Insulin activates rapidly a complex cascade of lipid and protein kinases leading to stimulation of mitogenic and metabolic events. Here we describe a renaturable kinase of 65 kDa (PK65) that becomes rapidly activated by insulin in differentiated L6 muscle cells (myotubes) and can phosphorylate histones immobilized in polyacrylamide gels. Insulin activation of PK65 was abolished by the tyrosine kinase inhibitor erbstatin and by the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor wortmannin, but was unaffected by inhibitors of protein kinases C and the activated ras GTPase p70S6K. Recently, a number of protein kinases have been described which become activated through interaction with the small GTP-binding proteins Rac and Cdc42 (p21-activated kinases, or PAKs) and lead to activation of the stress-induced mitogen-activated protein kinase (MAPK) p38 MAPK. Two different polyclonal antibodies recognizing the carboxyl-terminal or the Rac-binding domain of a 65-kDa PAK (PAK65) immunoprecipitated the myotube PK65. The insulin-induced activation of PK65 in myotubes was detectable following immunoprecipitation of the kinase. Furthermore, PK65 associated with and became activated by glutathione S-transferase-Cdc42Hs in the presence of GTPγS (guanosine 5′-3-O-(thio)triphosphate). In myotubes insulin also induced tyrosine phosphorylation of p38 MAPK. However, this phosphorylation was insensitive to wortmannin, indicating that p38 MAPK is not activated by PK65 in insulin-stimulated cells. The results suggest that insulin activates in muscle cells a renaturable kinase (PK65) closely related to PAK65. Tyrosine kinases and PI 3-kinase act upstream of PK65 in the insulin signaling cascade. Insulin activates p38 MAPK in myotubes, but this occurs by a pathway independent of PI 3-kinase and PK65.

Insulin mediates its metabolic and mitogenic effects by activation of a complex signaling cascade of protein tyrosine and serine/threonine kinases, as well as lipid kinases (1). Binding of insulin to its receptor leads to tyrosine phosphorylation of intracellular molecules such as the insulin receptor substrate-1 (IRS-1) and Shc, which act as docking sites for proteins containing SH2 domains (2). Two major branches of the insulin signaling cascade have been characterized, one controlled by PI 3-kinase, a dual protein and lipid kinase that phosphorylates phosphoinositides at the D-3 position (1, 3), and another controlled by the small GTP-binding protein p21ras (Ras) (1, 2). PI 3-kinase regulates membrane traffic, and can mediate metabolic and mitogenic effects initiated by cell surface receptors (3–5). The proposed downstream targets of PI 3-kinase include the ribosomal p70S6K (3, 6–9), some isoforms of PKC (10, 11), and a newly identified class of PKC-like serine/threonine kinases known as Akt/PKB (12, 13). In addition, IRS-1 and Shc bind Grb2, a small SH2 domain-containing molecule that induces Ras activation through the guanine nucleotide exchange factor Sos (14–16). Stimulated Ras activates a cascade of protein serine/threonine kinases which includes Raf, MEK and the MAPKs, Erk 1 and 2 (17–20).

Tyrosine kinase and serpentine receptor ligands can also activate other members of the Ras superfamily of small GTP-binding proteins. These include Rac and Cdc42, which have been shown to control the actin-mediated membrane ruffling and the formation of filopodia, respectively (21, 22). However, recent studies suggest that Rac and Cdc42, like Ras, also control signaling cascades of protein kinases leading to gene expression. In yeast, activation of Cdc42Sc by pheromone receptor-stimulated heterotrimeric G proteins leads to activation of a protein serine/threonine kinase, STE20, which is essential for the mating responses (23–25). A number of serine/threonine kinases homologous to STE20, known as p21-activated kinases (PAKs) have been identified in mammalian tissues (27–32). Recent data suggest that PAKs may be intermediate steps in the activation of the stress-induced MAPKs p38 and JNK/SAPK, by Cdc42 and Rac (33, 34).

PAK-like kinases were shown to become activated in response to chemoattractants and coagulants (31, 32) but the possible participation of these kinases in signaling by tyrosine kinase receptors, particularly the insulin receptor, has not been investigated. PAKs share with other kinases the ability to renature following conventional SDS-PAGE (32). Therefore, the activation of these kinases can be conveniently monitored by in gel assays, where substrates are trapped into polyacrylamide gels during polymerization. We took advantage of this assay to examine whether insulin activates PAKs or related kinases. In addition we investigated the effect of the hormone on p38 MAPK and the relationship between this kinase and PAK. These studies were performed in cultured muscle cells, which in vivo are a major target for the metabolic and growth effects of insulin (2).

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EXPERIMENTAL PROCEDURES

Materials— Erbsstatin was purchased from Biromol (Plymouth Meeting, PA). The monoclonal anti-phosphotyrosine antibody coupled to Sepharose beads was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The polyclonal anti-PAK65 antibody against the 19 amino acids of the PAK carboxyl terminus (aPAK19), the sPAK19 antigen peptide (C19 peptide), and the polyclonal anti-p38 MAPK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal anti-PAK Rac binding domain (aPAKRBBD) antibody and GST-Cdc42Hs precoupled to agarose beads were kind gifts from Dr. S. Pelech (University of British Columbia, Vancouver, BC). Protein A-Sepharose was purchased from Pharmacia (Uppsala, Sweden).

Cell Culture—Monolayers of L6 muscle cells were grown to the stage of myotubes as described previously (41). The cells were grown in 12-well plates for lysate preparations or in 10-cm diameter dishes for precipitations and were incubated in serum-depleted medium (α-minimal essential medium containing 0.1% fetal bovine serum) for 5 h prior to experimental manipulations.

Renaturable Kinase Assay—The method of Kameishi and Fujisawa (38) was used to measure the kinase activity of renaturable kinases, as reported previously (38, 39). Briefly, histone H2a (VI-S) or myelin basic protein (both at 0.5 mg of protein/ml of gel solution) were dissolved into the polyacrylamide gel solution before polymerization. Following polymerization of the gel, the samples were loaded and separated by electrophoresis, and then denatured in 7 M guanidine, renatured in buffer containing dithiothreitol, Nonidet P-40, and EDTA, and tested for kinase activity on histone VI-S or myelin basic protein using [γ-32P]ATP (25 μCi/gel). Use of Mg2+ and 25 μM non-radioactive ATP minimizes the contribution of autoprophosphorylation to the activity detected. The gels were dried and analyzed by phosphorimaging with a Molecular Dynamics Phosphorlmager system (Sunnyvale, CA), using ImageQuant software.

Immunoprecipitations—Serum-depleted myotubes were treated as described in the figure legends. For PAK immunoprecipitations, cells were washed and lysed in a Nonidet P-40-containing buffer by shaking for 25 min at 4°C as described earlier (29). Cell lysates were incubated with 10 μl of anti-PAK polyclonal antibodies for 12 h under rotation at 4°C. In some experiments the polyclonal aPAK19 was preincubated with 10 μg of the C19 peptide from the PAK65 carboxyl terminus for 1 h at 4°C before incubation with the cell lysates. The immunocomplexes were adsorbed with 10 mg of protein A-Sepharose for 4 h by rotation at 4°C. For phosphotyrosine immunoprecipitations, cell lysates were washed and lysed as described earlier (4). Cells lysates were incubated with a 20-μl suspension of the monoclonal anti-phosphotyrosine antibody coupled to agarose beads for 16 h under rotation at 4°C. At the end of these periods, the beads were washed five times with PBS, the associated proteins were eluted with 4× concentrated Laemmli sample buffer and subjected to in gel kinase assays.

Isolation of PAK65 with GST-Cdc42Hs-Agarose—Lysates of control myotubes were subjected to precipitations with GST-Cdc42Hs essentially as described previously (29). Briefly, GST-Cdc42Hs coupled to agarose beads (30 μl beed slurry/10 μl lysate) was incubated with 1 mM GDP or GTPγS 1 mM EDTA and 10 mM MgCl2 for 25 min at room temperature. The beads were washed three times with buffer II to remove unbound nucleotides. Cell lysates were prepared as described above for immunoprecipitation. Washed GST-Cdc42Hs-coupled beads were incubated with cell lysates for 1 h at 4°C under rotation. Proteins were eluted from beads with 4× concentrated Laemmli sample buffer and were subjected to in gel kinase assays.

Other Assays—Human neutrophils isolated from healthy donor blood were stimulated with FMLP and lysed as described previously (40). SDS-PAGE and immunoblotting with the ECL detection system were performed essentially as described (37). The primary antibodies were used at 1/500 dilution for anti-PAK19 and 1/1000 for anti-p38 and were detected with horseradish peroxidase-coupled anti-rabbit IgGs (1/5000 dilution). The fluorograms were analyzed by densitometry with the Discovery Series DNA 35 gel scanner as described previously (41).

RESULTS

We first examined the presence of insulin-responsive renaturable kinases in lysates of L6 myotubes using in gel kinase assays with histone VI-S or myelin basic protein as substrates. Insulin treatment of L6 myotubes induced phosphorylation of histone VI-S by a renaturable kinase of 65 kDa (Fig. 1A). We will refer to this histone kinase hereafter as PK65, for protein kinase of 65 kDa. PK65 was activated rapidly (5 min) upon stimulation of the cells with 100 nM insulin. The activation of PK65 persisted after 15 min, but declined following a 30-min incubation of myotubes with the hormone. As shown in Fig. 1A, the PK65 of myotubes comigrated with a renaturable kinase identified previously in fMLP-stimulated neutrophils (39, 40). PK65 was the major kinase observed in histone VI-S-containing gels. A second kinase of about 40 kDa was occasionally detected, but, when present, its activity was not altered by insulin (results not shown). In contrast, when myelin basic protein was used as the substrate, an insulin-induced broad band of 42-44 kDa was detected (Fig. 1B). Earlier immunoprecipitation experiments showed that this band includes the MAPKs Erk 1 and 2 (results not shown). No other major renaturable kinases could be detected in myelin basic protein gels, but modest kinase activity could be detected in the 65-kDa and 80-kDa regions (Fig. 1B). These results suggest that histone is preferred as a substrate by PK65, compared to myelin basic protein.

The possible dependence of the insulin activation of PK65 on tyrosine kinase activity and on signaling molecules acting downstream of the insulin receptor and IRS-1, such as PI 3-kinase, PKC, and p70S6K, was investigated. Treatment of myotubes with erbstatin (10 μg/ml) abolished activation of PK65 by insulin, implying that stimulation of PK65 requires tyrosine kinase activity (Fig. 2). We have observed previously that erbstatin treatment of myotubes (under the conditions used here) inhibited the insulin-induced tyrosine phosphorylation of IRS-1. To evaluate the role of PI 3-kinase, myotubes were treated with WM. This agent (100 nM) also abolished the insulin stimulation of PK65 (Fig. 2). We have shown that 100 nM WM abolishes the insulin stimulation of PI 3-kinase in myotubes (4). At this concentration WM does not affect the insulin-induced tyrosine phosphorylation of IRS-1 or the tyrosine phosphorylation and activation of Erk 1 and 2.2 In contrast to erbstatin and WM, pretreatment of the myotubes with the PKC inhibitor BIM (1 μM) did not prevent the stimulation of PK65 by insulin. Similarly, we observed no effect of rapamycin (30 ng/ml), an inhibitor of the activation of p70S6K, on the insulin stimulation of PK65 (Fig. 2).

Since Fig. 1A showed that PK65 of myotubes co-migrates with a 65-kDa fMLP-stimulated renaturable kinase from neu-
trophils and recent studies demonstrated that this chemo-
attractant activates a 65-kDa PAK isoform (PAK65) in neutro-
phils (31), we investigated whether the insulin-activated PAK65 
of myotubes is a PAK65-like enzyme. The reactivity of PAK65 
with anti-PAK65 antibodies was examined by immunoblotting 
and immunoprecipitation experiments using control and insu-
lin-stimulated myotubes. A commercially available antibody 
against the carboxyl-terminal 19 amino acids of PAK65 
(αPAKC19) recognized only a 65-kDa band in immunoblots of 
myotube lysates (Fig. 3A). This result suggests that PAK65 or 
homologous proteins are present in myotubes. Next, αPAKC19 
as well as a second antibody against the Rac-binding domain 
of PAK65 (αPAKRBD) were used to isolate PAK65 from lysates 
of control and insulin-treated myotubes. The immunoprecipitated 
proteins were subjected to in gel assays with histone VI-S as 
substrate. As was the case for cell lysates, the immunoprecipi-
tates showed renaturable kinase activity stimulated by insulin, 
which comigrated with the PAK65 detected in myotube lysates 
(Fig. 3B). To examine the specificity of the immunoprecipita-
tions, αPAKC19 was preincubated with its antigen, C19 pep-
tide, before incubation with the myotube lysates. This preincu-
bation completely abolished the precipitation of PAK65 by 
αPAKC19, suggesting that the precipitation of PAK65 by 
αPAKC19 was specific (Fig. 3C).

To demonstrate further the similarity of P65 with PAK-like 
kinesins, we examined whether P65 could bind to GST-
Cdc42Hs. GST-Cdc42Hs coupled to agarose beads was preincu-
bated with either GDP or GTPγS before incubation with cell 
lysates. The isolated proteins associated with the GST-
Cdc42Hs-agarose beads were subjected to in gel kinase assays. 
A significantly higher amount of P65 activity associated with 
GST-Cdc42Hs in the presence of GTPγS than in the presence 
GDP (Fig. 3D). Cdc42Hs did not only bind but also activated 
P65 in the presence of GTPγS. The results in Fig. 3D sug-
gested that P65 is probably a PAK-like kinase.

Transfection of dominant negative PAK was found to inhibit 
Rac and Cdc42 or interleukin-1 activation of p38 MAPK in 
COS-7 cells (33). Tyrosine phosphorylation is an essential step 
in the activation of MAPKs (16), and it is frequently used as an 
index of the stimulation of these enzymes. For this reason, we 
next examined whether insulin stimulates tyrosine phospho-
dylation of p38 MAPK in myotubes and whether this phos-
phorylation is sensitive to WM, as was the activation of P65. 
Anti-phosphotyrosine immunoprecipitations were performed 
from lysates of control and insulin-stimulated myotubes which 
had been pretreated with or without WM. The precipitates 
were probed with anti-p38 MAPK antibodies by immuno-
blotting. Some p38 MAPK was immunoprecipitated from untreated 
myotubes (Fig. 4). Insulin significantly increased the level of 
tyrosine phosphorylation of p38 MAPK within 5 min. However, 
unlike the activation of P65, the tyrosine phosphorylation of 
p38 MAPK in response to insulin was not blocked by pretreat-
ment with WM, suggesting that P65 and p38 MAPK are 
activated in myotubes by independent pathways.

**DISCUSSION**

Two different PAK isoforms, α-PAK and β-PAK of 68 and 65 
kDa, respectively, have been identified in rat brain (27, 30), 
while a third PAK isoform, mPAK3, was isolated from a mouse 
fibroblast cDNA library (29). Additionally, in human leuko-
cytes two PAK isoforms of 65 and 68 kDa were shown to be 
activated by chemotactic peptides (31), and a third 62-kDa PAK 
isoform (γ-PAK) was cloned from rat platelets and was found to 
be activated by thrombin (32). The results of Figs. 1–3 provided 
strong evidence that P65, a renaturable kinase activated by 
insulin in myotubes, is either P65 itself or a closely related 
member of the family of p21-activated kinases. The relation-
ship between these kinases is suggested by: (a) the cross-
reactivity of P65 with two different antibodies raised against 
distinct domains of P65, (b) the fact that both P65 and 
P65 can reanulate after SDS-PAGE, and (c) the association 
of P65 with the GTP-bound form Cdc42Hs, as had been de-
scribed for P65.

The results of Fig. 2 suggest that P65 lies downstream of 
tyrosine kinases and PI 3-kinase, since both erbstatin and WM
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