Purification and Characterization of a Cytosolic Insulin-stimulated Serine Kinase from Rat Liver*

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A cytosolic insulin-sensitive serine kinase has been purified to apparent homogeneity in parallel from livers of control or acutely insulin-treated rats. The kinase is labile and requires rapid purification for stability. The kinase migrates as a band of apparent M, = 90,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and renaturation, the 90-kDa band presumed to be the kinase shows kinase activity toward myelin basic protein in situ. Substrates of the kinase include Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide), ribosomal protein S6, S6 peptide, a proline-rich peptide substrate, microtubule-associated protein 2, and myelin basic protein. The kinase also phosphorylates histones H1 and H2B, but does not autophosphorylate to a significant extent. The activity of the kinase is inhibited by fluoride, glycerophosphate, p-nitrophenyl phosphate, p-nitrophenol, heparin, quercetin, poly-L-lysine, and potassium phosphate, but is unaffected by calcium, CAMP, spermine, protein kinase inhibitor peptide, phorbol myristate acetate, calcium plus phosphatidyserine, or vanadate. The kinase will utilize magnesium (10 mM) as well as manganese (1 mM) as a cofactor for maximal phosphotransferase activity. The kinase is not detected by immunoblotting with antibodies directed against protein kinase C or type II S6 kinase. Taken together, these properties distinguish this kinase from other insulin-sensitive kinases that have been described previously.

The purified kinase from livers of insulin-treated rats shows a 5–20-fold higher specific activity compared to enzyme prepared from control rats, suggesting a covalent modification as the mechanism of activation. Incubation of purified, insulin-stimulated kinase with purified phosphatase 2A leads to deactivation of the kinase activity, and the phosphatase inhibits nitrophenyl phosphate blocks this deactivation. The insulin-activated kinase fails to immunoblot with anti-tyrosine phosphate antibodies. Taken together, these results indicate that insulin activates this novel cytosolic protein kinase by a mechanism that causes its phosphorylation on serine or threonine residues.

Insulin regulates many metabolic pathways involved in protein, fat, and carbohydrate metabolism. The initial step in the regulation of these pathways is the binding of insulin to its cell-surface receptor and the subsequent activation of the receptor's tyrosine kinase domain (1, 2). This leads to receptor autophosphorylation on tyrosine residues and further activation of the receptor tyrosine kinase activity (3–6), leading to phosphorylation of several cellular proteins on tyrosine residues (6–11). Among the proteins thought to be substrates of the insulin receptor tyrosine kinase are phosphatidylinositol-3-kinase (12, 13) and a protein of apparent molecular weight of 160,000–180,000 (7, 11, 14) which has recently been cloned and named IRS-1 (15). The physiological relevance of the tyrosine phosphorylation of these proteins is not yet clear, but they may be involved in one or more of the pathways leading to the physiological end points of insulin action.

In addition to the increases in tyrosine phosphorylation, there is a rapid phosphorylation of some cellular proteins and dephosphorylation of others on serine and threonine residues in response to insulin (16). Indeed, protein serine/threonine phosphatase 1 (17) and several serine/threonine kinases (18, 19) have been shown to be activated in response to insulin. The activation of protein phosphatase 1 appears to be the result of serine phosphorylation of the regulatory G-subunit of the glycogen-bound form of phosphatase 1 (17) leading to the dephosphorylation and activation of glycogen synthase. The kinase involved in this pathway appears to be related to, or identical with, the type II S6 kinase (20). This suggests the hypothesis that phosphoprotein dephosphorylations that occur in response to insulin action are mediated by phosphatase activities that are regulated in turn by insulin-stimulated kinases.

Recent data suggest that sequential kinase reactions or cascades result from insulin receptor activation. The 42-kDa and 44-kDa forms of MAP1 kinase (21–24) have been shown to be activated by phosphorylation on threonine and tyrosine residues in response to insulin (25, 26), but are not believed to be phosphorylated directly by the insulin receptor (25). The mechanism by which the MAP kinases become phosphorylated remains controversial. Some investigators have suggested that the MAP kinases autophosphorylate on threonine and tyrosine (27–29), perhaps in response to an activator protein (27–31), while others have suggested that a MAP kinase with dual specificity for threonine and tyrosine

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Insulin-stimulated Cytosolic Serine Kinase

is responsible for activating MAP kinases in response to mitogens (32). The other insulin-stimulated serine/threonine kinases which have been purified and characterized including the type I (33-38) and type II ribosomal protein S6 kinase (39-41), raf-1 (42, 43), and an insulin-stimulated Kemptide kinase (KIK) (44) are believed to be activated by serine/threonine phosphorylation in response to insulin. Thus, protein serine/threonine phosphatasas can deactivates each of these insulin-stimulated kinases in vitro. Haystead et al. (45) showed that okadaic acid, a specific inhibitor of serine/threonine phosphatasas, can mimic the effects of insulin in stimulating serine kinase activity in isolated rat adipocytes, indicating that many of the insulin-stimulated serine/threonine kinases are activated by serine/threonine phosphorylation in response to insulin.

It has been shown in vitro that MAP kinase can phosphorylate and reactivate type II S6 kinase that has been previously dephosphorylated and inactivated by protein phosphatase 2A (20, 22, 29, 46-48). However, it has yet to be proven that this kinase cascade actually operates in vivo. Furthermore, although no activity studies have yet been done, MAP kinase has been shown to phosphorylate baculoviral-produced raf-1 in vitro (49) on a major site that is phosphorylated in vivo. These modifications may be an in vitro substrate for MAP kinase. Some data suggest that MAP kinase and the type I S6 kinase may be involved in different signalling pathways (50). How many pathways exist and how many of the insulin-stimulated serine/threonine kinases fit into these pathways remains to be determined. The mechanism by which activation of the insulin receptor tyrosine kinase leads to activation of these serine/threonine kinases by serine/threonine phosphorylation is also an important question.

In the course of studying insulin-stimulated serine kinase activities in crude rat liver preparations, we identified several apparently novel kinase activities. We report in this paper the purification and characterization of one of these kinases, a Mw 90,000 insulin-stimulated kinase (p90ISK) from rat liver. The characteristics of this kinase distinguish it from other known kinases. However, like the S6 kinases, raf-1, and KIK, this novel insulin-stimulated serine kinase appears also to be activated by serine/threonine phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Butyl-Sepharose was obtained from Pharmacia LKB Biotechnology Inc., Affi-Gel Blue from Bio-Rad, [γ-32P]ATP from Du Pont-New England Nuclear, and Optifluor from Packard. Phosphocellulose paper and No. 3 MM paper were from Whatman, histones and aprotinin were from Boehringer Mannheim, and ovalbumin, insulin, leupeptin, soybean trypsin inhibitor, myelin basic protein, p-nitrophenyl phosphate, poly-L-lysine (P2636, P0879, P6616, P6143), and bovine serum albumin were from Sigma. Ribosomal protein S6 was purified from rat liver (51), and MAP2 was purified from bovine brain (52). Peptides were synthesized at the University of Massachusetts Peptide Synthesis Facility. Catalytic subunit of CAMP-dependent protein kinase was the gift of Dr. William Benjamin (State University of New York at Stony Brook), and S6 kinase by Dr. Roger Davis (University of Massachusetts Medical Center and University). Anti-protein kinase P6516, P6143), and bovine serum albumin were from Sigma. Ribo-Subunit of CAMP-dependent protein kinase was the gift of Dr. Jackie

**Purification of p90ISK**—Two groups of 30 male Sprague-Dawley rats (175-200 g) were given subcutaneous injections of either 10 units per kg of bovine insulin in phosphate-buffered saline (19 mM NaH2PO4, 8.1 mM NaHPO4, 145 mM NaCl, 5.5 mM KCl) or phosphates buffered saline alone as has been described previously (53). Thirty minutes later, the rats were decapitated, and the livers were removed and placed in cold buffer A (25 mM Tris, pH 7.0, 20 mM p-nitrophenyl phosphate, 1 mM EDTA, 1 mM dithiothreitol) containing 1 mg of leupeptin/liter, 1 mg of aprotinin/liter, and 10 mg of soybean trypsin inhibitor/liter. All subsequent steps were carried out in parallel for livers from control and insulin-treated rats.

Each group of livers was homogenized in a Waring blender in 400 ml of buffer A containing the protease inhibitors listed above. The homogenates were centrifuged at 17,000 x g for 30 min at 4 °C. The supernatants were filtered through glass wool and mixed batch-wise with 1:10 (v/v) settled DEAE-Sepharose equilibrated in buffer A. The mixtures were swirled occasionally for 1 h at 4 °C, then filtered on a coarse sintered glass funnel. The DEAE-Sepharose was washed with 10 volumes of buffer A, then with 3 volumes of buffer A with 75 mM NaCl, and the enzyme was eluted with 5 volumes of buffer A with 300 mM NaCl. NaCl was added to the eluates to a final concentration of 2 M, and 1:20 (v/v) of settled butyl-Sepharose equilibrated in buffer A with 2 M NaCl was added. The mixtures were put on a rotating shaker for 1 h at 4 °C and filtered on a coarse sintered glass funnel. The butyl-Sepharose was washed with 10 volumes of buffer A with 2 M NaCl, and the enzyme was eluted with 20 volumes of buffer A. Triton X-100 was then added to a final concentration of 1%.

Settled Affi-Gel Blue (1:100, v/v) equilibrated in buffer A was then added to the eluates, and the mixtures were put on a rotating shaker for 1 h at 4 °C. The mixtures were filtered on a coarse sintered glass funnel. The Affi-Gel Blue was washed with 10 volumes of buffer A with 0.1% Triton X-100, and the enzyme was eluted with 3 volumes of buffer A with 0.1% Triton X-100 and 10% Optifluor. The samples were then concentrated to 5 ml in Centricon-30 units (Amicon), diluted to 50 ml with buffer A containing 0.1% Triton X-100, and injected onto an FPLC Mono Q HR 5/5 column equilibrated in buffer A with 0.1% Triton X-100. The column was run at a flow rate of 1.0 ml/min and eluted with a biphasic linear 21-ml gradient from 0-100 mM NaCl in buffer A containing 0.1% Triton X-100 over 1 min followed by 100 mM NaCl to 250 mM NaCl in buffer A containing 0.1% Triton X-100 over 20 min. Fractions (0.5 ml) were collected and assayed for kinase activity. The peak kinase fractions from the control and insulin-treated preparations were each pooled and concentrated in Centricon-30 units (Amicon) and were subsequently used for experiments.

**Assay of Kinase Activity**—Kinase activity was assayed by adding 10 μl of enzyme preparation to 20 μl of an assay mixture containing 25 mM Tris, pH 7.9, 15 mM MgCl2, 1 mM dithiothreitol, and 150 μM [γ-32P]ATP (0.5 μCi/ml). The reaction was linear up to 30 min, but the reaction was usually incubated for 15 min at 30 °C. The reaction was terminated by adsorbing 20 μl of each sample to PS1 phosphocellulose paper (1 X 2 cm). The phosphocellulose papers were washed with 180 mM phosphoric acid according to the method of Cicirrelli et al. (54) and counted in a liquid scintillation fluid. The activity is expressed as picomoles of 32P transferred per min per mg of enzyme sample.

**Assay of Protein Concentration**—Protein was determined by the BCA method (Pierce).

**Renaturation and Detection of Kinase Activity in SDS-Polyacrylamide Gels**—Purified p90ISK was subjected SDS-polyacrylamide gel electrophoresis, and kinase activity was measured in situ by a modification (55) of the method of Kameshita and Fujisawa (56). Electrophoresis was carried out using 5% acrylamide minigels (Hoeffer) in the buffer system of Laemmli (57). Myelin basic protein (0.5 mg/ml) was added to the separating gel immediately prior to polymerization. Two gel lanes were cut, and high range molecular weight markers (Bethesda Research Laboratories) were used to determine approximate molecular weights. Following electrophoresis at 10 mA for approximately 1 h, SDS was removed from the gel by equilibration in 20% 2-propanol, 50 mM Tris buffer, pH 8.0. The kinase was then fully denatured by incubating in 6 M guanidinium hydrochloride for 1 h at room temperature and allowed to renature overnight at 4 °C in 50 mM Tris, 14 mM 2-mercaptoethanol, 0.04% Tween 40, pH 8.0.

Gels were incubated in 3 ml of assay buffer (50 mM Tris, 10 mM MgCl2, 150 mM NaCl, 1 mM dithiothreitol, pH 7.0) for 20 min at room temperature in a sealed plastic bag. Kinase assays were then initiated by the addition of [γ-32P]ATP (50 μM, 106 cpm/nmol) and incubated for a further 60 min on an end-over-end mixer. The reaction was terminated by extensive washing with 5% trichloroacetic acid containing 1% sodium pyrophosphate. Gels were dried onto Whatman no. 3 MM paper and autoradiographed.

**Deactivation of p90ISK by Protein Phosphatase 2A**—Purified phosphatase 2A was the kind gift of Drs. Stephen Jaspers and Thomas
Insulin-stimulated Cytosolic Serine Kinase

Miller (University of Massachusetts Medical Center). One unit was defined as the amount of phosphatase that will release 1 nmol of Pi, per min from phosphate-labeled phosphorylase b (43). The phosphatase was in 25 mM Tris, pH 6.8, 25 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 50% glycerol. p90ISK from the final purification step was transferred to 20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM dithiothreitol, 0.2 mg of bovine serum albumin, (ml. 0.05%) Triton X-100, and 1 mM MgCl₂ using Centricron-30 units (Amicon). After incubation under the various conditions indicated in Fig. 8, the kinase activities were measured by incubation with 4 μl of phosphate incorporation into Kemptide as described under "Experimental Procedures." The concentrated Affi-Gel Blue eluates were diluted 10-fold with buffer A with 0.1% Triton X-100 and injected onto a Mono Q HR 5/5 FPLC column (not shown). Therefore, because of the instability of the enzyme, the more rapid batchwise elution is the method of choice at each of these steps.

The construction of a conventional purification table for p90ISK was not possible for several reasons. First, since MBP, the substrate used to assay p90ISK activity, is a good substrate for many kinases, the assay is not specific for p90ISK. Therefore, in the early steps of the purification, where many kinases are present that will phosphorylate MBP, p90ISK phosphorylation of MBP would represent only an indeterminable fraction of the total MBP phosphorylation. Second, as will be discussed in detail later, the presence of p-nitrophenyl phosphate (NPP) is necessary throughout the purification to maintain the insulin activation of kinase activity. However, NPP also reversibly inhibits p90ISK activity (Fig. 1) and, at the concentration of NPP that is necessary to maintain insulin activation of the kinase (20 mM), approximately 80% of p90ISK activity is inhibited. Therefore, activity measurements at all steps of the purification reflect only a small fraction (20%) of the total p90ISK activity. Third, during the last three steps of purification, the amount of protein present is very low and is in the minimum range for detection by conventional protein determination methods due to interference with dithiothreitol, NPP, and Triton X-100. Thus, accurate activity and total protein determinations could not be made at the various stages of purification of p90ISK. However, the final enzyme preparation can be concentrated and desalted to remove NPP, and accurate activity and protein determinations can be made to give a specific activity of the final enzyme preparations. The specific activity for p90ISK purified from insulin-treated rats is typically in the range of 1 nmol of Pi transferred to MBP per min per mg of kinase.

Fig. 2 shows the results of the FPLC Mono Q column in the final purification step. A single peak of insulin-stimulated kinase activity elutes from the Mono Q column between 150 and 200 mM NaCl. The peak is pooled, desalted, and concentrated for subsequent experiments. A silver-stained SDS-polyacrylamide gel of this final enzyme preparation is shown in Fig. 3. A single-stained band migrating with an apparent molecular weight of 90,000 is present in preparations derived from the livers of both control and insulin-treated rats. The elution pattern of this protein band correlates with the elution position of the kinase activity from the final Mono Q FPLC column (not shown).

When p90ISK purified either from control rats (not shown) or insulin-treated rats (Fig. 4) is chromatographed on an FPLC Superose 12 gel filtration column, a single peak of kinase activity is observed. The peak of kinase activity elutes between γ-globulin (M, 158,000) and bovine serum albumin (B, M, 67,000), suggesting that the kinase has an apparent molecular weight of about 90,000. A protein with an apparent molecular weight of 90,000 as determined by silver-staining of SDS-polyacrylamide gels co-elutes with the kinase activity (data not shown). This corresponds to the molecular weight of the kinase that elutes from the Mono Q column as determined by SDS-polyacrylamide gel electrophoresis (Fig. 3), suggesting that the native enzyme is monomeric.

Fig. 1. Inhibition of p90ISK activity by p-nitrophenyl phosphate. Ten μl of purified p90ISK from insulin-treated rats was added to 20 μl of assay mixture containing 75 mM Tris, pH 7.5, 15 mM MgCl₂, 150 μM [γ-32P]ATP, 0.15 mg/ml MBP, and varying concentrations of NPP. The reactions were allowed to proceed for 15 min at 30 °C and were terminated by adsorbing 20 μl of each sample to a piece of P81 paper as described under "Experimental Procedures."
control and acutely insulin-treated rats as described under "Experimental Procedures." Samples of 100 μl of the final Mono Q pools of enzyme from control (−) and insulin-treated (+) rats were boiled with 50 μl of SDS sample buffer (10% SDS, 0.1% bromphenol blue, 30% glycerol, 30% 2-mercaptoethanol), and 120 μl of each sample was electrophoresed on an 8% SDS-polyacrylamide gel according to the method of Laemmli (57). The gel was fixed in 45% methanol, 7% acetic acid for 1 h at room temperature and stained using a Bio-Rad silver stain kit.

FIG. 3. Electrophoresis and silver-staining of p90ISK on SDS-polyacrylamide gels. p90ISK was purified in parallel from control and acutely insulin-treated rats as described under "Experimental Procedures." Samples of 100 μl of the final Mono Q pools of enzyme from control (−) and insulin-treated (+) rats were boiled with 50 μl of SDS sample buffer (10% SDS, 0.1% bromphenol blue, 30% glycerol, 30% 2-mercaptoethanol), and 120 μl of each sample was electrophoresed on an 8% SDS-polyacrylamide gel according to the method of Laemmli (57). The gel was fixed in 45% methanol, 7% acetic acid for 1 h at room temperature and stained using a Bio-Rad silver stain kit.

FIG. 4. Gel filtration column profile of p90ISK. A volume of 200 μl of purified p90ISK from insulin-treated rats or Bio-Rad gel filtration standards plus 5 mg of bovine serum albumin/ml were injected onto a Pharmacia Superose 12 HR 10/30 FPLC column equilibrated in buffer A containing 10% glycerol and 0.5 M NaCl. The flow rate was maintained at 0.2 ml/min using a Pharmacia FPLC P-500 pump, and 0.5-ml fractions were collected. The kinase activity was measured by adding 10 μl of each fraction to 20 μl of assay mixture as described under "Experimental Procedures." The reaction was terminated after 15 min at 30 °C by adsorbing 20 μl of each sample onto P81 paper as described under "Experimental Procedures." The elution positions of the standards are indicated by arrows as follows: T, thyroglobulin (M, = 670,000); G, γ-globulin (M, = 158,000); B, bovine serum albumin (M, = 67,000); O, ovalbumin (M, = 44,000); M, myoglobin (M, = 17,000).

The physiological relevance of these complexes remains to be determined.

p90ISK Does Not Autophosphorylate—Many kinases have been shown to undergo autophosphorylation, an event which, for some kinases, leads to changes in kinase activity or other functions. Therefore, it was important to determine if p90ISK undergoes autophosphorylation. The autoradiograph in Fig. 5 shows that when purified p90ISK from control (lanes 1 and 2) or insulin-treated rats (lanes 3 and 4) is incubated with magnesium and [γ-32P]ATP in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of added substrate (MBP), no 32P is detected in the area of the gel where p90ISK migrates (see Fig. 3). The efficient phosphorylation of MBP by the kinases from control and insulin-treated rats indicates that the kinases are active. Thus, lack of kinase activity cannot account for the lack of autophosphorylation. It is possible, although unlikely, that the enzymes from both control and insulin-treated rats are fully autophosphorylated when they are isolated.

In Situ Gel Assay of Kinase Activity—In order to demonstrate that the M, = 90,000 band seen on silver stain of the final p90ISK preparations is actually the kinase, we attempted to assay MBP kinase activity after renaturation of p90ISK preparations that were run on SDS-polyacrylamide gels. Kinase preparations were loaded in sample buffer onto SDS-polyacrylamide minigels to which 0.5 mg of MBP/ml had been added immediately prior to polymerization. After electrophoresis, the proteins in the gel were completely denatured, then renatured, and magnesium and [γ-32P]ATP were added to assay kinase activity. As can be seen in Fig. 6, a band of apparent M, = 90,000 in the p90ISK preparations shows MBP kinase activity in the in situ gel assay. Significant insulin stimulation of the MBP kinase activity of the 90-kDa band is also evident. If inactive kinase is used, or if bovine serum albumin is used instead of kinase, no incorporation of 32P is seen (data not shown), suggesting that the 32P band that is seen when active kinase is used is not due to nonspecific adherance of the [γ-32P]ATP. Furthermore, as demonstrated above, the kinase does not autophosphorylate, so the 32P bands seen in the in situ gel assay are not due to p90ISK autophosphorylation. This indicates that the M, = 90,000
Anti-SGKZ Antibodies—Because p9OISK preparations from control band is the major, if not the only, MBP kinase present in the arrows be either of these other growth factor-stimulated kinases. The migration positions of prestarained high range molecular weight markers are indicated with arrows on the right side of the figure.

Western Blot of p9OISK with Anti-protein Kinase C and Anti-S6KII Antibodies—Because of the similarity in apparent size of p9OISK to that of protein kinase C and type II S6 kinase, it was necessary to determine whether p9OISK might be either of these other growth factor-stimulated kinases. Polyclonal antibodies that recognize isozymes α, β, and γ of protein kinase C on immunoblots were tested for their ability to recognize p9OISK. Protein kinase C from rat liver homogenates was easily detected on immunoblots, while nothing was detectable in the lanes containing p9OISK (data not shown). Similarly, polyclonal antibodies to type II S6 kinase easily recognized avian S6 kinase II without detecting anything in the lanes containing p9OISK (data not shown). These data suggest that p9OISK is structurally distinct from protein kinase C and from the type II S6 kinase.

Mechanism of p9OISK Activation in Response to Insulin—The recovery of similar amounts of the purified M₉ = 90,000 kinase from control and insulin-treated rats with 17-fold higher kinase activity in this particular preparation from insulin-treated rats as compared to control rats indicates that the effect of insulin is to increase p9OISK specific activity and not the amount of the kinase. This value varies from one preparation to another, but is generally between 5- and 20-fold. Fig. 7 is a representative experiment that shows that the activation of p9OISK in response to insulin is due to an increase in the Vₘₐₓ (100 pmol/min/mg for control versus 500 pmol/min/mg for insulin-treated in this experiment) of the enzyme rather than a decrease in the Kₘ for MBP (6 µM for control and insulin-treated). Similar results are also found using Kemptide or histone H2B as substrates (data not shown). The Kₘ for ATP is also similar for p9OISK from control (52 µM) or insulin-treated (50 µM) rats (data not shown), using MBP as a substrate.

The p9OISK preparation shown in Figs. 2 and 3 shows a 17-fold stimulation of kinase activity in response to insulin. The persistence of the insulin activation of p9OISK through several steps of purification suggests that covalent modification, rather than allosteric modulation, is the mechanism of activation of the kinase in response to insulin. Maintenance of p9OISK in an insulin-stimulated form during purification requires the presence of phosphatase inhibitors in all buffers (data not shown), suggesting that phosphorylation may be the mechanism of activation. Under conditions where autophosphorylated insulin receptor is detected, immunoblots using two different anti-phosphotyrosine antibodies (py-20 from ICN and anti-phosphotyrosine polyclonal IgG from Upstate Biotechnology) have failed to detect any phosphotyrosine in p9OISK purified from livers of control or insulin-treated rats (data not shown). Furthermore, when p9OISK purified from insulin-treated rats is treated with protein serine/threonine phosphatase 2A (Fig. 8), p9OISK activity is decreased 5-fold, almost to the level of p9OISK activity purified from control rats. This effect is not seen when the kinase preparations alone are incubated at 30 °C in the absence of NPP. The temperatures of the incubations were 0 °C in lanes 1 and 30 °C in the other lanes. After preincubation, 2 µl of deionized water was added to incubations 1–3, 2 µl of NPP was added to incubations 4, and the kinase activities were assayed as described under "Experimental Procedures." The values are means of the activities in three separate incubations, and the bars represent standard deviations.

Fig. 6. In situ gel assay of kinase activity. p9OISK purified from control (−) or insulin-treated (+) rats was electrophoresed on an 8% SDS-mini-polyacrylamide gel containing 0.5 mg of MBP/ml, the protein was renatured, and kinase activity was measured as described under "Experimental Procedures." The migration positions of prestained high range molecular weight markers are indicated with arrows on the right side of the figure.

Fig. 7. Lineweaver-Burk determination of Kₘ and Vₘₐₓ values for purified p9OISK using MBP as substrate. Samples of purified p9OISK from control or insulin-treated rats were assayed according to the method described under "Experimental Procedures" with varying concentrations of MBP as a substrate. Ten microliters of enzyme was added to 20 µl of assay mixture containing 75 mM Tris, pH 7.9, 15 mM MgCl₂, 150 µM [γ⁻³²P]ATP, and varying concentrations of MBP. The reaction was allowed to proceed for 15 min at 30 °C and was terminated by adsorbing 20 µl of each sample to P81 paper as described under "Experimental Procedures." The data are expressed in a double reciprocal (Lineweaver-Burk) plot, where the units for V are picomoles of P/mg/min, and the units for S are µM MBP. The lines were determined by linear least-squares analysis using the computer software program Cricket Graph.

Fig. 8. Deactivation of purified p9OISK by purified protein phosphatase 2A. p9OISK (2 µl) purified from either control or insulin-treated rats was preincubated for 40 min under various conditions. Lanes 1 and 2 are preincubations with 2 µl of buffer and 2 µl of NPP at 15 mM final concentration. Lanes 3 are preincubations with 6.4 units of phosphatase 2A in 2 µl of buffer and 2 µl of NPP. Lanes 4 are preincubations as in lanes 3 except that 2 µl of deionized water was added instead of NPP. The temperatures of the incubations were 0 °C in lanes 1 and 30 °C in the other lanes. After preincubation, 2 µl of deionized water was added to incubations 1–3, 2 µl of NPP was added to incubations 4, and the kinase activities were assayed as described under "Experimental Procedures." The values are means of the activities in three separate incubations, and the bars represent standard deviations.
by serine/threonine phosphorylation rather than tyrosine phosphorylation in response to insulin.

**Peptide and Protein Substrate Specificity of p90ISK**—The insulin-stimulated serine/threonine kinases which have previously been identified can be distinguished from each other by differences in *in vitro* substrate specificities. Therefore, several proteins and peptides which have been shown to be good substrates for other insulin-stimulated serine/threonine kinases were tested as substrates for purified p90ISK (Table I). Ribosomal protein S6, histone H2B, histone H1, and myelin basic protein (MBP) are very good substrates for phosphorylation by the kinase, with comparable rates of incorporation. The $K_v$ values obtained for MBP (6 $\mu$M) and histone H2B (2 $\mu$M) as substrates for p90ISK are low, while the stoichiometries of phosphorylation approach 1 mol of P$_i$/mol of substrate (0.7 for MBP and 0.6 for histone H2B), indicating that both are good substrates for p90ISK *in vitro*. The $K_v$ and phosphorylation stoichiometry for ribosomal protein S6 have not been determined. Histones H2A, H3, and H4 are poor substrates for p90ISK.

Several peptides were also tested as substrates for purified p90ISK. Kemptide, the substrate peptide used for assay of cAMP-dependent protein kinase (58), is a very good substrate, with a $K_v$ of 50 $\mu$M. The proline-rich ERT peptide substrate (59) is also a very good substrate, with a rate of phosphorylation comparable to that of Kemptide. The residue phosphorylated in the ERT peptide by p90ISK is not known. The peptides corresponding to phosphorylation sites in ATP citrate lyase (60) and ribosomal protein S6 (61) are also good substrates of the kinase, while the acidic substrate peptide for casein kinase 2 (62) is a poor substrate for p90ISK. These data suggest that p90ISK has a high affinity for substrates that contain basic residues and/or prolines surrounding the phosphorylated residue in the primary sequence. The precise primary sequence requirements that make a peptide or protein a good substrate for p90ISK have yet to be determined.

**Cofactors and Inhibitors of Purified p90ISK**—All known protein kinases require a metal cofactor, usually magnesium, for catalysis of kinase activity. Some kinases will utilize manganese instead of magnesium as a cofactor. Purified p90ISK will utilize either magnesium or manganese, with concentrations of 1 mM manganese or 10 mM magnesium being optimal for kinase activity under the assay conditions used (data not shown).

In order to characterize p90ISK and to distinguish it from other known kinases, a variety of agents which have been shown to affect the activity of other serine/threonine kinases were tested for their effects on p90ISK activity. For agents which were found to inhibit p90ISK activity, the concentration that causes 50% maximal inhibition of kinase activity ($IC_{50}$) is listed in Table II. Potassium phosphate, sodium fluoride, glyceraldehyde, poly-L-lysine, p-nitrophenol phosphate, p-nitrophenol, and heparin are all good inhibitors. The inhibition of kinase activity by sodium fluoride seems to be a direct effect on the kinase rather than precipitation of a magnesium fluoride complex and depletion of the magnesium required for kinase activity. This conclusion is drawn because the inhibition by fluoride is also seen when kinase activity is measured using manganese as a cofactor and under conditions where the catalytic subunit of cAMP-dependent protein kinase is unaffected (data not shown). Inhibition of the kinase activity by quercetin reaches a plateau at 50% of maximal inhibition between 100 and 600 $\mu$M quercetin, so it appears that maximum inhibition cannot be achieved by quercetin. Sodium vanadate (100 $\mu$M), calcium chloride (1 mM), cAMP (10 $\mu$M), spermine (500 $\mu$M), calcium plus phosphatidylserine (1 mM/300 $\mu$g/ml), phorbol myristate acetate (100 nM), and protein kinase inhibitor peptide (5 $\mu$M) (63, 64) have no effect on purified p90ISK activity.

**DISCUSSION**

The chromatographic properties of p90ISK described in this report are quite unique and distinguish it from most of the other known insulin-stimulated serine/threonine kinases.

| Table I | Substrate specificity of purified p90ISK |
| --- | --- |
| | Protiens | % MBP phosphorylation |
| Myelin basic protein | 100 |
| Histones | |
| H1 | 83 |
| H2A | 9 |
| H2B | 102 |
| H3 | 18 |
| H4 | 12 |
| S6 protein | 25 |
| ATP citrate lyase | 33 |
| MAP 2 | 5 |
| | % Kemptide phosphorylation |
| Kemptide (LRRASLG) | 100 |
| ATP citrate lyase peptide (RRTA/SfSfSfSfRADE) | 41 |
| S6 peptide (RRLSSSRA) | 27 |
| Casein kinase 2 substrate peptide (RRREEETEEEE) | 5 |
| ERT peptide substrate (KREL-VEpLTFSGAEFPNQALLR) | 98 |

**Table II**

| Inhibitors | IC$_{50}$ |
| --- | --- |
| Potassium phosphate | 15 mM |
| Sodium fluoride | 100 mM |
| p-β-Glycerophosphate | 30 mM |
| Heparin | 40 $\mu$g/ml |
| Poly-L-lysine | 10 $\mu$g/ml |
| Quercetin | >600 $\mu$M* |
| p-Nitrophenol phosphate | 10 mM |
| p-Nitrophenol | 5 mM |

* Inhibition of kinase activity plateaued at 50% maximal inhibition between 100 and 600 $\mu$M quercetin.
p90ISK and the other purified insulin-stimulated serine/threonine kinases, including the types I and II ribosomal protein S6 kinases (39, 65), MAP kinase (21), and KIK (53) all elute from DEAE-anion exchange resins within a similar range of salt concentrations (150 to 250 mM NaCl), although the type I S6 kinase may elute at slightly lower concentrations of salt (65). MAP kinase is very hydrophobic and binds to butyl-Sepharose under the conditions used to purify p90ISK, but KIK is present in the flow-through fraction. MAP kinase is separated from p90ISK at the Affi-Gel Blue step, as MAP kinase appears in the fractions that do not adsorb. It is not known where the types I and II S6 kinases elute during the butyl-Sepharose and Affi-Gel Blue steps, but it is likely that they elute in the flow-through fraction of the butyl-Sepharose column. p90ISK is different from both of the S6 kinases in that p90ISK will not bind to a Mono S cation exchange column (Pharmacia) under the conditions used to purify the S6 kinases. The chromatographic properties of both p90ISK and maf-1 are not known, but maf-1 is significantly lower in elution than that of p90ISK (Fig. 3). The observation that antibodies directed against type II S6 kinase and protein kinase C fail to recognize p90ISK suggests that p90ISK is distinct from these kinases. Taken together, these data provide strong evidence that p90ISK is a novel insulin-activated protein kinase.

The substrate specificities of purified p90ISK and of the other known insulin-stimulated serine/threonine kinases as assessed in cell-free conditions overlap a great deal, but there are some differences among them. Of the purified insulin-stimulated serine/threonine kinases, only KIK (53), p90ISK (Table I), and the type II S6 kinase (66) will phosphorylate Kemptide, and only KIK (53) and p90ISK (Table I) will phosphorylate histones. KIK and p90ISK differ in their substrate specificities in that histone H1 is a good substrate for P90 (Table I) but not for KIK (53). Also, S6 peptide is a much better substrate than is Kemptide for KIK (53), while the reverse is true for p90ISK (Table I). The primary structural requirements for substrates of KIK and p90ISK have not been determined specifically, but both apparently require the presence of basic residues in the proximity of the phosphorylated residue, as does type II S6 kinase (66). p90ISK also appears to be similar to the MAP kinases (67), cdc2 family of kinases (68), and ERT kinase (59, 69) in that it will phosphorylate proline-rich substrates. These broad requirements suggest that some in vivo overlap or possible redundancy of pathways leading to substrate phosphorylation may occur. The ability of p90ISK and the types I and II S6 kinases to phosphorylate ribosomal protein S6 in vitro may indicate a redundancy in intact cells, whereby several kinases phosphorylate the same protein. Differences in compartmentalization of the kinases, in time courses of activation of the kinases, or in the sites phosphorylated on the S6 protein by the kinases may underlie the need for this redundancy.

Where p90ISK and the other insulin-stimulated serine/threonine kinases fit into the signalling pathways that lead from the activation of the insulin receptor tyrosine kinase to the targets of insulin action is not clear. Only one of these kinases, MAP kinase, is activated by tyrosine phosphorylation, but it is not a substrate of the insulin receptor tyrosine kinase (25). Anti-phosphotyrosine antibodies fail to recognize p90ISK purified from livers of insulin-treated rats. Furthermore, protein serine/threonine phosphatase 2A can deactivate purified, insulin-stimulated p90ISK (Fig. 8). Thus, p90ISK is probably activated by serine/threonine phosphorylation, rather than tyrosine phosphorylation, in response to insulin.

All of the other known insulin-stimulated serine kinases, including KIK (44), maf-1 (42, 43), and the S6 kinases (33–40), also appear to be activated by serine/threonine phosphorylation. Thus, the link between the insulin receptor tyrosine kinase and activation of the known serine/threonine kinases does not appear to be a direct one. Furthermore, it is not clear if in intact cells these kinases lie in different positions along the same pathway in a complicated kinase cascade system, or at equivalent positions in different pathways, making up a network of independent kinase cascades. The latter possibility is probably correct, because in vitro evidence suggests that MAP kinase lies on the same cascade pathway as the type II S6 kinase (36, 46) but on a different pathway than the type I S6 kinase (50). The relationships among all of the insulin-stimulated serine/threonine kinases in vivo remains to be determined, and this issue is probably central to understanding the mechanism of insulin action.

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