotubes to insulin and its susceptibility to three structurally dissimilar actin-disrupting agents that can be used to investigate the role of the actin cytoskeleton in insulin signaling. Actin disassembly did not affect cell viability because even prolonged treatment (up to 16 h) of L6 cells with actin-disrupting agents did not alter the ability of cells to exclude trypan blue, nor did it alter their cellular ATP levels (results not shown).

Actin Filament Disassembly Decreases Insulin-induced Stimulation of DNA Synthesis in Myoblasts

Day 3 semiconfluent monolayers of L6 myoblasts were used for these studies because after 5–6 days in culture, L6 cells spontaneously differentiate into myotubes, which are nonproliferating cells. In these studies, serum-deprived cells were treated with or without insulin in the presence or absence of cytochalasin D and incubated with [3H]thymidine to examine the effects of these agents on DNA synthesis. Following a 24-h period of serum deprivation, untreated myoblasts exhibited a small but detectable amount of DNA synthesis, indicating that serum deprivation does not completely abrogate the proliferative capacity of these cells (see Fig. 2). However, addition of insulin increased this rate of synthesis 2.7-fold above basal levels. Pretreatment of the cells with cytochalasin D (Fig. 2) or latrunculin B (results not shown) abolished the ability of the hormone to mediate its mitogenic effects. Furthermore, treatment of myoblasts with cytochalasin D diminished the basal thymidine incorporation, suggesting that intact actin filaments are involved in both constitutive and insulin-induced proliferative events.

Effect of Actin Filament Disassembly on the Insulin-induced c-fos and c-jun Expression

It is well established that c-fos and c-jun expression are required for DNA synthesis and gene expression (28, 29). To investigate the role of the actin cytoskeleton in gene expression, we next examined the effects of actin disassembly on the insulin-induced expression of the two immediate early genes,
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**Effect of Actin Disassembly on the Insulin Phosphorylation and Activation of MAP Kinases**

Effects on Erk Phosphorylation and Activity—Erk 1 and Erk 2, the major isoforms of the extracellular signal-regulated kinases (Erks), are known to regulate c-fos transcription in response to a variety of mitogenic extracellular stimuli via phosphorylation of Elk-1 (30). Elk-1 is one of the ternary complex factors that, in conjunction with a dimer of the serum response factor, binds to the SRE element of the c-fos promoter (for a review, see Ref. 31). For their full activation, Erks require dual phosphorylation on residues Thr\(^{183}\) and Tyr\(^{185}\), mediated by their upstream regulator MAPK kinase (32, 33). Therefore, we next investigated the effects of cytoskeletal disruption on both phosphorylation and activation of Erk 1 and Erk 2. To examine the former, we used a polyclonal phosphospecific anti-Erk antibody that specifically recognizes phosphorylated Thr\(^{183}\) and Tyr\(^{185}\). To assay the latter, in gel kinase assays were employed. Fig. 4A illustrates that in serum-deprived myotubes, Erk phosphorylation was almost undetectable, and insulin induced a marked increase in their phosphorylation. Pretreatment of cells with cytochalasin D significantly attenuated the insulin-induced phosphorylation of Erk. Furthermore, pretreatment with either latrunculin B or C2 toxin also resulted in inhibition of insulin-induced Erk phosphorylation to an extent comparable to that achieved by cytochalasin D (Fig. 4A). In order to verify that the levels of Erk phosphorylation reflected the activity of the enzyme, in gel kinase assays were conducted to examine the ability of the enzyme to phosphorylate myelin basic protein in vitro (Fig. 4B). In accordance with their phosphorylation status, insulin treatment induced a marked increase in Erk activity that was almost completely abolished by pretreatment with cytochalasin D.

Effects on p38 MAPK Phosphorylation—Based on recent reports (34, 35) demonstrating involvement of not only Erk but also of p38 MAPK in the transcription of c-fos, we examined the effect of actin disassembly on insulin-induced phosphorylation of p38 MAPK. Fig. 4C shows a representative experiment demonstrating the effect of actin disassembly on the insulin-induced phosphorylation of p38 MAPK. As observed earlier (12), insulin induced a marked tyrosine phosphorylation of p38 MAPK in myotubes. As with Erk, phosphorylation of p38 MAPK was almost completely abolished by cytochalasin-induced actin disassembly.

**Effect of Actin Filament Disassembly on the Activation of PAK65**

We next investigated the involvement of the actin cytoskeleton in insulin-induced activation of PAK65. For these experiments, PAK65 activity was measured employing in gel assays with histone VI-S as the kinase substrate, which we have earlier observed to be a preferred substrate of PAK (12). Fig. 5 shows a representative autoradiogram (A) and quantitation of five independent experiments (B) examining the effects of insulin and cytochalasin D on PAK65 activity. Insulin induced robust activation of a 65-kDa renaturable kinase, which we have previously demonstrated to be PAK65 (12). Pretreatment of myotubes with cytochalasin D abolished the ability of the hormone to activate this kinase without significantly affecting the (low) basal activity of PAK65.

One potential explanation for the above results is that molecules participating in the insulin signaling cascade may interact directly or through intermediary proteins with actin filaments and that this interaction is specifically required for effective communication with and activation of downstream effectors. Alternatively, by profoundly altering the cell, disruption of the actin cytoskeleton could potentially nonspecifically
interrupt many signaling pathways. To exclude the latter possibility, we next examined the effects of actin disassembly on two steps of the insulin pathway that are known to be independent of MAPK activation, the insulin-induced activation of PI 3-kinase and p70S6k.

**Actin Filament Disruption Does Not Inhibit the Insulin-induced Stimulation of PI 3-Kinase and p70S6k**

In previous studies (19, 53), we demonstrated that the association of the p85 regulatory subunit of PI 3-kinase with IRS-1 was not a cytoskeleton-dependent event. Here, experiments were conducted to determine whether the activity (as opposed to protein interactions) of the enzyme could be affected by disruption of the actin cytoskeleton. IRS-1 and p85 were purified by immunoprecipitation from control, insulin-treated, and cytochalasin D-treated myotubes, and PI 3-kinase activity was evaluated using an in vitro assay. A representative autoradiogram of TLC analysis is shown in Fig. 6A. Insulin induced a rapid (within 5 min) association of PI 3-kinase with IRS-1 in addition to a significant activation of the enzyme, as judged by enhanced phosphorylation of PI to generate PI 3-phosphate in anti-IRS-1 immunoprecipitates. Similarly, activation of the enzyme was also observed in anti-p85 immunoprecipitates (Fig. 6A). In three independent experiments, insulin induced a 2.3 ± 0.3-fold (mean ± S.E.) increase in PI 3-kinase activity in anti-p85 immunoprecipitates compared with an 8.6 ± 0.9-fold increase in anti-IRS-1 immunoprecipitates. However, the cytochalasin D-induced actin filament disassembly did not inhibit the ability of insulin to activate PI 3-kinase, in either anti-IRS-1 or anti-p85 immunoprecipitates. Rather, actin disassembly appeared to potentiate insulin-induced activation of PI 3-kinase, particularly the fraction associated with IRS-1, which demonstrated a 15.8 ± 0.6-fold increase above basal levels. Actin disassembly did not significantly alter the basal activity of PI 3-kinase associated with either anti-p85 or anti-IRS-1 immunoprecipitates. A similar observation was made in 3T3-L1 adipocytes (53). These findings demonstrate that although this branch of the insulin signaling pathway does not require an intact actin cytoskeleton for its activation, intact microfilaments may curb the degree of PI 3-kinase activation, possibly by modulating association with regulatory molecules. Future studies will address this possibility.
To determine whether signaling events known to act downstream of PI 3-kinase (36–39) could be sensitive to cytoskeletal disassembly, we investigated the effects of cytochalasin D on insulin-induced activation of ribosomal p70S6k. Fig. 6 summarizes the results of five independent experiments in which the activity of p70S6k was measured in immunoprecipitates using an in vitro kinase assay. Paralleling the effects on activity of PI 3-kinase, actin filament disruption did not inhibit significantly the insulin-induced activation of p70S6k.

Intact Actin Filaments Are Required for Insulin Activation of Ras

The actin filament-dependent signaling pathways examined above have in common the small GTP binding protein Ras as an upstream regulator. On the basis of the above results, it may be inferred that actin is specifically involved in signaling events originating from an activated Ras. Accordingly, we next investigated the effects of cytoskeletal disruption on insulin-regulated Ras activation as determined by quantification of GTP loading of this protein. Cells treated in the presence or absence of insulin and/or cytochalasin D were lysed and incubated with a fusion protein containing the Ras binding domain of Raf coupled to GST (GST-RBD-Raf) in order to capture GTP-loaded Ras and estimate its activation (20). Fig. 7 illustrates that in serum-deprived myotubes, the levels of GTP-bound Ras were minimal and that insulin treatment induced a significant elevation of these levels. Importantly, actin filament disassembly abolished the ability of insulin to switch Ras from inactive to an active (GTP-bound) form. These findings are consistent with the prevailing concept that PAK65, Erks, and p38 MAPK lie downstream of Ras.
Involvement of Actin Filaments in the Insulin-induced Shc Phosphorylation and Its Binding to Grb2

To determine whether the actin cytoskeleton is involved in the activation of Ras itself or in upstream events, we examined the effects of cytoskeletal disassembly on insulin-induced tyrosine phosphorylation of Shc and binding of Grb2. Fig. 8 illustrates these results. As reported earlier (40), we detected three different isoforms of Shc in myotube cell lysates by immunoblotting with an affinity-purified polyclonal antibody against Shc (Fig. 8A). The estimated molecular masses of these isoforms were 52, 56, and 68 kDa. The molecular mass of Shc isoforms detected here are slightly higher than those observed in other cell types (41, 42), which might be accounted for by different degrees of phosphorylation of Shc in L6 myotubes. Indeed, phosphorylation of Shc was detectable even in untreated, serum-deprived cells (see Fig. 8B). From perusal of these immunoblots, it is apparent that the 56-kDa isoform is the most abundant in myotubes. Myotubes were treated with insulin and cytochalasin D and lysed, and anti-phosphotyrosine immunoprecipitates were analyzed by SDS-PAGE followed by blotting with anti-Shc antibody. In three experiments, insulin treatment resulted in enhanced phosphorylation of all three isoforms (21.9-, 5.1-, and 9.5-fold for the 52-, 56-, and 68-kDa isoforms, respectively). Fig. 8B shows a representative blot from these experiments, and a lower exposure of the same blot is also included in order to illustrate clearly the insulin-induced phosphorylation of the 56-kDa isoform. Interestingly, cytochalasin D affected neither the basal levels of Shc phosphorylation nor its enhancement in response to insulin treatment (phosphorylation levels for the three isoforms following actin disassembly were 19.8-, 5.3-, and 11.4-fold, respectively).

These observations, which are consistent with the lack of effect of cytochalasin D on insulin-induced IRS-1 tyrosine phosphorylation (19, 53), indicate that the very early events of the initiation of insulin action are not dependent on the actin cytoskeleton. Consequently, the possibility that the cytoskeletal-dependent step is located between Shc phosphorylation and Ras activation was considered. Consequently, we investigated the effects of actin disassembly on the association of Shc with Grb2 by probing anti-Shc immunoprecipitates with an anti-Grb2 antibody. Although Grb2 association with Shc was not detected in serum-depleted myotubes, insulin induced a strong association of Grb2 with Shc (Fig. 8C). Importantly, cytochalasin D-induced actin filament disassembly significantly inhibited (by 50%, p < 0.05, t test) the association of Grb2 with Shc (Fig. 8C). The results of three independent experiments are summarized in Fig. 8D. Latrunculin B caused a similar inhibition of Shc-Grb2 interaction (Fig. 8C).

Shc Associates with Actin-rich Triton X-100-insoluble Fractions

To investigate whether Shc interacts with the myotube actin cytoskeleton, the distribution of Shc in subcellular fractions was examined by a procedure utilizing Triton X-100 solubility and differential centrifugation (see under “Experimental Procedures”). This procedure allowed isolation of cytosol, Triton X-100-soluble membranes, a membrane skeletal fraction, and a Triton X-100-insoluble cytoskeletal fraction. The latter two fractions are known to be rich in actin. Fig. 9 illustrates the distribution of actin, Shc, and Grb2 in these subcellular fractions. It is apparent that this procedure successfully separates subcellular fractions rich in actin from membranes and cytosol. Probing the fractions with an anti-Shc antibody demonstrated localization of Shc isoforms in all fractions, including those of the cytoskeleton and membrane skeleton. The cytosolic fraction
contained the highest amounts of each of the three isoforms of Shc. Solubilized membranes, the membrane skeleton, and the cytoskeletal fraction appeared to contain mainly the 56-kDa Shc isoform and small amounts of the 52-kDa one. The 68-kDa isoform was present predominantly in the cytosol, and only small amounts were present in the cytoskeletal and membrane skeleton fraction. This isoform was absent from the soluble membrane fraction (Fig. 9). Interestingly, Grb2 could be detected only in the cytosolic fraction (Fig. 9). These observations suggest an association of Shc with the actin cytoskeleton in muscle cells. Our preliminary attempts to demonstrate an association of Grb2 with Shc in these cytoskeletal fractions were unsuccessful. However, future experiments will address this possibility using alternate methodology.

**DISCUSSION**

The cellular responses to insulin are diverse and include activation of transmembrane transport, cytoplasmic, and nuclear kinases and activation of transcription factors. In the present study, we have identified a potential mechanism by which the actin cytoskeleton regulates DNA synthesis and gene expression in insulin-responsive muscle cells. The results support an essential role for the actin cytoskeleton in the initiation and propagation of certain nuclear effects of insulin by facilitating molecular interactions occurring early in this pathway, specifically the interaction of Shc with Grb2.

Treatment of either undifferentiated (myoblasts) or differentiated (myotubes) L6 cells with insulin induced a marked reorganization of the actin filaments adjacent to the plasma membrane (Fig. 1), leading us to question whether the actin cytoskeleton is required for downstream signaling. Treatment of L6 cells with any of three structurally different actin-disrupting agents (cytochalasin D, latrunculin B, and botulinum C2 toxin) led to a net decrease in the amount of filamentous actin in both myoblasts and myotubes (Fig. 1), as has been observed by others (43–45). Moreover, this treatment pre-
vented insulin-induced alterations in the actin cytoskeleton (Fig. 1). The importance of the actin network in insulin-induced DNA synthesis was supported by studies in L6 myoblasts, in which we observed that F-actin disassembly inhibited both basal and insulin-stimulated DNA synthesis (Fig. 2). Currently, it is believed that DNA replication requires the expression of both immediate early genes, such as c-fos and c-jun, and cyclins of the D and E families (28, 29). Our results that a functional actin network is required for expression of c-fos (Fig. 3) support and extend previous studies in other systems in which the importance of the actin cytoskeleton in cell cycle progression has been described. For example, Bohmer et al. (29) and Seufferlein et al. (46) observed that cytochalasin D prevented the serum-induced and platelet-derived growth factor-induced expression of cyclins D and E, respectively. Taken together, these results suggest that insulin mediates its nuclear effects by induction of immediate (and possibly intermediate) early gene expression in an actin filament-dependent manner. Furthermore, it appears that intact actin filaments are also required to mediate c-fos expression, possibly destined to facilitate AP1-regulated gene expression.

It is noteworthy that whereas insulin treatment resulted in a rapid (within 1 h) induction of c-fos transcription, the levels of c-jun mRNA did not change appreciably during this period. As c-jun transcription was constitutive under these conditions, the expression of c-fos appeared to be the regulatory step in the formation of the AP1 complex and induction of gene expression. However, insulin may also exert a regulatory influence on the AP1 system at the protein level: insulin treatment can induce the phosphorylation of both c-Fos and c-Jun proteins in cultured adipocytes (47), which in turn regulates their DNA binding activity. Taken in the context of our data, it may be that a component of insulin regulation of c-Jun in L6 myotubes is exerted at the posttranslational level. This possibility is supported by reports that treatment of Rat 1 fibroblasts or skeletal muscles with insulin results in activation of Jun N-terminal kinase (48, 49), the kinase that is responsible for phosphorylation of c-Jun.

Members of the MAPK family have been implicated in regulation of c-fos expression. Harada et al. (16) reported that insulin-induced c-fos expression in 32D cells involves activation of Erk kinases via Shc and Ras. In addition, other reports have implicated p38 MAPK in c-fos expression (34, 35). Accordingly, we focused our attention on members of these pathways to identify the actin-dependent step or steps activated by insulin. We observed a rapid phosphorylation and activation of Erks in response to insulin treatment, which was abolished by treatment with actin filament-disrupting agents. Concurrently, we also observed that actin filament disassembly inhibited insulin-induced phosphorylation of p38 MAPK and activation of PAK65. These findings indicated participation of the actin cytoskeleton in signaling steps located upstream of these key regulatory enzymes. Taken together, these data demonstrate that both Erk and p38 MAPK, which are insulin-activated kinases capable of activating ternary complex factor and subsequently c-fos expression, depend on an intact actin network for their activation. Moreover, the results suggest that the actin cytoskeleton of myoblasts and myotubes is required for the propagation of insulin-induced signaling events at a prenuclear level.

Although the experimental conditions employed in the current study were not designed to assess the relative importance of Erk versus p38 MAPK in the insulin-induced c-fos expression, in preliminary experiments we observed that insulin-stimulated DNA synthesis in L6 myoblasts was inhibited by the MAPK kinase inhibitor PD 98059 (not shown). This is in agreement with reports in other cell types that insulin-induced c-fos expression was inhibited by the MAPK kinase inhibitor (16, 50), and it indicates that Erk is the member of the MAPK family most relevant to insulin-induced c-fos regulation in cells of diverse origin.

To determine whether other insulin-activated signaling events were similarly dependent on an intact cytoskeleton, we studied the insulin-induced activation of PI 3-kinase and its downstream target, p70S6K (37, 51–53). Our results indicate that intact microfilaments are not essential for propagation of this branch of insulin-stimulated signaling events. Actin disassembly did not inhibit (if anything, it facilitated) the activation of PI 3-kinase by insulin, detected in both anti-IRS-1 and anti-p85 immunoprecipitates, or the insulin-induced activation of p70S6K. There are several important implications of these findings: (i) in accordance with the currently accepted models (1–3) IRS-1 and PI 3-kinase appear to function in a signaling pathway clearly distinct from that leading to Erk activation, (ii) not all events initiated by the insulin receptor require participation of the actin network, and (iii) pharmacological actin disassembly does not exert a generalized inhibitory effect on all signaling events.

As Ras is a key regulator of cell proliferation and gene expression activated by different mitogenic stimuli (20, 54), we investigated the possible involvement of the cytoskeleton in signaling events propagated by this small GTPase. The finding that Ras activation was inhibited by actin disassembly indicated that signaling events localized close to the plasma membrane, possibly in the vicinity of the receptor, were regulated by the cytoskeleton. This role of the actin network in regulation of Ras activation has not been previously reported. Cell anchorage has been reported to facilitate the propagation of the cascade of protein kinases downstream of Ras but did not appear to be essential for the activation of Ras itself (55). In this context, our findings indicate that cytoskeletal involvement in insulin-induced activation of Ras may occur at steps earlier than those activated by cell anchorage.

To investigate such proximal events, we examined the effects of cytoskeletal disruption on insulin-induced tyrosine phosphorylation of Shc and binding of Grb2 to Shc. We observed that insulin-induced tyrosine phosphorylation of all three isoforms of Shc was unaffected, indicating that these events did not require an intact actin cytoskeleton. Shc is known to bind directly to the insulin receptor through its phosphotyrosine binding domain, which recruits Shc to the vicinity of the receptor and subsequently facilitates its phosphorylation by the kinase domain of the receptor (2, 41, 42, 56). Our results indicate that these initial events are unaltered by actin filament disassembly. However, actin filament disassembly inhibited the insulin-induced association of Shc with Grb2, suggesting that proper communication of Shc with its downstream effectors is dependent on an intact cytoskeleton. It is plausible that a particular spatial distribution or conformational state of Shc is required for successful propagation of downstream events, and this is achieved by interaction with the actin network. This hypothesis is strongly supported by the observations of Thomas et al. (57), who reported that Shc utilizes the actin network to propagate nerve growth factor signaling in PC12 cells and that the adaptor binds actin directly through the N-terminal portion of the molecule.

To examine potential interactions of Shc with the cytoskeleton in L6 cells, we utilized subcellular fractionation. Whereas the largest amount of Shc isoforms was detected in the cytosolic fraction in untreated cells, significant amounts were detected in both the cytoskeleton and membrane skeleton fractions in which the concentration of actin was highest. These data indicate that Shc associates with actin-rich compartments and provide indirect evidence that microfilaments may be involved...
in the subcellular distribution of this adaptor molecule, which could influence downstream signaling events. We attempted to investigate the effects of insulin and cytochalasin D on the subcellular distribution of Shc but were unable to detect any significant alterations in the distribution of this adaptor by these treatments (results not shown). Similar findings were also observed recently in 3T3-L1 adipocytes (58). It should be noted that Grb2 was localized to the cytosolic fractions of myo-fibroblasts, and we were also unable to detect translocation to the actin-rich fractions. However, it is possible that such a translocation is very transient, involves only a minor subtraction, or is of such low affinity that it was not detected under our experimental conditions.

From our results, it appears that Shc and the interaction between Shc and Grb2 are the earliest actin-dependent steps in the insulin signaling pathway. Immediately downstream of the Shc/Grb2 interaction is the activation of Sos by Grb2. Under these conditions of our experiments, an interaction of Grb2 with Sos has been observed in other cell types (59, 60). Our findings do not exclude an association of events acting downstream of Shc/Ras/MAPK pathway, unlike that of IRS-1/PI 3-kinase/aktors, insulin-like growth factor I and platelet-derived growth factor (57). It should be noted that Grb2 was localized to the cytosolic fractions of myo-fibroblasts, and we were also unable to detect translocation to the actin-rich fractions. However, it is possible that such a translocation is very transient, involves only a minor subtraction, or is of such low affinity that it was not detected under our experimental conditions.

In summary, we demonstrate that the signaling pathway that lies downstream of the insulin receptor requires an intact microfilament network for propagation of its effects leading to DNA synthesis and immediate early gene expression. Our investigations of early postreceptor events indicates that propagation of the Shc/Ras/MAPK pathway, unlike that of IRS-1/PI 3-kinase/p70S6k, is selectively dependent on an intact actin cytoskeleton at a very proximal site, specifically the Shc-Grb2 interaction. The results of the present study, together with those of Thomas et al. (57) and Seufferlein et al. (46) (discussed above), indicate that insulin mediates its cellular effects using signal transduction mechanisms that are similar to those of nerve growth factor but clearly distinct from those utilized by platelet-derived growth factor. These results may have important implications for the better understanding of the mechanisms of insulin resistance in diabetes and suggest that it may be important to investigate the actin cytoskeleton and associated (binding) proteins in cells from models of the diabetic state.

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REFERENCES

1. White, M. F., and Kahn, C. R. (1994) J. Biol. Chem. 269, 1–4
2. Cheatham, B., and Kahn, C. R. (1995) Endocrinology 131, 167–172
3. Yenush, L., and White, M. F. (1997) BioEssays 19, 491–500
4. Skolnik, E. Y., Batzer, A., Li, N., Lowenstein, E., Mohammadi, M., and Rapp, R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5753–5757
5. Goshima, K., Masuda, A., and Qwarke, K. (1984) J. Cell Biol. 98, 801–809
6. Kotani, K., Yonezawa, K., Hara, K., Ueda, H., Kitamura, Y., Sakaue, H., Ando, A., Chavanieu, A., Calas, B., Grigorescu, E., Nishiyama, M., Waterfield, M. D., and Kauga, M. (1994) EMBO J. 13, 2313–2321
7. Musgrove, R. A., and Sutherland, R. L. (1994) Semin. Cancer Biol. 5, 381–389
8. Böhm, R. M., Scharf, E., and Assoian, R. K. (1996) Mol. Cell. Biol. 17, 101–111
9. Stone, J., and Shalloway, D. (1996) Curr. Biol. 6, 1621–1627
10. Tresing, R. (1995) Curr. Opin. Cell Biol. 8, 205–215
11. Harada, S., Smith, R. M., Smith, J. A., White, M. F., and Jarett, L. (1996) J. Biol. Chem. 271, 30222–30226
12. Aktories, K., Barnmann, M., Ohshima, L., Tsyurya, S., Jakobs, K. H., and Habermann, E. (1996) Nature 382, 390–392
13. Reif, K., Burgering, B. M. T., and Cantrell, D. A. (1997) EMBO J. 16, 4905–4913
14. Ahmed, N. G., Seger, R., Bratlien, R. L., Diltz, C. D., Tonks, N. K., and Krebs, E. G. (1991) J. Biol. Chem. 266, 4220–4227
15. Ahmed, N. G., Seger, R., and Krebs, E. G. (1993) Curr. Opin. Cell Biol. 5, 992–999
16. Whitham, A. J. S., Shore, P., Sharrocks, A. D., and Krall, D. A. R. (1995) J. Biol. Chem. 270, 403–408
17. Haraldin, C. A., Cano, E., Cuesta, A., Baratti, M. S., Cohen, P., and Mahadevan, L. C. (1996) Curr. Biol. 6, 1028–1031
18. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) Mol. Cell. Biol. 14, 4902–4911
19. Reif, K., Burgering, B. M. T., and Cantrell, D. A. (1997) J. Biol. Chem. 272, 14426–14433
20. Kao, A. W., Waters, S. B., Okada, S., and Pessin, J. E. (1997) Endocrinology 138, 2474–2480
21. Cooper, A. J. (1987) J. Cell Biol. 105, 1473–1478
22. Spector, I., Shochet, N. R., Blasberger, D., and Kashman, Y. (1989) Cell Motil. Cytoskeleton 13, 127–144
23. Mclean, L., Downey, G. J., Aktories, K., and Roitman, C. M. (1991) J. Immunol. 147, 1139–1146
24. Seufferlein, T., Withers, D. J., Mann, D., and Rozenburg, E. (1996) Mol. Biol. Cell 7, 1865–1875
25. Kao, A. W., and Kahn, C. R. (1994) J. Biol. Chem. 269, 11887–11892
26. Miller, B. S., Shankavaram, U. T., Horney, M. J., Gere, A. C., Kurtz, D. T., and Rosenzweig, S. A. (1996) Biochemistry 35, 8769–75
27. Nohria, C. M., Tabrizchi, G., Davis, R. J., and Malbon, C. C. (1996) J. Biol. Chem. 271, 30765–30773
28. Pang, L., Sawada, T., Decker, S. J., and Saltiel, A. R. (1995) J. Biol. Chem. 270, 13585–13588
29. Chung, J., Granner, T. C., Lemon, K. P., Kazlauskas, A., and Blenis, J. (1994) Nature 371, 71–75
30. Myers, M. G., Jr., Grammer, T. C., Wang, L. M., Sun, X. J., Pierce, J. H., and Sutherland, R. L. (1994) Biochemistry 33, 28783–28789
31. Wang, G., Bilan, P., Tsakiridis, T., Hinek, A., and Klip, A. (1998) Biochem. J. 331, 917–928
32. Waldmann, V., and Rabie, H. M. (1996) Pathol. Res. Pract. 192, 883–891
33. Lin, Y. H., Chen, Q., Howe, A., and Juliano, R. L. (1997) J. Biol. Chem. 272, 8849–8852
34. Ishihara, H., Sasaki, T., Ishiki, M., Takata, Y., Imamura, T., Ueda, H., and Morita, M. (1997) Biochim. Biophys. Acta 1326, 19664–19667
35. Lin, T. H., Chen, G., Schel, J. A., Grinstein, S., Butler, J., and Downey, G. P. (1997) J. Cell. Physiol. 172, 94–108